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**(54) Title: FLUORESCENCE RESONANCE ENERGY TRANSFER ENZYME SUBSTRATES**

A.

No Energy Transfer

Energy Transfer

formula (I)

formula (A')

$E_{\max} = \lambda 1'$

$E_{\max} = \lambda 2$

$E_{\max} = \lambda 1$

$E_{\max} = \lambda 2$

$\lambda 1' < \lambda 1 < \lambda 2$

B.

No Energy Transfer

formula (I)

$$E_{\max} = \lambda_1'$$

Energy Transfer

formula (I')

$$E_{\max} = \lambda_2$$

**(57) Abstract:** Disclosed are compounds of formula (I) wherein D<sub>1</sub> is a first dye moiety whose fluorescence properties may be modulated so as to be suitable as a donor or an acceptor in an energy transfer arrangement; D<sub>2</sub> is a second dye moiety suitable as an acceptor or a donor in an energy transfer arrangement with said first dye; L is a linking group comprises 2-200 linked atoms, wherein said linking group optionally includes an enzyme cleavage site; and M is an enzyme cleavable group chosen to modulate the fluorescence properties of D<sub>1</sub>. The compounds of formula (I) may be used as reporter molecules for detecting biochemical cleavage events in assays that employ fluorescence resonance energy transfer.



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## Fluorescence Resonance Energy Transfer Enzyme Substrates

### Field of Invention

5 The present invention relates to fluorescence-based assays; and to reagents and methods for measuring enzyme activity, particularly enzyme cleavage assays.

### Background of Invention

10 Assays for measuring enzyme activity, particularly enzyme cleaving activity such as hydrolysis, are widely employed in the biological and pharmaceutical sciences. With the advent of combinatorial chemistry and high throughput screening, there is a growing need for simple, sensitive and  
15 cost-effective assays to screen for potential modulators of enzyme activity. Of particular interest to the pharmaceutical industries are methods for detecting proteolytic enzyme cleavage and phosphate cleavage.

Fluorescence-based assays offer significant advantages over  
20 radiochemical, ELISA, antibody and more traditional techniques for measuring enzyme cleaving activity in terms of simplicity of handling, sensitivity, cost and ease of automation. Fluorogenic substrates are routinely used for homogeneous enzyme assays to determine enzyme activity, or the effect of potential drugs on enzyme activity in drug discovery. A number of these  
25 enzyme substrates are commercially available, or have been reported in the literature. For example, US 4812409 (Babb, B., et al) discloses hydrolysable fluorescent substrates comprising blocked dye moieties derived from phenalenone or benzphenalenone, which when cleaved from the substrate during enzymatic hydrolysis, form fluorescent dyes having a fluorescence  
30 emission above about 530nm. Substrates responsive to hydrolytic enzymes are also available based on derivatives of fluorescein and rhodamine. For example, fluorescein diphosphate is a widely used, non-fluorescent and colourless substrate for alkaline phosphatase (PP2A), which when hydrolysed by this enzyme forms fluorescein (Vieytes M., et al, Anal. Biochem., (1997),

248, 258-64). However, substrates based on fluorescein usually incorporate two enzyme cleavable sites, and bi-phasic enzyme kinetics takes place through cleavage, firstly to a mono-substituted fluorescent analogue, then to the free fluorophore. Because the mono-substituted fluorescent substrate 5 absorbs and emits at the same wavelength as the free fluorophore, interpretation of the enzyme kinetics is more difficult. Fluorogenic substrates based on fluorinated derivatives of 4-methylumbelliflferone have been described for the assay of  $\beta$ -galactosidase activity and acid phosphatase activity (Gee, K.R., et al, (1999), 273, 41-8).

10

Many of the dyes used in fluorescence based enzyme assays, emit in the 350-550 nm range. Some of the dyes that do emit at higher wavelength, e.g. resorufin and DDAO are reactive towards thiols (including peptides/proteins with thiol functionalities) and lose their fluorescence 15 properties. They are therefore unlikely to be useful in *in vivo* applications.

Fluorescence-based enzyme substrates that have dyes with useful fluorescence properties that emit in the red or infrared region of the spectrum are nitro-substituted cyanine dye derivatives (EP 1086179 B1: Hamilton, A. et al). The fluorescence of these dyes is quenched by the presence in the 20 molecule of a substituted nitro group. Upon reduction of the nitro group by a nitroreductase, the dye becomes fluorescent. These fluorogenic substrates have proved to be useful and are currently used in live cell assays; however, they have only limited applications. Hence, there is a clear need for new 25 fluorogenic enzyme substrates with emission at higher wavelength (red or infra-red) that are non-reactive except towards the enzyme of interest and provide a high signal to background ratio.

The present invention describes new fluorogenic enzyme substrates that are substrates for a range of different enzymes and emit at different 30 wavelengths including red and near infra-red. Also provided, are dual enzyme substrates for determining the ratios of two enzyme activities. This is more relevant for *in vivo* systems, where two separate enzyme substrates cannot be relied upon to measure relative enzyme activities, due to differences in

absorption, distribution and excretion properties of individual single enzyme substrates. Finally, also provided are methods for determining enzyme activity, as well as methods of determining a metabolic state either *in vitro* or *in vivo*.

5

It is thus an object of the invention to provide a fluorescence-labelled enzyme substrate, particularly a fluorescence resonance energy transfer (FRET) labelled substrate and a method of measuring the activity of an enzyme in cleaving a substrate, the substrate comprising a FRET label and 10 an enzyme cleavable group.

It is also an object of the invention to provide a method of screening for a test agent which may affect enzyme cleaving activity.

15 **Summary of Invention**

Thus, in a first aspect of the invention, there is provided a compound of formula (I):



20

(I)

wherein:

D<sub>1</sub> is a first dye moiety whose fluorescence properties may be modulated so as to be suitable as a donor or an acceptor in an energy transfer arrangement;

D<sub>2</sub> is a second dye moiety suitable as an acceptor or a donor in an energy transfer arrangement with said first dye;

L is a linking group comprises 2-200 linked atoms, wherein said linking group 30 optionally includes an enzyme cleavage site; and

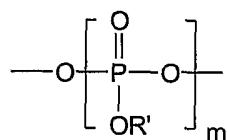
M is an enzyme cleavable group chosen to modulate the fluorescence properties of D<sub>1</sub>.

Suitably,  $D_1$  and  $D_2$  are linked by linking group  $L$  such that, under suitable conditions, fluorescence resonance energy transfer (FRET) may take place one with the other. FRET is a distance-related process in which the electronic excited states of two dye molecules interact without emission of a photon. See, Forster, T., "Intermolecular Energy Transfer and Fluorescence", Ann. Physik., Vol.2, p.55, (1948). One result of this interaction is that excitation of a donor molecule enhances the fluorescence emission of an acceptor molecule. The fluorescence quantum yield of the donor is correspondingly diminished. For FRET to occur, suitably, the donor and acceptor dye molecules must be in close proximity (typically between 10-100 Å), since energy transfer efficiency decreases inversely as the 6<sup>th</sup> power of the distance ( $r$ ) between the donor and acceptor molecules. Suitably, in the present invention, either  $D_1$  or  $D_2$  may be the donor or acceptor in the FRET relationship such that when  $D_1$  is the donor molecule,  $D_2$  is the acceptor and vice versa. By donor, it is meant that the dye moiety is capable of absorbing energy from light and emits light at wavelength frequencies which are at least partly within the absorption spectrum of the acceptor. By acceptor, it is meant that the dye moiety is capable of absorbing energy at a wavelength emitted by a donor dye moiety. Suitably, there is overlap between at least a portion of the emission spectrum of the donor dye molecule with the absorption spectrum of the acceptor dye molecule. Suitably, the wavelength of the emission maximum of the acceptor dye is longer than the wavelength of the emission maximum of the donor dye. In one format, one of the donor or acceptor molecules can be a non-fluorescent (or quenching) fluorophor which is in close proximity to a second fluorescent acceptor or donor fluorophor. Upon excitation of the non-fluorescent dye, energy is dissipated as heat rather than fluorescence energy and resonance energy transfer or fluorescence emission cannot take place.

Suitably, group  $M$  is chosen from enzyme cleavable groups that are capable of modulating the fluorescence properties of  $D_1$ , such that when  $M$  is covalently attached to  $D_1$ ,  $D_1$  is in a first fluorescence state, either having no, or substantially no fluorescence emission, or alternatively having a fluorescence emission with an emission wavelength maximum,  $E_{max} = \lambda 1'$ .

Suitably, M comprises a substrate for an enzyme, such that upon enzymatic cleavage of M from D<sub>1</sub> to form the compound of formula (I'), (see Figure 1), the fluorescence properties of D<sub>1</sub> are caused to be changed to a second fluorescence state such that D<sub>1</sub> emits fluorescence at an emission wavelength maximum, E<sub>max</sub> = λ1. Suitably, D<sub>2</sub> is a fluorescent dye having a fluorescence emission maximum, E<sub>max</sub> = λ2. Suitably, in the compound of formula (I), λ1' ≠ λ1 ≠ λ2.

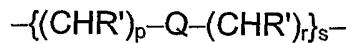
10 Suitably, linking group L comprises a group containing from 2-200 linked atoms selected from carbon atoms which may optionally include one or more groups selected from -C(O)-, -NR'-, -O-, -S-, -CH=CH-, -CO-NH-, phenylenyl and the group:



15 where R' is selected from hydrogen and C<sub>1</sub> – C<sub>4</sub> alkyl and m is an integer from 1 to 3.

In one preferred embodiment, linking group L contains from 2-50 linked atoms, having the structure:

20



where Q is selected from: -C(O)-, -NR'-, -O-, -S-, -CH=CH-, -CO-NH- and phenylenyl groups; R' is hydrogen or C<sub>1</sub> – C<sub>4</sub> alkyl, each p is independently 0 – 10, each r is independently 0 – 10, and s is 1, 2, or 3.

Preferably Q is selected from: -C(O)-, -CHR'-, -O- and -CO-NH-, where R', p, r and s are hereinbefore defined.

30

In another embodiment, linker group L may also comprise a peptide or an oligonucleotide fragment. When the linker is a peptide or contains a peptide fragment, it may contain from 2-20 natural or unnatural amino acids or a combination thereof. More preferably, L contains 2-12 amino acids and

5 most preferably, 2-8 amino acids. When the linker group L is an oligonucleotide or contains an oligonucleotide fragment, it may contain from 2-20 nucleoside bases or modified bases. Preferably the number of bases will be in the range from 4-15; more preferably in the range 4-10.

10 The linker may include part of the constituents extending from the fluorochrome. In other words, the linker is attached to the dye chromophore but is not a part of it. In the compounds of the present invention, none of the linkers includes a network of double bonds that permit conjugation of the donor and acceptor. For optimal energy transfer to occur, the transition

15 moments of the donor and acceptor fluorochromes are orientated relative to each other in a non-perpendicular direction, e.g. positioned generally parallel or in tandem relative to each other. With a relatively short linker and optimal orientation, there may be efficient resonance energy transfer even when the spectral overlap becomes small.

20

### Brief Description of the Figures

Figure 1 is a schematic diagram illustrating preferred embodiments of the invention.

25 Figure 2 illustrates the synthetic reaction scheme for the preparation of Compounds (V) and (VI).

Figure 3 shows the UV spectrum of Compound (II) before and after treatment with alkaline phosphatase.

30 Figure 4 shows the UV spectrum of Compound (III) before and after treatment with alkaline phosphatase.

Figure 5 shows the UV spectrum of Compound (IV) before and after treatment with alkaline phosphatase.

Figure 6 shows the emission spectrum of Compound (II) before and after treatment with Alkaline Phosphatase by excitation at 455 nm and 475 nm.

Figure 7 shows the emission spectrum of Compound (III) before and after treatment with Alkaline Phosphatase by excitation at 455 nm and 475 nm.

Figure 8 shows the emission spectrum of Compound (IV) before and after treatment with Alkaline Phosphatase by excitation at 455 nm and 475 nm.

5

### **Detailed Description of the Preferred Embodiments**

In one embodiment according to the first aspect of the invention,  $D_1$  is the donor dye and  $D_2$  is the acceptor dye. In this embodiment (shown in

10 Figure 1A), the emission wavelength maximum ( $\lambda 1$ ) of  $D_1$  is less than the emission wavelength maximum ( $\lambda 2$ ) of  $D_2$  and there is overlap between at least a portion of the emission spectrum of  $D_1$  with the absorption spectrum of  $D_2$ . When  $M$  is covalently attached to  $D_1$ ,  $D_1$  has either no, or substantially no fluorescence emission, or alternatively fluoresces having a fluorescence 15 emission wavelength maximum  $\lambda 1'$ , where  $\lambda 1'$  is less than  $\lambda 1$ . In this state, there is no, or substantially no, overlap between the emission spectrum of  $D_1$  and the absorption spectrum of  $D_2$ , such that there is no energy transfer between  $D_1$  and  $D_2$ . Upon cleavage of  $M$  from  $D_1$ , the fluorescence properties 20 of  $D_1$  are caused to be changed to a second fluorescence state such that  $D_1$  emits fluorescence at the emission wavelength maximum  $\lambda 1$ . As a result, overlap between the emission spectrum of the  $D_1$  and the absorption spectrum of  $D_2$  is restored and energy transfer between  $D_1$  and  $D_2$  can take place. Thus, upon excitation of the donor dye  $D_1$  at an appropriate excitation wavelength, an increase in fluorescence emission from  $D_2$  may be detected 25 and/or quantitated.

In a second embodiment  $D_2$  is the donor dye and  $D_1$  is the acceptor dye. In this embodiment (shown in Figure 1B), the emission wavelength maximum ( $\lambda 2$ ) of  $D_2$  is less than the emission wavelength maximum ( $\lambda 1$ ) of  $D_1$  and there is overlap between at least a portion of the emission spectrum of the  $D_2$  with the absorption spectrum of  $D_1$ . When  $M$  is covalently attached to  $D_1$ ,  $D_1$  has either no, or substantially no fluorescence emission, or alternatively fluoresces having a fluorescence emission wavelength maximum  $\lambda 1'$ , where  $\lambda 1'$  is greater than  $\lambda 1$ . In this state, there is no, or substantially no,

overlap between the emission spectrum of  $D_2$  and the absorption spectrum of  $D_1$ , such that there is no energy transfer between  $D_2$  and  $D_1$ . Upon cleavage of  $M$  from  $D_1$ , the fluorescence properties of  $D_1$  are caused to be changed to a second fluorescence state such that  $D_1$  emits fluorescence at an emission 5 wavelength maximum  $\lambda 1$ . As a result, overlap between the emission spectrum of the  $D_2$  and the absorption spectrum of  $D_1$  is restored and energy transfer can take place. Upon excitation of the donor dye  $D_2$  at an appropriate excitation wavelength, an increase in fluorescence emission from  $D_1$  may be detected and/or quantitated.

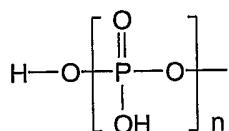
10

In the embodiment in which  $D_2$  is the donor dye and  $D_1$  is the acceptor, energy transfer may alternatively take place between the donor dye and two fluorescent states of the acceptor dye. In this arrangement, the acceptor dye in the first fluorescent state will emit at one wavelength  $\lambda 1'$  and upon cleavage 15 of  $M$  will generate a second fluorescence state that emits at a second wavelength  $\lambda 1$ .

In a further embodiment, the first and second fluorescence states of  $D_1$  may differ, not by their respective emission wavelength maxima  $\lambda 1$  and  $\lambda 1'$ , 20 but by the intensity of fluorescence emitted by the two states.

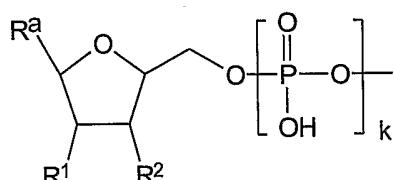
Compounds of formula (I) may be used as reporter molecules for detecting biochemical cleavage events in assays that employ fluorescence resonance energy transfer. Suitably, group  $M$  comprises a substrate for a 25 cleavage enzyme, preferably selected from the group consisting of a peptidase, a protease, a phosphatase, a dealkylase and a glycosidase. Treatment of the substrate with an enzyme under conditions suitable to cause cleavage of  $M$  from  $D_1$ , modulates the fluorescence properties of  $D_1$ , thereby switching  $D_1$  from the first fluorescent state to the second fluorescent state as 30 described hereinbefore.

In one preferred embodiment,  $M$  comprises a phosphate ester linkage having one or more phosphate groups covalently bonded to  $D_1$  and having the structure:



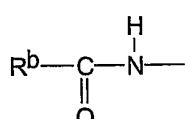
wherein n is an integer from 1 to 4. In this embodiment, the substrate is capable of being cleaved by a phosphatase such as bacterial alkaline phosphatase, or acid phosphatase, to yield an alcohol derivative of dye D<sub>1</sub>, and inorganic phosphate. As a consequence, D<sub>1</sub> is caused to be switched from a first fluorescent state to a second fluorescent state, thereby enabling transfer of excitation energy between donor and acceptor moieties. Upon excitation of the donor dye at its excitation wavelength, there is an increase in fluorescence intensity at the emission wavelength of the acceptor dye. The phosphate ester may be a pre-synthesised substrate or may be generated *in situ* by chemical hydrolysis or by an enzyme catalysed nucleoside monophosphate or nucleoside polyphosphate transfer from a terminal-phosphate labelled nucleoside polyphosphate having the structure:

15



wherein R<sup>1</sup> and R<sup>2</sup> are independently selected from H and OH; R<sub>a</sub> is a nucleoside base selected from adenine, guanine, cytosine, thymine, uracil, hypoxanthine and xanthine; and k is an integer from 1 to 6.

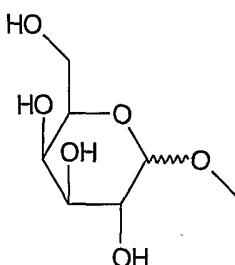
In another preferred embodiment, M comprises at least one peptide linkage ( $-\text{CO}-\text{NH}-$ ) covalently bonded to D<sub>1</sub>. In this embodiment, M typically has the structure:



25

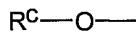
wherein  $R^b$  is a residue of a peptide or protein. Upon hydrolysis by a peptidase or protease, the M is cleaved from the fluorescence energy transfer label. Upon excitation of the donor moiety at its excitation wavelength, energy is transferred between the donor and acceptor dyes, allowing detection of an  
5 increase in fluorescence emission from the acceptor.

In a further embodiment, M comprises a glycosidic linkage and is a substrate for a glycosidase such as  $\alpha$ -glycosidases (e.g.,  $\alpha$ -amylase),  $\beta$ -glycosidases (e.g.  $\beta$ -glucosidase) and has the structure:



10

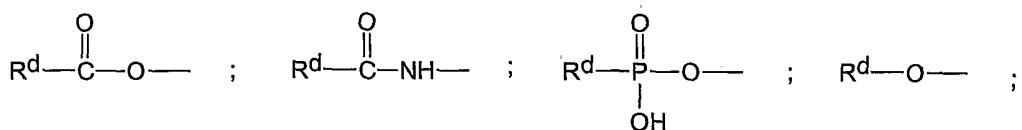
In a further embodiment, M comprises an ether linkage that is a substrate for a dealkylase and having the structure:



15

wherein  $R^c$  is  $C_1 - C_{20}$  straight or branched chain alkyl.

In a still further embodiment, group M further comprises a cell membrane permeabilising group, which may be selected from groups such as  
20 ether, ester, amide and phospho-diester groups of formula:



wherein  $R^d$  is  $C_1 - C_{10}$  straight or branched chain alkyl, either unsubstituted, or substituted with one or more halogen atoms, phenyl, either unsubstituted or  
25 substituted with one or more halogen atoms, or a peptide chain. Suitably, halogen atoms may be selected from fluorine, chlorine, bromine and iodine.

Useful examples of such membrane permeabilising groups include acetate ester, pivaloyl ester, acetoxyethyl ester, pentafluorobenzyl ether, pentafluorobenzyl amide, pentafluorobenzyl ester, phenyl phosphate, perfluoro-(C<sub>1</sub> – C<sub>6</sub>) alkyl ethers, perfluoro-(C<sub>1</sub> – C<sub>6</sub>) alkyl esters and perfluoro-(C<sub>1</sub> – C<sub>6</sub>) alkyl amide groups. Such groups are substrates for, and are hydrolysed by dealkylases, esterases such as proteases and phosphodiesterases as are found in cells. The skilled person will appreciate the variability of cell permeability of the reporter molecule and will be able to test for the same.

10

Suitably, the donor dye may be selected from coumarin dyes, benzocoumarin dyes, acridone dyes, xanthine dyes, phenoxazine dyes, rhodamine dyes, merocyanine dyes and cyanine dyes, preferably xanthine dyes and cyanine dyes, wherein the donor dye is capable of transferring energy to the acceptor dye.

15

Suitably, the acceptor dye may be selected from coumarin dyes, benzocoumarin dyes, acridone dyes, xanthine dyes, phenoxazine dyes, rhodamine dyes, merocyanine dyes and cyanine dyes, wherein the acceptor dye is capable of energy transfer with the donor dye.

20

Preferably, the donor dye is a xanthine dye or a cyanine dye and the acceptor dye is a rhodamine or a cyanine dye.

25

In one preferred embodiment, at least one of said donor and acceptor dye moiety is a cyanine dye. In another preferred embodiment, the donor dye is a xanthine dye and the acceptor dye is a rhodamine dye.

30

Suitable xanthine dyes include but are not limited to fluorescein and its derivatives, such as 5-carboxyfluorescein, 6-carboxyfluorescein and 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein.

Suitable cyanine dyes include but are not limited to CyA (3-( $\varepsilon$ -carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), Cy2 (3-( $\varepsilon$ -carboxypentyl)-3'-ethyl-oxa-carbocyanine), Cy3 (3-( $\varepsilon$ -carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine), Cy3.5 (3-( $\varepsilon$ -carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-( $\varepsilon$ -carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-( $\varepsilon$ -carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-dicarbocyanine, and Cy7 (1-( $\varepsilon$ -carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine.

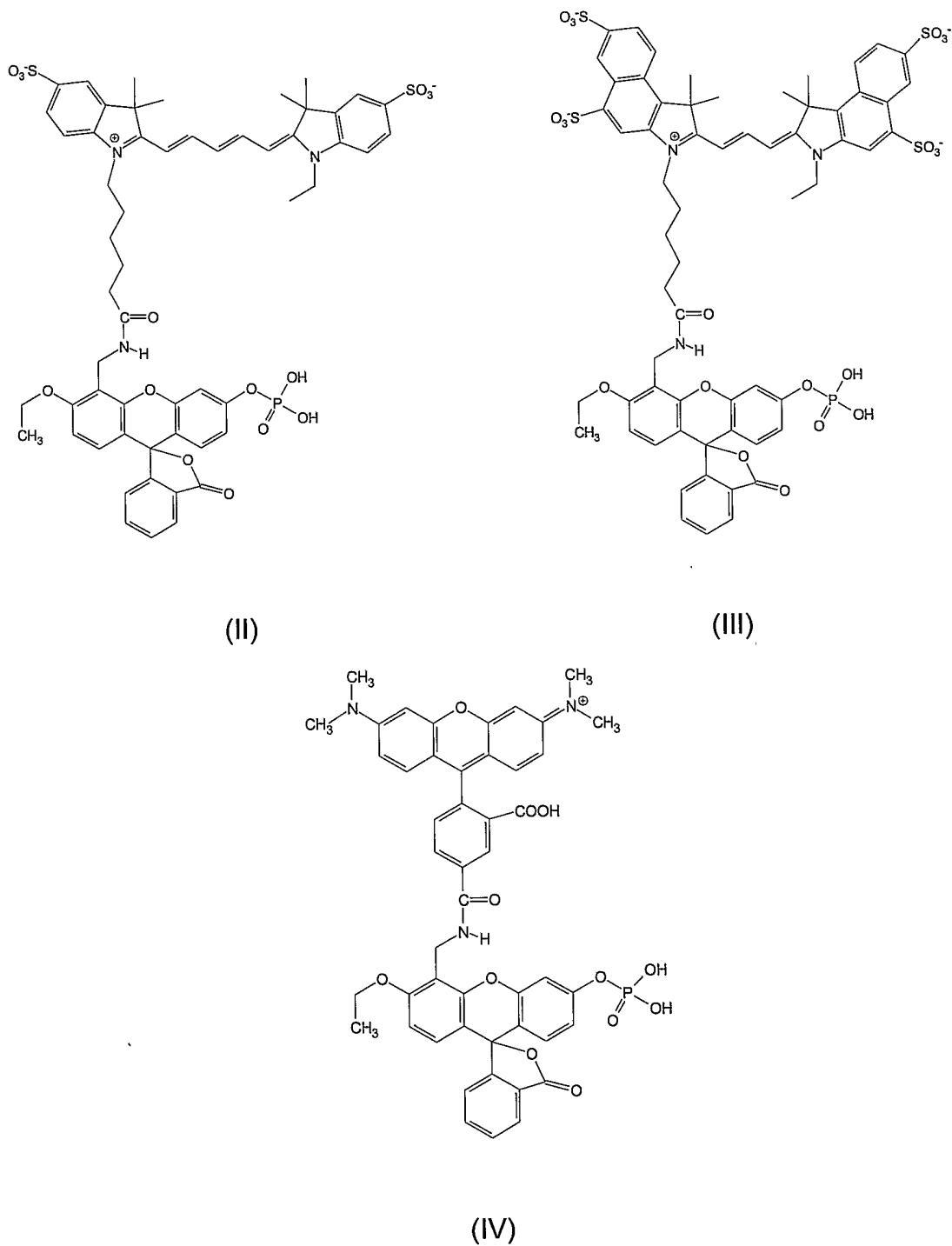
Suitable rhodamine acceptor dyes include but are not limited to: 5-carboxyrhodamine (Rhodamine 110-5), 6-carboxyrhodamine (Rhodamine 110-6), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-5-carboxyrhodamine, N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or TMR), 5-carboxy-X-rhodamine and 6-carboxy-X-rhodamine (ROX). Other classes of dyes include BODIPY<sup>TM</sup>, porphyrin dyes, rhodol dyes and perylene dyes.

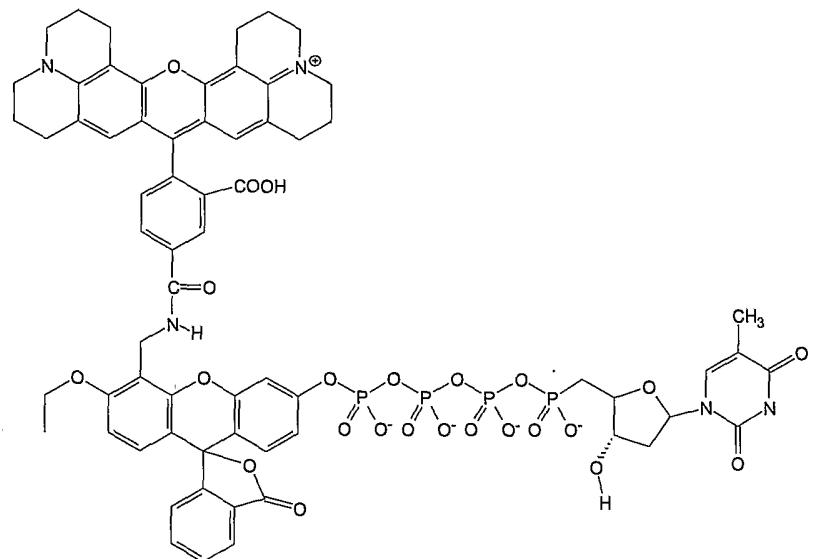
The fluorescent labelled enzyme substrates according to the first aspect may also include water solubilising constituents attached thereto for conferring a hydrophilic characteristic to the compound. They are preferably attached to aromatic ring systems of the donor or acceptor dye moieties. In the alternative, the linking group L may contain the water solubilising group.

Suitable solubilising constituents may be selected from the group consisting of amide, sulphonate, sulphate, phosphate, quaternary ammonium, hydroxyl, guanidinium and phosphonate. Sulphonate or sulphonic acid groups attached directly to the aromatic ring of the donor and/or acceptor fluorochromes are particularly preferred.

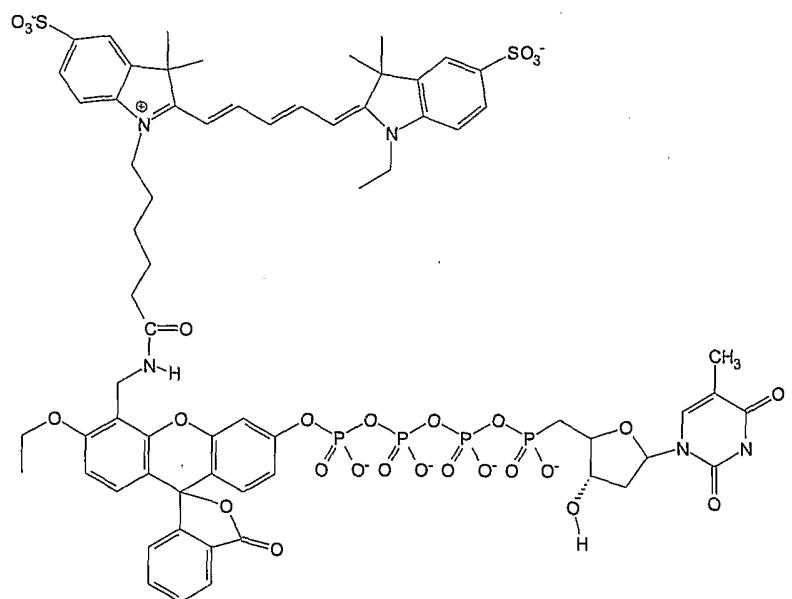
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Examples of energy transfer reporter molecules according to the invention are shown in Table 1.

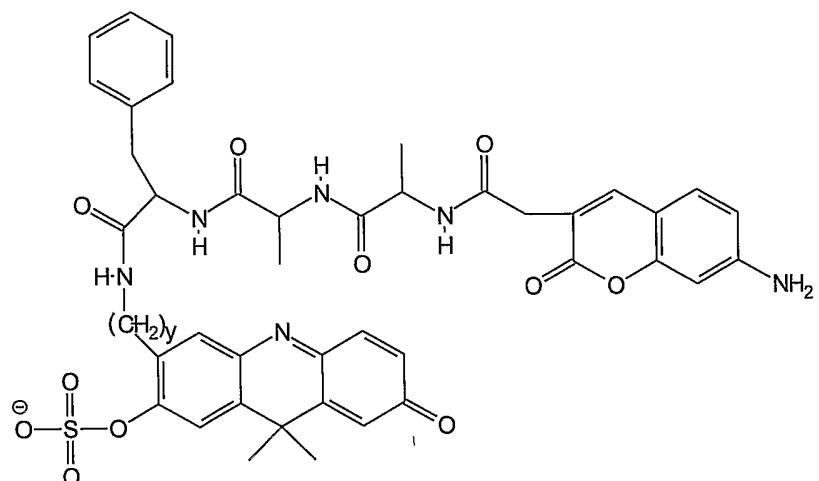
Table 1 Examples of FRET-coupled Reporter Molecules



(V)



(VI)



(VII)

In Examples (II), (III) and (IV), the reporter is a non-fluorescent energy transfer complex containing a quenched fluorescein donor dye and a Cy5, a Cy3 and a rhodamine acceptor dye respectively, the fluorescein moiety having an enzymatically cleavable phosphate group attached thereto. The phosphate group may be cleaved by the action of a phosphatase, thereby restoring fluorescence of the donor dye upon excitation of the fluorescein at its excitation wavelength. Detection of the cleavage event and measurement of phosphatase activity may be achieved by detecting fluorescence emission at the emission wavelength of the acceptor dye.

In Examples (V) and (VI), the reporter is a non-fluorescent energy transfer complex containing a quenched fluorescein donor dye and a Cy5 and rhodamine acceptor dye respectively, the fluorescein moiety having a nucleoside polyphosphate moiety attached thereto. Action of a nucleosidyl phosphotransferase generates a phosphatase substrate *in situ*, which in the presence of phosphatase, undergoes phosphate removal. The fluorescence of the fluorescein donor dye is restored. Subsequent energy transfer to the acceptor dye generates a red-shifted emission. When the nucleosidyl phosphotransferase enzyme is a nucleic acid polymerase, compositions of the present invention can be used for detection, characterization and quantitation of known and unknown nucleic acids.

In one embodiment, L is a cleavable linker and includes an enzyme cleavable group P which is different from the enzyme substrate group M. P may be selected from groups such as ether, ester, amide and phospho-diester groups. Such groups are substrates for, and are cleaved in aqueous buffer media by respectively, dealkylases, esterases such as proteases and phospho-diesterases. In this embodiment, it is preferable that D<sub>2</sub> emits at a first emission wavelength  $\lambda_2$  when attached to L, and at a second emission wavelength  $\lambda_2'$ , once D<sub>2</sub> is cleaved from L, where  $\lambda_2 \neq \lambda_2'$ . The reporter compounds of this embodiment are useful for measuring the relative activity of two enzymes in *in vivo* and cellular systems, where it is difficult to ensure equal delivery of two separate enzyme substrates. It is also possible for D<sub>1</sub> to emit at a first emission wavelength ( $\lambda_1$ ) when attached to M and at a different wavelength ( $\lambda_1'$ ) when M is cleaved, where  $\lambda_1 \neq \lambda_1'$ . Example (VII) shows such a dual enzyme reporter for an aryl sulfatase and Cathepsin G activity, which is potentially useful in detecting inflammation. When no enzyme activity is present, there is a weak emission at 610 nm when the compound of Example (VII) is excited at 360 nm. No emission is observed with excitation at 645 nm. With sulfatase activity only, strong emission is observed at 660 nm with excitation at 645 nm and weak emissions at 450 nm and 660 nm when excited at 360 nm. With Cathepsin G activity only, strong emission is observed at 450 nm when excited at 360 nm and no emission at 660 nm upon excitation at either 360 nm or 645 nm. With both enzymes present, strong emission is observed at 450 nm upon excitation at 360 nm and another strong emission at 660 nm upon excitation at 645 nm. No 660 nm emission is observed with excitation at 360 nm. Thus, by measuring emissions at 660 nm and 450 nm and by comparing with known standards it is possible to determine the relative enzyme activities of both enzymes at a particular time point or location in an *in vivo* system. Such dual enzyme activity substrates are useful for distinguishing between different disease states as well as determining the clinical stage or aggressiveness of a disease state.

Both MMP-2 and MMP-9 have been targeted for tumor detection and treatment, for example, in melanomas, expression of these proteins varies

significantly. While in very aggressive tumors, MMP-9 is expressed at high levels, no over-expression is observed in early stage tumors. Benign tumors show no MMP-9 activity. Similarly, MMP-2 activity also varies significantly with invasive tumors showing higher activity. Although this is true for the 5 majority of patients, it is not true for all. Therefore, measuring only MMP-2 or MMP-9 activity may lead to misdiagnosis of an aggressive tumor as being benign. For osteoporosis, Cathepsin K has been identified as a therapeutic target as it is over-expressed in osteoclasts; however this activity is also observed during normal bone turnover not associated with osteoporosis. In 10 osteoporosis however, high levels of MMP-9 are also observed. Measuring activity of MMP-9, however, is not a very specific indicator of osteoporosis. Therefore measuring both activities and their ratio provides a better diagnostic tool than a single enzyme activity measurement.

15 In a second aspect of the present invention, there is provided a method for determining the activity of an enzyme acting on a substrate molecule, the substrate comprising a compound of formula (I), wherein D<sub>1</sub>, D<sub>2</sub> and L are hereinbefore defined; and M comprises a substrate for a cleavage enzyme. The method comprises the steps of: i) measuring the fluorescence intensity of 20 the fluorescently labelled substrate; ii) combining an enzyme whose activity is to be determined with the substrate under conditions to cause cleavage of M from D<sub>1</sub>; and iii) measuring a change in fluorescence intensity of the fluorescent label following the combination of step ii); wherein the change in fluorescence intensity of the fluorescent label is used to determine the activity 25 of the enzyme.

Suitably, the compound of formula (I) is a substrate for the enzyme whose activity is to be determined, wherein M comprises an enzyme cleavable group preferably selected from a phosphate ester linkage, at least 30 one peptide linkage, an ether linkage and a glycosidic linkage as hereinbefore defined. Preferably, the enzyme is a hydrolase enzyme selected from the group consisting of phosphatase, peptidase, protease, dealkylase and glycosidase.

In one preferred embodiment according to the second aspect, the enzyme is a phosphatase, selected from the E.C. Class 3.1, including PTPase, PPTase-2A, PPTase-2B, or PPTase-2C. Examples are: tyrosine phosphatase, PP1 and PP-2B (serine/threonine phosphatase),

5

In a second preferred embodiment according to second aspect, the enzyme is a peptidase selected from the E.C. Class 3.4. Examples include, but are not limited to, angiotensin converting enzyme (ACE), caspase, cathepsin D, chymotrypsin, pepsin, subtilisin, proteinase K, elastase, 10 neprilysin, thermolysin, asp-n, matrix metallo proteinase 1 to 20, papain, plasmin, trypsin, enterokinase and urokinase.

15 In another embodiment, the enzyme is a glycosidase, preferably selected from the group consisting of E.C. Class 3.2, for example  $\alpha$ -amylase,  $\beta$ -amylase, glucan 1,4- $\alpha$ -glucosidase, cellulase, endo-1,3- $\beta$ -glucanase, oligo-1,6-glucosidase and lysozyme.

20 In a further embodiment, the enzyme is a dealkylase, for example different isozymes of cytochrome P-450.

20

Other enzymes, such as peroxidases,  $\beta$ -lactamase, nitroreductase, etc. may also be used with appropriate M or linker structures. Fluorogenic substrates for these enzymes are well known, e.g. Amplex red, a fluorescein and 6-chloro-7-hydroxy coumarin substituted cephalosporin, and 25 nitrocyanines, such as those described in (EP 1086179 B1, Hamilton, A. et al). Similar structures may be incorporated in the compositions described above.

30 Assays may be performed according to the present invention in high throughput screening applications, including those in which test agents are screened for their inhibitory effects, potentiation effects, agonistic, or antagonistic effects on the enzyme reaction under investigation. Thus, in a preferred embodiment according to the second aspect, there is provided a

method of screening for a test agent whose effect upon the activity of an enzyme in cleaving a substrate is to be determined. The method comprises the steps of: (a) performing the method according to the second aspect in the presence and in the absence of the test agent; and (b) determining the activity 5 of the enzyme in the presence and in the absence of the agent; wherein a difference between the activity of the enzyme in the presence and in the absence of said agent is indicative of the effect of the test agent on the activity of the enzyme. Alternatively, the screening can be carried out by performing the method in the presence of a test agent and comparing the value of the 10 activity of the enzyme with a control value for the enzyme activity in the absence of the test agent. The control value may be conveniently stored electronically in a database or other electronic format.

In a third aspect of the invention, there is provided a method of 15 determining relative activities of two enzymes acting on a substrate molecule, the substrate molecule comprising a compound of formula (I), wherein D<sub>1</sub> is a first dye moiety and D<sub>2</sub> is a second dye moiety as hereinbefore defined, M comprises a substrate for a first cleavage enzyme and L contains an enzyme cleavable group P which is substrate for a second cleavage enzyme different 20 from the first cleavage enzyme. The method comprises the steps of: i) measuring the fluorescence emission intensity of the fluorescently labelled substrate; ii) combining the first and second enzymes with the substrate under conditions to cause cleavage of M from D<sub>1</sub> and D<sub>2</sub> from D<sub>1</sub>; iii) measuring the fluorescence emission intensities of the first and second dye moieties 25 following the combination of step ii); and iv) utilising a change in fluorescence intensities of the first and second dye moieties to determine the relative activities of the enzymes.

Suitably, the first and second enzymes may be combined either 30 sequentially, in either order or, alternatively, simultaneously with the substrate molecule. Preferably, the first and second enzymes are selected from the group consisting of phosphatase, peptidase, protease, dealkylase and glycosidase, as hereinbefore described.

The method according to the third aspect of the invention may also be used to determine the effect of a test agent on the relative activities of two enzymes acting on a substrate molecule, in which the combining step ii) is performed in the absence and in the presence of the test agent. A difference 5 between the relative activities of the first and second enzymes in the presence and in the absence of said agent is indicative of the effect of the test agent on the relative activities of the first and second enzymes.

The test agent may be, for example, any organic or inorganic 10 compound such as a synthetic molecule or a natural product (e.g. peptide, oligonucleotide), or a may be an energy form (e.g. light or heat or other forms of electromagnetic radiation). Inhibitors of enzyme activity can be detected using the method according to the preferred embodiment; for example, the known inhibitor pepstatin A can be readily shown to inhibit cathepsin D 15 enzyme activity using the method of the invention. Thus, in the presence of 12 nM of pepstatin A, cathepsin D activity is some 5 fold less than in the absence of the inhibitor.

Suitably, the difference between the activity of the enzyme in the 20 absence and in the presence of the agent is normalised, stored electronically and compared with a reference value. Thus, for example, the difference in activity may be stored as a percentage inhibition (or percentage stimulation) on an electronic database and this value compared with the corresponding value for a standard inhibitor of the enzyme in question. In this way, only test 25 agents meeting a certain pre-determined threshold (e.g. as being as effective or more effective than the reference compound) may be selected as being of interest for further testing.

As an example, an assay for the detection of proteolytic enzyme 30 activity may be configured as follows. A reaction mixture is prepared by combining an enzyme and a fluorescent energy transfer reporter compound according to formula (I), wherein group M comprises a proteolytic enzyme cleavage site. The assay is suitably performed in the wells of a multiwell plate, e.g. a microtitre plate having 24, 96, 384 or higher densities of wells,

e.g. 1536 wells. The reaction may be performed with the enzyme substrate initially present in an aqueous assay buffer, suitably, 10mM MOPs, 50mM Tris or 50mM HEPES, containing 5 mM MgCl<sub>2</sub>. A test agent, such as a known or a putative inhibitor, may be optionally included in the reaction mixture. Upon

5 addition of enzyme, typically the reaction is allowed to proceed to completion, progress being monitored by observing the steady state fluorescence emission due to the fluorescent acceptor dye, which is recorded using a spectrofluorimeter. Alternatively, the assay may be performed under "stopped" conditions in which the reaction is allowed to proceed for a

10 predetermined time and then terminated with a stop reagent, normally an inhibitor of the enzyme activity, which is often non-specific. An example of a stop reagent is EDTA, which is used to sequester metal ions that are normally required for enzymatic activity.

15 The methods according to the present invention may also be employed to measure the activity of an enzyme acting on a substrate in a cellular environment, the substrate comprising a compound according to the present invention. Thus in a particular embodiment according to the second and third aspects the method comprises, before step i) the step of adding the substrate

20 to one or more cells in a fluid medium.

Typically, cultured cells are incubated with the FRET-coupled enzyme substrate at a concentration of 0.1 to 100 $\mu$ M in a suitable cell culture medium under conditions suitable for cell growth and for a time which may range from

25 0.5 to 24 hours. Cells are cultured according to standard cell culture techniques, e.g. cells are cultured in a suitable vessel in a sterile environment at 37°C in an incubator containing a humidified 95% air/5% CO<sub>2</sub> atmosphere. There are established protocols available for the culture of diverse cell types. (See for example, Freshney, R.I., Culture of Animal Cells: A Manual of Basic

30 Technique, 2<sup>nd</sup> Edition, Alan R.Liss Inc. 1987). When the substrate is required to be introduced into cells grown in cell or tissue culture, the substrate is simply added to the culture medium. Cells may also be contacted with the substrate in the presence of a test agent whose effect on the enzyme

activity is to be determined. In this embodiment, the detection step provides a measurement of the effect of the test agent on the activity of the enzyme under investigation.

5 Measurements of fluorescence intensity may be made using instruments incorporating photo-multiplier tubes as detectors. Changes in fluorescence intensity may be measured by means of a charge coupled device (CCD) imager (such as a scanning imager or an area imager) to image all of the wells of a microtitre plate. The LEADseeker<sup>TM</sup> system features a

10 CCD camera allowing fluorescence imaging of high density microtitre plates in a single pass. Imaging is quantitative and fast, and instrumentation suitable for imaging applications can now simultaneously image the whole of a multiwell plate. Where an assay is to be formatted for the determination of the activity of a test agent on enzyme activity, the assay may be performed under

15 continuous measurement of the fluorescence of the substrate. In this format, the intensity of the fluorescent labelled substrate changes continuously. The labelled substrate does not need separation from the product of the enzymatic reaction and thus, a time-course of the reaction may be obtained, allowing kinetic studies to be performed in real time.

20 In a fourth aspect of the present invention, there is provided a test kit for measuring the activity of at least one enzyme, the test kit comprising one or more different enzyme substrates, each of said substrates comprising a compound of formula (I), wherein D<sub>1</sub>, D<sub>2</sub>, L are hereinbefore defined and M

25 comprises a substrate for a cleavage enzyme. Preferably, the test kit further comprises at least one or more different enzymes each enzyme specific for a different said substrate.

30 The compounds of formula (I) may be prepared by covalent attachment of the fluorescence energy transfer moiety, D<sub>1</sub> – L – D<sub>2</sub>, to group M wherein D<sub>1</sub>, D<sub>2</sub>, M and L are hereinbefore defined, using direct chemical coupling methods that are well known to the skilled person. Alternatively, the compounds of the present invention may be prepared by *de novo* synthetic methods as illustrated in the examples. Group M may be initially attached to

D<sub>1</sub> to form the intermediate compound, M-D<sub>1</sub>, followed by attachment of D<sub>2</sub> via linker group L. The linker group L may be conveniently attached to M-D<sub>1</sub> prior to covalent attachment of D<sub>2</sub>, or alternatively L may be pre-attached to D<sub>2</sub>. Peptide, protein and oligonucleotide substrates for use in the invention 5 may be labelled at a terminal position, or alternatively at one or more internal positions. For reviews and examples of protein labelling using fluorescent dye labelling reagents, see "Non-Radioactive Labelling, a Practical Introduction", Garman, A.J. Academic Press, 1997; "Bioconjugation - Protein Coupling Techniques for the Biomedical Sciences", Aslam, M. and Dent, A., Macmillan 10 Reference Ltd, (1998). Protocols are available to obtain site specific labelling in a synthesised peptide, for example, see Hermanson, G.T., Bioconjugate Techniques, Academic Press (1996).

In order to clarify the principle and the function of the invention, 15 reference is now made to the following examples should not be construed as limiting the appended claims and the scope of the invention. The current invention should encompass any and all variations that become evident from the teachings provided herein.

20 **Examples**

1. Preparation of FRET-coupled Reporter Molecules (Compound (V) and Compound (VI) (See Figure 2)

25 1.1 4'(5')-Fluorescein diethylether-ester: Compound (2)

To a solution of NaOH (1.44 g) in methanol (145 ml) was added fluorescein (6.0 g) to give a dark colored solution which was concentrated to dryness *in vacuo*. This was co-evaporated with anhydrous DMF (2x50 ml) 30 and redissolved in anhydrous DMF (150 ml). Ethyl iodide (11.6 ml, 8 eq) was added and reaction stirred at room temperature for overnight. After concentration of the reaction mixture, it was dissolved in saturated sodium bicarbonate solution (150 ml) and washed with diethylether. A yellow

precipitate formed on cooling of the solution which was filtered and finally washed with ether (2x 25 ml) and ether/pentane (1:1, 50 ml).

1.2 4'(5')-Chloroacetylaminomethyl-3'(6')-ethylfluorescein diethylether-5 ester: Compound (3)

To a stirred solution of ethylfluorescein ester (Compound (2), 2.67 g) in 50 ml sulfuric acid, chloroacetylacetamide (850 mg) was added slowly over 5 minutes. The reaction mixture was stirred at room temperature overnight.

10 The reaction mixture was poured over crushed ice and the precipitate formed was filtered and washed with cold water. The TLC and mass analysis indicated the formation of mono and bis-chloroacetamido compounds. The products were purified by silica gel column chromatography using 2-15% methanol in methylene chloride. The bis-alkylated product was eluted first  
15 followed by two mono-alkylated products. The lower mono-alkylated product was the major product, which was used for further reactions.

1.3 4'(5')-Aminomethyl-3'(6')-ethylfluorescein: Compound (5)

20 Major monoalkylated product (Compound (3), 1g) was hydrolyzed with 6N HCl (15 ml) by heating for 2.5 hr. The product was extracted with CH<sub>2</sub>Cl<sub>2</sub>, washed with water and after drying over anhydrous sodium sulfate, purified by silica gel column chromatography to give 500 mg pure compound. MS ES+: 390.3

25 1.4 4'(5')-Trifluoroacetylaminomethyl-3'(6')-ethylfluorescein: Compound (6)

Compound (5) (390 mg) was taken up in anhydrous pyridine (5 ml) and TFA-NHS (650 mg) was added. The mixture was stirred overnight at room  
30 temperature. After concentration *in vacuo* and extraction with methylene chloride, the product was purified by silica gel chromatography to give 350 mg. pure product. MS ES+: 486.3

1.5. 4'-(5')-Trifluoroacetylaminomethyl-3'(6')-ethylfluorescein-6'(3')-phosphate: Compound (7)

To a solution of Compound (6) (200 mg) in acetonitrile (5 ml),  $\text{POCl}_3$  (189 mg, 3 eq) was added and the reaction mixture stirred at room temperature for 1 hour. Pyridine (290  $\mu\text{l}$ , 9 eq) was added and the reaction stirred for a further 2 hr. The reaction was quenched by adding TEAB (0.1 M, 10 ml). After 1 hr, the mixture was concentrated *in vacuo* and purified by silica gel chromatography using  $\text{CH}_2\text{Cl}_2$ /methanol gradient to give 130 mg pure monophosphate. MS ES $^-$ : 564.5

1.6 4'-(5')-Aminomethyl-3'(6')-ethylfluorescein-6'(3')- tetraphosphate-thymidine: Compound (8)

15 1.6.1 Synthesis of the imidazolide derivative of Compound (7)

To a solution of Compound (7) (60 mg) in anhydrous DMF (2 ml) was added tributylamine (30  $\mu\text{l}$ , 3 eq), and the mixture was concentrated to dryness *in vacuo*. The mixture was redissolved in anhydrous DMF (2 ml) and 20 carboonyliimidazole (85 mg, 5 eq) was added with stirring. The mixture was stirred at room temperature for 2 hr and checked for completion by HPLC-MS. The mixture was quenched with methanol (100  $\mu\text{l}$ ), stirred at room temperature for 1 hr, concentrated to dryness *in vacuo* and redissolved in anhydrous DMF (2 ml). MS ES $^-$ : 614.6

25

1.6.2 Addition of imidazolide derivative of Compound (7) to thymidine-5'-triphosphate

30 Thymidine-5'-triphosphate (triethylammonium salt, 100  $\mu\text{mol}$ ) was co-evaporated with tributylamine (400  $\mu\text{mol}$ ) in anhydrous DMF (2x2 ml) and finally redissolved in anhydrous DMF (2 ml).

To the above tributylammonium salt solution of thymidine-5'-triphosphate (TTP), imidazolide derivative (from step 1.6.1) was added and

the mixture stirred at room temperature for overnight. HPLC-MS indicated >90% complete reaction. This was further stirred for another 5 hr.

1.6.3 The reaction mixture from step 1.6.2 was concentrated and NH<sub>4</sub>OH

5 (30% solution, 10 ml) was added

The mixture was stirred at room temperature for 2.5 hr and left in a refrigerator overnight. The mixture was concentrated to dryness *in vacuo* and purified by anion exchange chromatography. This mixture was treated with  
10 snake venom phosphodiesterase (SVD) and alkaline phosphatase overnight (to remove any unreacted TTP) and purified further by reverse phase chromatography using 0.1 M TEAB (buffer A) to 25% acetonitrile in 0.1 M TEAB. Two major peaks were collected and analyzed by HPLC-MS. The first major peak gave mass at 931.8 and the second peak gave mass at required  
15 932.8.

It was apparent that the ammonia treatment during the hydrolysis of trifluoroacetyl group also reacted with the spirolactone and resulted in the formation of undesired amido compound. Peak 2 was concentrated to  
20 dryness *in vacuo*, co-evaporated with methanol (2x5 ml) and redissolved in H<sub>2</sub>O (1ml). The concentration was measured by UV. A total of 5.5 µmol pure compound was obtained.

1.7 Addition of Acceptor Dyes to Compound (8): Synthesis of Compounds

25 (V) and (VI)

To two separate reaction vessels of Compound (8) (2.2 µmol, 400 µl of 5.5 mM solution) was added 400 µl of 0.5 M sodium carbonate/sodium bicarbonate buffer (pH 8.5) and in one vessel 5-ROX-NHS ester (5.5 mg in 2  
30 ml DMF) and in another vessel Cy5-NHS ester (5 mg in 2 ml DMF) was added. Both reaction mixtures were stirred at room temperature for overnight and monitored by HPLC-MS for the completion of the reaction. The reaction mixtures were concentrated *in vacuo* and purified first on ion exchange column using 25% acetonitrile in 0.1 M TEAB (buffer A) and 25%

acetonitrile in 1M TEAB (buffer B). The peak containing the product was concentrated *in vacuo* and further repurified by reverse phase HPLC column. The correct fractions were collected and concentrated *in vacuo* and quantified by UV spectroscopy. Compound (V): MS ES<sup>+</sup>: 1449;

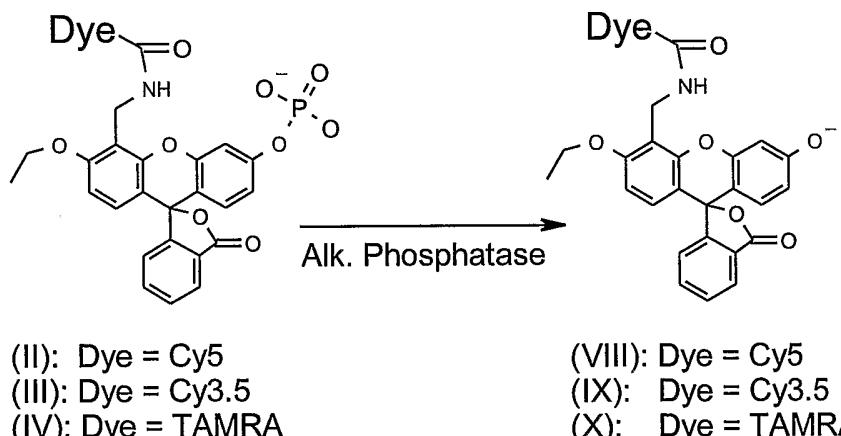
5 Compound (VI): MS ES: 1572

## 2. Synthesis of Compounds (II), (III) and (IV)

The synthesis was carried out as in the manner described reported above for Compounds (V) and (VI), except that the starting material was 4' (5')-aminomethyl-ethoxyfluorescein phosphate (obtained by hydrolysis of Compound (7) with  $K_2CO_3$  in methanol). The products were purified by HPLC and quantified by UV spectroscopy.

15 Compound (II): MS ES<sup>-</sup>: 1107.3;  
 Compound (III): MS ES<sup>-</sup>: 1342.4;  
 Compound (IV): MS ES<sup>-</sup>: 881.

### 3. Action of Alkaline Phosphatase on Compounds (II), (III) and (IV)



20

Compounds II, III and IV were taken up in 25 mM HEPES and 5 mM MgCl<sub>2</sub> buffer (pH 8.5) and treated with alkaline phosphates for 2 hours at room temperature. The reaction was followed spectroscopically, which indicated that the reaction was essentially complete within 30 minutes.

4. UV, Fluorescence and Energy Transfer Measurements on Compounds (II), (III), (IV), (VIII), (IX) and (X)

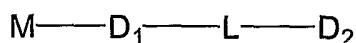
5 The UV and fluorescence spectra of phosphorylated (Compounds (II), (III) and (IV)) and non-phosphorylated dyes (Compounds (VIII), (IX) and (X)) are shown in Figures 2-8. UV spectra clearly show the appearance of peak(s) at about 472 nm upon treatment with alkaline phosphatase.

10 Having described the particular, desired embodiments of the invention herein, it should be appreciated that modifications may be made therethrough without departing from the contemplated scope of the invention. The true scope of the invention is set forth in the claims appended hereto.

What is claimed is:

1. A compound of formula:

5



wherein:

D<sub>1</sub> is a first dye moiety whose fluorescence properties may be modulated so as to be suitable as a donor or an acceptor in an energy transfer arrangement;

D<sub>2</sub> is a second dye moiety suitable as an acceptor or a donor in an energy transfer arrangement with said first dye;

L is a linking group comprises 2-200 linked atoms, wherein said linking group optionally includes an enzyme cleavage site; and

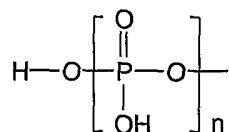
15 M is an enzyme cleavable group chosen to modulate the fluorescence properties of D<sub>1</sub>.

2. A compound according to claim 1 wherein M comprises a substrate for an enzyme.

20

3. A compound according to claim 2 wherein said enzyme is selected from the group consisting of a peptidase, a protease, a phosphatase, a dealkylase and a glycosidase.

25 4. A compound according to claim 1 to 3 wherein M is the group:



wherein n is an integer from 1 to 4.

30

5. A compound according to any of claims 1 to 3 wherein M comprises at least one peptide linkage ( $-\text{CO}-\text{NH}-$ ) covalently bonded to  $\text{D}_1$ .

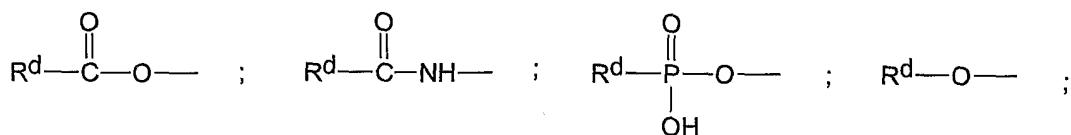
6. A compound according to any of claims 1 to 3 wherein M comprises a glycosidic linkage that is a substrate for a glycosidase.

5

7. A compound according to any of claims 1 to 3 wherein M comprises an ether linkage that is a substrate for a dealkylase.

10 8. A compound according to any of claims 1 to 3 wherein M further comprises a cell membrane permeabilising group.

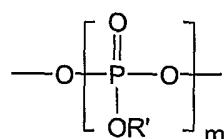
9. A compound according to claim 8 wherein said cell membrane permeabilising group is selected from groups:



wherein  $\text{R}^d$  is  $\text{C}_1 - \text{C}_{10}$  straight or branched chain alkyl, either unsubstituted, or substituted with one or more halogen atoms, phenyl, either unsubstituted or substituted with one or more halogen atoms, or a peptide chain.

20

10. A compound according to any of claims 1 to 9 wherein linking group L is selected from carbon atoms which may optionally include one or more groups selected from  $-\text{C}(\text{O})-$ ,  $-\text{NR}'-$ ,  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{CH}=\text{CH}-$ ,  $-\text{CO}-\text{NH}-$ , phenylenyl and the group:



where  $\text{R}'$  is selected from hydrogen and  $\text{C}_1 - \text{C}_4$  alkyl and  $m$  is an integer from 1 to 3.

11. A compound according to any of claims 1 to 9 wherein linking group L comprises a peptide or an oligonucleotide fragment.
12. A compound according to any of claims 1 to 9 wherein L is a cleavable linker and includes an enzyme cleavable group P which is different from the enzyme substrate group M.  
5
13. A compound according to any of claims 1 to 12 wherein said donor dye is selected from coumarin dyes, benzocoumarin dyes, acridone dyes, xanthine dyes, phenoxazine dyes, rhodamine dyes, merocyanine dyes and cyanine dyes, preferably xanthine dyes and cyanine dyes, wherein 10 said donor dye is capable of transferring energy to said acceptor dye.
14. A compound according to any of claims 1 to 12 wherein said acceptor dye is selected from coumarin dyes, benzocoumarin dyes, acridone dyes, xanthine dyes, phenoxazine dyes, rhodamine dyes, merocyanine dyes and cyanine dyes, wherein said acceptor dye is capable of energy transfer with the donor dye.  
15
- 20 15. A compound according to any of claims 1 to 12 wherein said donor dye is a xanthine dye or a cyanine dye and the acceptor dye is a rhodamine or a cyanine dye.
- 25 16. A compound according to any of claims 1 to 12 wherein at least one of said donor and acceptor dye moiety is a cyanine dye.
17. A compound according to any of claims 1 to 12 wherein said donor dye is a xanthine dye and said acceptor dye is a rhodamine dye.
- 30 18. A compound according to any of the previous claims further comprising water solubilizing constituents attached thereto, said water solubilizing constituents being selected from the group consisting of sulphonate, sulphonic acid, phosphate, phosphonate, quaternary ammonium and hydroxyl.

19. A method for determining the activity of an enzyme acting on a substrate molecule, said substrate comprising a compound of formula (I), wherein D<sub>1</sub>, D<sub>2</sub>, L are hereinbefore defined and M comprises a substrate for a cleavage enzyme, the method comprising the steps of:

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- i) measuring the fluorescence intensity of the fluorescently labelled substrate;
- ii) combining an enzyme whose activity is to be determined with the substrate under conditions to cause cleavage of M from D<sub>1</sub>; and
- iii) measuring a change in fluorescence intensity of the fluorescent label following the combination of step ii);

10

wherein said change in fluorescence intensity of the fluorescent label is used to determine the activity of said enzyme.

15

20. A method according to claim 19 wherein M comprises an enzyme cleavable group selected from a phosphate ester linkage, at least one peptide linkage, an ether linkage and a glycosidic linkage

20

21. A method according to claim 19 wherein said enzyme is a hydrolase enzyme selected from the group consisting of phosphatase, peptidase, protease, dealkylase and glycosidase.

25

22. A method of screening for a test agent whose effect upon the activity of an enzyme in cleaving a substrate is to be determined, said method comprising the steps of:

30

- (a) performing the method according to any of claims 19 to 21 in the presence and in the absence of said agent; and
- (b) determining the activity of the enzyme in the presence and in the absence of said agent;

wherein a difference between the activity of said enzyme in the presence and in the absence of said agent is indicative of the effect of said test agent on the activity of said enzyme.

23. A method of screening for a test agent whose effect upon the activity of an enzyme in cleaving a substrate is to be determined, said method comprising the steps of:

5 (a) performing the method according to any of claims 19 to 21 in the presence of said agent; and

(b) comparing the value of the activity of the enzyme with a control value for the enzyme activity in the absence of the test agent.

10 24. A method according to claim 23 wherein said control value is stored electronically in a database or other electronic format.

15 25. A method of determining relative activities of two enzymes acting on a substrate molecule, said substrate molecule comprising a compound of formula (I), wherein D<sub>1</sub> is a first dye moiety and D<sub>2</sub> is a second dye moiety as hereinbefore defined, M comprises a substrate for a first cleavage enzyme and L contains an enzyme cleavable group P which is substrate for a second cleavage enzyme different from said first cleavage enzyme, the method comprising the steps of:

20 i) measuring the fluorescence emission intensity of the fluorescently labelled substrate;

ii) combining said first and second enzymes with said substrate under conditions to cause cleavage of M from D<sub>1</sub> and D<sub>2</sub> from D<sub>1</sub>;

25 iii) measuring the fluorescence emission intensities of said first and second dye moieties following the combination of step ii); and

iv) utilising a change in fluorescence intensities of said first and second dye moieties to determine the relative activities of said enzymes.

30 26. A method according to claim 25 wherein said first and said second enzymes are selected from the group consisting of phosphatase, peptidase, protease, dealkylase and glycosidase.

27. A method according to claim 25 or claim 26 wherein said combining step ii) is performed in the absence and in the presence of a test agent whose effect on the relative activities of said first and second enzymes is to be determined; wherein a difference between the relative activities of said first and second enzymes in the presence and in the absence of said agent is indicative of the effect of said test agent on the relative activities of said first and second enzymes.

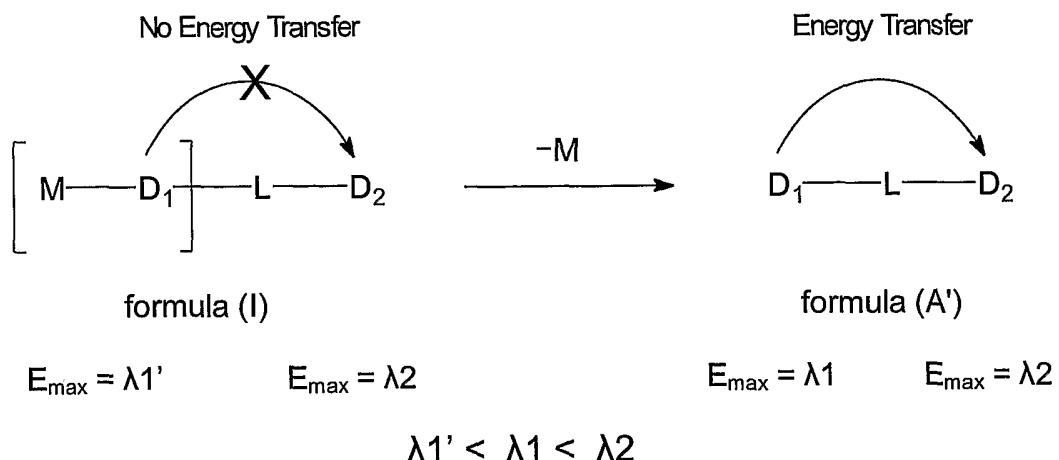
5

28. A test kit for determining the activity of an enzyme, said kit comprising one or more different enzyme substrates, each of said substrates comprising a compound of formula (I), wherein D<sub>1</sub>, D<sub>2</sub>, L are hereinbefore defined and M comprises a substrate for a cleavage enzyme.

10

15 29. A test kit according to claim 28 further comprising one or more different said enzymes each enzyme specific for a different said substrate.

A.



B.

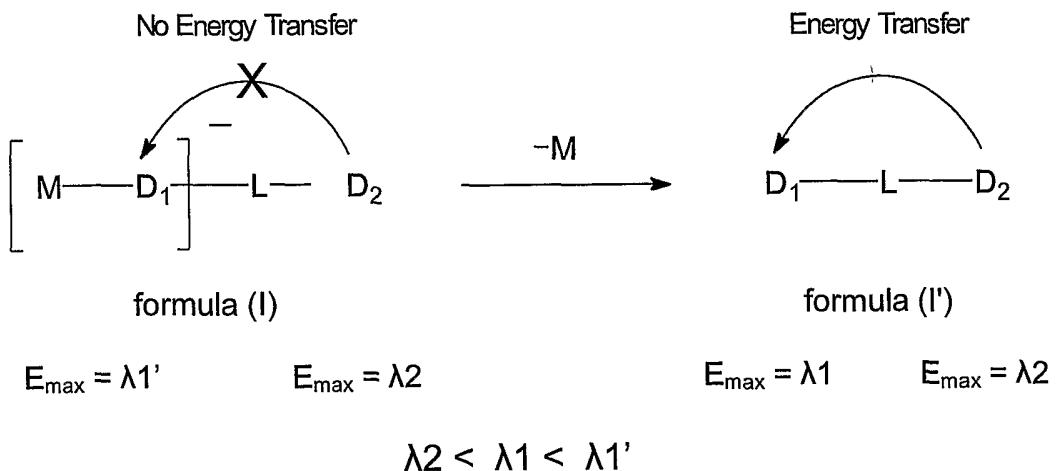


Figure 1

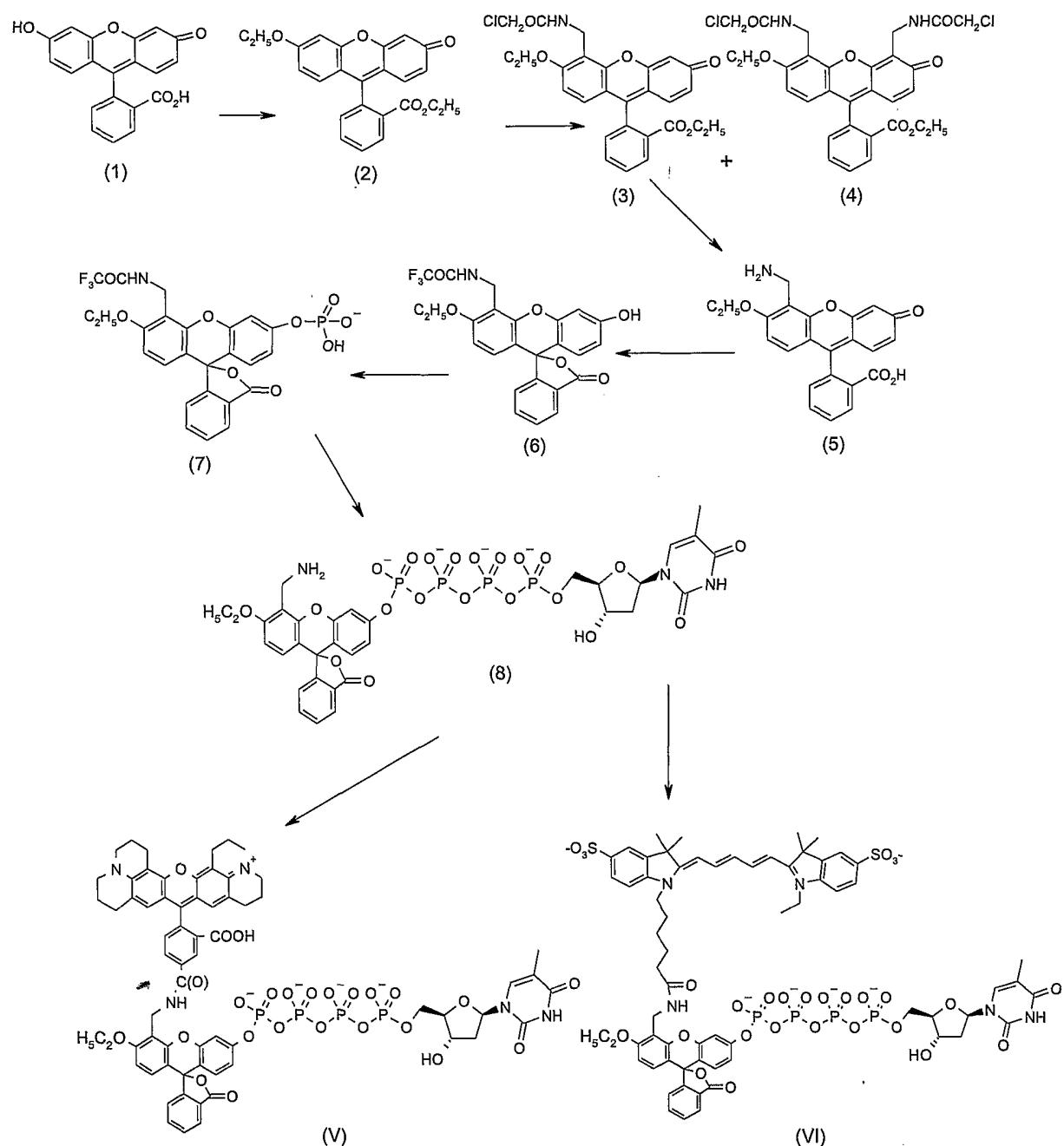
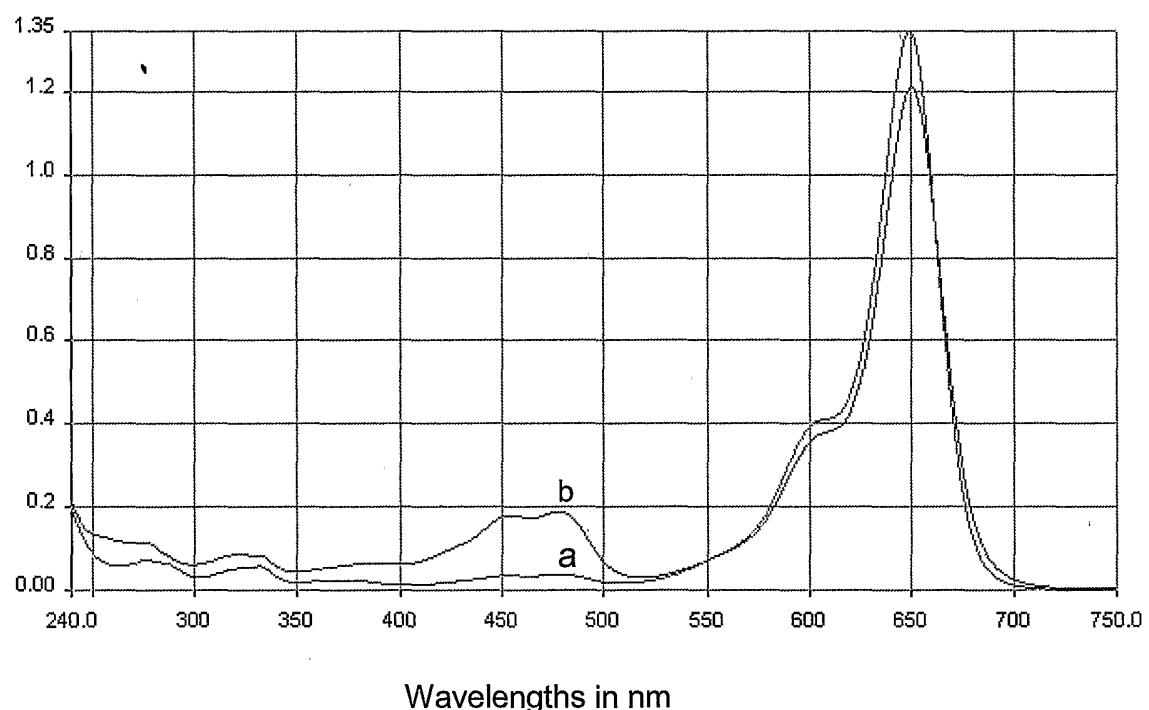


Figure 2

UV spectrum of Compound (II): (a) before and (b) after treatment with alkaline phosphatase



**Figure 3**

4/8

UV spectrum of Compound (III): (a) before and (b)  
after treatment with alkaline phosphatase

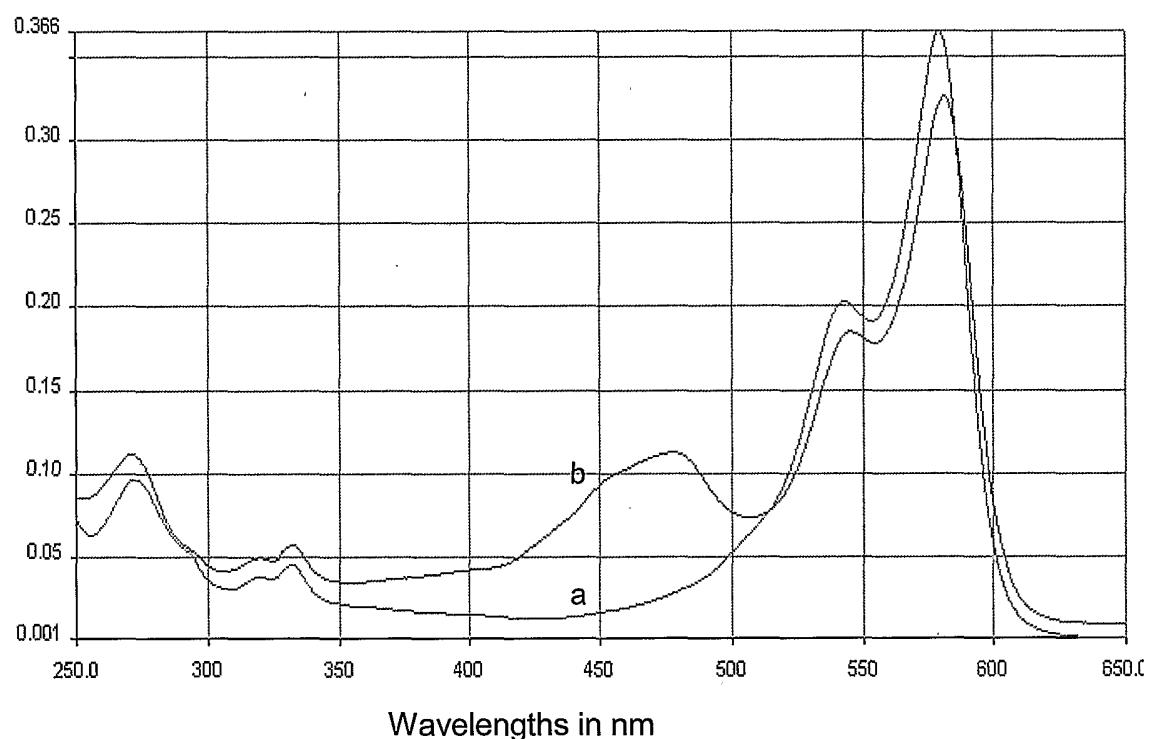


Figure 4

5/8

UV spectrum of Compound (IV): (a) before and (b)  
after treatment with alkaline phosphatase

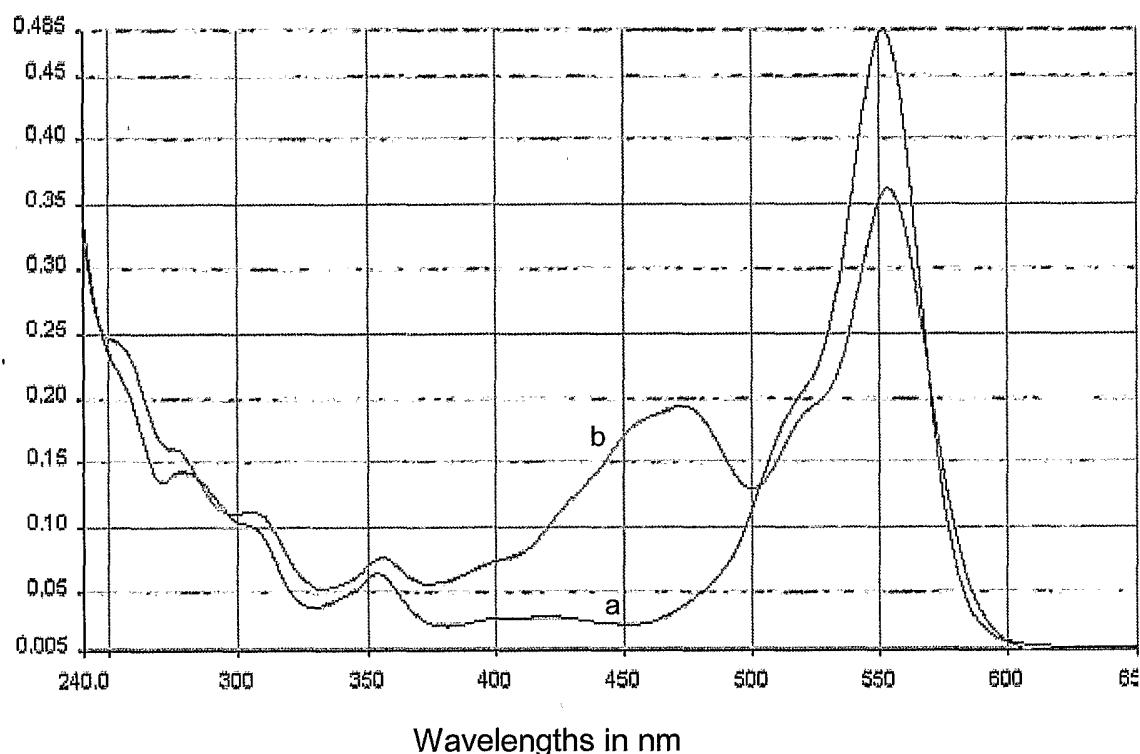
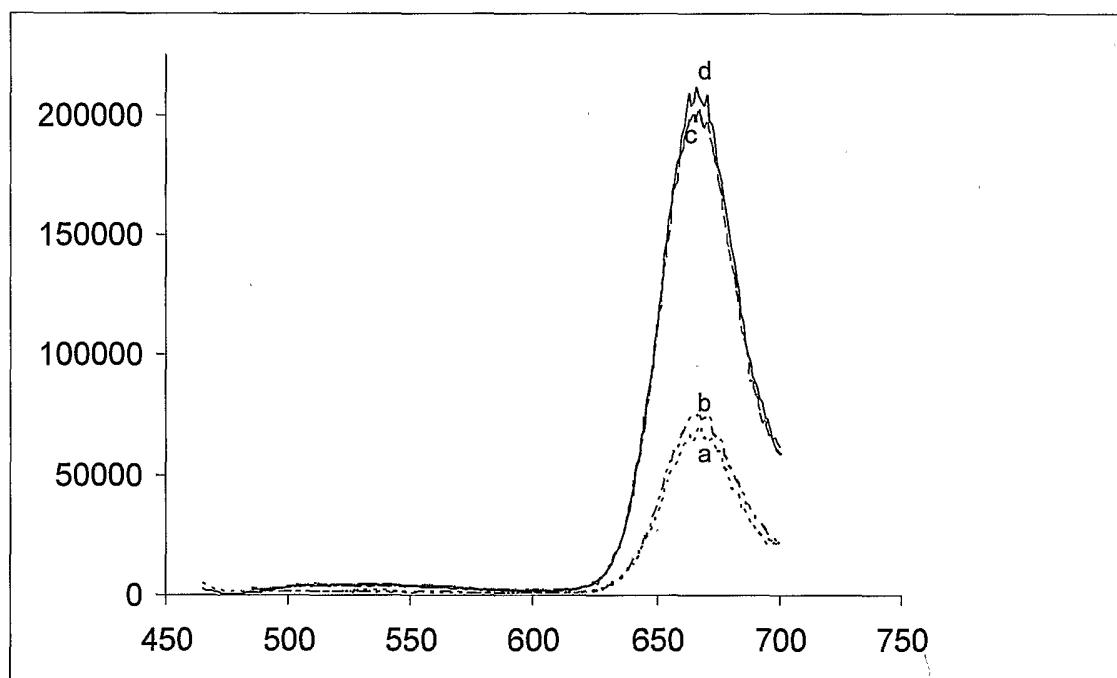


Figure 5

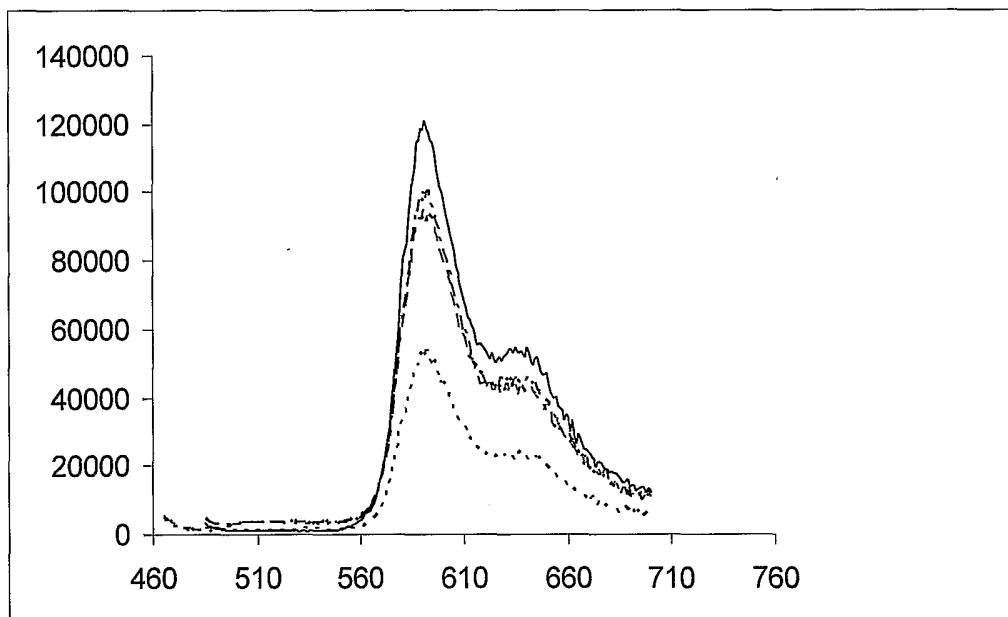
Emission spectrum of Compound (II) before and after treatment with  
Alkaline Phosphatase by excitation at 455 nm and 475 nm



Compound (II): a) Excitation at 455 nm; b) Excitation at 475 nm;  
Compound (VIII): c) Excitation at 455 nm; d) Excitation at 475 nm.

**Figure 6**

Emission spectrum of Compound (III) before and after treatment with  
Alkaline Phosphatase by excitation at 455 nm and 475 nm

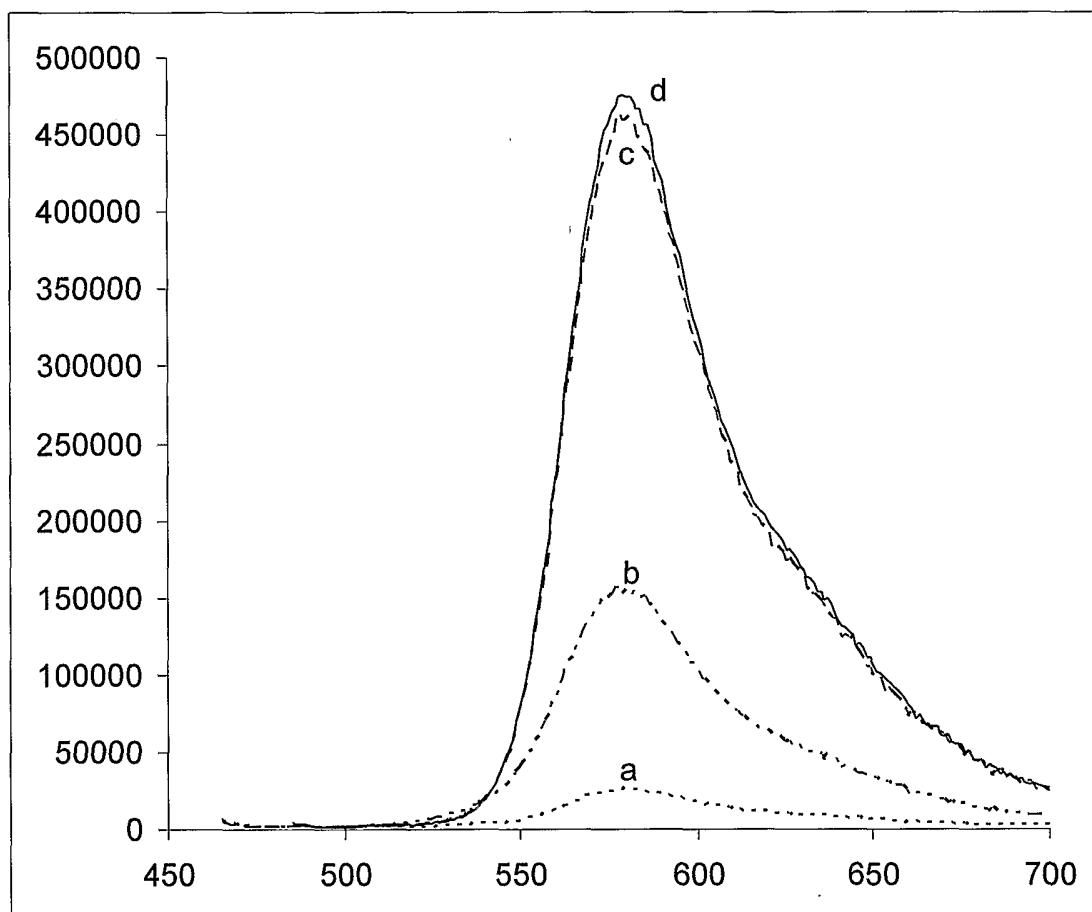


Compound (III): a) Excitation at 455 nm; b) Excitation at 475 nm;  
Compound (IX): c) Excitation at 455 nm; d) Excitation at 475 nm.

**Figure 7**

8/8

Emission spectrum of Compound (IV) before and after treatment with Alkaline Phosphatase by excitation at 455 nm and 475 nm.



Compound (IV): a) Excitation at 455 nm; b) Excitation at 475 nm;  
Compound (X): c) Excitation at 455 nm; d) Excitation at 475 nm.

Figure 8

# INTERNATIONAL SEARCH REPORT

PCT/US2005/013141

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/542 C12Q1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 795 729 A (LEE ET AL) 18 August 1998 (1998-08-18)  column 3, lines 26-43,53-67 column 4, lines 1-18 column 5, lines 5-10,34-44 column 6, lines 10-30,56-67 column 7, lines 1-63  Y claims 1-4,18,21-26; figure 1 -----	1-11, 13-24, 28,29
Y	US 2003/186348 A1 (THOMAS NICHOLAS ET AL) 2 October 2003 (2003-10-02) paragraphs '0088! - '0101!; claims 23-26; example 8; compound V -----	1-29
		-/--

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search  20 September 2005	Date of mailing of the international search report  10/10/2005
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Weber, P

## INTERNATIONAL SEARCH REPORT

PCT/US2005/013141

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WAHLER D ET AL: "High-throughput screening for biocatalysts." CURRENT OPINION IN BIOTECHNOLOGY. DEC 2001, vol. 12, no. 6, December 2001 (2001-12), pages 535-544, XP002345702 ISSN: 0958-1669 the whole document -----	1-29
A	GERSHKOVICH ALEXANDER A ET AL: "Fluorogenic substrates for proteases based on intramolecular fluorescence energy transfer (IFETS)" JOURNAL OF BIOCHEMICAL AND BIOPHYSICAL METHODS, vol. 33, no. 3, 1996, pages 135-162, XP002345703 ISSN: 0165-022X the whole document -----	1-29

**INTERNATIONAL SEARCH REPORT**

PCT/US2005/013141

**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: **19-27 (partially)** because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 19-27 relate at least partially to a diagnostic method practised on the human/animal body. The search has been carried out in as far as ex vivo methods are concerned.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest** The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/US2005/013141

Patent document cited in search report	Publication date		Patent family member(s)		Publication date
US 5795729	A 18-08-1998	AU WO	2254097 A 9728277 A1		22-08-1997 07-08-1997
US 2003186348	A1 02-10-2003	AU AU CA EP WO JP	782381 B2 3037601 A 2399368 A1 1252520 A2 0157237 A2 2003522333 T		21-07-2005 14-08-2001 09-08-2001 30-10-2002 09-08-2001 22-07-2003