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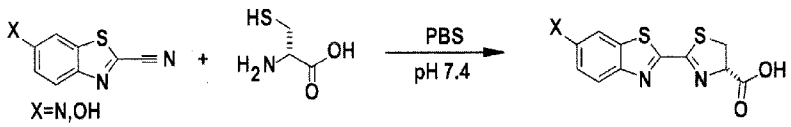
(54) Title: PRECURSOR MOLECULE FOR THE SYNTHESIS OF D-LUCIFERIN

Fig. 1:



(57) Abstract: The present invention relates to precursor molecules for the synthesis of D-luciferin, or blocked D-luciferin and derivatives thereof, which are functionalized in different positions. Further, the use of functionalized precursor molecules for the synthesis of D-luciferin or blocked D-luciferin and methods of synthesising blocked D-luciferin and D-luciferin or derivatives thereof are described. Also described is a kit of parts for screening assay comprising said functionalized precursor molecules to D-luciferin or blocked D-luciferin and derivatives thereof and the uses of such a screening assay for the detection of molecules for the study of molecular uptake and for the detection of a reducing environment. Methods of detection of bio- molecules such as metabolites, activity of enzymes and proteases and methods for determining a sequence within a peptide of protein cleaved by specific enzymes are described. Also a method of determining the cysteine and derivatives thereof concentration in a cell or tissue sample is described. Precursor molecules of D-luciferin according to the invention are advantageous as they are small and travel easily around live systems and allow tissue cells or organs to be targeted specifically. Further, the methods and screening assays described allow the simultaneous study of multiple biological processes described above in one experiment only.

PRECURSOR MOLECULE FOR THE SYNTHESIS OF D-LUCIFERIN

The present invention relates to functionalized precursor molecules for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blocked amino-D-luciferin and derivatives of thereof. Further, the use of functionalized precursor molecules for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blocked amino-D-luciferin and methods of synthesising D-luciferin, amino-D-luciferin, blocked D-luciferin or derivatives of thereofand blocked amino-D-luciferin are described. Also described is a kit of parts for a screening assay comprising said functionalized precursor molecules to D-luciferin, amino-D-luciferin, blocked D-luciferin, blocked amino-D-luciferin or derivatives of thereof and the uses of such a screening assay for the detection of molecules, for the study of cellular uptake of molecules,
for the detection of a reducing hypoxic environment and for the determination of peptide or protein sequences cleaved by a specific enzyme. Also a method of determining the cysteine concentration in a cell or tissue sample is described.

Enzymes play a critical role in drug discovery as therapeutic targets besides being indispensable components in bioanalytical applications such as ELISA, PCR and reporter gene assays. Genetic reporter constructs have contributed greatly to the study of gene expression and regulation and played a significant role in numerous applications both in vitro and in vivo. For example in high throughput screening both in biochemical and cell based assays novel approaches to interrogate Kinases, GPCRs, HDACs, PDEs, CYPs, and Proteases have been devised where there is readout of target activation or frequently inhibition either directly or indirectly by enzymatic assays. Reporters are usually assayed by endogenous characteristics such as

fluorescence when they happen to be engineered fluorescent proteins but frequently enzymes are preferred as they not only afford greater sensitivity by virtue of substrate turnover with time but also depending on the substrate can be assayed by absorbance spectrometry, fluorescence and luminescence. A potential limitation of enzymatic assays is endogenous activity but frequently in absorbance based methods it is the substrate instability that cause background signal and limit assay sensitivity with respect to dynamic range from Lambert Beer law that only has a two log window. In fluorescence and luminescence based readouts photon production is realized from excited state species that are created by virtue of light excitation or exothermic chemical reactions, respectively. Fluorescence based assays in principle are brighter as the exciting photons can be pumped at a much faster rate and their power can be varied in contrast to luminescence where the chemically excited state is generated at a much slower rate and it decomposes to yield photons at a much slower rate and with less energy. Fluorescence assays have much higher backgrounds as the detector needs to distinguish between the high influx higher energy (lower wavelength) of excitation photons versus less of the lower energy emitted photons. Typically the discrimination of excitation and emission is accomplished by optical filtration and geometry of the detectors as the excitation and emitting photons are oriented perpendicular to one another. Although optical filters are not perfect there are several analytical fluorophores, quantum dots and various fluorescent proteins that enable multiplexing whereby several of these can be interrogated by virtue of their differences in fluorescence properties i.e. excitation and emitting photons. Luminescence has the inherent advantage that since photons are not required to create the excited state, there is very low background and special optical arrangement are not necessary. The resulting low backgrounds not

only enable precise measurements at much smaller changes in emitted photons from the excited state but also yield a dynamic range comparable with radioactivity without the inherent problems of danger, environmental hazards and regulatory hazards plus the economics of disposal. Luminescence based methods have thus become the predominant assay readout both in vitro and in vivo. Luminescent substrates for several different enzymes have thus supplanted the colorimetric and fluorimetric substrates, for example luminol is the preferred substrate for horse radish peroxidase in ELISA and western blot assays requiring higher sensitivity. Dioxetane- based derivatives are well-known substrates for b-galactosidase, alkaline phosphatase and other hydrolytic enzymes, which utilize the chemically excited species to release photons post hydrolysis. Since the photons emitted from these chemiluminescent species are of lower wavelength, appropriate energy acceptors are used to excite fluorophores to shift the wavelength higher in order to overcome compound interference during high throughput screening.

Multiple enzymes need to be assayed when one is a target, under interrogation or endogenous and another is a reporter (Martin et al., "Dual Luminescence-Based Reporter Gene Assay for Luciferase and β-galactosidase," BioTechniques, 1996; vol. 21, No. 3:520-524; O'Connor et al., "Quantitation of Two Histochemical Markers in the Same Extract Using Chemiluminescent Substrates," Biotechniques, 1994; vol. 17, No. 3:502-509; Bronstein et al., "Combined Luminescent Assays for Multiple Enzymes," Bioluminescence and Chemiluminescence. Molecular Reporting with Photons (Hastings, et al., eds) Chichester:Wiley, 1997, 451-457).

US 6586196 describes an assay for measuring the activity of multiple enzymes in a single aliquot of a sample comprising quantifying the activity of first enzyme by measuring the light

signal produced by the degradation of the first enzyme substrate specific for first enzyme and repeating steps for each subsequent enzyme present in the aliquot that is to be measured. Reporter enzymes are typically luciferases specially firefly, renilla and cypridinia as they do not occur endogenously invivo. Target or endogenous enzymes are proteases like caspases and matrix metalloproteinases, kinases like serine/threonine and receptor tyrosine kinases, phosphatases, cytochrome P450s, phosphodiesterases and epigenetic histone modifiers like HDACs, HMTs and oxidoreductases like NADH/LDH, peroxidases and nitroreductases. US 6602657, US 6602658 and US 2012/0102581 A1, which interrogate multiple enzymes, teach altering the activity of a first enzyme prior to quantifying the activity of a second enzyme by a myriad of methods that involve altering the pH of a reaction mixture, heating the aliquot, inactivatinga first enzyme by adding an inhibitor and waiting for the first enzyme reaction to reach completion or waiting until the substrate is degraded. US 7951550 and US 7741067 describe adding accelerators and enhancers in order to modulate light output.

US 7582417 provides asequential reporter enzyme luminescence (SRL) method where, the activity of a reporter enzyme ß-galactosidase is evaluated using a secondary reporter system using firefly luciferase that uses luciferin produced by the hydrolysis of galactoside-functionalized luciferin. The functionalized galactoside-luciferin derivative is not a substrate of luciferase but is converted to such a substrate post hydrolysis. The enzyme luciferase, most notably from firefly and beetle that use D-luciferin as a substrate, have revolutionized the field of biotechnology by enabling preclinical imaging of small animals, through advancement of luciferase expression in individual cells, promoter-luciferase gene constructs and fusion gene products that enable elucidation

of complex biological pathways (LF Greer Luminescence 2002. 17, 43-74 and Luminescence Biotechnology, Instrument and Applications, K van Dyke, C van Dyke K Woodstock, CRC Press 2001).

Detection of light from transgenic animals carrying a gene encoding a light-generating protein is a powerful tool in diagnostics, drug discovery and medicine that allows for the identification of disease pathways, determination of mechanisms of action, evaluation of efficacy of drug compounds, and monitoring lead candidates' effects on disease progression in living animals and is described in US 7449615, US 7255851,US 7198774,US 6939533,US 6923951,US 6916462,US 6908605,US 6890515,US 6649143,US 6217847 and US 5650135.

In the case of bioluminescent proteins a substrate is typically administered to the animal prior to the evaluation. For example, luciferase, e.g. encoded by eukaryotic luc gene, catalyses the oxidation of D-luciferin in the presence of ATP, O2 and Mg^{+2} to generate light signals. The availability of the substrate has been shown to effect photon emission efficiency (Lee et al. (2003) Nuclear Medicine Communications 24: 1003-1009; Berger et al. (2008) Eur. J. Nuclear Medicine and Mol. Imaging 35(12):2275-2285).

Various derivatives of luciferin have been prepared, including preparations in which luciferin is covalently bonded to a targeting moiety as described by US 4665022 or a fluorescent label (5-fluoroluciferin available from Promega) as well as 6-substituted D-luciferin esters for use evaluation of pesticides as described by US 5374534. Despite the wide-spread use of bioluminescent imaging techniques, there remains a need for improved methods for detecting, quantifying and validating bioluminescence in living animals.

Several synthetic routes and numerous patents in the last 40 years since the seminal work by Emil White (The Chemi- and Bioluminescence of firefly Luciferin , Bioorganic Chemistry, 1, 92-122, 1971) have appeared describing the mechanisms and substrate requirements of various luciferins and functionalized luciferins (G Meroni etal, ARKIVOC, 1, 265-286, 2009). US 2011/0213124 describes methods for the synthesis of D-amino-luciferins from functionalized precursor protected 6-amino-benzothaizoles and cysteines. US 8216550 describes multiple luciferin derivatives substituted on the benzothiazole moiety to yield a plethora of derivatives.

US 2008/0003627 describes the synthesis of the L-luciferin from 2-cyano-6-hydroxy-benzothiazole and L-cysteine, the naturally occurring amino acid. However L-luciferin is not a substrate for the luminescent beetle luciferase (e.g. firefly luciferase, railroad worm (*Phrixothrix hirtus*) luciferase, *Pynophorus notilucus* luciferase and *Rhagophthalmus ohbai* luciferase) and so it had to be racemized through an esterase to yield the correct isomer D-luciferin.

There is a need to synthesize D- luciferin invivo that has the right functionality and stereochemistry to be a substrate for the above mentioned luciferase through the condensation of substituted benzothiazoles and or substituted cysteine derivatives. These functionalized precursors must directly yield a D-luciferin or aminoluciferin molecule to retain its function as a substrate for luciferase. Many enzymes of interest that are the target or under interrogation do not recognize luciferin derivatives modified to include the appropriate substrate for a variety of reasons, including the size of the derivative and a lack of interaction or activity with respect to modification at the carboxyl group of luciferin or the benzothiazole moiety. Further, certain cell-based assays may be limited due to low permeability of luciferin derivatives and instability of

luciferin derivatives.

WO/2010/030343 A1 describes functionalized derivatives of the benzothiazole moiety that have been used in vitro to yield D-luciferin derivatives, however, there is a need to assemble such derivatives in vivo for multiplexing with other luciferases as physiological conditions are a hindrance to manipulating the enzymatic activities of several enzymes through pH, heat, accelerators and inhibitors.

D-luciferin is a molecule that is oxidized by the enzyme luciferase in the presence of adenosine triphosphate (ATP), oxygen and a magnesium cation source, giving rise to luminescence in this reaction. Amino-D-luciferin also undergoes the same reaction. Thus the presence of oxygen, magnesium cations, ATP, luciferase and D-luciferin or amino-D-luciferin is indicated by a luminescent signal. If the functional groups of D-luciferin or amino-D-luciferin are blocked (for example by enzymatically cleavable groups), oxidation of D-luciferin or amino-D-luciferin cannot be catalyzed by the enzyme luciferase, i.e. no luminescent signal occurs.

The terms functionalized, substituted, protected, masked and blocked are used interchangeably and refer to functionalities that prevent the luciferin derivative from being a substrate of the reporter luciferase enzyme.

By functionalized D-luciferin or amino-D-luciferin herein and below the derivatives of both D-luciferin and amino-D-luciferin functionalized at any possible position of the molecule should be understood. By functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole herein and below the derivatives of 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole functionalized at any possible position of the molecule should be understood.

Known methods of utilizing the reaction between luciferase and D-luciferin or amino-D-luciferin involve the synthesis of blocked D-luciferin or blocked amino-D-luciferin prior to addition to the system to be studied. In terms of the usage of blocked D-luciferin or blocked amino-D-luciferin in the prior art, usually only one biological process, i.e. the activity of one biomolecule, is studied at a time.

Investigation of such a process occurs by reaction of the molecule studied with the help of the blocked D-luciferin or blocked amino-D-luciferin in such a way that the group or the groups blocking the OH, NH_2 or carboxyl functionalities within blocked D-luciferin or blocked amino-D-luciferin is removed to yield unfunctionalized D-luciferin or amino-D-luciferin. Unfunctionalized D-luciferin or amino-D-luciferin can then undergo a reaction with luciferase. The luminescent signal observed indicates that the molecule under investigation is in fact present in the system studied. Said molecule or a biological process associated to it removes the groups blocking the functional groups of D-luciferin or amino-D-luciferin, which originally prevented a reaction between D-luciferin or amino-D-luciferin and luciferase.

It is the object of the present invention to overcome the drawbacks of the prior art. In particular the present invention aims to provide small precursor molecules that travel easily around live systems due to their small size, allow certain tissue cells or organs to be targeted specifically and only react to form D-luciferin, amino-D-luciferin, blocked D-luciferin, blocked amino-D-luciferin, derivatives thereof or other conjugates with biological or radioactive or fluorescent reporting activity once they have been added to the cell or tissue sample or organism to be studied. Another object is the

simultaneous study of two or more biological processes in one experiment only.

It has surprisingly been found that this object is achieved by the subject matter of present claim 1. Claim 1 describes a precursor molecule for the synthesis of D-luciferin or amino-D-luciferin or blocked D-luciferin or blocked amino-D-luciferin, chosen from the group consisting of

a) functionalized D-cysteine or derivatives thereof, wherein the functionalization does not prevent the reactivity towards 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof in vitro and in vivo; and wherein when reacting with a molecule according to b) or when reacting within unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof in vitro and in vivo a fuctionalized D-luciferin or a functionalized amino-D-luciferin or derivatives thereof is obtained, which is not readily reactive towards luciferase;

b) functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole, wherein the functionalization does not prevent the reactivity in vitro and in vivo towards D-cysteine or derivatives thereof or other functionalized or unfunctionalized aminothiols, or functionalized or unfunctionalized 2-aminobenzylamines; and wherein, when reacting with a molecule according to a) or when reacting within unfunctionalized D-cysteine or derivatives thereof, a functionalized D-luciferin or a functionalized amino-D-luciferin molecule is obtained, which is not readily reactive towards luciferase;

c) functionalized D-cysteine or derivatives thereof, wherein the functionalization prevents the reactivity towards6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof in vitro and in vivo;

d) functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole, wherein the functionalization prevents the reactivity towards D-cysteine or derivatives thereof, or other functionalized or unfunctionalized aminothiols or functionalized or unfunctionalized 2-aminobenzylamines.

A common synthesis route of unfunctionalized D-luciferin or amino-D-luciferin, which is oxidized by the enzyme luciferase in the presence of ATP, oxygen and magnesium cations to yield light in a bioluminescent reaction, involves as precursors an unfunctionalized 2-cyano-6-hydroxybenzothiazole (OH-CBT) or an unfunctionalized 2-cyano-6-aminobenzothiazole (NH_2-CBT) and an unfunctionalized D-cysteine (D-Cys) as shown in Fig. 1 and 2, hereinbelow.

Instead of synthesizing D-luciferin or amino-D-luciferin using the unfunctionalized precursors depictedin Fig. 1 and 2 hereinbelow, there are a number of conceivable other synthesis routes that can be used to prepare functionalized or unfunctionalized D-luciferin or amino-D-luciferin molecules in vivo or in vitro using several combinations of functionalized and unfunctionalized precursor molecules, i.e. functionalized or unfunctionalized D-cysteine and functionalized or unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or functionalized or derivatives thereof or unfunctionalized aminothiols other than D-cysteine or derivatives thereof or functionalized or unfunctionalized 2-

aminobenzylamines or derivatives thereof, examples of which are depicted in Fig.3 A to C and Fig. 4 A and B.

While the term functionalization with regard to the D-luciferin or amino-D-luciferin molecule means that the molecule can be functionalized at any possible position available within the molecule, it particularly means that the molecule does not possess a free NH_2- or OH- group in the 6-position of its phenyl ring and/or no free carboxyl-group on the 5-membered ring of the molecule that is derived from cysteine or derivatives thereof. Rather, these groups are blocked or masked, i.e. rendered unreactive, by reacting with compounds such as chloroformates, carboxylic acids, organic acid anhydrides, alkylating agents for hydroxyl- or amino groups of luciferin and alcohols and amines in case of carboxylic groups of luciferin to yield groups such as esters, ethers and amides.Alternatively, blocking of these groups can be achieved by adding onto them a sequence of amino acids which may be part of a peptide or protein. Such blocking or masking is reversible under certain conditions, for instance when biomolecules such as specific enzymes are present that are capable of removing the functionalities that block the groups attached to D-luciferin or amino-D-luciferin. A D-luciferin or amino-D-luciferin molecule functionalized in this way is termed “blocked” or “caged” or “functionalized” D-luciferin or amino-D-luciferin.These terms can be used interchangeably. A blocked D-luciferin or amino-D-luciferin cannot readily react with the enzyme luciferase to undergo a bioluminescent reaction by oxidation that emits light, which can then be detected and measured.

The precursors for the synthesis of blocked derivatives of D-luciferin or amino-D-luciferin or derivatives thereof according to the invention are: D-cysteine, L-cysteine or derivatives

thereof possessing a blocked carboxyl-group, D-cysteine L-cysteine or derivatives thereof possessing a blocked carboxyl-group as well as a blocked thiol group and/or as well as a blocked amino-group, 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof possessing a blocked amino or hydroxyl-group, 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof possessing a blocked amino or hydroxyl-group as well as a blocked nitrile-group.In order to obtain a blocked D-luciferin or a blocked amino-D-luciferin molecule, the specific D-cysteine and cyanobenzothiazole or derivatives thereof listed above may or may not be used with each other in the same reaction.

As the structure of the enzyme luciferase (e.g. E. Conti, N. P. Franks and P. Brick, Cyrstal Structure of Firefly Luciferasethrows Light on a Superfamily of adenylate-forming Enzymes, Structure, 1996, Vol 4, 3, 287-298)) as well as techniques such as molecular modelling and active site access analysis are well known, it is possible to predict and determinewhich groups will block functionalities and thus prevent formation of D-luciferin derivatives or D-amino-luciferin derivatives. Further, these techniques are able to indicate if a D-luciferin derivative or D-amino-luciferin derivative is capable of accessing the enzyme luciferase's active site.

Alternatively, either one of the blocked D-cysteine, L-cysteine or derivatives thereof molecules listed or the blocked 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole and derivatives thereof listed can be used and added to the respective unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof or unfunctionalized D-cysteine molecule or derivatives thereof.

The precursors for the synthesis of D-luciferin or amino-D-luciferin according to the invention are: D-cysteine, L-cysteine or derivatives thereof possessing a blocked thiol-group and/or a blocked amino-group and 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof possessing a blocked nitrile-group.In order to obtain a D-luciferin or an amino-D-luciferin molecule, the specific D-cysteine and 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole and derivatives thereof listed above may or may not be used with each other in the same reaction.

Alternatively, a blocked D-cysteine, L-cysteine or derivatives thereof described may be added to an unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof molecule or a blocked 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof may be added to an unfunctionalized D-cysteine or derivatives thereof molecule.

Also encompassed by the present invention is the use of a functionalized D-cysteine or derivatives thereof according to compounds a) or c) as described above or a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) as described above for the synthesis of D-luciferin, amino-D-luciferin, a blocked D-luciferin or a blocked amino-D-luciferin.

For example, blocking of OH or NH_2 in the 6-position of cyanobenzothiazoles can include but is not limited to acyl-linked triphenylphosphines, fatty acid chains, monosaccharides or peptides. Blocking of the NH_2-group of D-cysteine can include but is not limited to peptide sequences. Blocking the thiol

functionality of the D-cysteine can include but is not limited to disulfide bonds. Blocking of the COOH functionality can include but is not limited to esterification or amide bond formation.

Another aspect of the invention is the method of synthesizing blocked D-luciferin or blocked amino-D-luciferin or derivatives thereof, wherein

i) a functionalized D-cysteine or derivatives thereof according to a) as described for the precursors above is mixed and allowed to react with either a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to b) as described for the precursors above; or with unfunctionalized6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole;

or, wherein

ii) a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to b) as described for the precursors above is mixed and allowed to react with either a functionalized D-cysteine or derivatives thereof according to a) as described for the precursors above; or withunfunctionalized D-cysteine or derivatives thereof.

The functionalized D-cysteine or derivatives thereof of method step i) is a D-cysteine or derivatives thereof whose carboxyl function is blocked and which is not prevented from reacting with a 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-

cyanobenzothiazole or derivatives thereof molecule via its amino and thiol-groups.

The functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof of method step ii. is 6-hydroxy-2-cyanobenzothiazole, 6-amino-2-cyanobenzothiazole or a derivative thereof whose OH or NH_2group is blocked and which is not prevented from reacting with D-cysteine or derivatives thereof via its nitrile group.

In order to obtain blocked D-luciferin or blocked amino-D-luciferin, the functionalized D-cysteine or derivatives thereof of route i) can be added to either a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or to an unfunctionalized6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazoleor derivatives thereof. Blocked D-luciferin or blocked amino_D-luciferin is also synthesized when a blocked 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole of routei i) is added to either a functionalized D-cysteine or to an unfunctionalized D-cysteine or derivatives thereof.

Further, a precursor molecule for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blocked amino-D-luciferin, or derivatives thereof, of the general formula X-Y-Z, wherein X is a moiety of up to 1000 Da, in particular in the range of 100 to 1000 Da; Y is a linker of 6 to 30 atoms, in particular comprising a disulfide moiety; and Z is a 2-cyano benzothiazole or a derivative thereof can be used.
X is preferably selected from the group of carbohydrates and derivatives thereof, in particular monoaccharides and derivatives thereof; peptides; folate; lactate; lipids; triglycerides; enzyme inhibitors.

The invention further encompasses a method of synthesizing functionalized D-luciferin or functionalized amino-D-luciferinin vitro and in vivo comprising the steps of

i) providing either a functionalized D-cysteine or derivatives thereof according to compounds a) or c) as described for the precursors above or a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) as described for the precursors above,

ii) providing the respective complementary unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof or unfunctionalized or functionalizedcysteine or derivatives thereof,

iii) adding the components provided in steps i) and ii) to a system containing one or more biomolecules capable of cleaving functional groups off functionalized cysteine or derivatives thereof and /or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof and allowing them to react.

A functionalized cysteine or derivatives thereof of method step i) is a cysteine molecule that is blocked at its carboxylic group and/or its SH- group and/or its NH_2-group.

A functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to method step ii) is a cyanobenzothiazole, which is blocked at its NH_2 or OH-group and/or at its nitrile group.

Both the blocked functional groups of the functionalized D-cysteine or derivatives thereof and the functionalized 6-hydroxy-2-cyanobenzothiazole or functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to steps i) and ii) must be unblocked prior to formation of blocked D-luciferin or blocked amino-D-luciferin or after such formation in order to yield free D-luciferin or free amino-D-luciferin by one or more suitable molecules, preferably biomolecules, more preferably by enzymes, for subsequent reaction with luciferase and detection of the emitted light.

When the reactions between 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof and D-cysteine or derivatives thereof in vivo or in vitro involve unblocking of the precursors prior to a reaction yielding D-luciferin or amino-D-luciferin, the formation of D-luciferin or amino-D-luciferin takes place only gradually.vCurrently used approaches in vivo use injectinon of already synthesized D-luciferin or amino-D-luciferin, which provides a rapid boost of light emission with the subsequent rapid decay of signal due to the enzyme luciferase inhibition. Alternatively, adrug pump is surgically implanted containing free D-luciferin or free amino-D-luciferin. This procedure is stressful for the animal. Injecting separately blocked D-cysteine or derivatives thereofandblocked 6-hydroxy-2-cyanobenzothiazole or blocked 6-amino-2-cyanobenzothiazole or derivatives thereof will provide gradual formation of D-luciferin or amino-D-luciferin or derivatives thereof over time, thus overcoming the drawback of luciferase inhibition and avoiding the exposure of the animal to a stressful situation.

Another aspect of the invention is akit of parts for a screening assay for the detection of molecules in vivo or in vitro, which comprises

- either a functionalized D-cysteine or derivatives thereof according to compounds a) or c) as described for the precursors above or a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) as described for the precursors above,
- the respective complementary unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or unfunctionalized or functionalized D-cysteine and derivatives thereof,
- optionally, luciferase,
- optionally, ATP,
- optionally, a magnesium cation source, and
- instructions for use.

This kit of parts is characterized in that it contains separately the above mentioned component of a functionalized cysteine or derivatives thereof according to compounds a) or c) as described for the precursors above or a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole according to compounds b) or d) from the second component of a respective complementary functionalized or unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or unfunctionalized or functionalized cysteine and derivatives thereof. Further, if comprised within the kit, luciferase is also kept separately from the previous two kit components. Luciferase is an optional part of the kit, i.e. it may be contained within the kit or it may be expressed by the cells of a sample to be studied.Also, optionally comprised within the kit and kept separately from other kit

components and each other are adenosine triphosphate (ATP) and a magnesium cation source, which may be a magnesium cation solution. The kit also comprises instructions for use explaining how the individual kit components are to be used; moreover, such instruction for use may also prompt the user to obtain further material (or material indicated as "optional" herein) from other sources and/or separately. This definition of "instructions for use" applies wherever it is used herein.

Molecules to be screened by the screening assay kit of parts are preferably biomolecules, more preferably enzymes, which react with blocked groups of the precursors and/or the blocked groups of functionalized D-luciferin or functionalized amino-D-luciferin thereby releasing the respective free precursors or free D-luciferin or free amino-D-luciferin. Typical enzymes are hydrolases such as proteases and peptidases, more specifically caspase, thrombin, trypsin, SARS-protease, cathepsins, kallikrein, aminopeptidase, prostate specific antigen, dipeptidyl peptidase, caplain, beta-galactosidase and phosphatase. Other molecules that could be detected in this way by the screening assay are azides and hydrogen peroxides.

Yet another aspect of the invention are the uses of a kit of parts for a screening assay as described above.

Said kit of parts can be used for the detection of molecules in vivo or in vitro. Detection of one type of molecule can be achieved by adding to the cell or tissue sample or organism to be investigated one precursor blocked in one position, followed by the unfunctionalized corresponding precursorthat is needed to synthesize D-luciferin or amino-D-luciferin. Optionally, i.e. if necessary, luciferase and/or a magnesium cation source and/or ATPare added to the sample studied. If the molecule necessary to

remove the blocking is present, D-luciferin or amino-D-luciferin will form upon removal of the blocking and then react with luciferase to give rise to luminescence. The luminescent signal may vary in intensity depending on the concentrations of the molecule investigated and depending on those of the different kit components.

It is also possible to investigate the presence of several molecules at the same time within the same sample. This is done by adding D-luciferin precursors or amino-D-luciferin precursors to the sample under investigation, which may be blocked in any number of available positions, i.e. in any of five different positions in total. These can then be unblocked by the molecules present and undergo the reaction to form D-luciferin or amino-D-luciferin, followed by the reaction with the enzyme luciferase.

Alternatively, the presence of two or more different molecules at the same time within a cell or tissue sample or the occurrence of two or more biological processes such as reactions at the same time within a cell or tissue sample can be studied by employing the reactions to form functionalized or unfunctionalized D-luciferin or functionalized or unfunctionalized amino-D-luciferin as bio-orthogonal reactions alongside the well-known Staudinger reaction in vitro or in vivo. The term bio-orthogonal reaction describes the reaction between two reagents, which can only react with each other in living systems or cell or tissue samples but not with any other components found in the living system or cell or tissue sample, i.e. the reagents of a bio-orthogonal reaction do not interfere with the reactions and biological processes occurring in the system that is being studied.
Further, the reagents that participate in one bio-orthogonal reaction do not interact with the reagents belonging to another

bio-orthogonal reaction, such as the Staudinger reaction, that occurs simultaneously in a living system or cell or tissue sample.

A kit of parts for a screening assay as described above can further be used for the study of cellular uptake of molecules in vitro or in vivo.For instance, the uptake into a cell, both through endocytosis and receptor-mediated pathways, of a protein having a cysteine side-chain can be shown by first binding a cysteine or derivatives thereof to said protein thus forming a disulfide bond. Said protein is then added to cells pre-treated with 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole. The reductive environment of the cell's cytosol containing glutathione will reduce the disulfide bond, which will release a free cysteine or derivatives thereof. Free cysteine or derivatives thereof and free 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole will then react to yield D-luciferin or amino-D-luciferin and derivatives thereof. If the cells investigated express luciferase or if luciferase is also added to the cell or tissue sample under investigation, D-luciferin or amino-D-luciferin and luciferase will react and give rise to luminescence, which can be detected and measured.

It is also conceivable that the uptake of other proteins having other kind of bonds cleaved by other cytosolic enzymes could be studied in this way.

Further, the use of a kit of parts for a screening assay as described above for the detection of a hypoxic environment in vivo or in vitro is encompassed by the present invention. In particular the presence of hypoxic reducing environments in cells, for instance caused by hypoxic tumours, can be

studied.For instance, disulfide bonds or azo-compounds are known to be reduced into respectively thiols and amines in hypoxic environments. A cysteine or derivatives thereof blocked in both or either of its thiol- or amino-positions by hypoxia-induced removable groups can be added to cell or tissue samples to investigate hypoxia. If hypoxic conditions are present, both will be unblocked and can then react with unblocked 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof also added to sample. If necessary, luciferase and/or a magnesium cation source and/or ATP is also added. If a hypoxic environment is present, cysteine or derivatives thereof and 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof will react to yield D-luciferin or amino-D-luciferin, which will then react with luciferase to give rise to luminescence.

Yet another aspect of the invention is the method of detecting biomolecules comprising the steps of

i) providing either a functionalized cysteine or derivatives thereof according to compounds a) or c) as described for the precursors above or a functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole according to compounds b) or d) as described for the precursors above,

ii) providing the respective complementary unfunctionalized or functionalized cysteine or derivatives thereof or unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof,

iii) providing luciferase,

iv) contacting the components provided in steps i. and ii. and allowing them to react in the presence of one or more biomolecules studied,and

v) detecting and measuring the luminescence signal generated.

The above described method can be carried out in vivo or in vitro. Luciferase is either present in the sample investigated through cellular expression of this enzyme or will be added to the sample.Luminescent signals are detected and measuredin accordance with routine procedures and equipment generally known in the art.

Further, a kit of parts for a screening assay for the determination of a sequence cleaved by a specific enzyme within a peptide or protein comprising

i) the peptide or protein bearing at the C-terminal end the sequence cleaved by the specific enzyme followed by a cysteine residue or derivatives thereof,
ii) providing an unfunctionalized6-hydroxy-2-cyanobenzothiazole or an unfunctionalized 6-amino-2-cyanobenzothiazole,
iii) optionally, an enzyme capable of cleaving a sequence within the peptide or protein,
iv) optionally, luciferase,
v) optionally, magnesium cation source,
vi) optionally, ATP
vii) optionally, a means of detecting luminescence and measuring its intensity, and
viii) instructions for use

is comprised by the present invention.

This kit of parts is characterized in that it contains separately the above mentioned component of a peptide or protein bearing at the C-terminal end the sequence cleaved by the specific enzyme followed by a cysteine residue or derivatives thereof, which could react with the second kit component, i.e. a

free 6-hydroxy-2-cyanobenzothiazole or a free 6-amino-2-cyanobenzothiazole. Optionally and also separately, the kit may contain an enzyme capable of cleaving a sequence within the peptide or protein and a means of detecting luminescence and measuring its intensity. Also optionally and separately, luciferase may be contained within the kit. Alternatively, it is possible that luciferase is expressed by cells within a sample to be studied. Optionally, separately, ATP and/or a magnesium cation source may be comprised within the kit. Another component of the kit of parts are instructions for use explaining in what sequence the individual kit components are to be added to tissue or cell samples or organisms.

Another aspect of the invention is the use of a kit of parts for a screening assay for the determination of a sequence within a peptide or protein cleaved by a specific enzyme as described above, whereby the individual kit components are utilized as is described for the method below. By using the kit of parts according to the invention, the best performing peptide sequence for a specific protease can be determined. Peptides with the desired sequence bearing at the C-terminal a cysteine after the cleavage site can be synthesized. Upon protease activity, the cleavage of the peptide will release a free cysteine or derivatives thereof, allowing the formation of luciferin or amino-luciferin if 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof is present in solution. Luciferase will have to be present in the cells investigated or may optionally be added. The resulting reaction between D-luciferin or amino-D-luciferin and luciferase will give rise to luminescence. Intensity of light output will be proportional to the efficiency of peptide cleavage, allowing the determination of the best specific peptide sequence substrate for a particular enzyme.

Also, the method of determining a sequence within a peptide or protein cleaved by a specific enzyme comprising

i) providing a peptide or protein bearing at the C-terminal end the sequence cleaved by the specific enzyme followed by a cysteine residue or derivatives thereof,

ii) providing an unfunctionalized 6-hydroxy-2-cyanobenzothiazole or an unfunctionalized 6-amino-2-cyanobenzothiazole or derivatives thereof,

iii) providing an enzyme capable of cleaving a sequence within the peptide or protein,

iv) mixing the compounds of steps i., ii. and iii. and allowing them to react,

v) providing information on proteolytic cleavage of the peptide

vi) providing luciferase, and

vii) detecting and measuring a luminescence signal

is comprised by the invention.

Further, a method of determining the cysteine or derivatives thereof concentration in a cell or tissue sample comprising

i) lysing a tissue sample and adding a reducing agent to it;

ii) adding a functionalized or an unfunctionalized6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole according or derivatives thereof to b) or d) of the precursors described above;

iii) adding luciferase;

iv) allowing the mixture obtained in step iii) to react;

v) detecting and measuring a luminescence signal;

vi) extrapolating from said signal the cysteine or derivatives thereof concentration;

is encompassed by the present invention.

The above method describes effectively a method of carrying out a new bioorthogonal click reaction forming a covalent bond of two molecules in biological and physiological environments, in vitro and in vivo.

It can be used in concert with the well-known Staudinger ligation. These two reactions are totally independent, which means that neither functionalized nor unfunctionalized 6-hydroxy-2-cyanobenzothiazole nor functionalized or unfunctionalized 6-amino-2-cyanobenzothiazole or derivatives thereof nor functionalized or unfunctionalized cysteine or derivatives thereof can react with the Staudinger ligation reagents, i.e. modified phosphine and azides. This approach can allow the study or more complex systems or biological processes using the possibility of selectively modifying two different kinds of biomolecules at the same time, in vitro and in vivo (see Fig. 40).

Suitable reducing agents according to step i) are glutathione, thioredoxin, tris(2-carboxyethl)phosphine and dithiothreitol.The reducing agent serves to cleave disulfide bonds present in cell or tissue samples. Any cysteine or derivatives thereof that are potentially present will be set free. In steps ii. and iii. of the above method, functionalized or unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof and luciferase are added.6-Hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof reacts with all forms of free cysteine such as L-, D- and N-terminal cysteine or other derivatives thereof. A luminescent substrate is only obtained from the reaction with D-cysteine to give D-luciferin or amino-D-luciferin, which can then react with luciferase.Provided blocked 6-hydroxy-2-cyanobenzothiazole or blocked 6-amino-2-cyanobenzothiazole or

derivatives thereof is used in step ii, biomolecules capable of unblocking these precursors will need to be present in the sample in order to obtain D-luciferin or amino-D-luciferin or corresponding derivatives thereof.
The concentration of cysteine or its derivatives can then be extrapolated from the luminescent signal.

This method of determining a cysteine concentration can specifically be used to determine the presence, concentration, location of bacteria, which express cysteine or derivatives thereof. In this way sepsis or any bacterial infection can be detected. In this way changes at the molecular level within deceased cells, i.e. the activities and concentrations of biomolecules present can be detected and help to develop selective therapeutics.

Another aspect of the invention involves a method for determining the presence of bacteria in food by

i) adding unfunctionalized or functionalized6-hydroxy-2-cyanobenzothiazole or unfunctionalized or functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof and unfunctionalized or functionalized cysteine or derivatives thereof to a sample to be investigated,
ii) optionally, adding luciferase,
iii) optionally, adding a magnesium cation source,
iv) allowing the components according to step i), optionally according to step i) and ii) or according to step i), ii) and iii), to react, and
v) detecting and measuring a luminescence signal.

This method also allows to test food samples for the presence of bacteria, as the reaction between D-luciferin or amino-D-luciferin and luciferase also requires adenosine triphosphate

(ATP), which is normally absent in food unless living bacteria are present. The emitted light detected and measured is proportional to the amount of ATP present in the food. Thus the presence of living bacteria can be tested.

Yet another aspect of the invention is a method amenable to the study of isomerization of L- to D-luciferin, which is catalyzed by luciferase, in order to search for conditions and/or enzymes that can avoid this isomerization.

Also, protein or peptide degradation in cell or tissue samples or in organisms can be studiedby using the method for determining cysteine or derivatives thereof concentration described above. In this case, step i. of the method would be omitted. Rather, any free D-cysteine present will have been released by degradation of the amino acid chains of proteins or peptides within the sample by processes such as autophagy. Addition of 6-hydroxy-2-cyanobezothiazoleor 6-amino-2-cyanobenzothiazole or derivatives thereof in its unfunctionalized form will give rise to D-luciferin or amino-D-luciferin or derivatives thereof synthesis. Following the addition of luciferase, a luminescent signal can be detected and measures allowing the assessment of the degree of protein or peptide degradation occuring within the sample studied.

The bioorthogonal reactions described above could also be applied to fluorescent or radioactive studies of the glycosylation processes in the cell in vitro and in vivo. To this end, 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof functionalized with a sugar moiety should be incubated with thecells or injected into animals. The cell glycan pathway incorporates the sugar derivative of 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-

cyanobenzothiazole or derivatives thereof as a normal sugar into different glycans. Following this, cysteine, an aminothiol or a 2-aminobenzylamine or derivatives thereof functionalized by fluorescent or radioactive labels are incubated with the cells or injected into the animals. Upon bioorthogonal reaction with the cyanobenzothiazole moiety, labelling of glycans with fluorescent or radioactive label is achieved. Thus glycosilation processes in vitro and in vivo can be studied.

Another aspect of the invention involves a method of measuring the cellular uptake of a small molecule, comprising the steps of:

i) Providing cells that contain both cysteine, in particular D-cysteine, or a derivative thereof; and a luciferase;

ii) Subjecting the cells of step i) to molecules according to any one of the precursor molecule for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blockedamino-D-luciferin, or derivatives thereof described above;

iii) Determining the reaction product within the cells of the molecule provided in step ii) and the cysteine or the derivative of cysteine provided in step i), preferably by means of determination of the intensity of luminescence.

In particular, the small molecule whose uptake is measured is glucose.

The invention is described in more detail hereinbelow, by means of illustrative figures and embodiments.

Fig.1 Condensation reaction between unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole and unfunctionalized D-cysteine yielding unfunctionalized D-luciferin or amino-D-luciferin.

Fig.2 Mechanism of the reaction yielding D-luciferin.

Fig.3A Condensation reaction between a functionalized cyanobenzothiazole and a functionalized D-cysteine.

Fig.3B Condensation reaction between a functionalized cyanobenzothiazole and an aminothiol derivative.

Fig.3C Condensation reaction between a functionalized cyanobenzothiazole and a 2-aminobenzylamine.

Fig.3D Cyanobenzothiazoles derivatives containing modification on the atoms of the six-membered and/or five-membered rings, which can be used with the reaction described on Fig. 1 and Fig. 3A to C.

Fig 4A Reaction mechanism between 6-hydroxy-2-cyanobenzothiazole and an aminothiol derivative.

Fig.4B Reaction mechanism between 6-hydroxy-2-cyanobenzothiazole and 2-aminobenzylamine.

Fig.4C Scheme describing the principle of blocked-D-cysteine for enzyme, protease or biomolecule imaging.Blocked D-cysteine (peptide-D-Cys, molecule a)) is composed of an amminoacid (aa) sequence that contains on the C-

terminal a D-cysteine residue. The aa sequence situated in front of the D-cysteine is known to be cleaved-off after reaction with a specific enzyme. Once the free cysteine (molecule b)) released by enzymatic reaction, cyanobenzothiazole(CBT, molecule c)) will selectively react with the D-cysteine forming D-luciferin (molecule d)). OH-CBT will not react with the peptide-D-Cys construct as free 1,2-aminothiol is necessary for the reaction to occur. Light emission proportional to the quantity of D-luciferin formed will be emitted if luciferase is present.

Fig.5 Graph showing the luminescence intensity [RLU] as a function of time [s] for solutions containing different concentrations of thrombin.Curves a) to e) depict results for the following mixtures, wherein CBT stands for cyanobenzothiazole:

a) thrombin (20U) + peptide1 + aminoCBT + luciferase
b) thrombin (10U) + peptide 1 + aminoCBT + luciferase
c) thrombin (5U) + peptide 1 + aminoCBT + luciferase
d) peptide 1 + aminoCBT + luciferase
e) luciferase assay buffer (control)

Fig.6 Diagram of total photon flux for solutions containingdifferent concentrations of thrombin measured over a time period of 2h 4min 48s. Columns 6.1 to 6.5 represent the following samples, wherein CBT stands for cyanobenzothiazole:

6.1 thrombin (20U) + peptide1 + aminoCBT + luciferase
6.2 thrombin (10U) + peptide 1 + aminoCBT + luciferase
6.3 thrombin (5U) + peptide 1 + aminoCBT + luciferase
6.4 peptide 1 + aminoCBT + luciferase

6.5 luciferase assay buffer (control)

Fig.7 Diagram of total photon flux [ph/cm2/sr]for SKOV3-LUC-D3 living cell samples containing various combinations and concentrations of D-luciferin, OH-CBT, NH_2-CBT, D-Cys, L-Cys and NH_2-D-luciferin.Columns7.1 to 7.21 correspond to the following sample solutions, wherein CBT stands for cyanobenzothiazole and Cys stands for cysteine:

7.1 NH_2-CBT (75 µM)
7.2 NH_2-CBT (75 µM), no washing step
7.3 NH_2-CBT (75 µM) + L-Cys (75 µM)
7.4 NH_2-CBT (0.75 µM) + D-Cys (0.75 µM)
7.5 NH_2-CBT (7.5 µM)+ D-Cys (7.5 µM)
7.6 NH_2-CBT (7.5 µM) + D-Cys (15 µM)
7.7 NH_2-CBT (7.5 µM) + D-Cys (37.5 µM)
7.8 NH_2-CBT (7.5 µM) + D-Cys (75 µM)
7.9 NH_2-CBT (75 µM) + D-Cys (75 µM)
7.10 amino-luciferin (7.5 µM)
7.11 OH-CBT (75 µM)
7.12 OH-CBT (75 µM), no washing step
7.13 OH-CBT (75 µM) + L-Cys (75 µM)
7.14 OH-CBT (0.75 µM) + D-Cys (0.75 µM)
7.15 OH-CBT (7.5 µM) +D-Cys (7.5 µM)
7.16 OH-CBT (7.5 µM) +D-Cys (15 µM)
7.17 OH-CBT (7.5 µM) +D-Cys (37.5 µM)
7.18 OH-CBT (7.5 µM) +D-Cys (75 µM)
7.19 OH-CBT (75 µM) +D-Cys (75 µM)
7.20 luciferin (75 µM)
7.21 D-Cys (75 µM)

Fig.8 Diagram of total photon flux [ph/cm2/sr] for SKOV3-LUC-D3 living cell samples containing various combinations

and concentrations of D-luciferin or amino-D-luciferin, D-Cys, L-Cys .Columns8.1 to 8.11 correspond to the following sample solutions, wherein Cys stands for cysteine:

8.1 luciferin (0.75 µM)

8.2 luciferin (7.5 µM)

8.3 luciferin (7.5 µM) + D-Cys (7.5 µM)

8.4 luciferin (75 µM)

8.5 luciferin (75 µM) + D-Cys (75 µM)

8.6 aminoluciferin (0.75 µM)

8.7 aminoluciferin (0.75 µM) + D-Cys (0.75 µM)

8.8 aminoluciferin (7.5 µM)

8.9 aminoluciferin (7.5 µM) + D-Cys (7.5 µM)

8.10 aminoluciferin (75 µM)

8.11 aminoluciferin (75 µM) + D-Cys (75 µM)

Fig.9 Diagram of total photon flux [ph/cm2/sr] for SKOV3-LUC-D3 living cell samples incubated with solutions of different concentrations of D-Cys, NH_2-CBT or OH-CBT for 20 min followed by a washing step with PBS and by addition of solutions of equal concentrations of complementary D-Cys, NH_2-CBT or OH-CBT, respectively.Columns 9.1 to 9.16 correspond to the following sample solutions, wherein CBT corresponds to cyanobenzothiazole and Cys to cysteine:

9.1 D-Cys (7.5 µM) 20 min incubated, washed + NH_2-CBT (7.5 µM)

9.2 NH_2-CBT (7.5 µM) 20 min incubated, washed +D-Cys (7.5 µM)

9.3 D-Cys (75 µM) 20 min incubated, washed + NH_2-CBT (75 µM)

9.4 NH_2-CBT (75 µM) 20 min incubated, washed +D-Cys (75 µM)

9.5 D-Cys (7.5 µM) 20 min incubated, no washing step + NH_2-CBT (7.5 µM)

9.6 NH_2-CBT (7.5 µM) 20 min incubated, no washing step + D-Cys (7.5 µM)

9.7 D-Cys (75 µM) 20 min incubated, no washing step + NH_2-CBT (75 µM)

9.8 NH_2-CBT (75 µM) 20 min incubated, no washing step + D-Cys (75 µM)

9.9 D-Cys (7.5 µM) 20 min incubated, washed + OH-CBT (7.5 µM)

9.10 OH-CBT (7.5 µM) 20 min incubated, washed +D-Cys (7.5 µM)

9.11 D-Cys (75 µM) 20 min incubated,washed +OH-CBT (75 µM)

9.12 OH-CBT (75 µM) 20 min incubated,washed +D-Cys (75 µM)

9.13 20min incubated, no washing step +OH-CBT (7.5 µM)

9.14 OH-CBT (7.5 µM) 20 min incubated,no washing step +D-Cys (7.5 µM)

9.15 D-Cys (75 µM) 20 min incubated, no washing step + OH-CBT (75 µM)

9.16 OH-CBT (75 µM) 20 min incubated, no washing step +D-Cys (75 µM)

Fig.10 Diagram of total photon flux [ph/cm2/sr] for MDA-MB-231-Luc living cell samples containing various combinations and concentrations of D-luciferin, amino-D-luciferin, OH-CBT, NH_2-CBT, D-Cys and L-Cys. Columns 10.1 to 10.17 correspond to the following sample solutions, wherein CBT stands for cyanobenzylthiazole and Cys stands for cysteine:

10.1 D-Cys (75 µM)

10.2 NH_2-CBT (75 µM)

10.3 NH_2-CBT (75 µM) + L-Cys (75 µM)
10.4 NH_2-CBT (7.5 µM) + D-Cys (7.5 µM)
10.5 NH_2-CBT (7.5 µM) +D-Cys (15 µM)
10.6 NH_2-CBT (7.5 µM) +D-Cys (37.5 µM)
10.7 NH_2-CBT (7.5 µM) +D-Cys (75 µM)
10.8 NH_2-CBT (75 µM) +D-Cys (75 µM)
10.9 aminoluciferin (75 µM)
10.10 OH-CBT (75 µM)
10.11 OH-CBT (75 µM) + L-Cys (75 µM)
10.12 OH-CBT (7.5 µM) + D-Cys (7.5 µM)
10.13 OH-CBT (7.5 µM) + D-Cys (15 µM)
10.14 OH-CBT (7.5 µM) + D-Cys (37.5 µM)
10.15 OH-CBT (7.5 µM) + D-Cys (75 µM)
10.16 OH-CBT (75 µM) + D-Cys (75 µM)
10.17 luciferin (75 µM)

Fig.11 Diagram of total photon flux [ph/cm2/sr] for MDA-MB-231-Luc living cell samples containing various combinations and concentrations of D-luciferin, amino-D-luciferin and D-Cys.Columns 11.1 to 11.10 correspond to the following sample solutions, wherein Cys stands for cysteine:

11.1 luciferin (0.75 µM)
11.2 luciferin (7.5 µM)
11.3 luciferin (7.5 µM) + D-Cys(7.5 µM)
11.4 luciferin (75 µM)
11.5 luciferin (75 µM) + D-Cys (75 µM)
11.6 amino-D-luciferin (0.75 µM)
11.7 amino-D-luciferin (7.5 µM)
11.8 amino-D-luciferin (7.5 µM) + D-Cys (7.5 µM)
11.9 amino-D-luciferin (75 µM)
11.10 amino-D-luciferin (75 µM) + D-Cys (75 µM)

Fig.12 Diagram of total photon flux [ph/cm2/sr] forMDA-MB-231-Luc living cell samples incubated with solutions of different concentrations of D-Cys, NH_2-CBT or OH-CBT for 20 min followed by a washing step with PBS and by addition of solutions of equal concentrations of complementary D-Cys, NH_2-CBT or OH-CBT, respectively. Columns 12.1 to 12.16 correspond to the following sample solutions, wherein Cy stands for cysteine and CBT stands for cyanobenzothiazole:

12.1 D-Cys (7.5 µM) + 20 min incubated, washed + NH_2-CBT (7.5 µM)

12.2 NH_2-CBT (7.5 µM) + 20 min incubated, washed + D-Cys (7.5 µM)

12.3 D-Cys (75 µM) + 20 min incubated, washed + NH_2-CBT (75 µM)

12.4 NH_2-CBT (75 µM) + 20 min incubated, washed + D-Cys (75 µM)

12.5 D-Cys (7.5 µM) + 20 min incubated, no washing step + NH_2-CBT (7.5 µM)

12.6 NH_2-CBT (7.5 µM) 20 min incubated, no washing step +D-Cys (7.5 µM)

12.7 D-Cys (75 µM) 20 min incubated, no washing step +NH_2-CBT (75 µM)

12.8 NH_2-CBT (75 µM) 20 min incubated, no washing step +D-Cys (75 µM)

12.9 D-Cys (7.5 µM) + 20 min incubated, washed + OH-CBT (7.5 µM)

12.10 OH-CBT (7.5 µM) 20 min incubated, washed+D-Cys (7.5 µM)

12.11D-Cys (75 µM) 20 min incubated, washed+ OH-CBT (75 µM)

12.12OH-CBT (75 µM) 20 min incubated, washed +D-Cys (75 µM)

12.13D-Cys (7.5 µM) 20 min incubated, no washing step +OH-CBT (7.5 µM)

12.14OH-CBT (7.5 µM) 20 min incubated, no washing step +D-Cys (7.5 µM)

12.15D-Cys (75 µM) 20 min incubated, no washing step +OH-CBT (75 µM)

12.16OH-CBT (75 µM) 20 min incubated, no washing step +D-Cys (75 µM)

Fig.13 Diagram of total photon flux [ph/cm2/sr] for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally with either D-luciferin or D-Cys + OH-CBT or OH-CBT only or with a control solution.Columns 13.1 to 13.4 correspond to the following sample solutions, wherein CBT stands for caynobezothiazole, Cys for cysteine and PBS and DMS for phosphate buffered saline and dimethylsulfide, respectively:

13.1 luciferin

13.2 OH-CBT + D-Cys

13.3 PBS + DMS (control solution)

13.4 OH-CBT

Fig.14 Graph of time vs. total photon flux [ph/cm2/sr]for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally with either D-luciferin or D-Cys + OH-CBT or OH-CBT only or with a control solution.Curves a) to d) represent, wherein CBT stands for cyanobezothiazole and Cys for cysteine and PBS and DMS for phosphate buffered saline and dimethylsulfide, respectively:

a) luciferin

b) b) OH-CBT + D-Cys

c) OH-CBT

d) PBS + DMS (control)

Fig.15 Diagram of total photon flux [ph/cm2/sr]for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally first with either a phosphate buffered saline (PBS) solvent sample or D-Cys, followed by another injection with either D-luciferin or OH-CBT. Columns 15.1 to 15.4 correspond to the following sample solutions, wherein CBT stands for cyanobezothiazole andCys for cysteine, respectively:

15.1 OH-CBT + PBS

15.2 OH-CBT + D-Cys (1:1)

15.3 OH-CBT + D-Cys (1:10)

15.4 D-luciferin

Fig.16 Graph of time vs. total photon flux [ph/cm2/sr]for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally first with eithera phosphate buffered saline (PBS) solvent sample or D-Cys, followed by another injection with either D-luciferin or OH-CBT.Curves a) to d) represent the following, wherein CBT stands for cyanobezothiazole and Cys for cysteine:

a) D-luciferin

b) OH-CBT + D-Cys (1:10)

c) OH-CBT + D-Cys (1:1)

d) OH-CBT

Fig.17 Diagram of total photon flux [ph/cm2/sr] for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally first with either a phosphate buffered saline (PBS) solvent sample or D-

Cys, followed by another injection with either6-amino-2-cyanobenzothiazole (NH_2-CBT), amino-D-luciferin or a solvent control sample.Columns 17.1 to 17.4 correspond to the following sample solutions, wherein CBT stands for cyanobezothiazole andCys for cysteine, respectively:

17.1 NH_2-CBT

17.2 NH_2-CBT + D-Cys (1:1)

17.3 NH_2-CBT+ D-Cys (1:10)

17.4 Amino-D-luciferin

Fig.18 Graph of time vs. total photon flux [ph/cm2/sr]for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally first with eithera phosphate buffered saline (PBS) solvent sample or D-Cys, followed by another injection with either 6-amino-2-cyanobenzothiazole (NH_2-CBT), amino-D-luciferin or a solvent control sample.Curves a) to d) represent, wherein CBT stands for cyanobezothiazole and Cys for cysteine:

a) Amino-D-luciferin

b) NH_2-CBT + D-Cys (1:10)

c) NH_2-CBT + D-Cys (1:1)

d) NH_2-CBT

Fig.19 Diagram of total photon flux [ph/cm2/sr] for FVB-luc+ healthy mice with ubiquitous D-luciferase expression, where the columns represent:

19.1 the total photon flux of a group of four mice injected with lipopolysaccharide (LPS) and D-(+)-Galactosamine (D-GaIN), followed after 6 hours by an injection with DEVD-amino-D-luciferin

19.2 the total photon flux of a group of four mice injected with the solvent vehicle (PBS), followed after 6 hours by an injection with DEVD-amino-D-luciferin

19.3 the total photon flux of a group of four mice injected with lipopolysaccharide (LPS) and D-(+)-Galactosamine (D-GaIN), followed after 6 hours by An injection with DEVD-D-Cys, then NH_2-CBT

19.4 the total photon flux of a group of four mice injected with the solvent vehicle (PBS), followed after 6 hours by an injection with DEVD-D-Cys, then NH_2-CBT

Fig. 20 Graph of time vs. total photon flux [ph/cm2/sr] for FVB-luc+ healthy mice with ubiquitous D-luciferase expression injected intraperitoneally with

a) lipopolysaccharide (LPS) and D-(+)-Galactosamine (D-GaIN), followed after 6 hours by an injection with DEVD-amino-D-luciferin

b) lipopolysaccharide (LPS) and D-(+)-Galactosamine (D-GaIN), followed after 6 hours by an injection with DEVD-D-Cys, then NH_2-CBT

c) the solvent vehicle (PBS), followed after 6 hours by an injection with DEVD-amino-D-luciferin

d) the solvent vehicle (PBS), followed after 6 hours by an injection with DEVD-D-Cys, then NH_2-CBT

Fig.21: Graph showing the luminescence intensity [p/s] as a function of time [min] for solutions containing

Prostate Specific Antigen (PSA) enzyme. Curves a) to c) depict results for the following mixtures, wherein CBT stands for hydroxy-cyanobenzothiazole and peptide stand for z-His-Ser-Ser-Lys-Leu-Gln-(D-Cys)-OHamino acid sequence(Exposure time 10 sec.):

a) PSA (0.1mg/mL) + peptide + CBT + luciferase

b) peptide + CBT + luciferase

c) PSA (0.1mg/mL) + CBT + luciferase

Fig.22: Diagram of total photon flux [p] for solutions containing Prostate Specific Antigen (PSA) enzyme measured over a time period of 10min. Columns a) to c) represent the following mixtures, wherein CBT stands for hydroxy-cyanobenzothiazole and peptide stand for z-His-Ser-Ser-Lys-Leu-Gln-(D-Cys)-OH amino acid sequence(Exposure time 10 sec.):

a) PSA (0.1mg/mL) + peptide + CBT + luciferase

b) peptide + CBT + luciferase

c) PSA (0.1mg/mL) + CBT + luciferase

Fig.23: Diagram of luminescence intensity [p/s] (only one time point, 11min after addition of luciferase solution) for solutions containing Prostate Specific Antigen (PSA) enzyme. Columns a) to c) represent the following mixtures, wherein CBT stands for hydroxy-cyanobenzothiazole and peptide stand for z-His-Ser-Ser-Lys-Leu-Gln-(D-Cys)-OH amino acid sequence(Exposure time 10 sec.):

a) PSA (0.1mg/mL) + peptide + CBT + luciferase

b) peptide + CBT + luciferase

c) PSA (0.1mg/mL) + CBT + luciferase

Fig.24: Scheme describing the strategy for imaging multiple biomolecules of interest (BOI) using blocked luciferin precursors. By modifying differently the D-Cysteine (D-Cys) moiety at the three blockable positions (Cage 1 to 3 in molecules a), b) and c)) as well as the cyanobenzothiazole (CBT, molecule b)) moiety on the phenolic atom, 4 BOI activities could be imaged at the same time. The cage 1 (molecule a)) and 2 (molecules a) and b)) will avoid the D-Cys moiety to react with the CBT (molecule d)). The cage 3 (molecules a), b) and c)) and 4 (molecule d)) will not avoid the formation of a luciferin derivative (molecule e)) but will result in a luciferin moiety that is no substrate for luciferase enzyme. Only when cage 3 and 4 will be selectively cleaved-off by the BOI 3 and 4, free luciferin (molecule f)) will be produced, resulting in light emission after oxidation by the luciferase enzyme (step g)). Even if only one of the four cages is present and all the other position are not functionalized, the resulting modified luciferin is not a substrate for luciferase enzyme. One, two, three or four BIO activities can then be imaged and quantified using this strategy.

Fig. 25: Use of Gly-Gly-Arg-D-Cys peptide (molecule a)) for imaging and quantification with bioluminescence of thrombin enzyme via condensation reaction of 1,2-animothiol (molecule b)) with cyanobenzothiazole (CBT, molecule c))) forming D-luciferin (molecule d)) as product.

Fig. 26: Use of Asp-Glu-Val-Asp-D-Cys (DEVD-D-Cys) peptide (molecule a)) for imaging and quantification with

bioluminescence of caspase-3/7 enzyme via condensation reaction of 1,2-animothiol (molecule b)) with cyanobenzothiazole (CBT, moleculec))) forming D-luciferin (moleculed))as product.

Fig. 27: Use of His-Ser-Ser-Lys-Leu-Gln-D-Cys (HSSKLQ-D-Cys) peptide (molecule a)) for imaging and quantification with bioluminescence of Prostate Specific Antigen (PSA) enzyme via condensation reaction of 1,2-animothiol (molecule b)) with cyanobenzothiazole (CBT, molecule c))) forming D-luciferin (molecule d)) as product.

Fig. 28: Use of β-Galactoside-Cyanobenzothiazole(B-Gal-CBT) molecule (molecule a)) for imaging and quantification with bioluminescence of β-galactosidase enzyme via condensation reaction of 1,2-animothiol (molecule b)) with cyanobenzothiazole (CBT, molecule c))) forming D-luciferin (molecule d)) as product.

Fig. 29: Graph showing the luminescence intensity [RLU] as a function of time [min] for β-galactosidase enzyme test-tube assay. Curves a), b), c) and e) depict results for the following mixtures, wherein Gal-CBT stands for β-galactoside-cyanobenzothiazole and Cys stand for D-cysteine. All solutions contains luciferase enzyme with appropriate:

a) GalCBT (1x) + Cys (1x) + β-Galactosidase
b) GalCBT (1x) + Cys (10x) + β-Galactosidase
c) GalCBT (1x) + Cys (1x)
d) GalCBT + Cys + heat-killed β-Galactosidase

Fig. 30: Diagram of total photon flux [RLU] for solutions containing β-galactosidase enzyme measured over a time period of 45 min. Columns a)to f) depict results for the following mixtures, wherein Gal-CBT stands for β-galactoside-cyanobenzothiazole and Cys stand for D-cysteine and CBT stands for hydroxy-cyanobenzothiazole. All solutions contains luciferase enzyme with appropriate cofactors:

a) GalCBT (1x) + Cys (1x) + β-Galactosidase
b) GalCBT (1x) + Cys (10x) + β-Galactosidase
c) GalCBT (1x) + Cys (1x)
d) CBT (1x) + Cys (1x) + β-Galactosidase
e) GalCBT + Cys + heat-killed β-Galactosidase
f) D-luciferin + β-Galactosidase

Fig. 31: Picture of the 96-well plate for test-tube experiments ofβ-galactosidase enzyme experiments. Wells A)to F) (done in triplicates) depict results for the following mixtures, wherein Gal-CBT stands for β-galactoside-cyanobenzothiazole and Cys stand for D-cysteine and CBT stands for hydroxy-cyanobenzothiazole. All solutions contain luciferase enzyme with appropriate cofactors. Blue represent a low light emission and red-orange a strong one:

A) GalCBT (1x) + Cys (1x) + β-Galactosidase
B) GalCBT (1x) + Cys (10x) + β-Galactosidase
C) GalCBT (1x) + Cys (1x)
D) CBT (1x) + Cys (1x) + β-Galactosidase
E) GalCBT + Cys + heat-killed β-Galactosidase
F) D-luciferin + β-Galactosidase

Fig. 32: Overall representation of caspase-3/7activity imaging with DEVD-(D-Cys) peptide (molecule a)) and NH_2-CBT (molecule c)) in living transgenic animals.

In step 1) Lipopolysaccharides (LPS) and D-Galactosamine (d-GalN) are injected into a transgenic animal in order to achieve capase-3 activation. This step is followed by step 2), which involves injection of DEVD-(D-Cys) (molecule a)) and NH_2-CBT (molecule c)) into the animal. Molecule a) is converted to D-Cys (molecule b)) by the capase-3 enzyme. Molecule b) then reacts with NH_2-CBT (molecule c)) to yield D-amino-luciferin (molecule d)). In step 3) reaction of D-amino-luciferin (molecule d)) with luciferase enzyme results in luminescence.

Fig. 33: Synthetic scheme of cyanobenzothiazolyl β-D-galactopyranoside.
The reaction scheme schows treatment of galactopyranoside with H_2SO_4 in MeOH and CH_2Cl_2 to obtain Cyanobenzothiazolyl β-D-Galactopyranoside.

Fig. 34: Representation of the Cytochrome CYP3A4enzyme activity imaging strategy.Cytochrome CYP3A4enzyme can be measured by presenting a functionalized D-cysteine, such as (*s*)-2-amino-3,3-diisopropoxypropane-1-thiol (molecule a) in reaction A.) that upon enzymatic oxidationby CYP3A4 releases free D-luciferin (molecule d)in reaction A.) in the presence of cyanobenzothiazole (molecule c) in reaction A.). Either the functionalized D-cysteine is uncaged and then reacted with cyanobenzothiazole to form D-luciferin (reaction A.),

or the functionalized D-cysteine (molecule a) in reaction B.)is allowed to react with cyanobenzothiazole to form D-Luciferin-IPA (molecule b) in reaction B.), in vitro or in vivo, followed by oxidation by CYP3A4 to form D-luciferin (molecule c) in reaction B.).

Fig. 35: Synthetic scheme of (S)-4-(diisopropoxymethyl)-2,2-dimethylthiazolidine (molecule b)).

Fig. 36: Reaction between cyanobenzothiazole (molecule a)) and (*2S,2'S*)-3,3-disulfanediylbis(1,1-diisopropoxypropan-2-amine (molecule b)) in the presence of TCEP.

Fig. 37: Chromatogram (a)) and mass spectrum (b)) of the D-luciferin-IPA. LCMS confirmed the product to be the functionalized luciferin (MH+ 367.0, MNa+ 389.2).

Fig. 38: Scheme showing the removal of blocking groups from blocked functionalities of D-Luciferin or D-amino-luciferin

Fig. 39: Scheme showing unblocking (uncaging) of the cyanobenzothiazole carrying a biomolecule via intracellular reduction by glutathione.
The released cyanobenzothiazole or derivative thereof will react with separately added D-cysteine to form D-luciferin or derivatives thereof that in turn are substrates for the luciferase enzyme. The D-cysteine is added prior to or after the addition of the blocked cyanobenzothiazole

Fig. 40: Scheme showing unblocking (uncaging) of the cyanobenzothiazole via the Staudinger ligation

performed by a separately added biomolecule carrying an azide functionality.

The released cyanobenzothiazole or derivative thereof will react with separately added D-cysteine to form D-luciferin or derivatives thereof that in turn are substrates for the luciferase enzyme. The D-cysteine is added prior to or after the addition of the blocked cyanobenzothiazole and/or the addition of the biomolecule carrying an azide.

Fig 41: Reaction constants determined by HPLC for nitriles 1-17 upon their incubation with free cysteine.

Fig.42: HRMS results for HPLC-fractions containing product of reactions of nitriles 1-11 with cysteine.

Fig. 43: Total photon flux during 2 hours incubation of SKOV3 Luc-D3 living cells with GlcCBT and D-Cysteine.
Error bars are ± Standard Deviation. Numbers represent the difference in signal between GlcCBT+D-Cysteine versus GlcCBT+D-Cysteine+50 mM D-Glucose.

a) D-Lufiferin
b) GlcCBT + D-Cys
c) GlcCBT + D-Cys + D-Glu
d) GlcCBT
e) D-Cys

Fig.44: Synthetic scheme of the 1-hydroxypropyl-((3-((2-cyanobenzo[d]-thiazol-6-yloxy)-carbonyloxy)propyl) disulfanyl) D-Glucose (GlcCBT) Probe.

The two steps leading to compound 4 were achieved by following the procedure: Compound **2** was synthesized as previously described (Altamore, T. M.; Fernandez-

Garcia, C.; Gordon, A. H.; Huebscher, T.; Promsawan, N. Angewandte Chemie, International Edition, 2011 , 50, 11167-11171) and compound 1 was synthesized according to the reported procedure (Henkin, A. H.; Cohen, A. S.; Dubikovskaya, E. A.; Park, H. M.; Nikitin, G. F.; Auzias, M. G.; Kazantzis, M.; Bertozzi, C. R.; Stahl, A. *ACS Chemical Biology*, **2012**, *11*, 1884-1891).

To a stirred solution of 1 (1eq, 0.039 mmol, 16 mg) in DMF (0.5 ml) a solution in DMF (0.5 ml) of reagent 2 (1eq, 0.039 mmol, 10 mg) was added at once. Thus, to the yellowish solution triethylamine (1.6 eq, 0.062 mmol, 8.7 ul) was slowly added. The reaction mixture turned. After three hours PBS pH = 7.2 (1ml) was added follow by addition of D-Cys (1.2 eq, 0.047 mmol, 5.67 mg). The crude material was then directly purified by HPLC, to yield the compound 1-hydroxypropyl-((3-((2-cyanobenzo[d]-thiazol-6-yloxy)-carbonyloxy)propyl)disulfanyl) D-Glucose (compound 4).

Example 1 – Determination and quantification of enzyme (Thrombin) activity by using functionalized D-cysteine as a precursor to D-luciferin (Fig. 25)

A 1000 U/ml stock solution of thrombin (product number T1063, from human plasma, Sigma-Aldrich) in storage buffer (50mM sodium citrate pH 6.5, 200mM NaCl, 0.1% PEG-6000, 50% Glycerol) was prepared.

The solution containing Gly-Gly-Arg-DCys (410 µL, peptide 1) and thrombin (200 U/mL) in cleavage buffer (20mM Tris-HCL pH 8.4, 150mM NaCl, 2.5 mM $CaCl_2$) was incubated for three hours at room temperature. The same was done for a 100 U/mL and a 50 U/mL

solution of thrombin. 100 µL portions of each of the three solutions were mixed with 2-cyano-6-aminobenzothiazole (amino CBT, 17.08 µL) solution in 2:1 EtOH/MeOH solvent. The mixtures were kept under an air atmosphere at room temperature for 4 hours.

5 µL portions of each of the solutions were then diluted with luciferase assay buffer (0.1mM Tris-HCl pH 8.0, 2mM adenosine triphosphate (ATP), 5mM $MgSO_4$) and mixed with a 60 µg/mL D-luciferase solution (100 µL, D-luciferase from Photinuspyralis (firefly) product No. L9506, Sigma-Aldrich) in luciferase assay buffer.

Luminescent intensity in relative light units (RLU) was measured immediately with a luminometer for an integration period of 13 seconds.

The luminescent intensity as well as the total photon flux were the highest for the solution containing the largest thrombin concentration and became smaller when this concentration decreased. The luminescent intensity and the total photon flux were then proportional to the thrombin concentration. When no thrombin is present, the luminescent signal is the same as for the blank (luciferase assay buffer without any luciferase substrate). The luminescent signal obtained for solutions containing thrombin were significantly higher than for the ones that do not contain thrombin.

The results of this experiment are also depicted in Fig.5 and 6.

<u>Example 2a – Bioluminescent evaluation of cyanobenzothiazole condensation reaction with D-Cysteine in luciferase expressing SKOV3-Luc-D3 living cells</u>

Luciferase-expressing SKOV3-Luc-D3 cells were grown in 10 cm round culture plates with McCoy's media (Gibco) containing 10% fetal bovine serum (FBS), penicillin/streptomycin and glutamax.

On a black 96-well-plate, 10000 cells per well were seeded. After 48 hours the cells were first washed with phosphate buffered saline (PBS, 200 µL) and then incubated for 5 minutes in a solution containing D-cysteine (D-Cys) or PBS (100 µL). Then a solution of 6-Hydroxy-2-cyanobenzothiazole (OH-CBT), 6-amino-2-cyanobenzothaizole (NH_2-CBT) or D-luciferin (100 µL) was added immediately before imaging using an IVIS spectrometer. For each reagent, solutions of three different concentrations were used, namely 150 µM, 15 µM and 1.5 µM, giving rise to final concentrations for all reagents of 75 µM, 7.5 µM and 0.75 µM.

The following reagent combinations were used:

- NH_2-CBT (75µM) + D-Cys(75µM)
- NH_2-CBT (7.5µM) + D-Cys (7.5µM)
- NH_2-CBT (0.75µM) + D-Cys (0.75µM)
- NH_2-CBT (7.5µM) + D-Cys (15µM)
- NH_2-CBT (7.5µM) + D-Cys (37.5µM)
- NH_2-CBT (7.5µM) + D-Cys (75µM)
- NH_2-CBT (75µM) + L-Cys(75µM)
- NH_2-CBT (75µM)
- D-Luciferin (75µM)
- D-Luciferin (7.5µM)
- D-Luciferin (0.75µM)
- D-Luciferin (7.5µM) + D-Cys (7.5µM)
- D-Luciferin (75µM) + D-Cys (75µM)
- D-Cys (75µM)
- OH-CBT (75µM) + D-Cys(75µM)

- OH-CBT (7.5µM) + D-Cys (7.5µM)
- OH-CBT (0.75µM) + D-Cys (0.75µM)
- OH-CBT (7.5µM) + D-Cys (15µM)
- OH-CBT (7.5µM) + D-Cys (37.5µM)
- OH-CBT (7.5µM) + D-Cys (75µM)
- OH-CBT (75µM) + L-Cys(75µM)
- OH-CBT (75µM)
- amino-D-Luciferin (75µM)
- amino-D-Luciferin (7.5µM)
- amino-D-Luciferin (0.75µM)
- amino-D-Luciferin (7.5µM) + D-Cys (7.5µM)
- amino-D-Luciferin (75µM) + D-Cys (75µM)
- PBS

Each of the experiments with the above reagent combinations was performed in triplicate.

Immediately after addition of the OH-CBT, NH_2-CBT or D-luciferin solutions, IVIS spectra were recorded every minute for one hour on an IVIS spectrometer using living image software on auto settings.

The results recorded are shown in Fig.7 and 8.

Example 2b

In order to determine if the reactions of Example 2a above occurred inside or outside the cells, the cells were first incubated with either D-Cys or OH-CBT or NH_2-CBT (75 or 7.5 µL) for 20 minutes. The cells were then washed with PBS. Following this, OH- or NH_2-CBT or D-Cys solutions of 75 µL or 7.5 µL concentrations were added.

The following reagent combinations were used with "20 min" indicating the reagent with which the cells were incubated over the 20 minute period:

- NH_2-CBT 20min (7.5µM) + D-Cys(7.5µM)
- NH_2-CBT 20min (75µM) + D-Cys (75µM)
- D-Cys 20min (7.5µM) + NH_2-CBT (7.5µM)
- D-Cys20min(75µM)+NH_2-CBT(75µM)
- OH-CBT 20min (7.5µM) + D-Cys (7.5µM)
- OH-CBT 20min (75µM) + D-Cys(75µM)
- D-Cys 20min (7.5µM) + OH-CBT (7.5µM)
- D-Cys 20min (75µM) + OH-CBT (75µM)

Each of the experiments with the above reagent combinations was performed in triplicate.

Immediately after addition of the OH-CBT, NH_2-CBT or D-Cys solutions to the incubated cells, IVIS spectra were recorded every minute for one hour on an IVIS spectrometer using living image software on auto settings.

The results recorded are shown in Fig.9.

Example 2c

The experiments described in Example 2b were repeated without the washing step.

The results recorded are shown in Fig.9.

Example 3a – Bioluminescent evaluation of cyanobenzothiazole condensation reaction with D-Cysteine in luciferase expressing MDA-MB-231-Luc living cells

D-Luciferase-expressing MDA-MB-231-Luc cells were grown in 10cm round culture plates with MEM alpha media (Gibco) containing 10% fetal bovine serum, penicilline/strepomycin and glutamax.

On a black 96-well plate, 10000 cell/well were seeded. 48h after the seeding, the cells were first washed with phosphate buffer solution (200 µL) and then incubated for 5 min in solutions containing D-Cys or PBS (100µL).
Then a solution of OH-CBT, NH_2-CBT or D-luciferin (100µL) was added.
Solutions of three different concentrations for each reagent were used, namely 150 µM, 15 µM and 1.5 µM, giving rise to final concentrations of 75µM, 7.5µM or 0.75 µM for all the reagents.

The following reagent combinations were used:

- NH_2-CBT (75µM) + D-Cys(75µM)
- NH_2-CBT (7.5µM) + D-Cys (7.5µM)
- NH_2-CBT (0.75µM) + D-Cys (0.75µM)
- NH_2-CBT (7.5µM) + D-Cys (15µM)
- NH_2-CBT (7.5µM) + D-Cys (37.5µM)
- NH_2-CBT (7.5µM) + D-Cys (75µM)
- NH_2-CBT (75µM) + L-Cys(75µM)
- NH_2-CBT (75µM)
- D-Luciferin (75µM)
- D-Luciferin (7.5µM)
- D-Luciferin (0.75µM)
- D-Luciferin (7.5µM) + D-Cys (7.5µM)

- D-Luciferin (75µM) + D-Cys (75µM)
- D-Cys (75µM)
- OH-CBT (75µM) + D-Cys(75µM)
- OH-CBT (7.5µM) + D-Cys (7.5µM)
- OH-CBT (0.75µM) + D-Cys (0.75µM)
- OH-CBT (7.5µM) + D-Cys (15µM)
- OH-CBT (7.5µM) + D-Cys (37.5µM)
- OH-CBT (7.5µM) + D-Cys (75µM)
- OH-CBT (75µM) + L-Cys(75µM)
- OH-CBT (75µM)
- amino-D-Luciferin (75µM)
- amino-D-Luciferin (7.5µM)
- amino-D-Luciferin (0.75µM)
- amino-D-Luciferin (7.5µM) + D-Cys (7.5µM)
- amino-D-Luciferin (75µM) + D-Cys (75µM)
- PBS

Each of the experiments with the above reagent combinations was performed in triplicate.

Immediately after addition of the D-Cys or L-Cys solutions, IVIS spectra were recorded every minute for one hour on an IVIS spectrometer using living image software on auto settings.

The results recorded are shown in Fig.10 and 11.

Example 3b

In order to determine if the reactions of Example 3a above occurred inside or outside the cells, the cells were first incubated with either D-Cys or OH-CBT or NH_2-CBT (75 or 7.5 µL) for 20 minutes. The cells were then washed with PBS. Following

this, OH- or NH_2-CBT or D-Cys solutions of 75 µL or 7.5 µL concentrations were added.

The following reagent combinations were used with "20 min" indicating the reagent with which the cells were incubated over the 20 minute period:

- NH_2-CBT 20min (7.5µM) + D-Cys(7.5µM)
- NH_2-CBT 20min (75µM) + D-Cys (75µM)
- D-Cys 20min (7.5µM) + NH_2-CBT (7.5µM)
- D-Cys 20min (75µM) + NH_2-CBT (75µM)
- OH-CBT 20min (7.5µM) + D-Cys (7.5µM)
- OH-CBT 20min (75µM) + D-Cys(75µM)
- D-Cys 20min (7.5µM) + OH-CBT (7.5µM)
- D-Cys 20min (75µM) + OH-CBT (75µM)

Each of the experiments with the above reagent combinations was performed in triplicate.

Immediately after addition of the OH-CBT, NH_2-CBT or D-Cys solutions to the incubated cells, IVIS spectra were recorded every minute for one hour on an IVIS spectrometer using living image software on auto settings.

The results recorded are shown in Fig.12.

Example 3c

The experiments described in Example 3b were repeated without the washing step.

The results recorded are shown in Fig.12.

Example 4a - Bioluminescent evaluation of cyanobenzothiazole condensation reaction with D-Cysteine in ubiquitously luciferase expressing FVB-luc+healthy mice

Four groups of two healthy FVB-luc+ mice((FVB-Tg(CAG-luc-GFP)L2G85Chco/J) mice) each expressing ubiquitously luciferase were injected intraperitoneally with either a 30 mg/mL solution of D-luciferin (150 mg/kg or 0.535 mmol/kg) or with a 12.966 mg/mL solution of D-Cys (64.83 mg/kg) in PBS followed by a 60 mg/mL solution of OH-CBT (94.28 mg/kg or 0.535 mmol/kg)in DMSO three minutes later or with a 60 mg/mL solution of OH-CBT (94.28 mg/kg or 0.535 mmol/kg)in DMSO only or a solvent control sample of PBS and DMSO only, which contained the same solvent volume as the other samples depending on mouse weight.

The animals were anesthetized using isofluorane and one image per minute was recorded over a period of 50 minutes for each mouse using a Caliper life science IVIS spectrometer on auto settings. The data were treated with living image software (Caliper Life Science) using the ROI tool.

The results recorded are depicted in Fig.13 and 14.

Example 4b

A group of five healthy FVB-Luc+ mice ((FVB-Tg(CAG-luc-GFP)L2G85Chco/J) mice), each expressing ubiquitously luciferase, ere injected intraperitoneally with D-cysteine (D-Cys) (0.268 mmol/kg), which was formulated as a 0.0536 mmol/mL solution in phosphate buffered saline (PBS). A second group of the same type and number of mice was injected with a ten-fold amount of D-Cys,

namely 2.68 mmol/kg. Two further groups of five mice each were injected with a PBS control sample.

Three minutes later, the same mice were injected with either 6-hydroxy-2-cyanobenzothiazole (OH-CBT), or D-luciferin or a PBS or dimethylsulfoxide (DMSO) solvent control sample. Each of these samples was administered in an amount of 0.268 mmol/kg. D-luciferin was formulated in PBS at a concentration of 0.0536 mmol/mL and OH-CBT was formulated in DMSO at a concentration of 0.268 mmol/mL.

Immediately after the second injection, luminescence images were acquired using a Xenon IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA, USA). One image per minute over a period of 60 minutes was recorded using the automatic setting available on the instrument.

The results recorded are shown in Fig. 15 and Fig.16.

Example 4c

The same experiment as described in example 4b was repeated using in the second injection step 0.268 mmol/kg of either 6-amino-2-cyanobenzothiazole (NH_2-CBT), amino-D-luciferin or a PBS or dimethylsulfoxide (DMSO) solvent control sample. NH_2-CBT was formulated in DMSO at a concentration of 0.268 mmol/mL.

Immediately after the second injection, luminescence images were acquired using a Xenon IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA, USA). One image per minute over a period of 60 minutes was recorded using the automatic setting available on the instrument.

The results are shown in Fig. 17 and Fig. 18.

Example 5 - (Fig.26, Fig.32) - Non-invasive Bioluminescent imaging of Caspase-3/7 activity in ubiquitously luciferase expressing FVB-luc+healthy mice using the cyanobenzothiazole condensation reaction with D-Cysteine

Two groups of four healthy FVB-Luc+ mice ((FVB-Tg(CAG-luc-GFP)L2G85Chco/J) mice), each expressing ubiquitously luciferase, were injected with 100 µg/kg of a lipopolysaccharide (LPS) solution in phosphate buffered saline (PBS) of a concentration of 3 µg LPS in 50 µL PBS and with 267 mg/kg of a solution of D-(+)-Galactosamine (D-GaIN) in PBS of a concentration of 8 mg in 50 µL PBS in order to induce hepatic apoptosis and thus Capase-3/7 activation.

After six hours, 22 mg/kg of a Asp-Glu-Val-Asp-D-Cysteine (DEVD-D-Cys) in PBS solution having a concentration of 0.68 mg/100 µL was injected intraperitoneally into the mice belonging to one of the groups of mice treated with LPS and D-GaIN, followed 10 minutes later by 6.8mg/kg of a6-amino-2-cyanobenzothiazole (NH_2-CBT)in DMSO solution having a concentration of 0.205 mg/20 µL. (See Fig. 32)
As a positive control, 34 mg/kg of a DEVD-amino-D-luciferin solution in PBS having a concentration of 1.02 mg/100 µL was injected into the other group of mice treated with LPS and D-GaIN after six hours instead of DEVD-D-Cysteine (DEVD-D-Cys) and 6-amino-2-cyanobenzothiazole (NH_2-CBT).

Another two groups of four untreated FVB-Luc+ mice each were injected with 22 mg/kg of a DEVD-D-Cysteine (DEVD-D-Cys) in PBS solution having a concentration of 0.68 mg/100 µL, followed by 6.8mg/kg of a 6-amino-2-cyanobenzothiazole (NH_2-CBT)in DMSO solution having a concentration of 0.205 mg/20 µL and with 34

mg/kg of a DEVD-amino-D-luciferin solution in PBS having a concentration of 1.02 mg/100 µL, respectively.

The presence of capase-3/7 activity in the mice is indicated by cleavage of the DEVD-peptide off DEVD-D-Cys or DEVD-amino-D-luciferin followed by luminescence arising from the reaction between luciferase and amino-D-luciferin.

A Xenogen IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA, USA) was used to acquire luminescence images. One image per minute over a period of 60 minutes was recorded using the automatic setting available on the instrument.

The results are shown in Fig. 19 and Fig. 20.

Also, apart from studying the protease activity of capase-3/7 in cell death signalling, the activities of other proteases in cell death signalling or upon metabolic change can be studied in vivo and in vitro. To this end, a reaction of either 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole with the D-cysteine released from the C-terminal of a peptide sequence specific to the protease of interest is studied and controlled via the bioluminescent signal. Only if D-cysteine is released by the specific protease, it can react with 6-hydroxy-2-cyanobenzthiazole or 6-amino-2-cyanobenzothiazole, giving rise to D-luciferin or amino-D-luciferin, i.e. the substrate for luciferase.
The following table shows the proteases whose activities can be studied and controlled and the respective imaging kits comprising either 6-hydroxy-2-cyanobenzothiazole (6-OH-CBT) or 6-amino-2-cyanobenzothiazole (6-NH2-CBT) and a D-cysteine attached to a peptide sequence, which is a cleavage site for the specific protease studied:

Imaging kit	Protease
Z-DEVD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-3 and -7
Z-LETD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-8
Z-LEHD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-9
GP-dCys + 6-OH-CBT or 6-NH2-CBT	dipeptidyl peptidase 4 (DPPIV)
Suc-dCys + 6-OH-CBT or 6-NH2-CBT	calpain- and chymotrypsin-like activities of proteasome
Z-LRR-dCys + 6-OH-CBT or 6-NH2-CBT	trypsine-like activity of proteasome
Z-nLPnLD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-like activity of proteasome
Z-QEVY-dCys + 6-OH-CBT or 6-NH2-CBT	clapain and proteasome chymotrypsine-like activity
VP-dCys + 6-OH-CBT or 6-NH2-CBT	dipeptidyl peptidase 4 (DPPIV)
Z-VDAVD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-2
Z-VEID-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-6
Z-ATAD-dCys + 6-OH-CBT or 6-NH2-CBT	caspase-12
Z-IEPD-dCys + 6-OH-CBT or 6-NH2-CBT	granzyme B
Z-IETD-dCys + 6-OH-CBT or 6-NH2-CBT	granzyme B and caspase-6
Z-TSAVLQ-dCys + 6-OH-CBT or 6-NH2-CBT	SARS protease
Z-VNSTLQ-dCys + 6-OH-CBT or 6-NH2-CBT	SARS protease

Example 6 - Detection of Prostate Specific Antigen (PSA) enzyme activity by using functionalized D-cysteine as a precursor to D-luciferin

His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) amino-acids sequence is known to be selectively cleaved-off by active PSA enzyme. Free D-Cysteine can be selectively released using the z-His-Ser-Ser-Lys-Leu-Gln-(D-Cys)-OH (HSSKLQ-DCys) construct. Once the free

cysteine released, OH-CBT will selectively react with the D-Cysteine forming D-Luciferin as OH-CBT will not react with the HSSKLQ-DCys construct (free 1,2-aminoithiol are necessary for the reaction to occur). Light emission proportional to the quantity of D-luciferin formed will be emitted if Luciferase is present.

2.0976 uL of Active PSA commercial stock solution (2.86 mg/mL, Calbiochem) was added to 60 uL of a 500 uM HSSKLQ-DCys peptide solution in PSA buffer (50 mM Tris, pH 7.5, 150 mM NaCl) and incubated for 4h at 37°C. After 4h, a 15 uL sample is taken of the reaction mixture and added to 15uL of a 500 uM HO-CBT solution in PSA buffer containing 10% DMSO and incubated for 15min.
On a black 96 well plate, 80 uL of a Luciferase solution (Promega, 60 ug/mL in 0.1M Tris-HCl, 2mM ATP, 5mM MgSO4) is added to 20 uL of the reaction mixture right before measuring the bioluminescence emission.
Luminescent intensity (p/s) was acquired on the IVIS-50 () using living images software (0 min sequence imaging: exposure 10 sec. Delay between acquisition 1 min. binning Hi. No emission filters.).

The results of this experiment are also depicted in Fig.21, Fig.22 and Fig.23.

Example 7 - Detection of the β-galactosidaseenzyme by using a Galactose-functionalized cyanobenzothiazole (Gal-CBT) as a precursor to D-luciferin.

β-galactosidase activity can be measured by presenting a functionalized cyanobenzothiazole (here: β-Galactoside-CBT) that upon enzymatic hydrolysis releases free D-luciferin in the presence of D-cysteine (see Fig.28).

A 1000 U/ml stock of β-galactosidase (product number G4155-1KU, from Escherichia coli, from Sigma-Aldrich) in storage buffer (1 mM MgCl2, 1 mM TRIS in aqueous 50% glycerol suspension) was obtained from the manufacturer. (5 uL) of the enzyme solution was diluted in 45 uL of PBS (pH 7.4) and 10 uL of this solution (ca 1 U enzyme) was transferred to the reaction vessel, which contained 70 uL PBS (pH 7.4) and 10 uL 1 M NaCl.

Five (A-E) reactions were run in parallel, where A-B were experiments using different ratios of D-cysteine (1:1 or 10:1, with respect to Gal-CBT) and C-E were control experiments. Experiment C was run without any β-galactosidase present (negative control), D was run with unfuncionalized CBT (positive control) and E was run with heat-killed β-galactosidase (negative control). An additional positive control (F) was also run, where D-luciferin was used. A summary of the reactions is shown in Fig.B.

For the heat-kill, β-galactosidase was heated to 100 °C for 30 min prior to experiment in order to see if the presence of a deactivated enzyme can still catalyse the reaction.

A solution of 1 mM Gal-CBT or CBT was prepared and 10 uM (10 nmol) was put in reaction vessels A, B, D and E and the mixture was incubated for 1 h at 37 °C. After 1 h, the solution was cooled to room temperature, upon which 1 mM (A, C, D, E) or 10 mM (B) D-Cys. The reactions were left at room temperature for 20 min.

A luciferase assay buffer (0.1mM Tris-HCl pH 8.0, 2mM adenosine triphosphate (ATP), 5mM $MgSO_4$) was prepared and mixed with a 60 µg/mL D-luciferase solution (100 µL, D-luciferase from

Photinuspyralis (firefly) product No. L9506, Sigma-Aldrich) in luciferase assay buffer. The luciferase assay was plated on a 96-well plate (80 uL per well). Then reaction A-E plus the positive control, F, D-luciferin in PBS (pH 7.4), were added to three wells each. Reaction A, B, C, D and E contain 20 uM CBT derivatives and 20 uM D-Cys. Reaction F contains 10 uM D-Luciferin. Photon flux was immediately measured by either IVIS (Caliper) or a Plate reader (Tecan).

All reactions were performed under air using freshly prepared stock solutions of all reagents. Caution was taken to protect reactions and stocks from light.

Luminescent intensity in relative light units (RLU) was measured immediately with a luminometer for an integration time of 100 ms over 60 kinetic cycles for one hour.
The luminescent intensity as well as the total photon flux were highest for reactions containing free D-luciferin (F) and the reaction containing unfunctionalized CBT (D), which was to be expected. The reactions containing functionalized CBT and b-gal enzyme (A and B) nevertheless showed strong luminescent activity, especially when compared to the reactions having no b-gal or heat-killed b-gal (C and E) (see Fig.29 and Fig.30).
Photon flux measurement of the first 15 min of reactions A, B, D and E were difficult due to saturation of the detector. After 15 min, all reactions could be measured, however. A picture of the first minute in an IVIS of the plate can be seen in Fig.31.

Synthesis of Cyanobenzothiazolyl β-D-Galactopyranoside (Fig.33)

Previous reports have claimed that basic deacetylation works poorly due to the electrophilic nitrile of the cyanobenzothiazole (Amess et al, Carb. Res., 205 (1990)225-233).

We also found this to be the case and for this reason an acidic deacetylation protocol was here developed.

Cyanobenzothiazolyl 2,3,4,6-tetra-O-acetyl-β-D-Galactopyranoside (Amess et al, Carb. Res., 205 (1990)225-233) (100 mg, 0.197 mmol) was dissolved in CH_2Cl_2 (5 mL). Conc. H_2SO_4 (0.5 mL) was diluted in MeOH (5 mL) at 0 °C and slowly added to the CH_2Cl_2 solution. Over 4 h, steady formation of product was seen by LCMS analysis, but also an increase of formation of cyanobenzothiazole (by-product) and the reaction was carefully quenched at 0 °C using NH_4OH (25%) under rigorous stirring, trying to avoid any yellow/green colouration, which indicated by-product formation. This could be avoided by keeping pH at 6 or lower. The white slurry was concentrated *in vacuo* (co-evaporating with toluene, 5 mL) resulting in a white solid. 2-Propanol (10 mL) was added and the suspension was sonicated to fully dissolve the product. The remaining white solid (salts) were filtered off. The clear 2-propanol solution is then concentrated *in vacuo* to give a white solid. The crude material was purified by column chromatography (CHCl3:MeOH 8:1) to give cyanobenzothiazolyl β-D-galactopyranoside (66.3 mg, 99%) as a white solid, which was lyophilized. ^{1}H NMR (400 MHz, D_2O) δ 7.98 (d, *J* = 9.1 Hz, 1H), 7.71 (d, *J* = 2.5 Hz, 1H), 7.39 (dd, *J* = 9.2, 2.5 Hz, 1H), 5.14 (d, *J* = 7.5 Hz, 1H), 4.04 (dd, *J* = 3.2, 1.0 Hz, 1H), 3.97 - 3.79 (m, 5H). ^{13}C NMR (101 MHz, D_2O) δ 157.1 (s), 146.8 (s), 137.0(s), 135.6 (s), 125.0 (d), 119.4 (d), 112.9 (s), 107.5 (d), 100.8 (d), 75.6 (d), 72.4 (d), 70.4 (d), 68.4 (d), 60.7 (t). HRMS calcd for $C_{14}H_{14}N_2O_6S$ [MH^+] 339.0651, found 339.0657

Example 8 - Detection of the enzyme CYP3A4 by using a functionalized D-Cysteine as a precursor to D-luciferin.

Cytochrome enzyme CYP3A4 activity can be measured by presenting a functionalized D-Cysteine (here: isopropyl acetal functionalized) that upon enzymatic oxidationby CYP3A4 releases free D-luciferin in the presence of cyanobenzothiazole (see Fig.34). Either the functionalized D-Cysteine is uncaged and then reacted with cyanobenzothiazole to form D-Luciferin (see Fig.34, A), or the functinalized D-Cysteine is allowed to react with cyanobenzothiazole to form D-Luciferin-IPA (Fig.34, B), in vitro or in vivo, followed by oxidation of CYP3A4 to form D-luciferin.

Synthesis of (S)-4-(diisopropoxymethyl)-2,2-dimethylthiazolidine (Fig.35).

Aldehyde tert-Butyl 4-formyl-2,2-dimethylthiazolidine-3-carboxylate (22 mg, 0.090 mmol)(seeMeisenheimer et al, *Drug Metabolism and Disposition* (2011) 39, 2403-2410.) was dissolved in 2-propanol (2.5 mL) and put under N_2. Add 4M HCl in dioxane (0.288 mL) dropwise at room temperature. After 18 h, the complete consumption of aldehyde (Rf 0.6) and formation of a product (Rf 0.4) could be seen by TLC (Toluene/Ethyl Acetate 6:1). The reaction mixture was concentrated by co-evaporation with toluene and the crude material was purified by column chromatography (DCM + 1% Et_3N → DCM:MeOH + 1% Et_3N 5:1) to give (S)-4-(diisopropoxymethyl)-2,2-dimethylthiazolidine (12 mg, 54%) as an oil. Upon storage -20 °C, disulfide analogue (*2S,2'S*)-3,3-disulfanediylbis(1,1-diisopropoxypropan-2-amine was formed, presumably via acetal hydrolysis followed by oxidative disulfide

formation. This disulfide compound was also the compound seen on HRMS.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80 (d, *J* = 3.0 Hz, 1H), 3.87 - 3.76 (m, 2H), 3.45 (ddd, *J* = 9.5, 6.6, 3.0 Hz, 1H), 3.01 (dd, *J* = 8.1, 3.1 Hz, 2H), 1.60 (s, 3H), 1.47 (s, 3H), 1.19 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 98.4 (d), 75.0 (s), 69.9 (d), 69.5 (d), 36.4 (t), 33.1 (q), 31.3 (q), 29.9 (4 x q). HRMS calcd for $C_{18}H_{40}N_2O_4S2$ [MH^+] 413.2508, found 413.2525 (disulfide).

In order to verify that the functionalized cysteines indeed form a functionalized D-luciferin, a test reaction between cyanobenzothiazole and (*2S,2'S*)-3,3-disulfanediylbis(1,1-diisopropoxypropan-2-amine in the presence of TCEP was carried out (Fig.36). (*2S,2'S*)-3,3-disulfanediylbis(1,1-diisopropoxypropan-2-amine (0.54 mg, 0.0013 mmol) was dissolved in 2-propanol (0.4 mL) and TCEP*HCl (0.64 mg, 0.0022 mmol) dissolved in PBS (0.1 mL, pH 7.4) was added to reduce the disulfide bond. Cyanobenzothiazole (0.25 mg, 0.0014 mmol) was then dissolved in THF (0.1 mL) and transferred to the reaction. One drop of $NaHCO_3$ (sat.) was added to get the pH up to 7. The reactions was stirred at room temperature and after 1 h, LCMS confirmed the product to be the functionalized luciferin (MH+ 367.0, MNa+ 389.2) (Fig.37).

<u>Example 9 - Kinetics investigation of condensation reaction of D-Cysteine with cyanobenzothiazole-derivatives</u>

General procedure for rate constant determination for 1-6: To 50 µL of 0.01M solution of nitrile in freshly degassed MeCN 450 uL of 0.1, 0.125, 0.15 or 0.2M freshly prepared solution of cysteine in freshly degassed PBS was added, and the mixture was shaken. Afterwards, the aliquot of 75 µL of reaction mixture was taken at 5 min, 10 min, 15 min, 20 min, 25 min time points and quenched with 200 µL of 10% aqueous HCl to stop the reaction.

100 µL of quenched solution were analyzed by HPLC to determine the degree of conversion.

General procedure for rate constant determination for 7-17: To 950 µL of freshly degassed PBS, 20 µL of 0.01M solution of nitrile in freshly degassed MeCN were added. The mixture was shaken and to the resulting solution, 30 µL of 0.1, 0.125, 0.15 or 0.2 M of freshly prepared solution of cysteine in freshly degassed PBS was added, and the mixture was shaken. Afterwards, the aliquot of 100 µL of reaction mixture was taken at 1 min, 2 min, 3 min, 4 min, 5 min time points and quenched with 200 µL of 10% aqueous HCl to stop the reaction. 100 µL of quenched solution were analyzed by HPLC to determine the degree of conversion.
The degree of conversion was determined by intensity of UV absorbance at characteristic wavelength of initial nitrile HPLC fraction. The nature of product was determined by separation of small HPLC fraction containing product and analyzing it by HRMS. The HRMS results are given in Fig. 42. The fact that quenching with HCl stopped the reaction was proved by stability of UV absorbance intensity of initial nitrile peak after 1-2 days. The pseudo-first order constants were determined for each concentration of cysteine by linearization in ln [C(nitrile)] – t coordinates. Second order constants were determined for each concentration of L-cysteine by dividing the pseudo-first order values to the corresponding cysteine concentration in the resulting reaction mixture. (Fig.41 and 42)(Reddy, G. R.; Thompson, W. C.; Miller, S. C. Journal of the American Chemical Society 2010, 132, 13586-7).

Example 10 - Non-Invasive Bioluminescent Imaging and Quantification of Glucose Uptake in Living Cells and Animals

using a Glucose-functionalized cyanobenzothiazole (GlcCBT) as a precursor to D-luciferin.

SKOV-3 Luc D-3 cell line (human ovarian cancer cell line) was chosen due to its known high glucose metabolism. SKOV-3 Luc D-3 were grown in DMEM Media uplemented with 10 % FBS and 1% Penicillin/Streptomycin. Two days before imaging cells were plated at density $5x10^4$ cells/ml in a black 96-well plates with clear bottom. After cells became confluent, they were incubated in Low-Glucose DMEM media for 4 hours. Cells were then washed once with ice-cold PBS, and incubated with D-Cysteine solutions (50, 20 and 10 µM) in glucose-depleted DMEM media for 20 min. Cells were then loaded with solutions of GlcCBT (50, 20, 10 µM) in glucose-depleted DMEM media. Bioluminescence emission was immediately acquired for two hours using A Xenogen IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA, USA). One image per minute over a period of 120 minutes was recorded using the automatic setting available on the instrument.

To study glucose-inhibition, cells were treated with solutions of GlcCBT (50, 20, 10 µM) in mixture with D-Glucose (50 mM). Corresponding bioluminescent signal was measured for 120 min. D-Luciferin was also used as a positive control.

We were able to detect the signal from a reaction in concentrations up to 10 µM. We also found that uptake of GlcCBT was significantly inhibited in the presence of D-Glucose, that indicates that GlcCBT can be taken up by itself in a glucose-specific manner (Fig. 43).

GlcCBT was synthesized according to the scheme shown in Fig. 44.

CLAIMS

1. A precursor molecule for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blocked amino-D-luciferin, or derivatives thereof chosen from the group consisting of

a) functionalized D-cysteine or derivatives thereof, wherein the functionalization does not prevent the reactivity towards 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole; and wherein, when reacting with a molecule according to b) or when reacting with an unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole, a functionalized D-luciferin or a functionalized amino-D-luciferin or any derivatives thereof is obtained which is not readily reactive towards luciferase;

b) functionalized 6-hydroxy-2-cyanobenzothiazole or functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof, wherein the functionalization does not prevent the reactivity towards D-cysteine or derivatives thereof, functionalized or unfunctionalized aminothiols or functionalized or unfunctionalized 2-aminobenzylamines or derivatives thereof; and wherein, when reacting with a molecule according to a) or when reacting with an unfunctionalized cysteine, a functionalized D-luciferin or a functionalized amino-D-luciferin or derivatives thereof is obtained, which is not readily reactive towards luciferase;

c) functionalized D-cysteine or derivatives thereof, wherein the functionalization prevents the reactivity towards 6-

hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof;

d) functionalized 6-hydroxy-2-cyanobenzothiazole or functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof, wherein the functionalization prevents the reactivity towards cysteine or derivatives thereof.

2. A molecule in particular according to claim 1, of the general formula X-Y-Z, wherein X is a moiety of up to 1000 Da, in particular in the range of 100 to 1000 Da; Y is a linker of 6 to 30 atoms, in particular comprising a disulfide moiety; and Z is a 2-cyano-benzothiazole or a derivative thereof.

3. A molecule according to claim 2, wherein X is selected from the group of carbohydrates and derivatives thereof, in particular monoaccharides and derivatives thereof; peptides; folate; lactate; lipids; triglycerides; enzyme inhibitors.

4. Use of a functionalized D-cysteine or derivatives thereof according to compounds a) or c) of any one of claims 1 to 3 or a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) of any one of claims 1 to 3 for the synthesis of D-luciferin, amino-D-luciferin, blocked D-luciferin or blocked amino-D-luciferin, and any derivatives thereof, respectively.

5. Method of synthesizing blocked D-luciferin or blocked amino-D-luciferin, wherein

a) a functionalized D-cysteine or derivatives thereof according to a) of any one of claims claims 1 to 3 is mixed and allowed to react with either a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to b) of any one of claims 1 to 3; or with unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof;

or, wherein

b) a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to b) of any one of claims 1 to 3 is mixed and allowed to react with either a functionalized D-cysteine or derivatives thereof according to a) of any one of claims 1 to 3; or withunfunctionalized D-cysteine or derivatives thereof.

6. Method of synthesizing D-luciferin or amino-D-luciferin or derivatives thereof comprising the steps of

i) providing either a functionalized cysteine or derivatives thereof according to compounds a) or c) any one of claims 1 to 3 or a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) of any one of claims 1 to 3;

ii) providing the respective complementary unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazoleor unfunctionalized or functionalized cysteine or any derivatives thereof;

iii) adding the components provided in steps i) and ii) to a system containing one or more biomolecules capable of cleaving functional groups off functionalized D-cysteine or derivatives thereof and/or functionalized 6-hydroxy-2-cyanobenzothiazole or functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof and allowing them to react.

7. Kit of parts for a screening assay for the detection of molecules or for the detection of the activity of proteases and enzymes in vivo or in vitro comprising
   - either a functionalized D-cysteine or derivatives thereof according to compounds a) or c) of any one of claims 1 to 3 or a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) of any one of claims 1 to 3;
   - the respective complementary unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazoleor unfunctionalized or functionalized cysteine or derivatives thereof;
   - optionally, luciferase;
   - optionally, ATP,
   - optionally, magnesium cation source and
   - instructions for use.

8. Use of a kit of parts for a screening assay according to claim 7 for the detection of molecules.

9. Use of a kit of parts for a screening assay according to claim 7 for the study of the uptake of molecules through endocytosis or receptor-mediated pathways.

10. Use of a kit of parts for a screening assay according to claim 7 for the detection of an environment reducing disulfide bonds and azo-compounds.

11. Method of detecting biomolecules comprising the steps of
    i) providing either a functionalized D-cysteine or derivatives thereof according to compounds a) or c) of any one of claims 1 to 3 or a functionalized 6-hydroxy-2-cyanobenzothiazole or a functionalized 6-amino-2-cyanobenzothiazole or derivatives thereof according to compounds b) or d) of any one of claims 1 to 3;
    ii) providing the respective complementary unfunctionalized or functionalized D-cysteine or derivatives thereof or unfunctionalized or functionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole or derivatives thereof;
    iii) providing luciferase;
    iv) contacting the components provided in steps i. and ii. and allowing them to react in the presence of one or more biomolecules studied; and
    v) detecting and measuring the luminescence signal generated.

12. Kit of parts for a screening assay for the determinationof a sequence cleaved by a specific enzyme within a peptide or protein in vivo or in vitro comprising
    i) the peptide or protein bearing at the C-terminal end the sequence cleaved by the specific enzyme followed by a D-cysteine residue or derivatives thereof,
    ii) an unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole,

iii) optionally, an enzyme capable of cleaving a sequence within the peptide or protein,
iv) optionally, luciferase,
v) optionally, ATP,
vi) optionally, magnesium cation source,
vii) optionally, a means of detecting luminescence and measuring its intensity, and
viii) instructions for use.

13. Use of a kit of parts according to claim 12 for the determination of a sequence within a peptide or protein cleaved by a specific enzyme.

14. Method of determining a sequence within a peptide or protein cleaved by a specific enzyme comprising
i) providing a peptide or protein bearing at the C-terminal end the sequence cleaved by the specific enzyme followed by a D-cysteine residue or derivatives thereof,
ii) providing an unfunctionalized 6-hydroxy-2-cyanobenzothiazole or 6-amino-2-cyanobenzothiazole;
iii) providing an enzyme capable of cleaving a sequence within the peptide or protein,
iv) mixing the compounds of steps i), ii) and iii) and allowing them to react,
v) providing information on proteolytic cleavage of the peptide
vi) providing luciferase, and
vii) detecting and measuring a luminescence signal.

15. Method of determining the D-cysteine, L-cysteine, or derivatives thereof concentration in a cell or tissue sample comprising the steps of:
i) lysing a tissue sample and adding a reducing agent to it,

ii) adding unfunctionalized 6-hydroxy-2-cyanobenzothiazole or unfunctionalized 6-amino-2-cyanobenzothiazole,
iii) adding luciferase,
iv) allowing the mixture obtained in step iii) to react,
v) detecting and measuring a luminescence signal,
vi) extrapolating from said signal the D-cysteine or derivatives thereof concentration.

16. A method of measuring the cellular uptake of a small molecule, comprising the steps of:
i) Providing cells that contain both cysteine, in particular D-cysteine, or a derivative thereof; and a luciferase;
ii) Subjecting the cells of step i) to molecules according to any one of claims 1-3;
iii) Determining the reaction product within the cells of the molecule provided in step ii) and the cysteine or the derivative of cysteine provided in step i), preferably by means of determination of the intensity of luminescence.

Fig. 1:

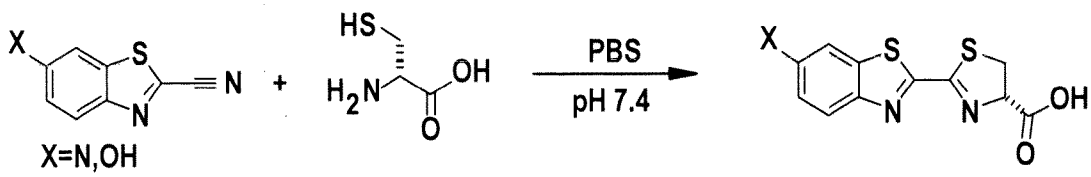


Fig. 2:

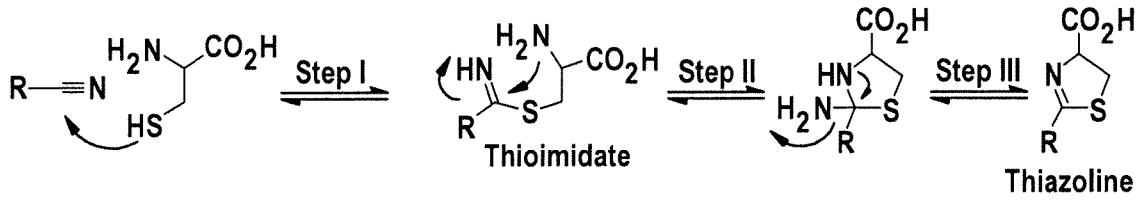


Fig. 3A:

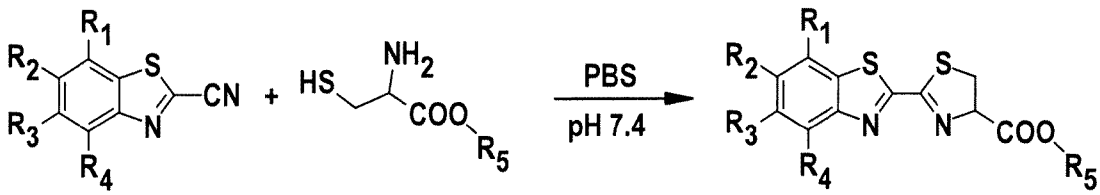


Fig. 3B:

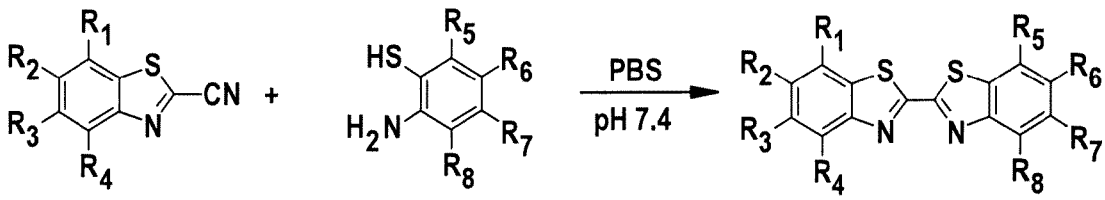


Fig. 3C:

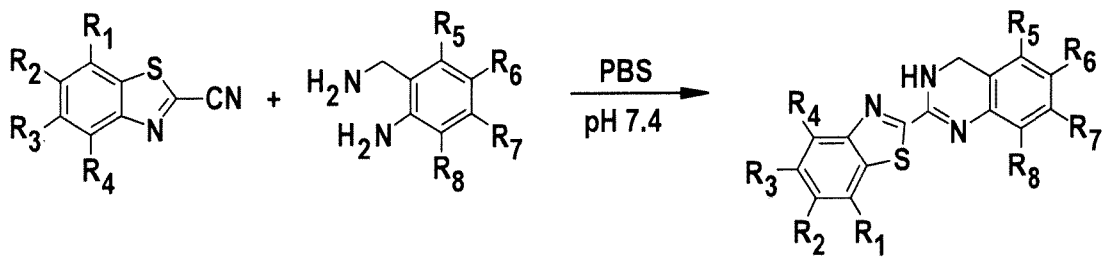


Fig. 3D:

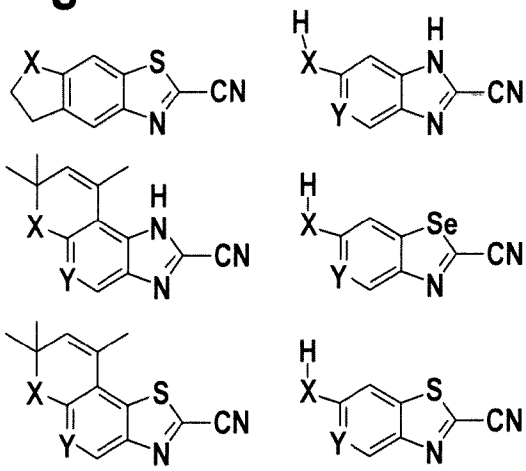


Fig. 4A:

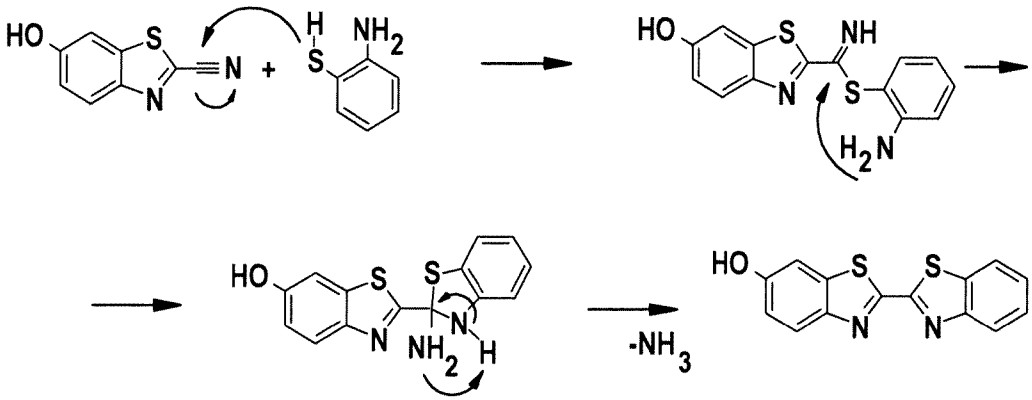


Fig. 4B:

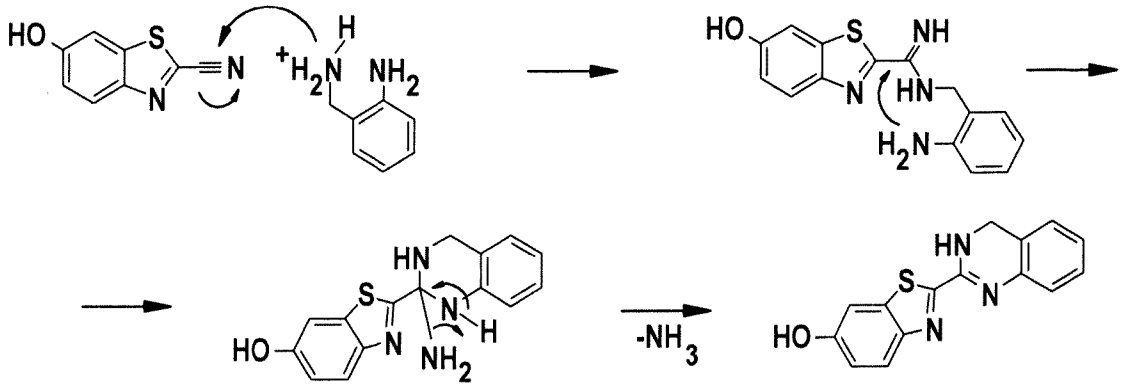


Fig. 4C:

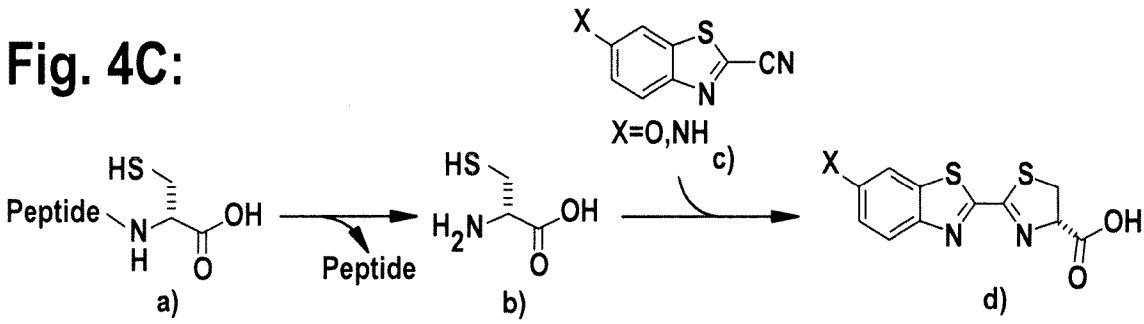

X
S
CN
N
X=O,NH
c)
HS
Peptide
N
H
OH
O
a)
Peptide
HS
H_2N
OH
O
b)
X
S
S
N
N
OH
O
d)

Fig. 5:

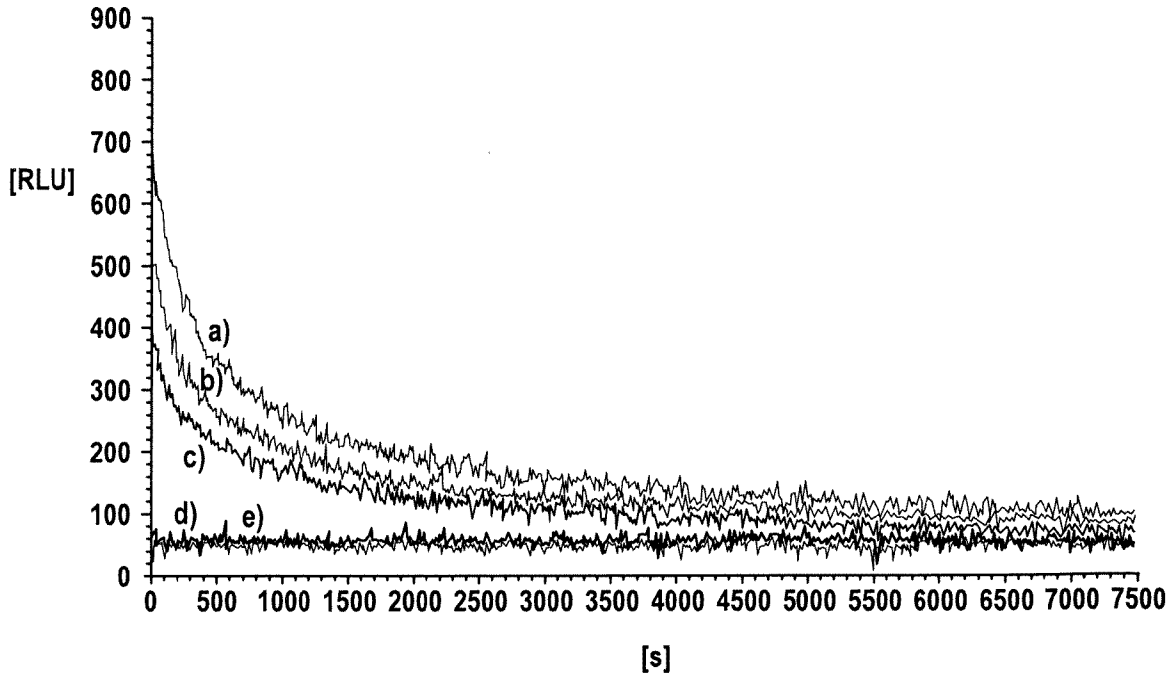

900
800
700
[RLU]
600
500
400
300
200
100
0
a)
b)
c)
d)
e)
0 500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500 6000 6500 7000 7500
[s]

Fig. 6:

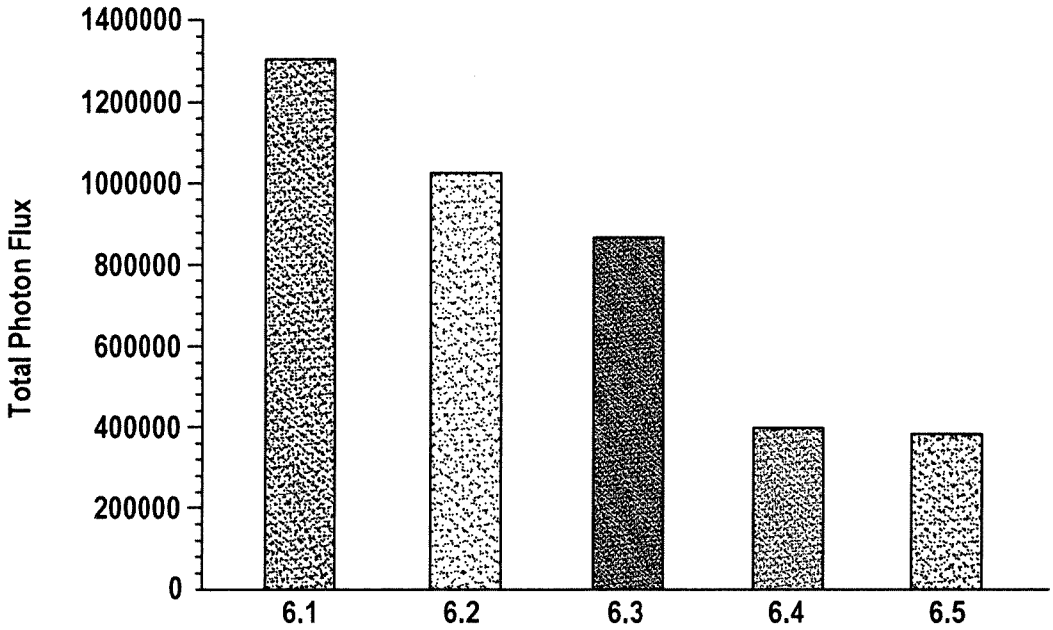

Total Photon Flux
1400000
1200000
1000000
800000
600000
400000
200000
0
6.1
6.2
6.3
6.4
6.5

Fig. 7:

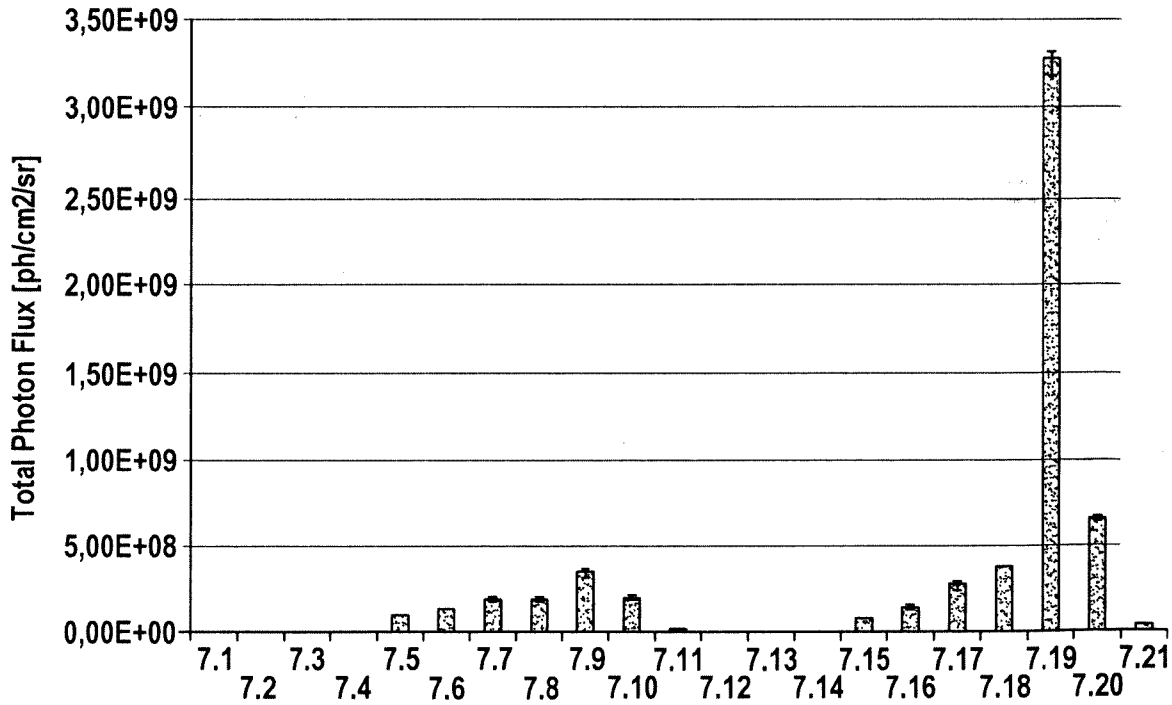

Total Photon Flux [ph/cm2/sr]
3,50E+09
3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
7.1
7.2
7.3
7.4
7.5
7.6
7.7
7.8
7.9
7.10
7.11
7.12
7.13
7.14
7.15
7.16
7.17
7.18
7.19
7.20
7.21

Fig. 8:

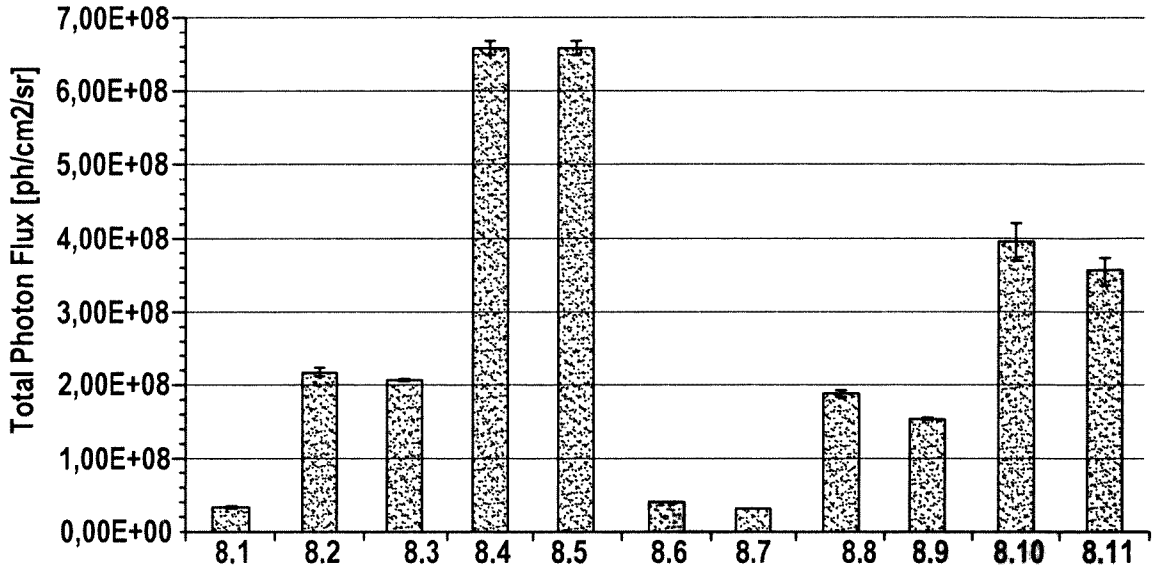

Total Photon Flux [ph/cm2/sr]
7,00E+08
6,00E+08
5,00E+08
4,00E+08
3,00E+08
2,00E+08
1,00E+08
0,00E+00
8.1
8.2
8.3
8.4
8.5
8.6
8.7
8.8
8.9
8.10
8.11

Fig. 9:

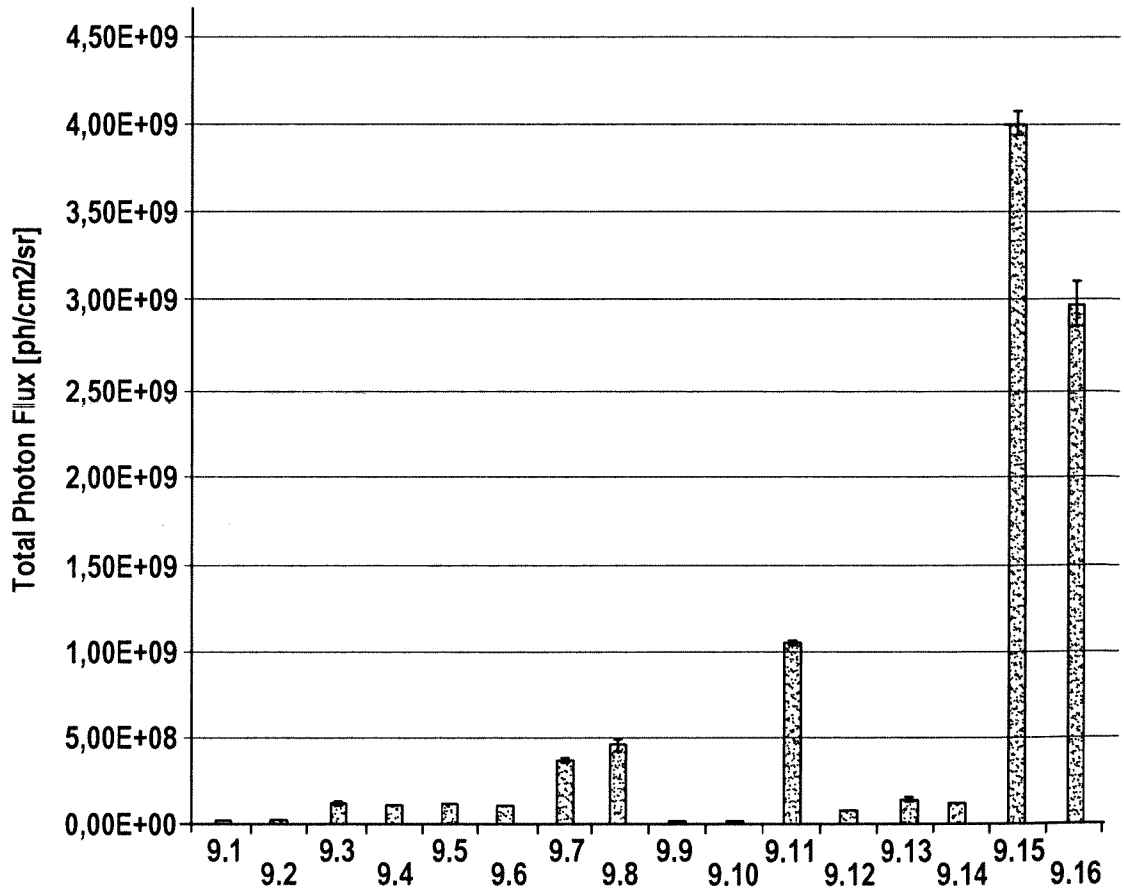

Total Photon Flux [ph/cm2/sr]
4,50E+09
4,00E+09
3,50E+09
3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
9.1
9.2
9.3
9.4
9.5
9.6
9.7
9.8
9.9
9.10
9.11
9.12
9.13
9.14
9.15
9.16

Fig. 10:

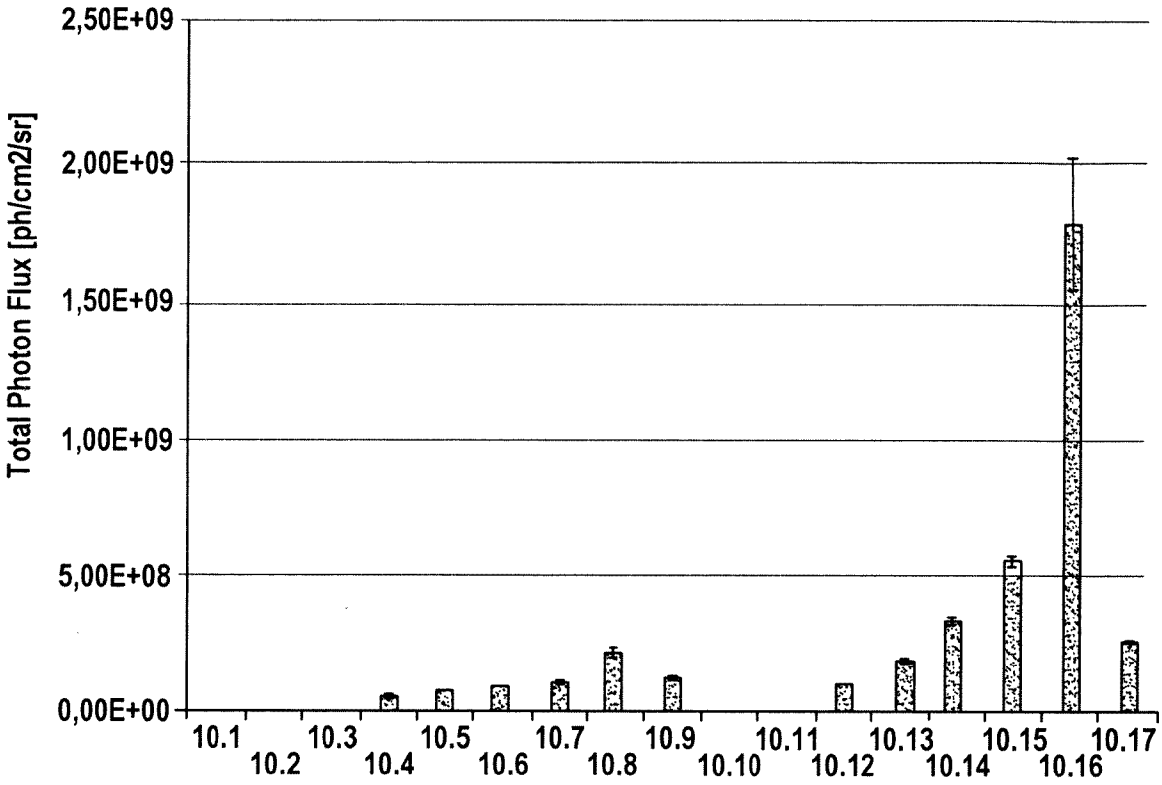

Total Photon Flux [ph/cm2/sr]
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
10.1
10.2
10.3
10.4
10.5
10.6
10.7
10.8
10.9
10.10
10.11
10.12
10.13
10.14
10.15
10.16
10.17

Fig. 11:

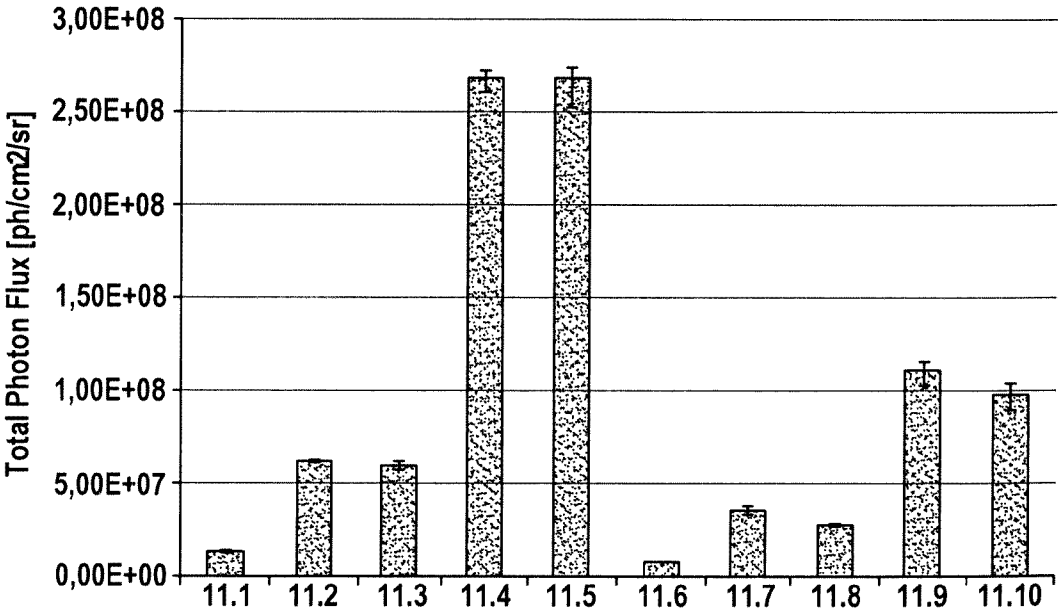

Total Photon Flux [ph/cm2/sr]
3,00E+08
2,50E+08
2,00E+08
1,50E+08
1,00E+08
5,00E+07
0,00E+00
11.1
11.2
11.3
11.4
11.5
11.6
11.7
11.8
11.9
11.10

Fig. 12:

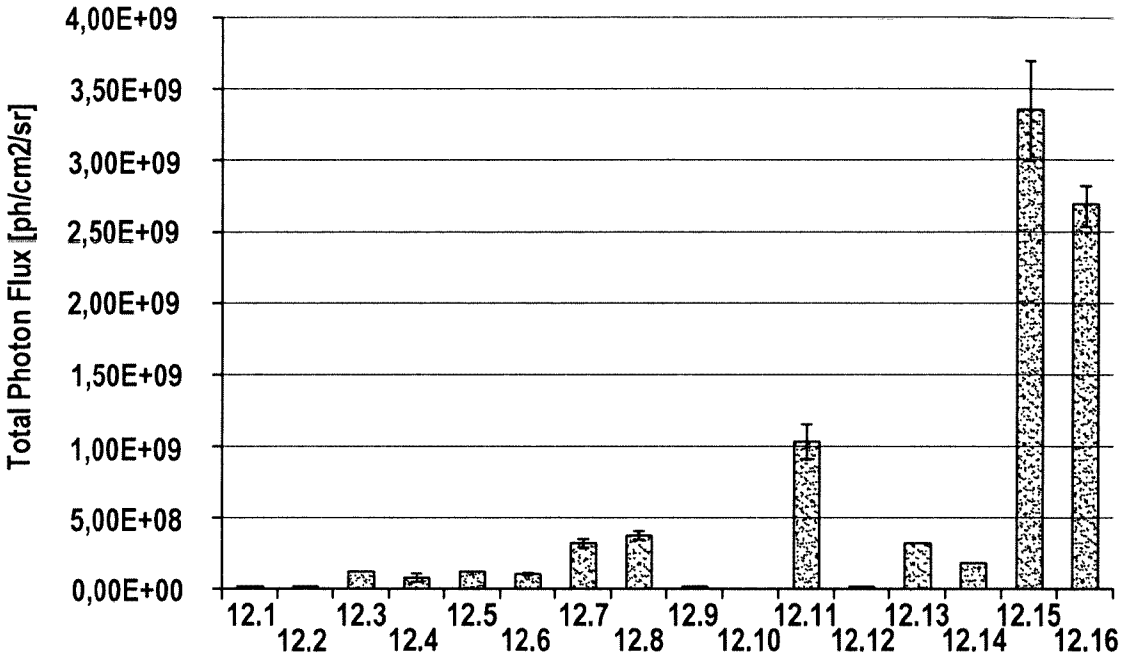

Total Photon Flux [ph/cm2/sr]
4,00E+09
3,50E+09
3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
12.1
12.2
12.3
12.4
12.5
12.6
12.7
12.8
12.9
12.10
12.11
12.12
12.13
12.14
12.15
12.16

Fig. 13:

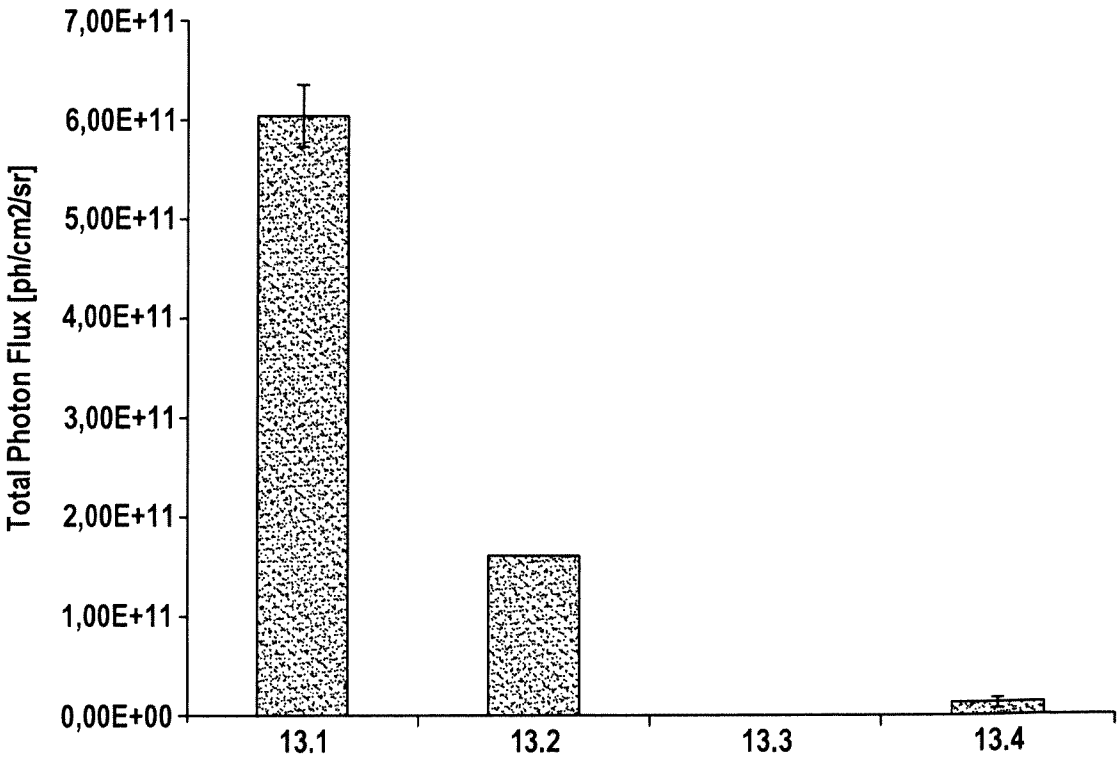

Total Photon Flux [ph/cm2/sr]
7,00E+11
6,00E+11
5,00E+11
4,00E+11
3,00E+11
2,00E+11
1,00E+11
0,00E+00
13.1
13.2
13.3
13.4

Fig. 14:

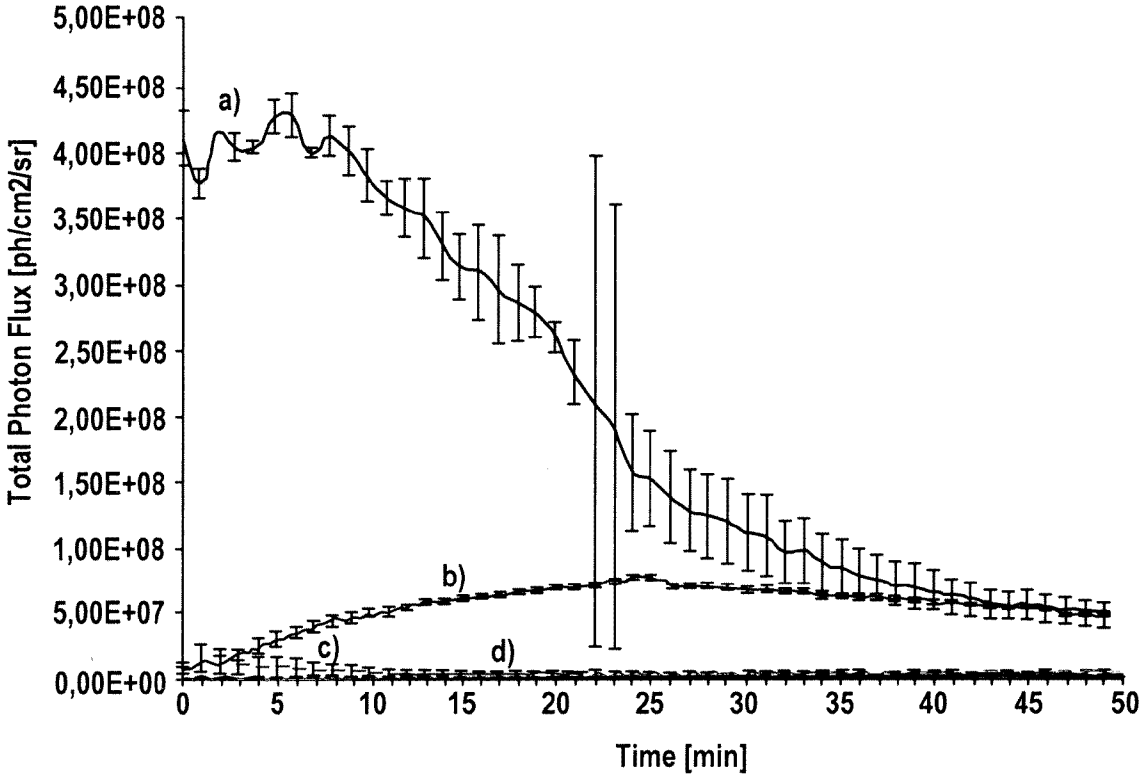

5,00E+08
4,50E+08
4,00E+08
3,50E+08
3,00E+08
2,50E+08
2,00E+08
1,50E+08
1,00E+08
5,00E+07
0,00E+00
Total Photon Flux [ph/cm2/sr]
a)
b)
c)
d)
0
5
10
15
20
25
30
35
40
45
50
Time [min]

Fig. 15:

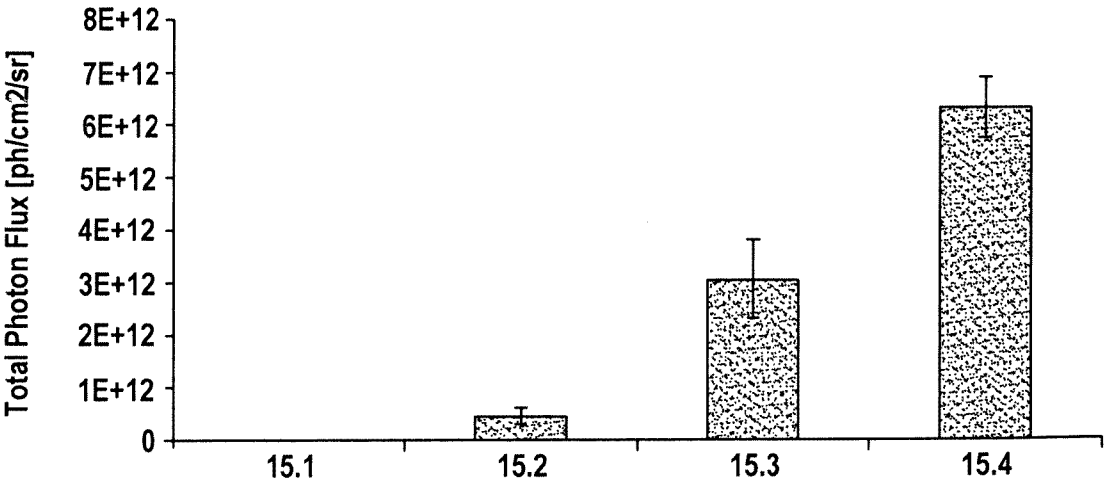

8E+12
7E+12
6E+12
5E+12
4E+12
3E+12
2E+12
1E+12
0
Total Photon Flux [ph/cm2/sr]
15.1
15.2
15.3
15.4

Fig. 16:

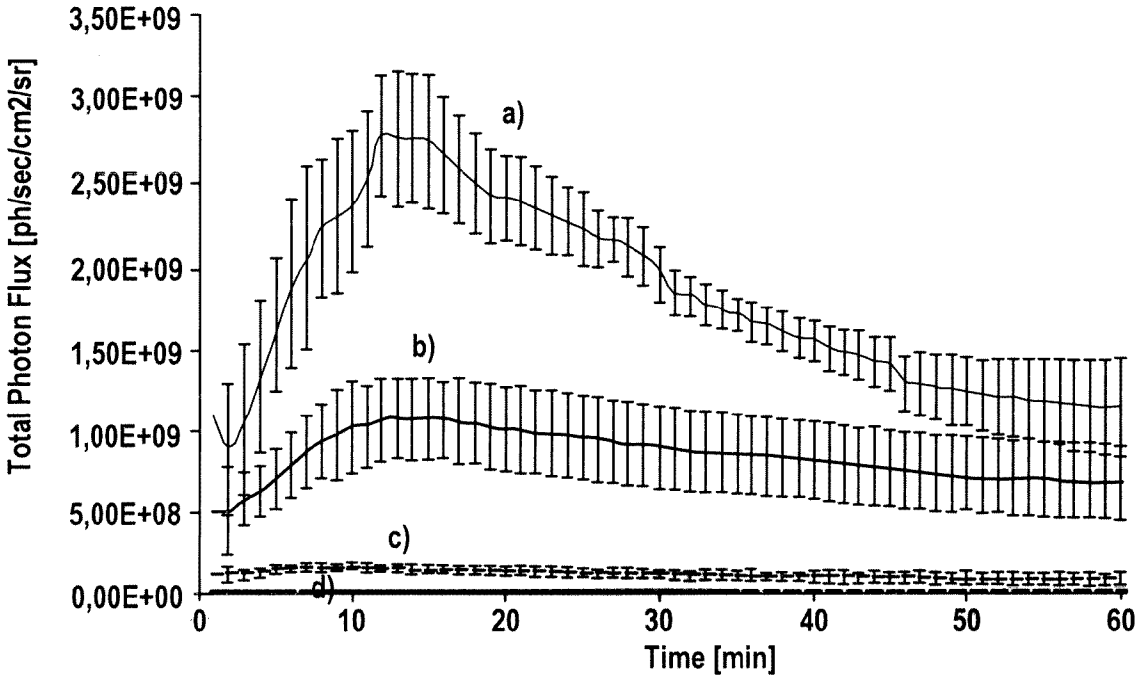

3,50E+09
3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
Total Photon Flux [ph/sec/cm2/sr]
a)
b)
c)
d)
0
10
20
30
40
50
60
Time [min]

Fig. 17:

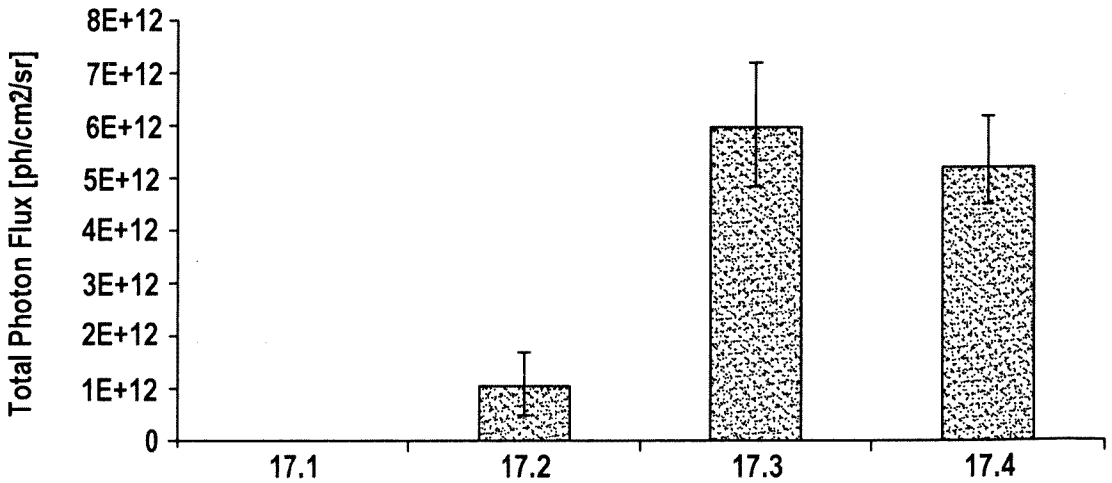

8E+12
7E+12
6E+12
5E+12
4E+12
3E+12
2E+12
1E+12
0
Total Photon Flux [ph/cm2/sr]
17.1
17.2
17.3
17.4

Fig. 18:

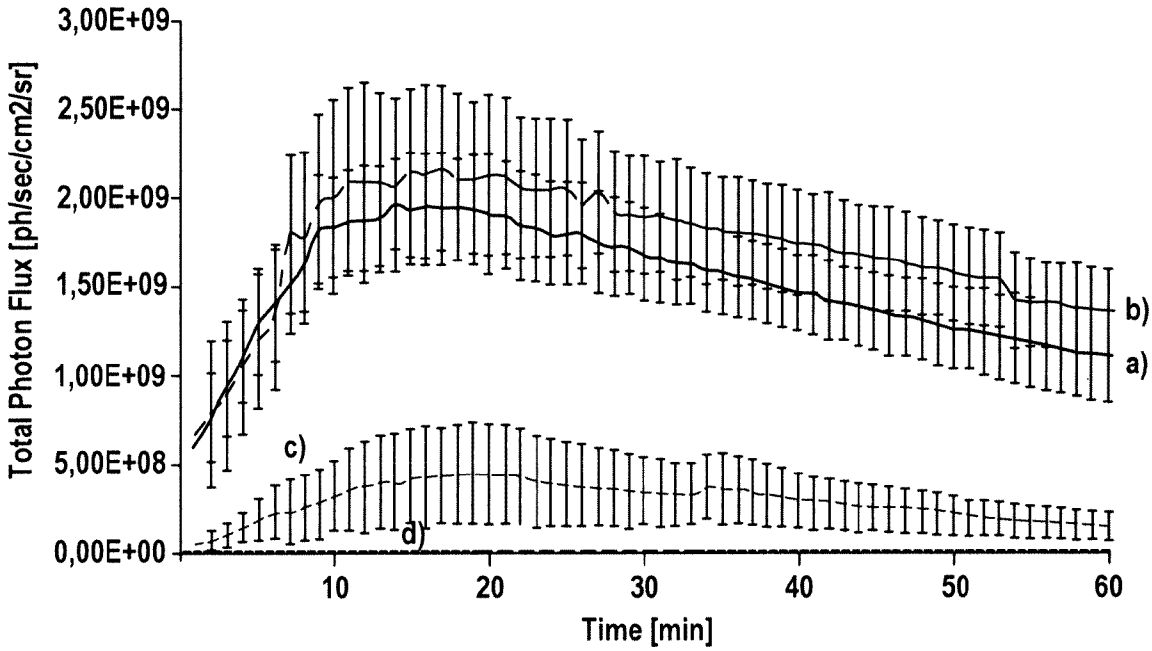

3,00E+09
2,50E+09
2,00E+09
1,50E+09
1,00E+09
5,00E+08
0,00E+00
Total Photon Flux [ph/sec/cm2/sr]
10
20
30
40
50
60
Time [min]
a)
b)
c)
d)

Fig. 19:

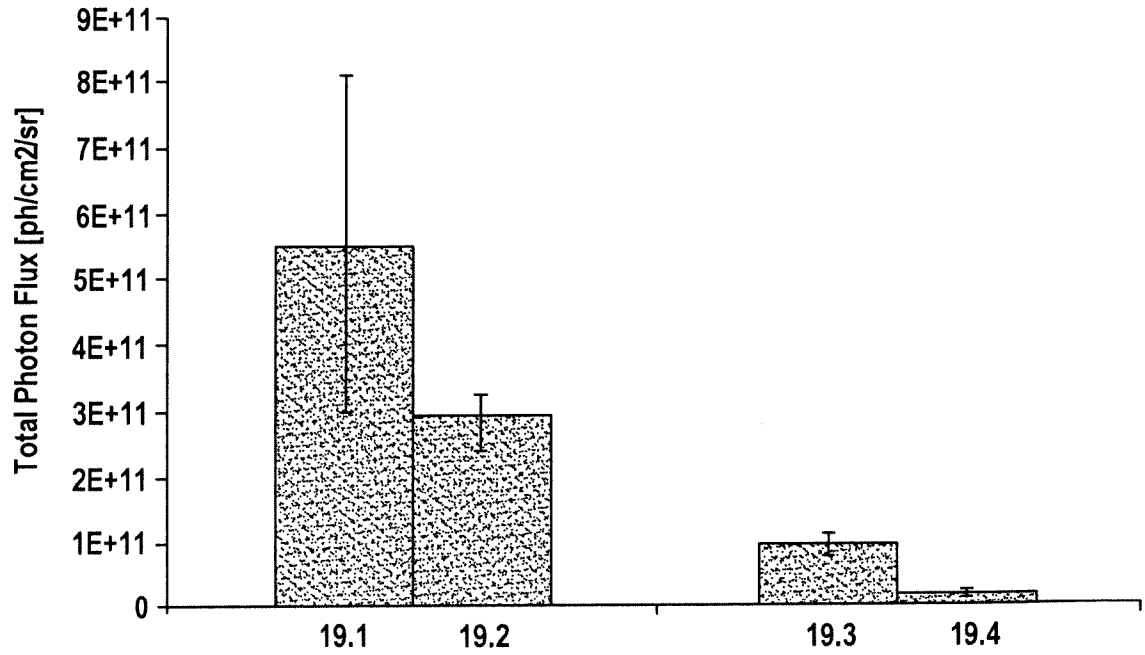

9E+11
8E+11
7E+11
6E+11
5E+11
4E+11
3E+11
2E+11
1E+11
0
Total Photon Flux [ph/cm2/sr]
19.1
19.2
19.3
19.4

Fig. 20:

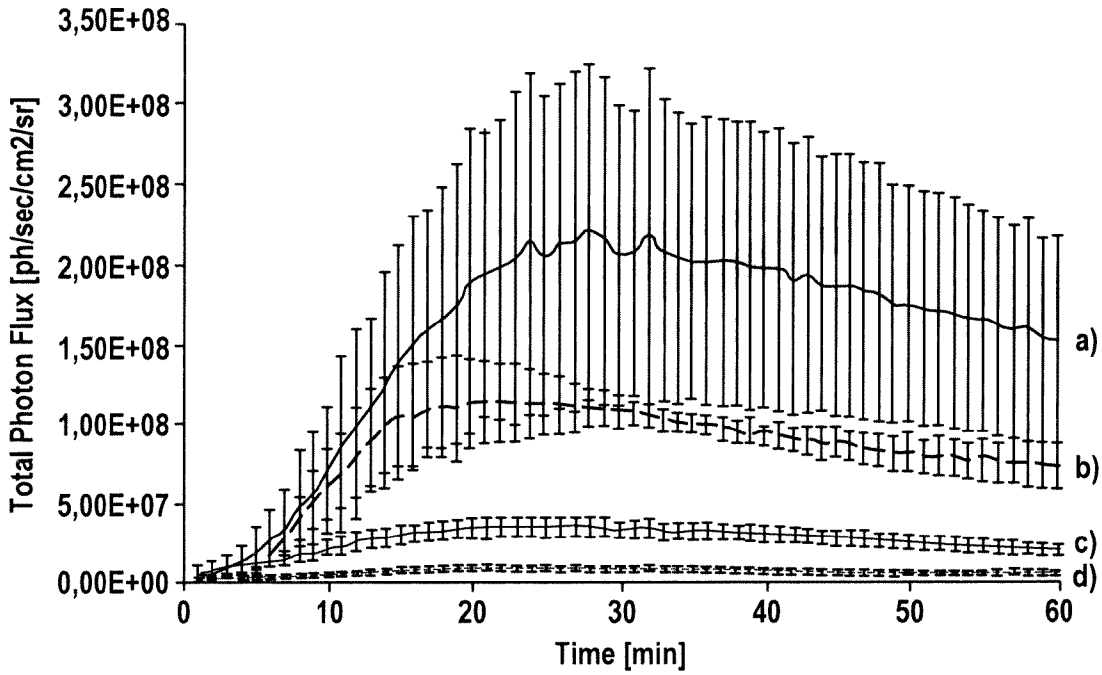

3,50E+08
3,00E+08
2,50E+08
2,00E+08
1,50E+08
1,00E+08
5,00E+07
0,00E+00
Total Photon Flux [ph/sec/cm2/sr]
0
10
20
30
40
50
60
Time [min]
a)
b)
c)
d)

Fig. 21:

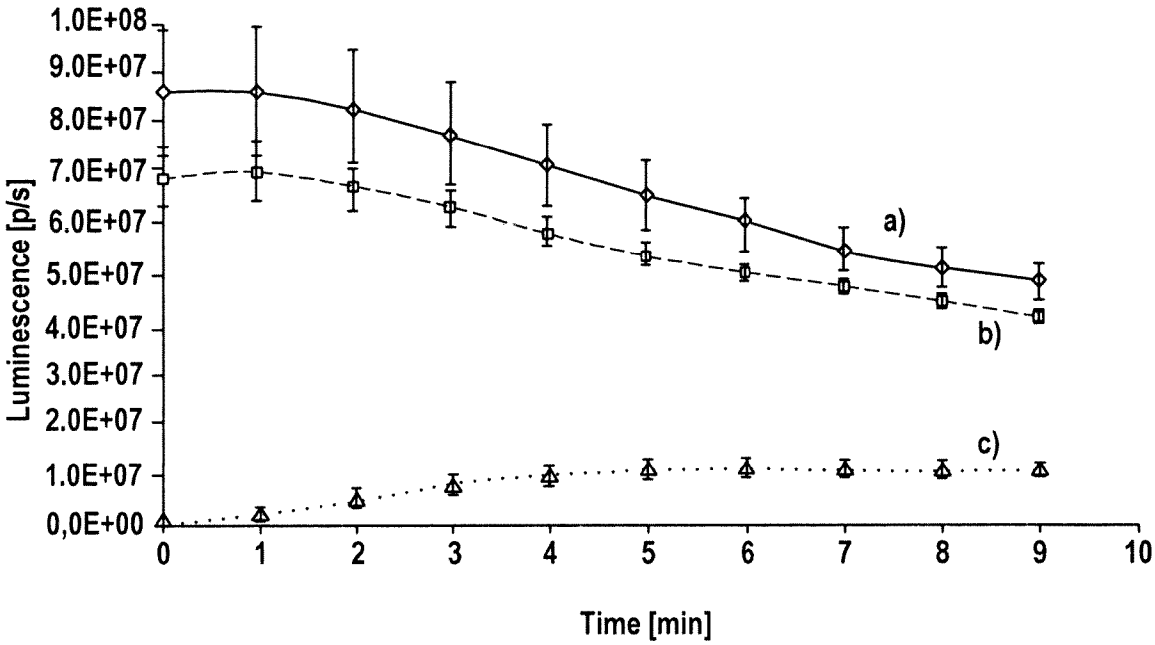

1.0E+08
9.0E+07
8.0E+07
7.0E+07
6.0E+07
5.0E+07
4.0E+07
3.0E+07
2.0E+07
1.0E+07
0,0E+00
Luminescence [p/s]
0
1
2
3
4
5
6
7
8
9
10
Time [min]
a)
b)
c)

Fig. 22:

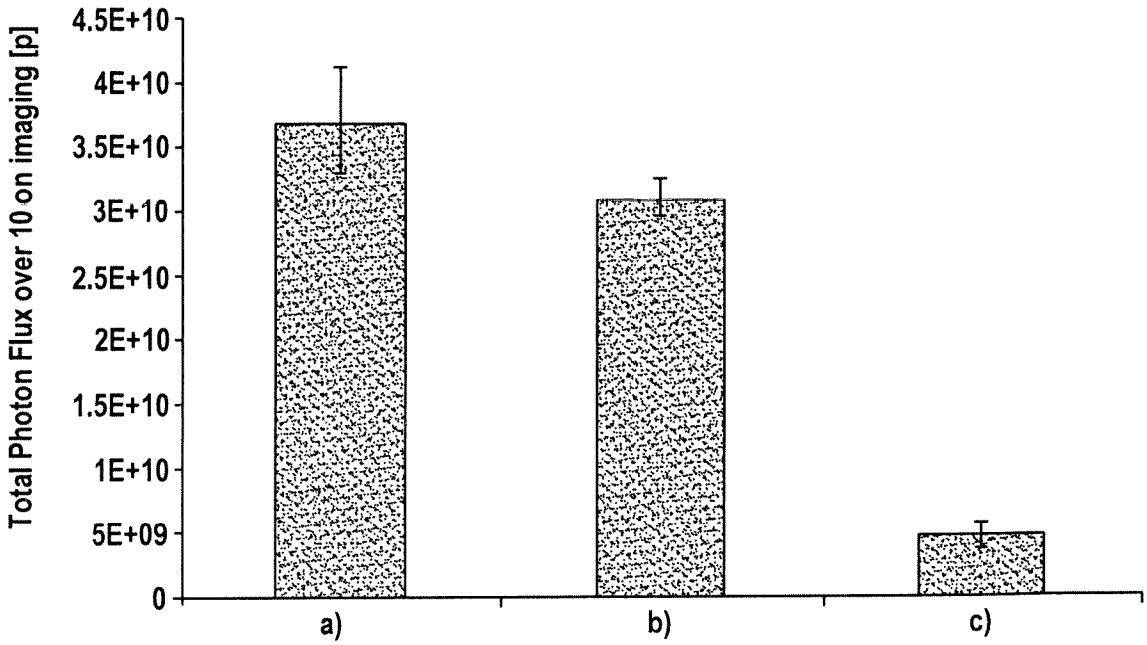

4.5E+10
4E+10
3.5E+10
3E+10
2.5E+10
2E+10
1.5E+10
1E+10
5E+09
0
Total Photon Flux over 10 on imaging [p]
a)
b)
c)

Fig. 23:

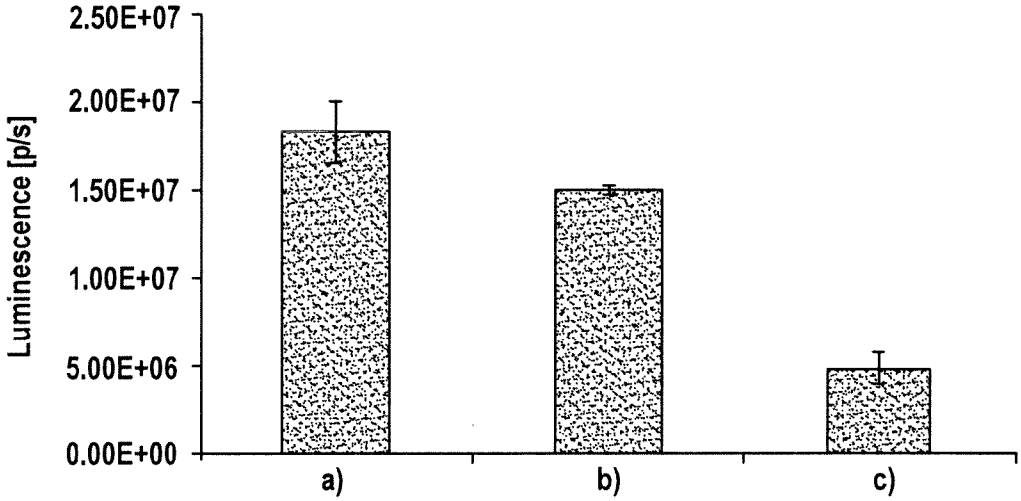

2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Luminescence [p/s]
a)
b)
c)

Fig. 24:

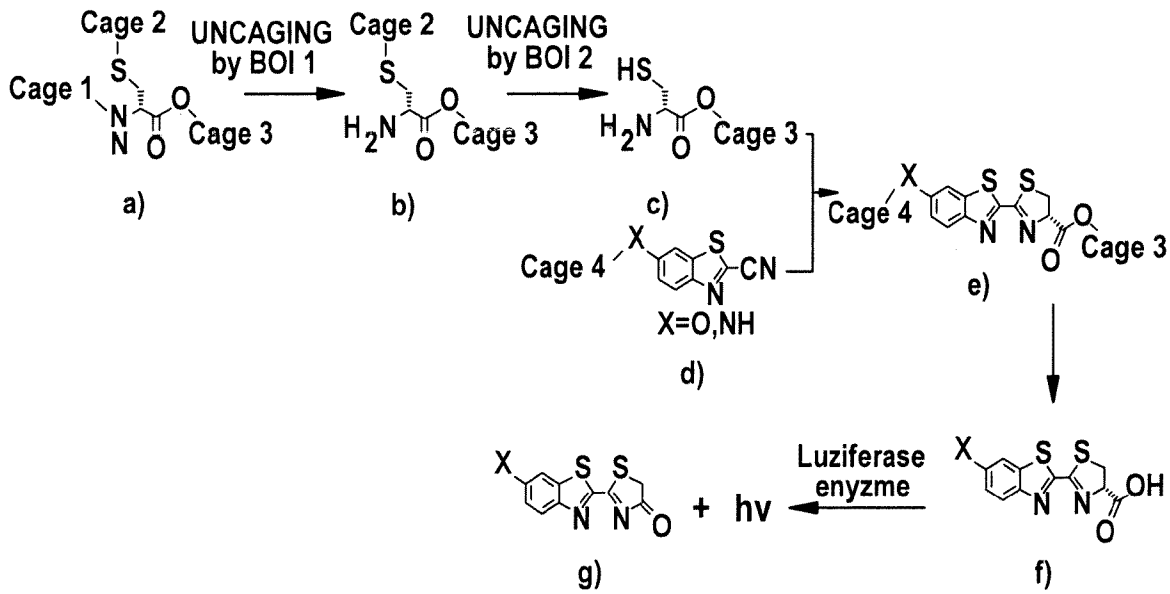

Cage 2
Cage 1
Cage 3
UNCAGING by BOI 1
UNCAGING by BOI 2
HS
a)
b)
c)
Cage 4
X=O,NH
d)
e)
Luziferase enyzme
+ hv
f)
g)

Fig. 25:

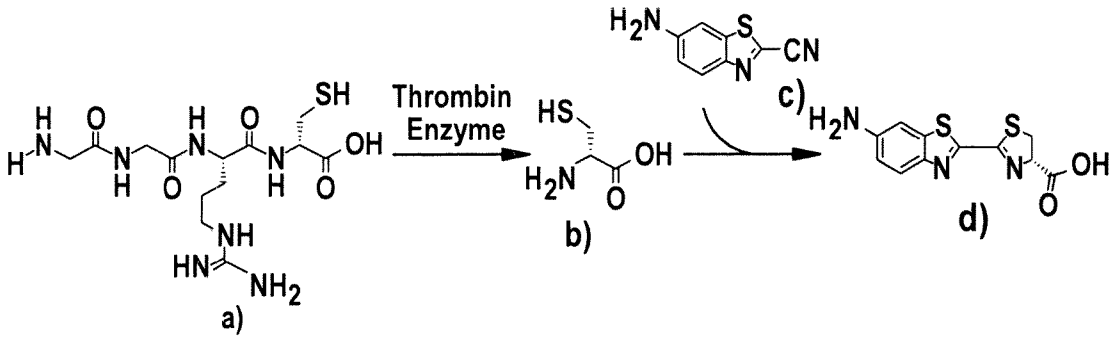


Fig. 26:

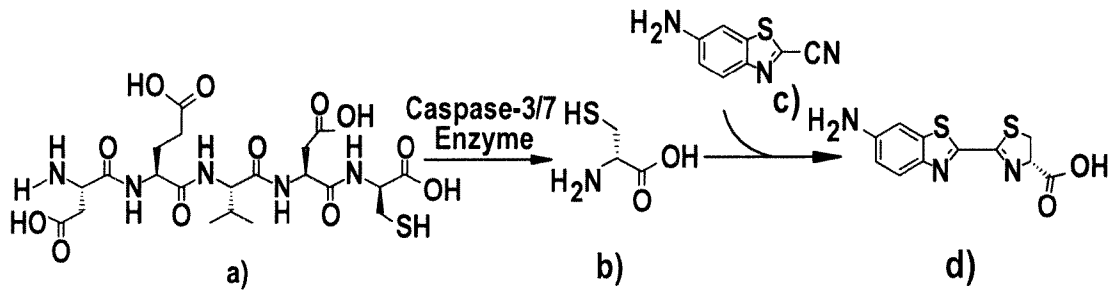


Fig. 27:

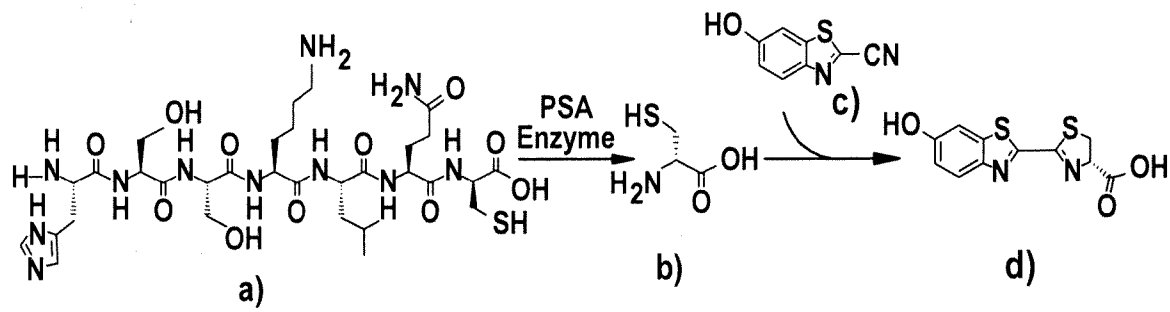


Fig. 28:

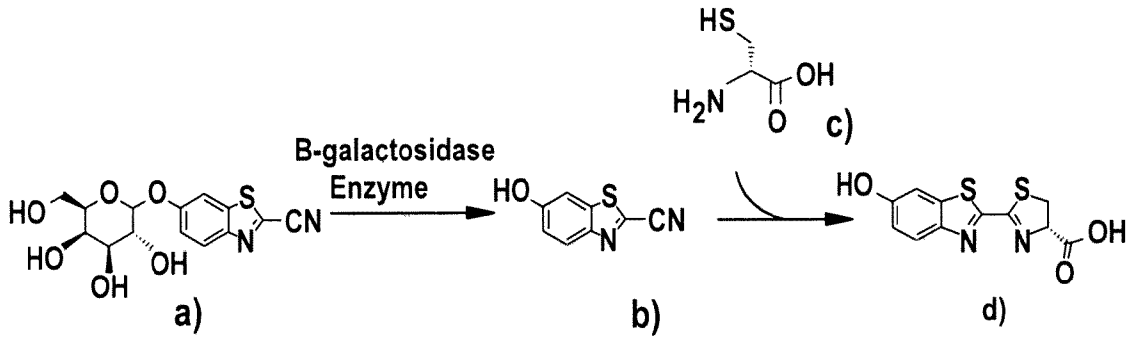

HS
OH
H_2N
O
c)
B-galactosidase
Enzyme
HO
HO
OH
OH
O
O
S
N
CN
a)
HO
S
N
CN
b)
HO
S
S
N
N
OH
O
d)

Fig. 29:

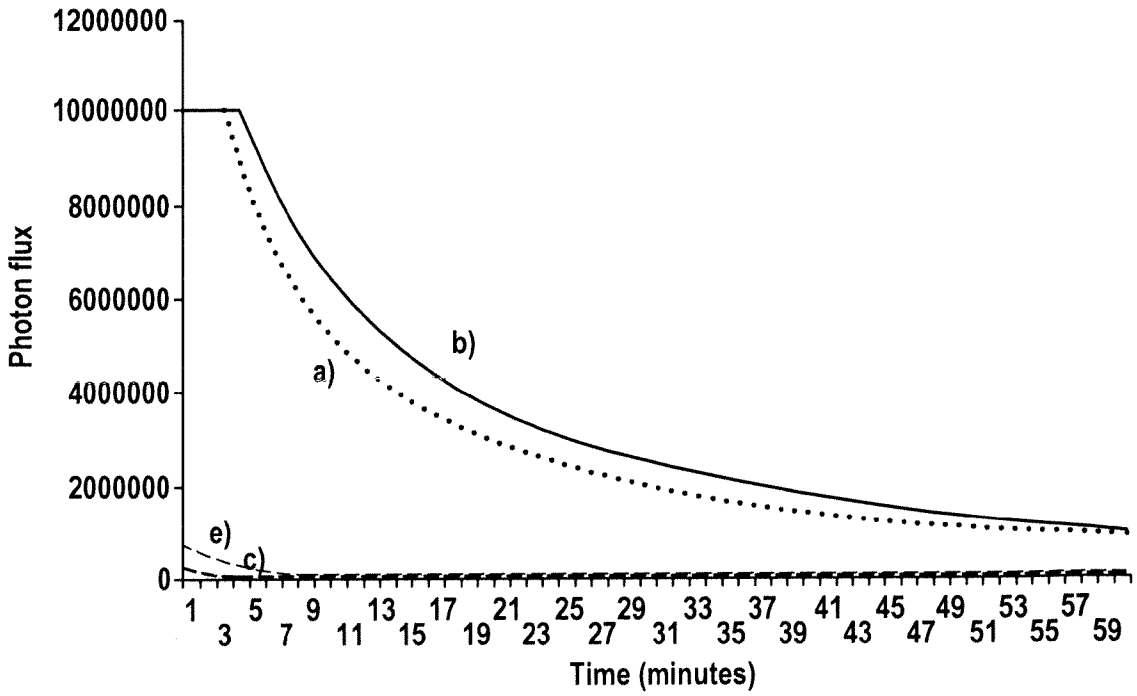

12000000
10000000
8000000
6000000
4000000
2000000
0
Photon flux
a)
b)
c)
e)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Time (minutes)

Fig. 30:

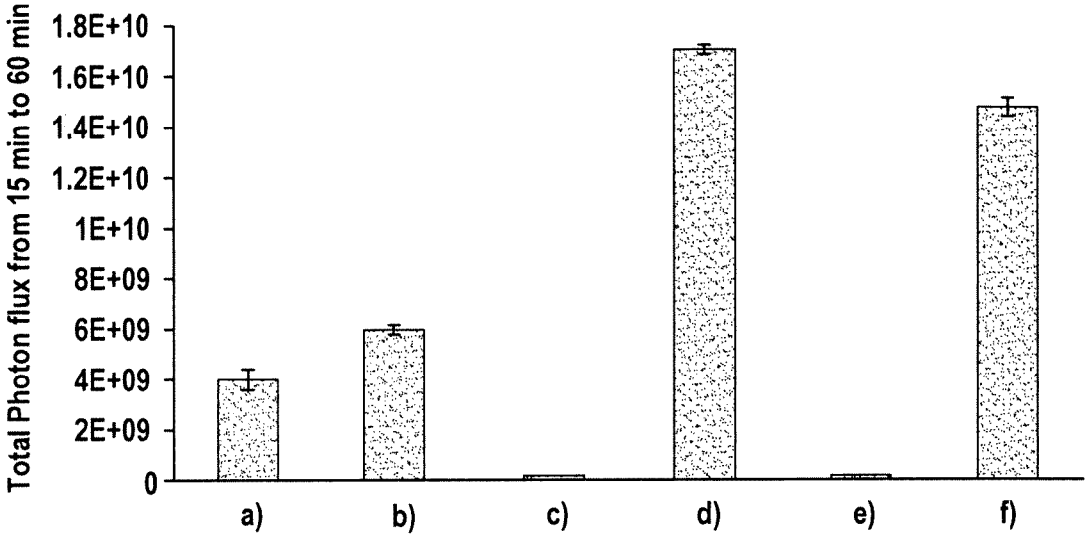

Total Photon flux from 15 min to 60 min
1.8E+10
1.6E+10
1.4E+10
1.2E+10
1E+10
8E+09
6E+09
4E+09
2E+09
0
a)
b)
c)
d)
e)
f)

Fig. 31:

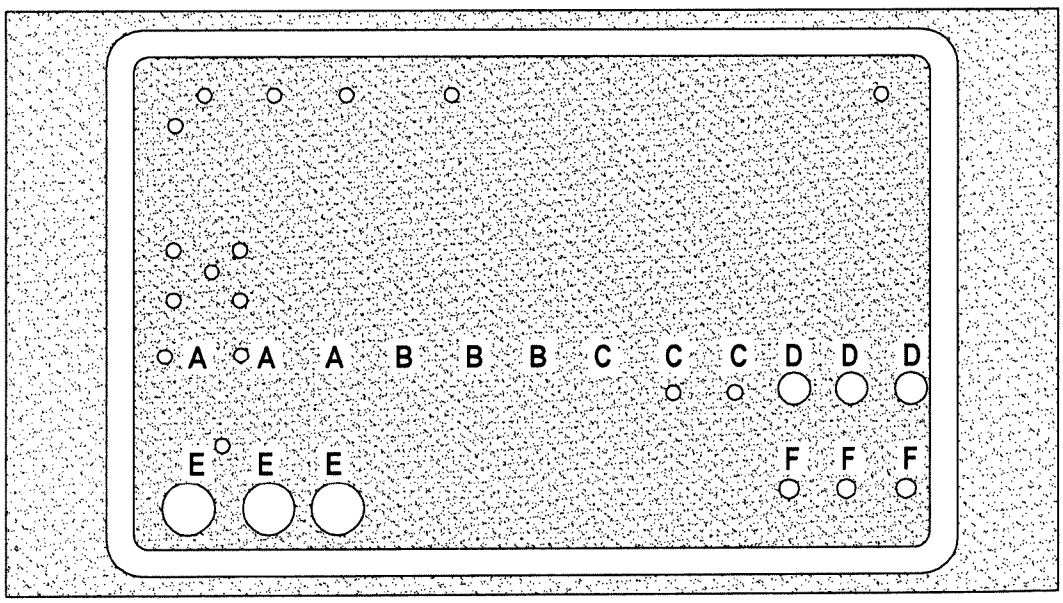

A A A B B B C C C D D D
E E E
F F F

Fig. 32:

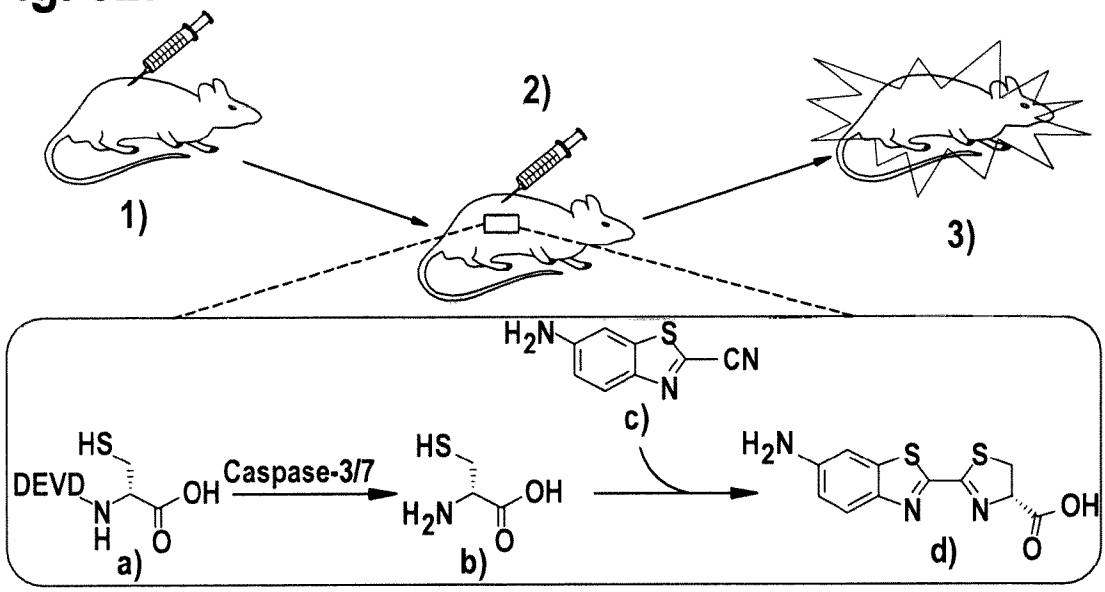


Fig. 33:

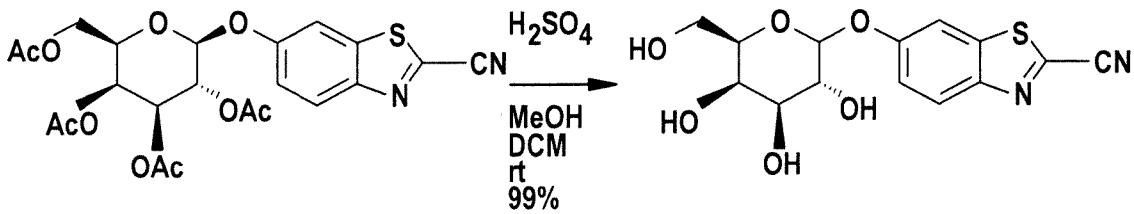


Fig. 34:

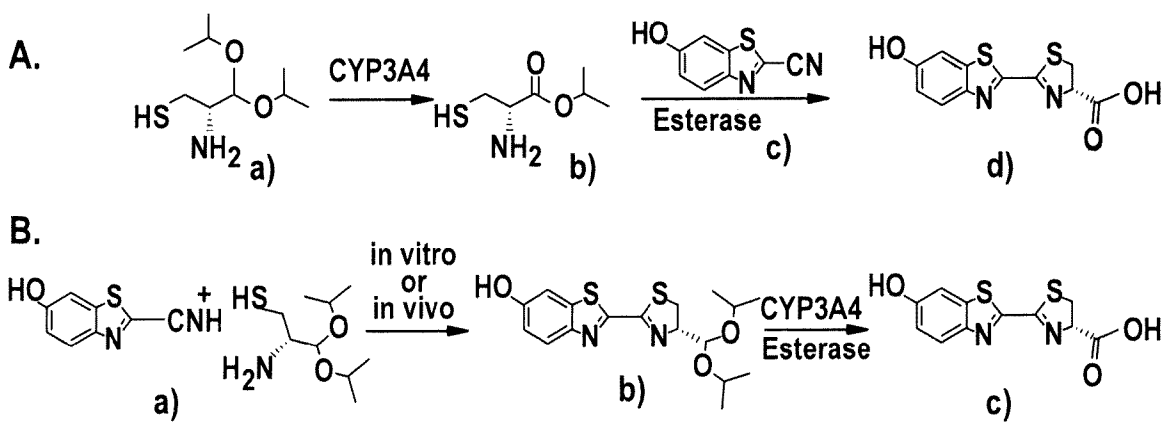


Fig. 35:

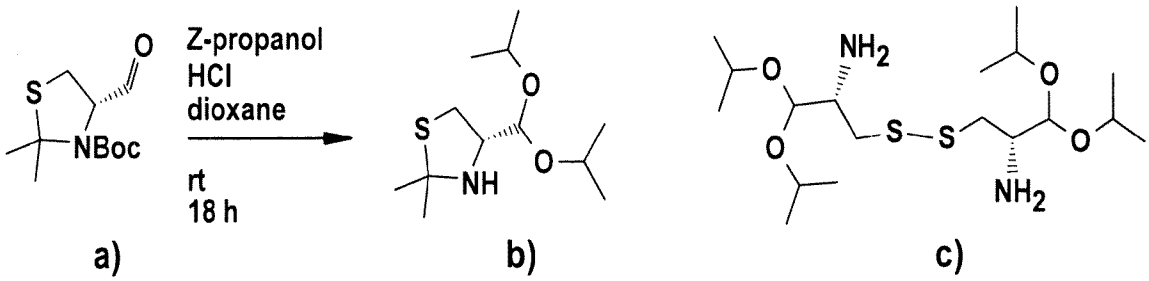


Fig. 36:

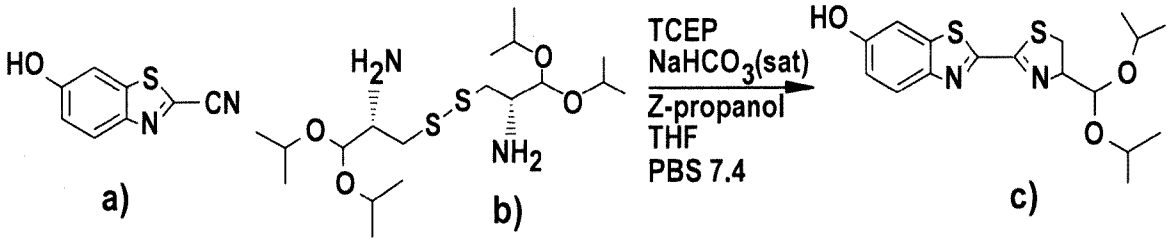


Fig. 37:

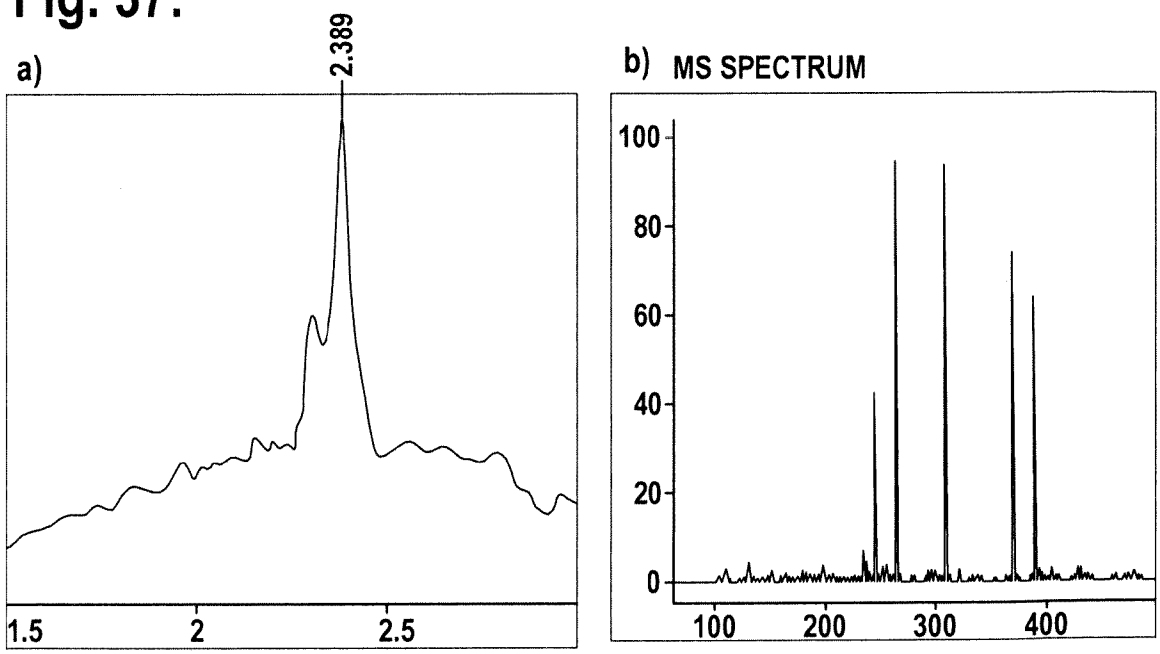


Fig. 38

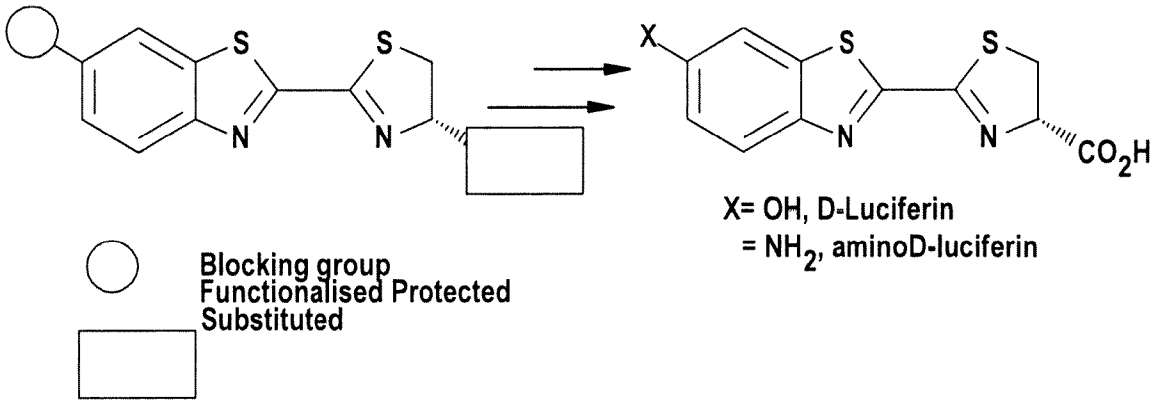

X= OH, D-Luciferin
= NH2, aminoD-luciferin
Blocking group
Functionalised Protected
Substituted

Fig. 39

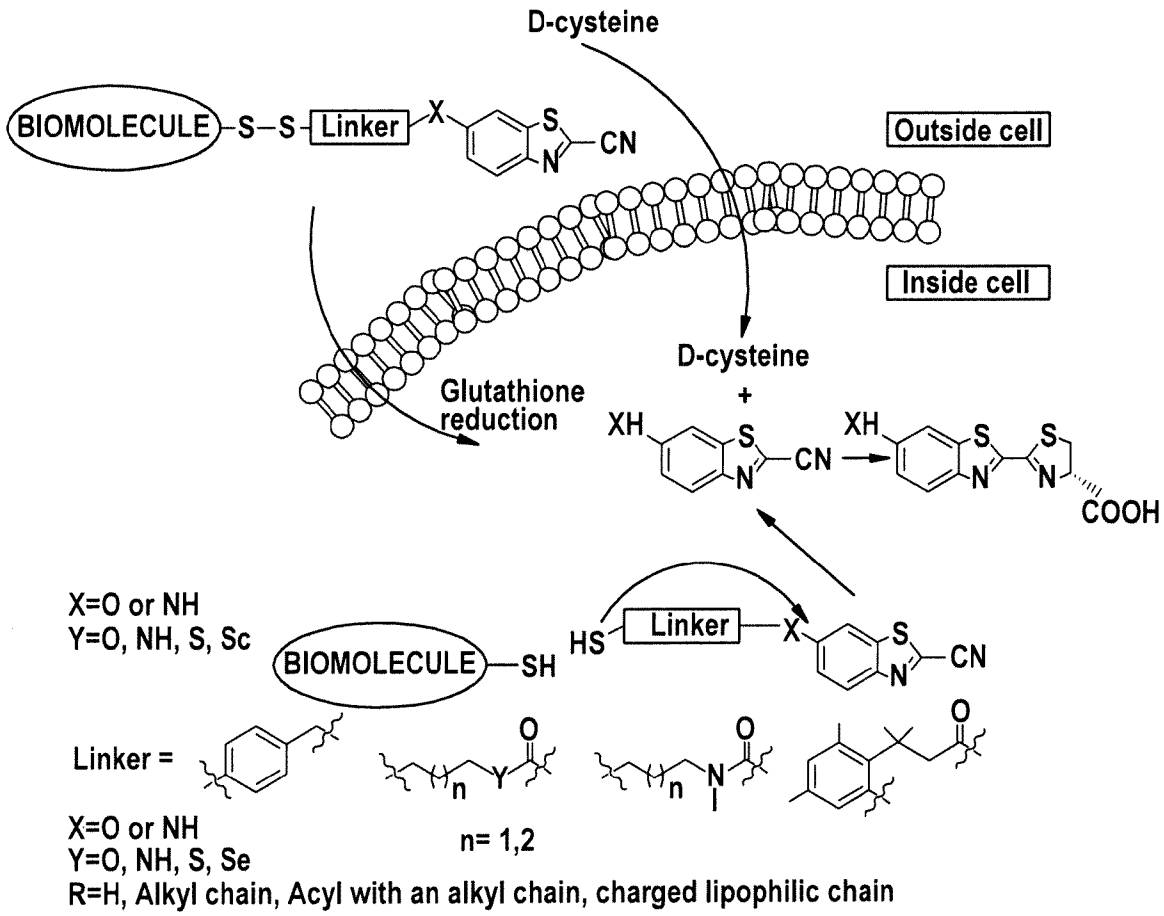
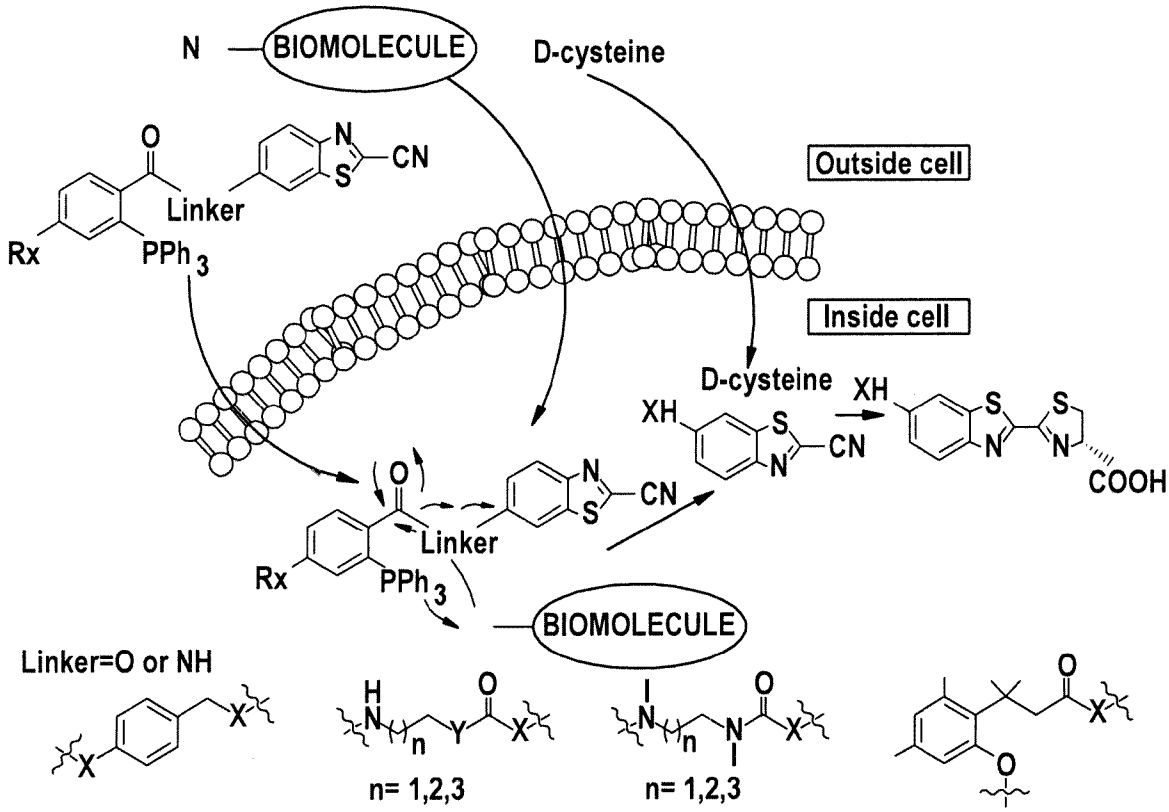

D-cysteine
BIOMOLECULE
Linker
Outside cell
Inside cell
Glutathione reduction
D-cysteine
+
X=O or NH
Y=O, NH, S, Sc
Linker =
X=O or NH
Y=O, NH, S, Se
n= 1,2
R=H, Alkyl chain, Acyl with an alkyl chain, charged lipophilic chain

Fig. 40



X=O or NH
Y=O, NH, S, Se
R=H, Alkyl chain, Acyl with an alkyl chain, charged lipophilic chain

Fig. 41

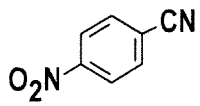
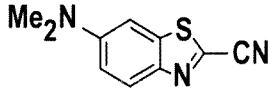
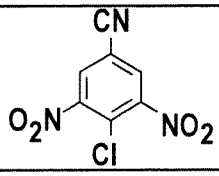
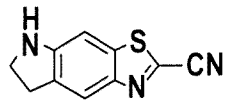
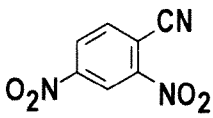
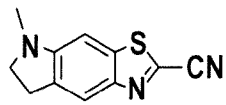
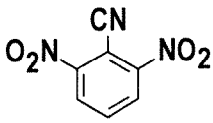
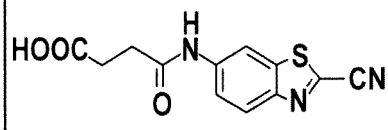
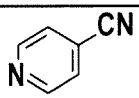
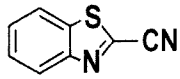
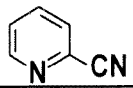
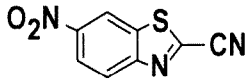
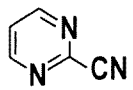
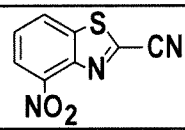
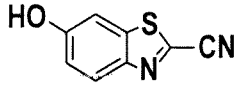
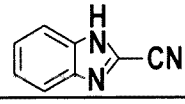
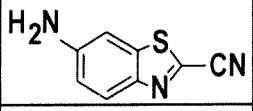
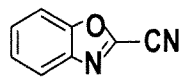
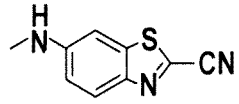
1		0.00117± 0.000196	11		1.22= 0.332
2		0.00248± 0.000316 (S_NAr reaction constant)	12		1.81= 0.089
3		0.0155= 0.00034 (S_NAr reaction constant)	13		0.688± 0.1479
4		0.00132± 0.000124 (S_NAr reaction constant)	14		7.10 ± 0.106
5		0.00512± 0.000397	15		n.d.
6		0.00617± 0.000558	16		n.d.
7		0.572±0.0797	17		n.d.
8		3.24±0.117	18		n.d.
9		2.63±0.264	19		n.d.
10		1.23±0.164			

Fig. 42

Nitrile Compound (Fig. 41) reacted with cysteine	[M+H] + of product, found	[M+H] + of product, calculated
1	253.0291	253.0283
5	209.0417	209.0385
6	209.0370	209.0385
7	210.0324	210.0332
8	280.9991	281.0055
9	280.0200	280.0209
10	294.0366	294.0365
11	308.0536	308.0517
12	306.0353	306.0365
13	320.0509	320.0522
14	380.0372	380.0369

Fig. 43

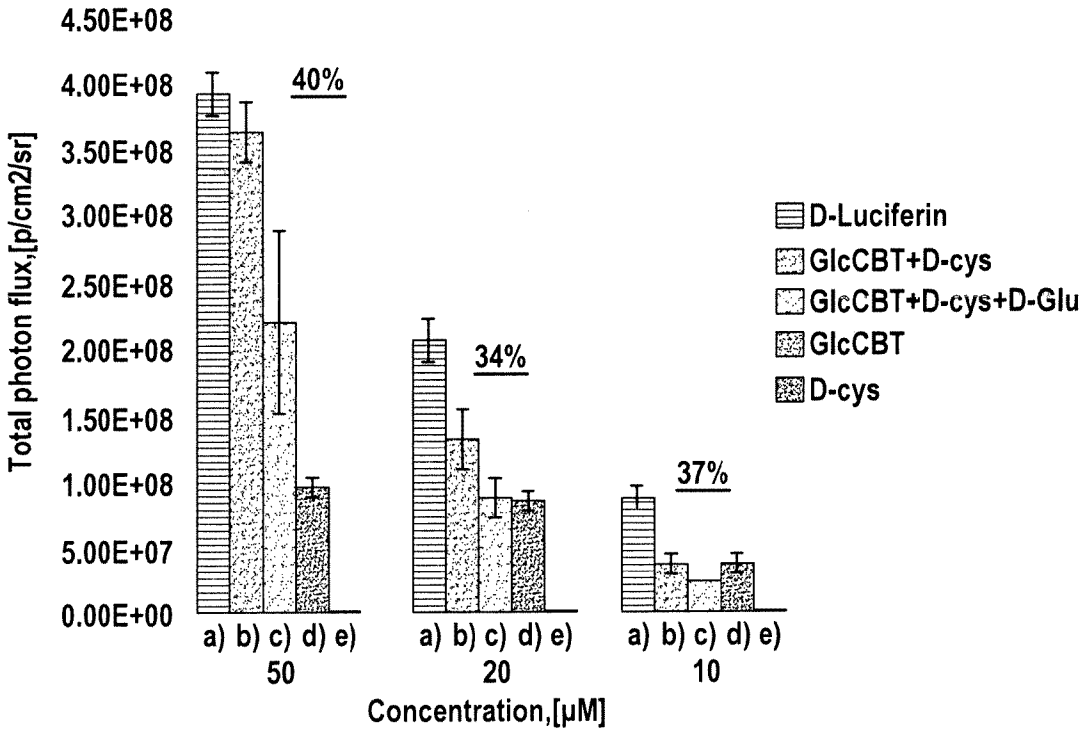


Fig. 44