Chem Soc Rev

RSC Publishing

REVIEW ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 662

Cage the firefly luciferin! – a strategy for developing bioluminescent probes

Jing Li, Laizhong Chen, Lupei Du and Minyong Li*

Received 9th July 2012

DOI: 10.1039/c2cs35249d

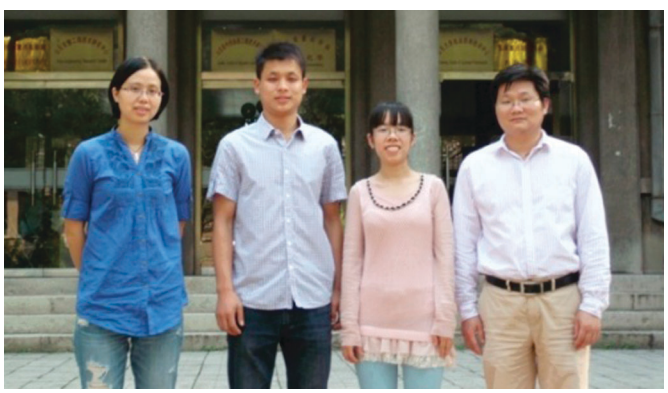
www.rsc.org/csr

Bioluminescent imaging (BLI) has been widely applicable in the imaging of process envisioned in life sciences. As the most conventional technique for BLI, the firefly luciferin–luciferase system is exceptionally functional *in vitro* and *in vivo*. The state-of-the-art strategy in such a system is to cage the luciferin, in which free luciferin is conjugated with distinctive functional groups, thus accommodating an impressive toolkit for exploring various biological processes, such as monitoring enzymes activity, detecting bioactive small molecules, evaluating the properties of molecular transporters, *etc.* This review article summarizes the rational design of caged luciferins towards diverse biotargets, as well as their applications in bioluminescent imaging. It should be emphasized that these caged luciferins can stretch out the applications of bioluminescence imaging and shed light upon understanding the pathogenesis of various diseases.

1. Introduction

Bioluminescence is a natural phenomenon that emits visible light produced by a chemical reaction within a living organism. This occurrence can be found in various living organisms, including bacteria, marine organisms and insects.[1–5] The North American firefly (*Photinus pyralis*) is one of the main luminous species found in insects.[6] As the most studied bioluminescent system, the firefly luciferin–luciferase reaction has been well studied for at last 50 years. In such a firefly luciferase–luciferin system, at least two chemicals are demanded: one is called luciferin that produces the light after being oxidized by an enzyme, and the other is called luciferase, which can catalyze the oxidation of luciferin. Other molecules, such as oxygen, ATP

Department of Medicinal Chemistry, Key Laboratory of Chemical Biology of Natural Products (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China. E-mail: mli@sdu.edu.cn; Fax: +86-531-8838-2076; Tel: +86-531-8838-2076



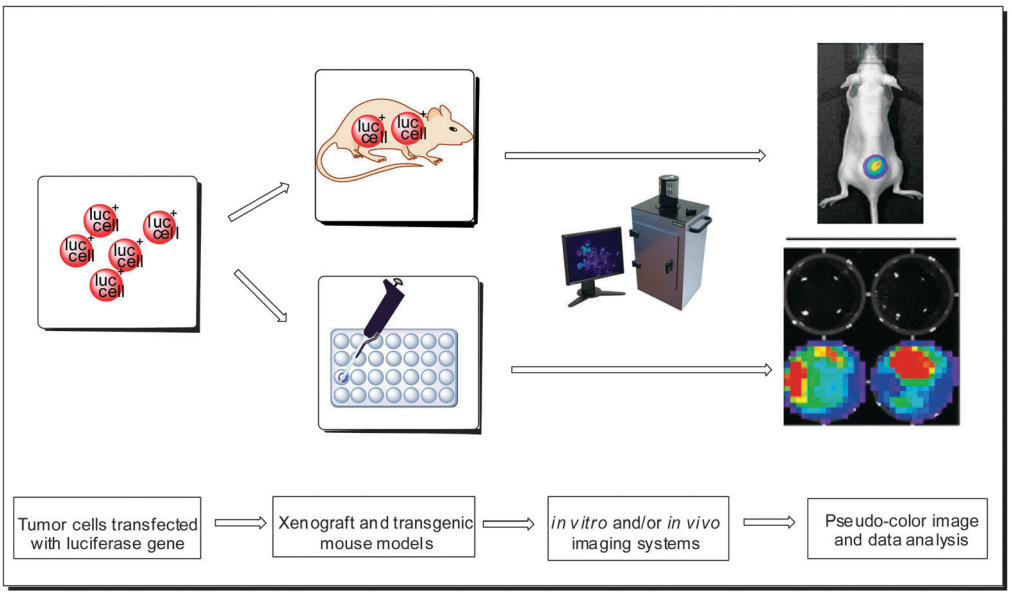
Lupei Du, Laizhong Chen, Jing Li and Minyong Li

Jing Li received her bachelor's degree at the School of Pharmacy, Shandong University in 2010. She joined the Li group and pursued her master's degree at the same school. Her research interests mainly focus on the design and bioactivity study of bioluminescent probes.

Laizhong Chen obtained his bachelor's degree at the School of Pharmacy, Shandong University in 2009. He stayed at Shandong University to complete his PhD in the Li lab. His main research interests include the design of APN (aminopeptidase N) probes and their biological application.

Lupei Du is currently an associate professor at the School of Pharmacy, Shandong University. In 2006 she obtained her PhD degrees from China Pharmaceutical University, and got her postdoctoral training at the Department of Chemistry, Georgia State University (2006–2009). She joined Shandong University in 2009. Her main research interests include the rational design and synthesis of medicinal molecules and bioactive probes.

Minyong Li is a professor at the School of Pharmacy, Shandong University. He received his PhD degree from China Pharmaceutical University in 2005. He began his academic career in 2005 with Dr Binghe Wang at the Department of Chemistry, Georgia State University as a postdoctoral research associate. From 2007 to 2009, he was a research assistant professor at the Department of Chemistry, Georgia State University. In 2009, he moved to his current institution as a professor. His research interests are in the general areas of medicinal chemistry and chemical biology.



Scheme 1 Scheme of bioluminescent imaging.

and magnesium ions acting as co-factors, may also be necessitated. Firefly luciferase (Fluc) can catalyze a two-step oxidation of luciferin (LH_2), thus emitting yellow to green light, as well as releasing oxyluciferin, CO_2 and AMP. It needs to be underlined that the light can be emanated, ranging from 530 to 640 nm, depending on the pH,[7,8] polarity of the solvent,[9] microenvironment of the enzyme[10] and a manifold of other reasons.[11,12] A highly sensitive charge-coupled device (CCD) camera can capture such light shedded by oxyluciferin, noninvasively, to create a pseudo-color image based on the light intensity that is superimposed on a reference photograph of the animals. For that reason, the location and mass of luciferase-labeled cells can be visualized very well (Scheme 1).

The reliable, sensitive, convenient and non-invasive bioluminescent imaging has had a profound impact on the fundamental understanding of *in vivo* biology and will particularly upgrade the application of small animal models in the laboratory. Previously, considering that BLI enables diverse features to be visualized, a number of implementations of BLI have been extensively described for monitoring cells and biomolecular processes in living subjects, including pathogen detection,[13] tumor growth[14] and responses to therapy patterns of gene regulation,[15] measurements of protein–protein interactions[16] and ADMET (absorption, distribution, metabolism, excretion and toxicity).[17] Furthermore, BLI allows for longitudinal and duplicate imaging without killing the animal models, therefore, the processes of the living can be detected in real time and noninvasively other than an endpoint determination by sacrificing the animals.

Firefly luciferin is a highly specific substrate for firefly luciferase, therefore, the modification at the 6′- or 4-position inhibits the interaction between luciferin and luciferase. Consequently, numerous luciferin derivatives as luminogenic substrates provide a new pathway into the bioluminescent imaging *in vitro* and *in vivo*, and thereafter expand the scope of BLI.

The current review article summarizes caged luciferins and their application in detecting vital processes. A brief discussion on the bioluminescent advantages and a survey of current native and synthesized substrates catalyzed by luciferase will also be presented in this review. Furthermore, exceptional endeavours have been concentrated on the present-day advances in bioluminescent probe design and quantitative mechanism determination by strength of output luciferase.

2. The advantage of bioluminescent imaging (BLI)

In recent years, some noninvasive imaging methods, such as magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), BLI, and fluorescence imaging (FLI), have been well introduced. Compared with other imaging methods, BLI and FLI are more sensitive, convenient and easily-handled. Since a vast number of fluorescent and bioluminescent probes have been described and much attention has been drawn to the investigation of new probes and new assay methods in the past few decades, the following two paragraphs will mainly focus on the comparison of BLI and FLI.

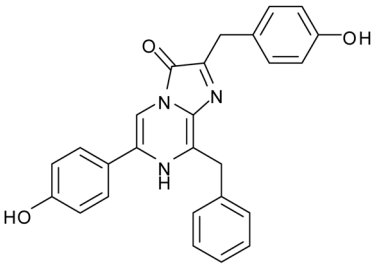
Thus far, numerous commercial fluorescent probes are available to boost the development of life sciences significantly; hence FLI has been immensely used in detecting diversified living processes due to its low cost and high speed. The noninvasive FLI needs excitation light, which is ordinarily <600 nm, that can lead to tissue auto-fluorescence and cell damage and can be quenched by tissue components vigorously. Therefore, it cannot be applied for deep tissue imaging. The sensitivity of the FLI can also be influenced by various factors, and the background may be high. Accordingly, much effort has

been paid to discover probes with excitation and emission wavelengths between 600 and 900 nm, especially to locate fluorescent probes that emit far-red and near infra-red light. It needs to be emphasized that these novel fluorescent molecules can provide optimal and safe profiles for live animals, thus resulting in high tissue penetration and low auto-fluorescence.[18]

Compared with FLI, BLI does not demand an excitation light source. Considering that most of the cell- or animal-based models do not express luciferase, thus ensuring the absence of background signals, BLI can be carried out in absolute darkness to avoid the effect of the emission light. What is more, the broad spectrum of oxyluciferin with a large component above 600 nm drives the absorption of hemoglobin and melanin to be relatively low. The sensitivity of BLI detection is determined by the emission spectra of bioluminescent reporters as well as the interaction with mammalian tissue. However, some drawbacks have also emerged during the study of bioluminescent imaging. One of the key limitations is the lack of alternative luciferase substrates. Only luciferin and aminoluciferin are widely applied in animal bioluminescent imaging. What is more, the luciferase–luciferin reaction requires many cofactors, such as ATP and Mg^{2+}. Thus, this method is limited in engineered cells or small animal models. It is a long way off from being used in humans. The half time of luciferin and aminoluciferin is relatively short, so it is not suitable for approaches that require long integration times such as three-dimensional animal bioluminescent imaging.[19] The visible light produced by luciferase can also be attenuated due to the absorption and scattering of light by mammalian tissues.[20] In the longer term, there are ongoing efforts to overcome these disadvantages and improve the BLI method.

3. The chemistry and biological processes of firefly bioluminescence

Several luciferases have been well identified in nature, and most of them have been facilitated for BLI studies in mammals, such as firefly (*Photinus pyralis*) luciferase, green or red click beetle (*Pyrophorus plagiophthalamus*) luciferases, *Renilla* luciferase, *Gaussia* luciferase and bacteria luciferase. Firefly luciferase (FLuc) from *Photinus pyralis* is a monomeric enzyme of 61 kD that catalyzes the oxidation of D-luciferin in the presence of ATP, Mg^{2+} and oxygen.[21] This reaction system is the main method used for current animal imaging research with an emission wavelength at about 600 nm. Another luciferase isolated from the click beetle (*Pyrophorus plagiophthalamus*) is an enzyme of 61 kD, which also employs D-luciferin to produce green-orange (544 nm) or red (611 nm) light.[22] *Renilla* (sea pansy) and *Gaussia* (marine copepod) from the sea both utilize coelenterazine (Fig. 1) as a substrate and produce blue light with a peak intensity around 480 nm. The auto-oxidation of coelenterazine leads to high background signals. Consequently, the facilitation of these luciferases is restricted.[23–25] The light-emitting reaction in bacteria produces blue light through the oxidation of reduced riboflavin phosphate (FMNH2) and a long chain fatty aldehyde.[26] The substrates employed by bacteria luciferase are accessible in normal cells. The nonluminescent



coelenterazine

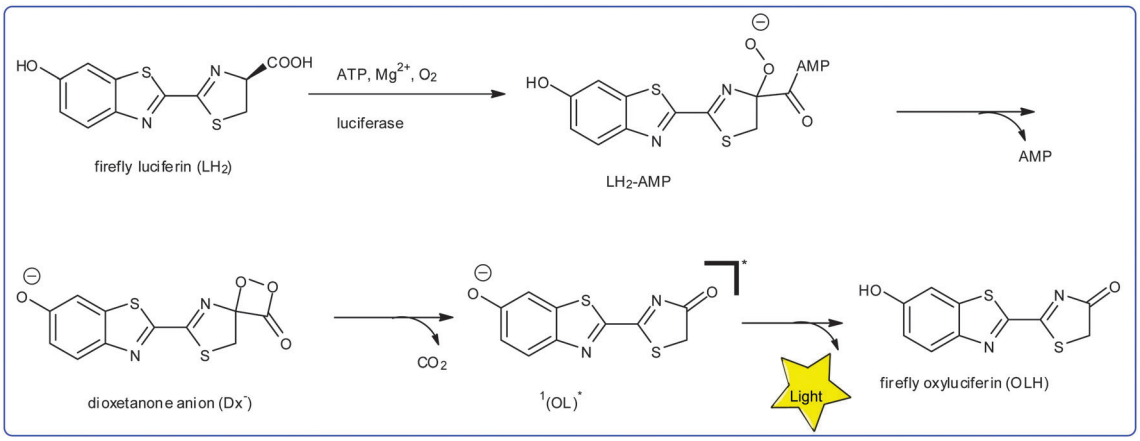
Fig. 1 The structure of coelenterazine.

bacteria can glow in the dark by marking with the lux gene cluster that encodes the bacteria luciferase. These bioluminescent bacteria infect the host animal and throw light upon the study of pathogenic processes of infection.[27] In view that the firefly luciferase–luciferin system is the most studied in bioluminescence imaging, we will focus on the chemical and biological processes of the firefly luciferase–luciferin reaction hereinafter.

Fluc is able to catalyze the oxidation of firefly luciferin (LH_2) in the presence of ATP, Mg^{2+} and oxygen. In the first step, Fluc catalyzes the formation of the luciferin–adenylate conjugate, followed by oxygenation and cyclization, upon which the dioxetanone anion (Dx^-) is formed. Subsequently, a light emitter intermediate, the excited singlet state of OL^- [$^1(OL^-)^*$] is generated. Luminescent light is produced with a peak intensity of around 600 nm upon $^1(OL^-)^*$ relaxation to the ground state. Firefly oxyluciferin (OLH), CO_2 and AMP are released at the same time (Scheme 2).[28]

While all known insect luciferases can apply D-luciferin as a substrate, the light emitted by the same substrate can be diverse, ranging from red to yellow to green, and the emission wavelength maxima in the range 530–640 nm. The color of light is affected by different environmental factors, such as pH and temperature *in vitro*. The key to explaining the multiple colors of firefly bioluminescence *in vivo* is the light emitter species of oxyluciferin. Nevertheless, the actual light emitter species continues to be a particularly intriguing aspect of firefly bioluminescence. The development of this concept should be based on the investigation of a detailed luciferase–luciferin interaction mechanism. As mentioned above, the color of the bioluminescence is closely correlated with the subtle structural differences in luciferase. As a result, it will be particularly helpful to determine the crystal structure of luciferase and its complex with the substrate. Based on the crystallographic result, novel luciferase substrates can be well designed and discovered, thus encouraging the application of bioluminescence significantly.

The gene for *P. pyralis* luciferase was cloned and the nucleotide sequence of cDNA and genomic DNA was determined in 1987.[21] In 1996, the *apo*-form crystal structure of firefly luciferase was determined at 2.0 Å resolution. The luciferase protein is folded into two compact domains, a large *N*-terminal domain (4–436) that consists of a distorted antiparallel β-barrel and two β-sheets, and the small *C*-terminal portion (440–544), which is separated from the *N*-terminal domain by a wide cleft.[29] Afterwards, in order to determine the interaction between luciferase and its substrates and reveal the conformational changes of the enzyme–substrate



Scheme 2 The mechanism of firefly bioluminescence.

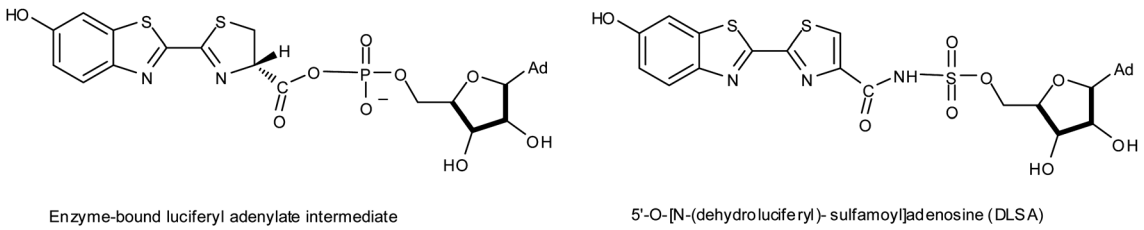


Fig. 2 The structural comparison between the luciferyl adenylate intermediate and its analogue DLSA.

complex, a high-energy intermediated analogue, 5′-*O*-[*N*-(dehydroluciferyl)-sulfamoyl] adenosine (DLSA) was synthesized (Fig. 2). The co-crystal structure of the wild-type luciferase from *Luciola cruciata* [LcrLuc (WT)] complexed with DLSA was determined (PDB ID: 2D1S)[10] (Fig. 3a). Details of the interactions of DLSA with luciferase are shown in Fig 3b. The O18 of the DLSA forms hydrogen bonds with the Ser 200 and Thr 345 at distances of 3.08, 3.00, and 3.11 Å, respectively. The O19 of DLSA forms hydrogen bonds with the residues Ser 201 and His 247 at distances of 2.98, 3.30, and 2.85 Å, respectively. In addition, N38 of the DLSA can form hydrogen bonds with Gly 341 (2.84 Å). The benzothiazole ring of DLSA makes important hydrophobic interactions with the side chains of Phe 249, Thr 253, Ile 288 and Ala 350, and with the main chains of the β13 and β14 strands. While the adenosine moiety utilizes Ala 319, Tyr 342, Asp 424, Ile 436 and Lys 531 for hydrophobic interactions.

Their research *de facto* proposed a relationship between luciferase structure and spectral difference in luciferase bioluminescence. The transient movement of hydrophobic residue Ile 288 can affect the molecular rigidity of the excited state of oxyluciferin by contacting benzothiazole ring of DLSA, and then decide the color of bioluminescence.

4. The substrates of firefly luciferase

The native substrate of firefly luciferase, luciferin **1**, was first isolated from the North American firefly *Photinus pyralis* by Bitler and McElroy.[30] Firefly luciferin has two isomers, while only the D-(−) isomer is biologically active, the L-(+)-luciferin could not produce light.[31] In 1966, White and McElroy claimed that the 6-hydroxyl moiety of D-(−)-luciferin can be replaced with an amino group to generate aminoluciferin **2**.[32] Aminoluciferin emits light at a longer wavelength (596 nm), and has a higher affinity with luciferase than natural luciferin. Since then, multiple firefly luciferin analogues have been synthesized for extensive mechanistic determination and luciferase structure–function studies. However, because of the high substrate specificity of luciferase, none of these derivatives demonstrate a more reasonable property than native luciferin or aminoluciferin. Therefore, D-luciferin and aminoluciferin are still the most commonly-used substrates in the BLI assay.

Through the structure–activity studies of luciferase substrates, it has been found that a strongly electron-donating substituent at the 6′-position is required for light emission. Alkylation or acylation of luciferin at the 6′-phenol position, or acylation of aminoluciferin at the 6′-amino position can affect the luciferase–substrate reaction. Thus, these analogues exhibit no luminescence properties.[32] However, a series of N-alkylated aminoluciferins, such as compound **3** in Fig. 4, can act as luciferase substrates. *N*-Monosubstituted aminoluciferins with straight-chain alkyl substituents of increasing length were prepared for structure–activity relationship studies. Several substituted compounds emitted stronger light than the unsubstituted aminoluciferin.[33] In another instance, a group of conformation-restricted cyclic alkylaminoluciferin substrates **4** acting as the substrates of the modified luciferase Ultra-Glo was synthesized.[34] The increment of rigidity and restricted bond rotation of these cyclic alkylaminoluciferins leads to an increase in the relative quantum yield.

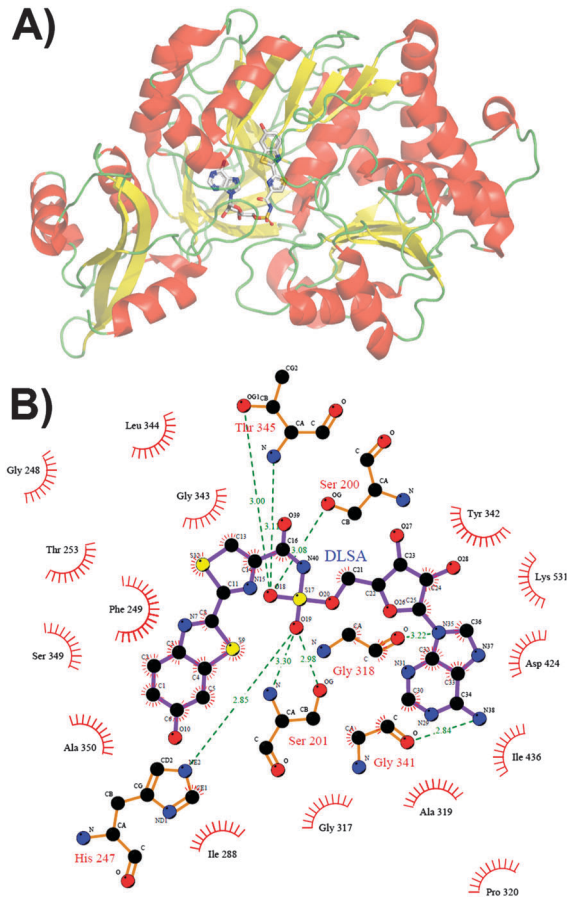


Fig. 3 (A) Ribbon diagram of luciferase (PDB entry: 2D1S) complexed with ligand DLSA (white sticks) plotted by using the Pymol program; (B) a schematic illustration of the interactions of DLSA with luciferase generated by using the HBPLUS and Ligplot programs.

However, Ultra-Glo cannot be expressed in cells or *in vivo*, so they can only be applied in *in vitro* high-throughput screening but are not available for *in vivo* imaging.

Three functional luciferase substrates based on the aminoluciferin scaffold can extend the applicability of the luciferin–luciferase system.[35] One is Glu-AL **5** that conjugates aminoluciferin with glutamate (Glu-AL). Compound **5** is the first membrane-impermeable bioluminescent substrate; thereafter it can be applied to distinguish between intra- and extracellular events. The other is Cy5-AL **6** that links aminoluciferin with a NIR-emitting cyanine (Cy) dye. When Cy5-AL reacts with luciferase, bioluminescence resonance energy transfer (BRET) can be observed and the luminescence can be seen in the near infra-red region (λ_{max} = 673 nm). This probe can be applied to deep tissue imaging in view of its ideal tissue penetration. The third is biotin-DEVD-aminoluciferin **7** (DEVD is the amino acid sequence Asp-Glu-Val-Asp) derived from biotin-aminoluciferin. Biotin-aminoluciferin can complex with avidin with high affinity and specificity. This complex can block access to luciferase, resulting in the loss of bioluminescence. Biotin-DEVD-aminoluciferin **7** does not exhibit bioluminescence when complexed with avidin, while Lys-aminoluciferin, which is also a substrate of luciferase, is released after the molecule is degraded by caspase-3 and time-dependent bioluminescence appears. It needs to be highlighted that such a strategy can be applied to the detection of various proteases and *in vivo* imaging. Therefore, the observation of a variety of bioluminescent substrates can extend the contemporary application of the bioluminescent assay *in vivo*.

To overcome the limitation that it is restricted mainly to small animals at superficial depths, a red-shift luciferin analogue has been designed. This luciferin analogue contains a selenium atom in place of the native sulfur atom on the thiazole position of compound **8**.[36] Different from the Cy5-AL mentioned above, this simple modification would not alter the distribution and other physicochemical properties compared with the native substrate. The bioluminescent imaging emission maximum for such a molecule was 600 nm with red light, while that of D-luciferin was 558 nm and that of aminoluciferin was 588 nm. However, the selenium analogue **8** emitted approximately 74% of the number of photons that were emitted by aminoluciferin. Thus, this analogue shows a lower quantum yield compared with aminoluciferin *in vitro*. However, the red-shifted bioluminescent signal can enhance the tissue penetration.

The notorious difficulty associated with producing luciferase substrates also causes a lack of alternative luciferase substrates. Therefore, it may be necessary to design a novel general and rapid strategy for synthesizing luciferin along with modified analogues **9** and **10**. Two of the electronically modified luciferin analogues turned out to be the substrates of luciferase and can emit luminescence.[37] Most importantly, the luciferin scaffold can be prepared from aniline starting materials and Appel's salt. A variety of other heterocyclic luciferin analogues, without a benzothiazole moiety, will be synthesized and expand the imaging toolkit. The substrates of luciferase identified and prepared till now are shown in Fig. 4.

5. Caged firefly luciferins as bioluminescent probes

So far, almost all reported luciferase substrates are based on D-luciferin and aminoluciferin scaffolds. It has been found that the 6′-hydroxy (6′-amino) group of D-luciferin (aminoluciferin) is crucial for enzyme combinations. Therefore, modification at the hydroxyl (amino) group of luciferin often prohibits its recognition with luciferase, and causes quenching of the bioluminescence emission. These caged luciferins can produce luminescence when reacting with specific biological molecules by following two procedures: firstly, the caged substrates are cleaved by corresponding enzymes or bioactive molecules; subsequently, the free luciferin is oxidized by luciferase to emit light (Scheme 3). The intensity of the light output is closely related to the amount of free luciferin and therefore with the activity of the enzymes or the small molecules in the first reaction. Thus, modified probes have been designed for the detection of target enzyme activity, or the functions of small molecules. This review summarizes a panel of caged luciferins as bioluminogenic substrates, which can release the free luciferin after being triggered

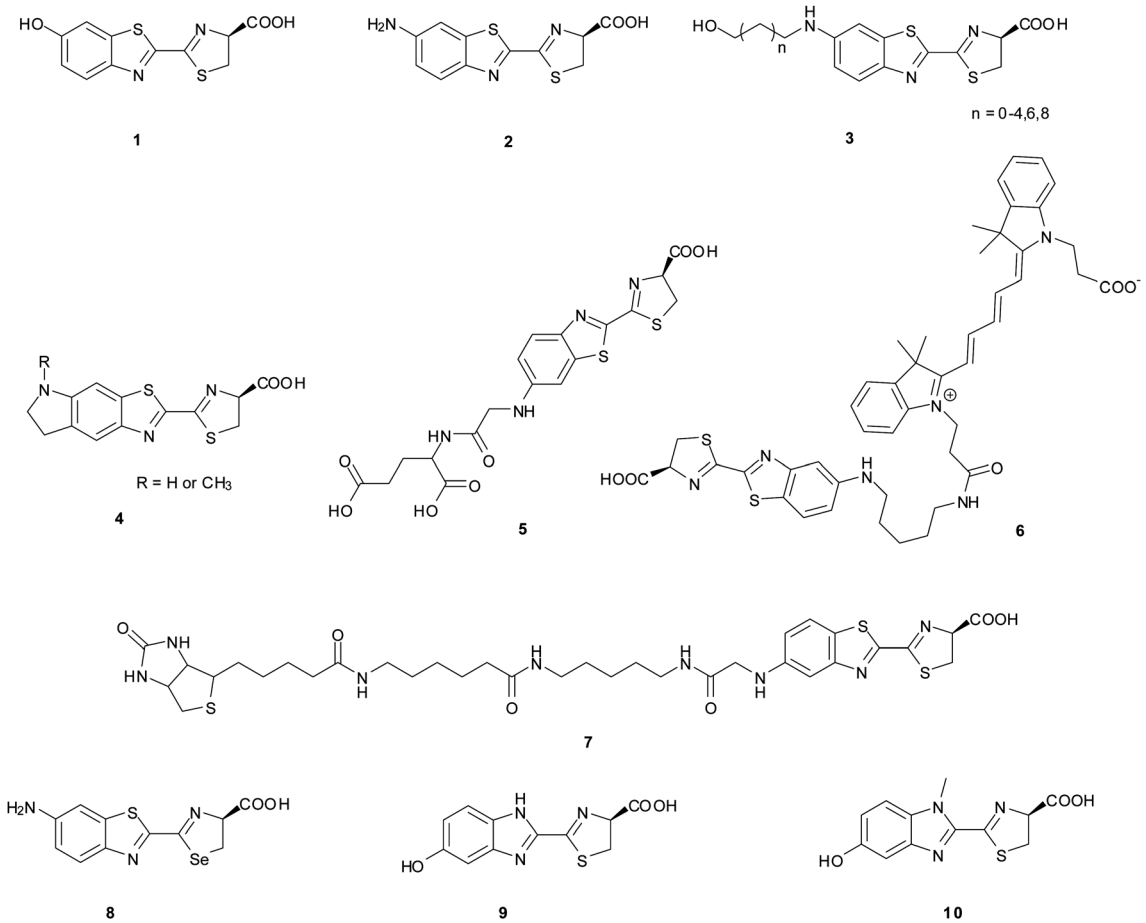
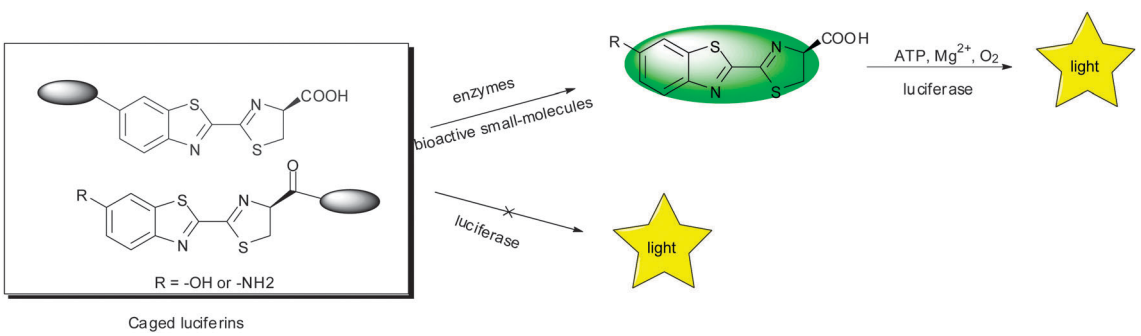


Fig. 4 Substrates of firefly luciferase. (**1**) Firefly luciferin;[30] (**2**) aminoluciferin;[32] (**3**) hydroxyalkyl aminoluciferins;[33] (**4**) cyclic alkylaminoluciferin;[34] (**5**) Glu-AL;[35] (**6**) Cy5-AL;[35] (**7**) biotin-AL;[35] (**8**) selenium analogue of D-luciferin;[36] (**9**)–(**10**) electronically modified luciferins.[37]



Scheme 3 Design idea of caged luciferins.

by various biopolymers, bioactive small molecules and the cellular uptake and release process, and then react with the luciferase to shed the bioluminescence light.

5.1 Probes for imaging enzymes

Caged firefly luciferins are particularly useful for longitudinal studies of enzyme activities. In a typical experiment, these probes can be catalyzed to release firefly luciferin by specific enzymes and then emit light in the presence of luciferase. High selectivity and specificity between the enzyme and luciferin-based substrate drives this system to be potentially useful for monitoring various enzyme activities.

5.1.1 Protease assay. Apoptosis or programmed cell death (PCD) plays a crucial role in normal development and tissue homeostasis, but the disorder of apoptosis may cause various diseases. Since proteases are involved in numerous disease processes, they are substantial biotargets for high-throughput screening (HTS) and cell-based apoptosis assays. As mentioned previously, the classical protease assay, by using peptide-conjugated fluorophores, may be very limited in view of the

high background signals. Fortunately, the bioluminescent assay can overcome this obvious drawback in the fluorescent assay. What is more, a number of luminogenic protease substrates can be obtained from commercial sources.

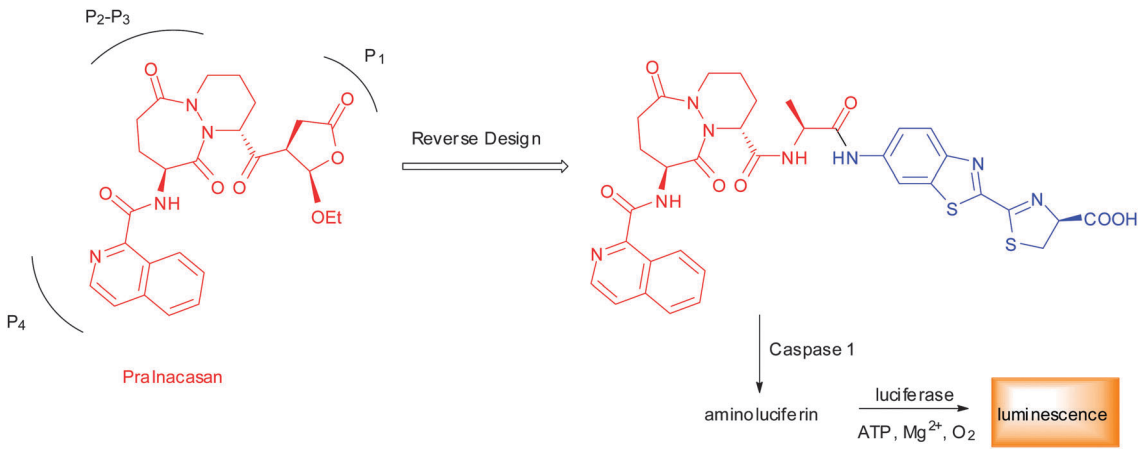
5.1.1.1 Bioluminescence imaging of caspase activity. In the bioluminescent protease assay, one important point is the development of caspase bioluminogenic substrates. Cysteine-aspartic proteases or caspases are a family of cysteine proteases with 14 family members that have been identified till now. Caspases play key biological roles in apoptosis, necrosis, and inflammation. The ability to monitor the activity of caspases will enable us to observe the disease burden and progression in various therapeutic models.

It is well known that the DEVD (amino acid sequence Asp-Glu-Val-Asp) tetrapeptide is the conserved recognition sequence for caspase-3 and -7.[38] Conjugated aminoluciferin, Z-DEVD-aminoluciferin, can be recognized and cleaved to generate free aminoluciferin in the presence of caspase-3 or -7. As a result, the intensity of light emitted by aminoluciferin can indicate the activity of caspase-3 and -7. Taking caspase-3 as an example, a variety of procedures for the bioluminescent assay have been developed, including a two-step nonhomogeneous assay and a one-step homogeneous assay.[39,40,46] The sensitivity, speed and stability is increased, while the false hit rate in a test inhibitor screening is down. This noninvasive molecular imaging method can be used for detecting drug efficacy and therapeutic programs.

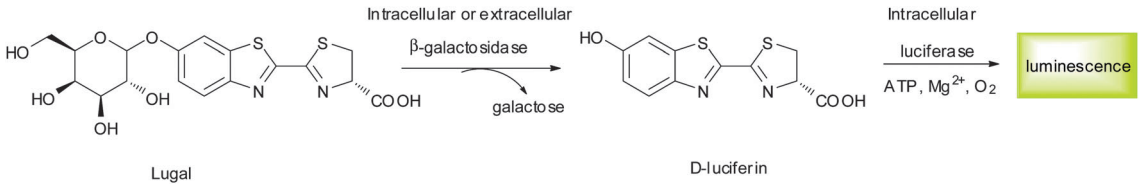
In another case, aminoluciferin derivatives were used as the caspase-1 substrates for producing luminescence. Caspase-1 performs a crucial role involved in the process of inflammation. Thus, it was important to monitor the abundance and activity of caspase-1. The caspase-1 inhibitor pralnacasan was turned into a new bioluminescent ABP for caspase-1 (CM-269) based on the concept of reverse design, where chemically optimized protease inhibitors were turned into selective substrate activity-based probes (ABPs). These probes made it possible for direct detection of caspase-1 activity (Scheme 4).[41] It should be noted that such a strategy can provide a feasible train-of-thought, of which different recognizable peptides can be combined with luciferin to give other protease assays, such as measuring chymotrypsin (succinyl-Leu-Leu-Val-Tyr-aminoluciferin), trypsin (Z-Leu-Arg-Arg-aminoluciferin), or caspase-like (Z-Nle-Pro-Nle-Asp-aminoluciferin) activities of proteasomes.[42–44]

5.1.1.2 Bioluminescence imaging of furin activity. Furin, a critical member of the protein convertase family, exists in mammalian cells. Since recent research has determined that furin is overexpressed in cancer and inflammatory conditions, such as HIV, influenza and dengue fever virus infection, the inhibition of furin turns out to be closely intertwined with the absence or decrease of invasiveness and tumorigenicity of several human cancer cells *in vivo*.[45] The group led by Rao designed two furin probes, acetyl-RVRR-aminoluciferin (Ac-RVRR-AmLuc) and acetyl-RYKR-aminoluciferin (Ac-RYKR-AmLuc), by coupling aminoluciferin to the previously reported C-terminus of the furin recognition peptide sequences, RVRR and RYKR.[46] These caged aminoluciferins can be hydrolyzed in the presence of furin to release recognition peptides and aminoluciferin, and to subsequently generate bioluminescence with firefly luciferase. A quantitative relationship between the bioluminescence intensity and the activity of furin has been confirmed herein as well. In the mean while, a control probe acetyl-RRKY-aminoluciferin (Ac-RRKY-AmLuc) was synthesized, in which such a peptide sequence cannot be recognized by furin to release the free aminoluciferin. The bioluminescent imaging data indicated that Ac-RVRR-AmLuc produced a higher signal than Ac-RYKR-AmLuc, while Ac-RRKY-AmLuc generated negligible light emission. It should be emphasized that these highly selective and sensitive furin probes could allow for the investigation of the role of furin in hypoxia *in vivo*, thus providing a way of imaging tumor hypoxia.

5.1.2 β-Galactosidase assay. A conjugate of luciferin, D-luciferin-*O*-β-galactoside (Lugal), was a sensitive and specific substrate of β-galactosidase (β-gal).[47] This galactoside moiety could be cleaved by β-galactosidase to generate free luciferin for bioluminescence emission. This caged luciferin could be applied in different fields for detecting the activity of β-galactosidase. Considering that the detection of β-galactosidase in cultured bacteria can indirectly reflect the presence of coliforms, a rapid positive/negative bioluminescence test for coliforms was developed.



Scheme 4 The reverse design of the caspase-1 reporter.



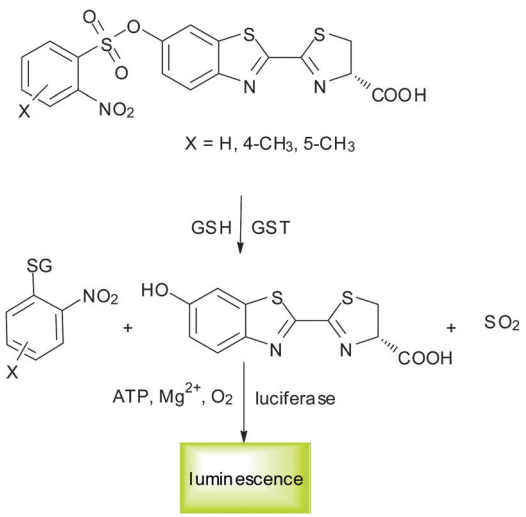
Scheme 5 Lugal acting as a β-galactosidase probe.

The sensitivity of this bioluminescent assay was approximately 50-fold higher than the fluorescent assay; what is more, the assay takes only 7 h, compared to 24 h by conventional methods. Therefore, it can be used for rapid identification of low-level bacterial contamination in food stocks.[48]

In another example, D-luciferin-*O*-β-galactoside, with a one-step cloned enzyme donor immunoassay (CEDIA) system, provides a rapid and sensitive technique for immunochemical detection and quantification of analytes.[49] Both the above-mentioned examples are terminal examinations, requiring lysis of the cells. The firefly luciferase–luciferin reaction needs cofactors, thus firefly luciferase activity cannot be detected outside the cell. Important applications of *in vivo* imaging, such as antibody labeling, or serum protein monitoring, are precluded. Sequential reporter-enzyme luminescence (SRL) made it possible for *in vivo* imaging of both intracellular and extracellular β-galactosidase activity.[50] Lugal must first be cleaved by β-gal before it can be catalyzed by luciferase. The strength of the luminescent signal is correlated with β-gal activity (Scheme 5). Thus, when using SRL, the activity of other enzymes can be detected by taking advantage of the luminescent properties of luciferase. This caged luciferin is nontoxic, so as to enable daily luminescent imaging without weight loss or other illness effects. The concept of coupling the activity of luciferase and β-gal for *in vivo* imaging greatly extends the applications of bioluminescent imaging.

5.1.3 Glutathione S-transferase assay. Recently, Zhou and his colleagues claimed that a series of nitrophenyl luciferin ether and sulfonate compounds were bioluminescent substrates for the glutathione S-transferase assay.[51] They anticipated that electrophilic *o*-nitrophenyl luciferin, quinolinyl luciferin ether derivatives or luciferin sulfonates could exhibit reactivity with GSH (Scheme 6). The free luciferin or quinolinyl luciferin could be released after nucleophilic attack of GSH. After a number of compounds were designed and detected, the results indicated that the GST activities are not only influenced by the electron-directing group(s), but also highly depend on the leaving ability of the nucleophilic group. Moreover, luminescent signals produced with luciferin sulfonates were much higher than for the luciferin ether derivatives. Therefore, the electrophilic aromatic substituted luciferins could provide a low background and high sensitivity approach for rapidly and effectively detecting GST and its activity as well as the catalytic mechanism.

5.1.4 Monoamine oxidase assay. Monoamine oxidases (MAOs), which catalyze the oxidative deamination of several biogenic and xenobiotic amines to the corresponding aldehydes, play extremely meaningful roles in metabolism.[52] A novel two-step monoamine oxidase probe based on a bioluminescent assay for monitoring

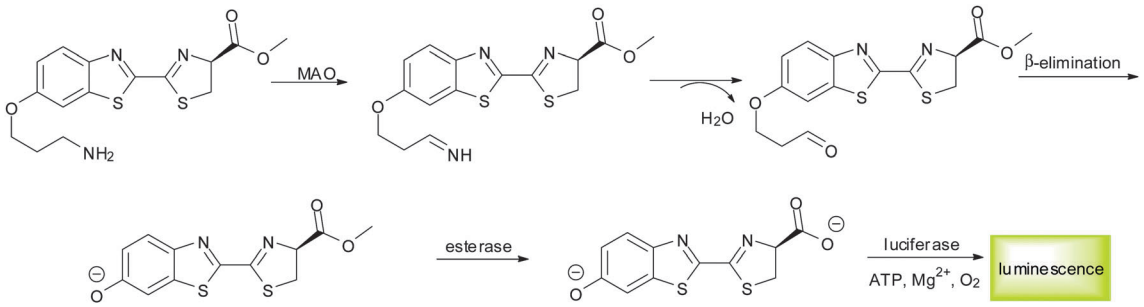


Scheme 6 The release of free luciferin catalyzed by GSH and GST.

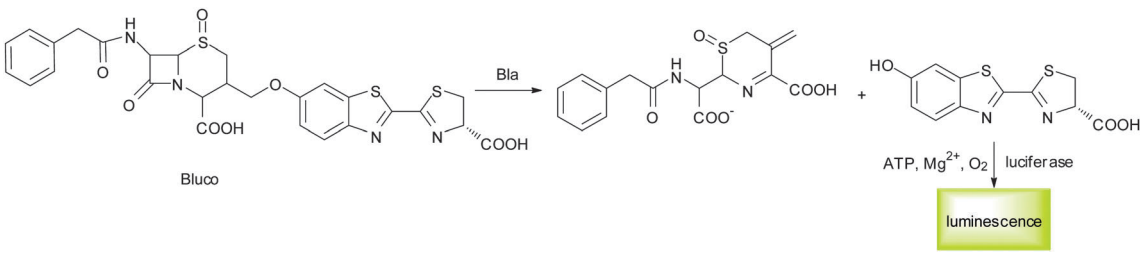
MAOs activity was more sensitive than current fluorescent methods. Based on the function of MAOs, this novel methyl ester luciferin (as an aminopropylether analogue), first reacted with MAOs and then the detection reagents stopped the MAO reaction and converted the product of the first step to luminescence (Scheme 7).[53,54] The bioluminescent assay demonstrated lower limits of detection (LOD), of 1 and 6 ng of microsomal protein per reaction for MAO A and MAO B, respectively, compared with the LOD of between 170 and 190 ng in the fluorescent assay. Accordingly, high-throughput screening and drug metabolism can be conducted considering the activity of MAOs.

5.1.5 β-Lactamase assay. β-Lactamase expressed by the bacteria can efficiently hydrolyze β-lactam antibiotics, such as penicillin and cephalosporin, thus resisting β-lactam antibiotics.[55] Bluco, a β-lactam and D-luciferin conjugate, was the first bioluminogenic substrate for TEM-1 β-lactamase (Bla).[56] It could not react with luciferase and emit light without Bla. However, the free D-luciferin could be released after opening of the β-lactam ring by Bla, it would eventually then be oxidized by luciferase in a light-producing reaction to image the Bla activity. Overall, this probe could be applied to the imaging of β-lactam resistant bacteria that express β-lactamase *in vivo* (Scheme 8). The combination of bioluminescent and fluorescent assay allows the Bla reporter assay to achieve *in vitro* evaluation, cell imaging and living animal imaging.

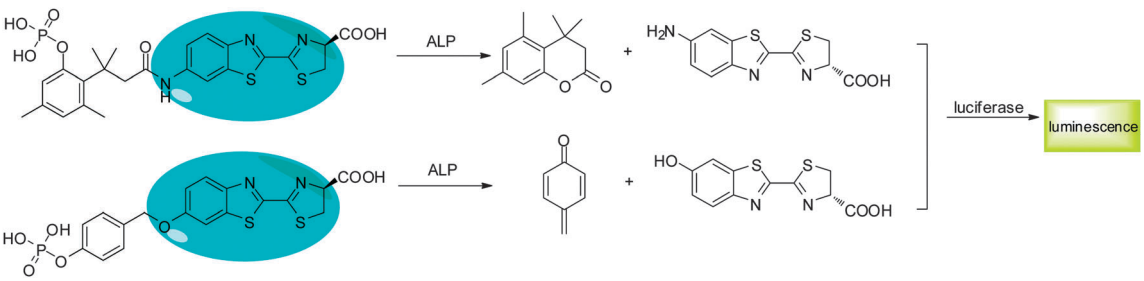
5.1.6 Alkaline phosphatase assay. Alkaline phosphatase (ALP), as the name suggests, is a hydrolase with high activity for specifically removing phosphate groups from numerous



Scheme 7 Caged luciferin monitoring monoamine oxidase activity.



Scheme 8 *In vivo* imaging of β-lactamase activity with Bluco.

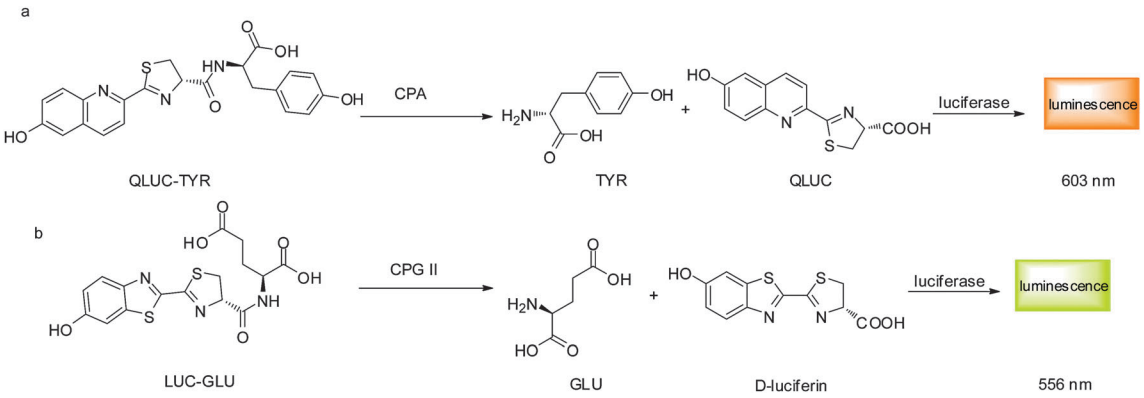


Scheme 9 Luciferin phosphates as alkaline phosphatase reporters.

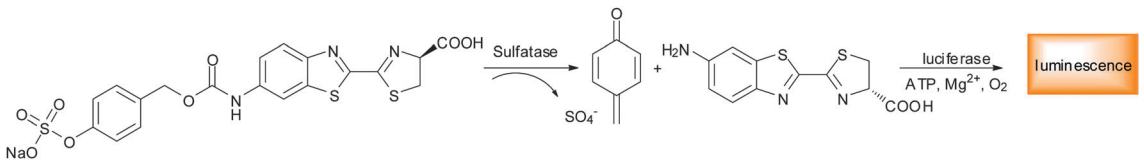
types of molecules.[57] A couple of 6-luciferin phosphates acted as luciferase substrates, after being sequentially activated when the phosphate moieties are selectively removed by ALP (Scheme 9).[58] However, the authors noticed that the pK_a of the luciferin phenol was low enough to lead to the phosphate hydrolysis by both nonenzymatic hydrolysis of 6-luciferin phosphate, such as nucleophilic attack, and P–O bond fission, and as a result, the sensitivity is low. Therefore, a self-immolative chemical adaptor, which is connected between the luciferin group and phosphate group, was used to optimize the pK_a of the hydroxyl group at the phosphate-bonding site for enhancing the stability. The self-immolative chemical adaptor strategy should bring out a meaningful avenue to develop novel fluorogenic and/or bioluminogenic probes.

5.1.7 Carboxypeptidase assay. Carboxypeptidases can cleave amide linkages of certain amino acid compounds at specific C-terminal residues in polypeptides and proteins.[59] QLUC-TYR and LUC-GLU are highly sensitive and selective luminescent probes for the carboxypeptidase assay.[60] Different from the probes mentioned above, the modification groups of these two probes were not blocked at the hydroxyl group, but at the 4-carboxyl group to yield additional biological information. QLUC-TYR and LUC-GLU can be cleaved during enzymatic degradation to release free QLUC and luciferin, catalyzed by two different types of carboxypeptidases, CPA and CPG II. Considering that the emission λ_{max} of QLUC is 603 nm, which is longer than that of luciferin at 556 nm, the authors proposed that a specific enzyme could be detected using a mixture of substrates based on the specific wavelengths (Scheme 10). Nevertheless, the result suggested that mainly the light emitted by luciferin was determined because of the dramatic low luminescent intensity of QLUC and substrate specificity.

5.1.8 Sulfatase assay. Considering that sulfatases are enzymes that can hydrolyze sulfate esters in association with enormous disease states, it is notable to develop a sensitive and selective optical probe that reports on sulfatase activity. The development of a sulfatase probe can be helpful for the understanding of the biological functions of sulfatases, as well as for drug screening and disease diagnosis. Recently, a sulfatase activity caged luciferin probe based on bioluminescence



Scheme 10 (A) QLUC-TYR for monitoring CPA activity. (B) LUC-GLU for monitoring CPG II activity.



Scheme 11 General strategy for the design of sulfatase activated probes.

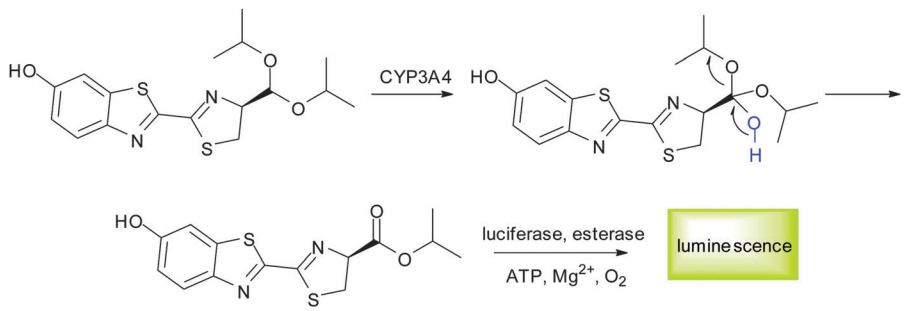
has thrown light upon the evaluation of sulfatase activity.[61] This probe was comprised of two parts, an associated substrate with reporter functions (Scheme 11). While the substrate is hydrolyzed by sulfatases, firefly luciferin was released to produce luminescence in the presence of luciferase, Mg^{2+}, O_2 and ATP. It needs to be noted that the reporter herein is aminoluciferin other than luciferin, in which the amino group becomes a critical bridge between the sulfatase substrate and the reporter function.

5.1.9 Cytochrome P450 activity test. Cytochrome P450 (P450) enzymes catalyze the oxidation of thousands of substrates, including metabolic intermediates and xenobiotic substances such as drugs and other toxic chemicals. P450 enzymes play critical roles in drug metabolism and drug interaction. Drugs can affect the activity of P450 enzymes and induct the *CYP* gene in turn. These changes may affect the elimination of drugs and result in adverse drug–drug interactions.[62] The influence of new chemical entities toward P450 enzymes should be evaluated by probes.

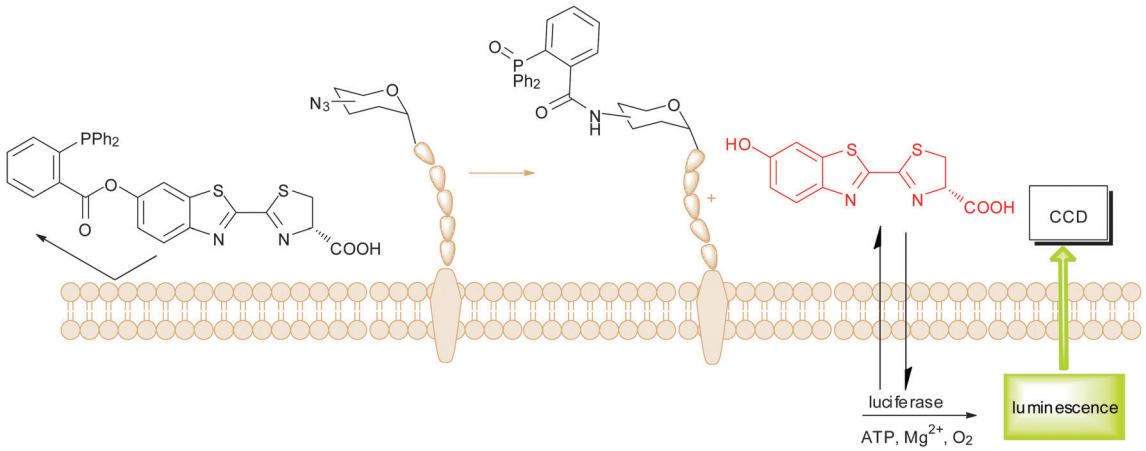
Recently, a series of proluciferin acetals for the P450 activity assay have been disclosed.[63] These chemotypes of bioluminogenic proluciferin can be oxidized by P450 enzymes to generate luciferin. After screening the luciferin acetals against a panel of purified P450 enzymes, a proluciferin can be oxidized by CYP3A4, sensitively and selectively, releasing the free luciferin, and then producing light. So this proluciferin is used as a probe to measure IC_{50} values of CYP3A4 inhibitors (Scheme 12).

5.2 Bioluminescence imaging of glycans in live cells

In the post-genomics era, proteomics and glycomics have emerged as fields of enormous importance. However, detecting and differentiating such modifications rapidly is not a trivial issue. This is especially true for glycosylation, which differs from phosphorylation, methylation, ubiquitination, and acetylation in that many different modifications can be achieved at a single site by the use of different glycosylation patterns.[64] It is well known that changes in glycosylation are often associated with disease states, such as cancer and chronic inflammation, and new therapeutic and diagnostic strategies are based on



Scheme 12 Proluciferin acetals measuring IC_{50} values of CYP3A4 inhibitors.



Scheme 13 Real-time imaging of glycans on living cells. (Figure adapted with permission from ref. 66. Copyright (2010), American Chemical Society.)

the underlying glycobiology. Therefore, glycans are attractive targets for molecular imaging.

Since glycans are not directly encoded by the genome, they are difficult to make accessible to genetically encoded reporters, thus presenting a challenge for the quick detection of glycosylation.[65] Classical methods for imaging glycans rely on metabolic labeling with chemical reporters and subsequent ligation to fluorescent probes. It is interesting that caged firefly luciferin can been employed for glycan imaging in live cells.

Bertozzi and coworkers reasoned that a phosphine–luciferin conjugate (Scheme 13), which was designed to release luciferin upon Staudinger ligation, would enable sensitive detection of azidosugars with deeply low background signal and high sensitivity.[66] This approach provides novel bioluminescent imaging technology that can help the development of glycomic tools.

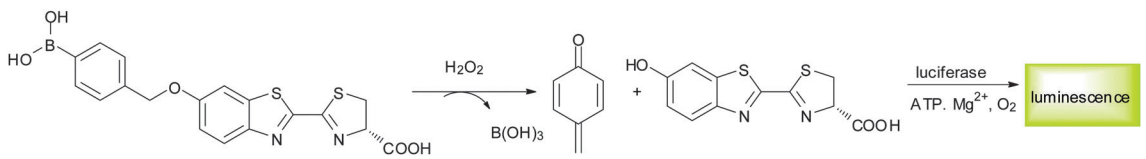
5.3 Probes for imaging bioactive small molecules

Bioactive small molecules are numerous functional small metabolites inside cells or drugs that can regulate biological functions. Small metabolites produced in cells, such as vitamins,[67] NO,[68] or H_2S,[69] may act as extracellular and intracellular messengers for signal transduction. Drugs can regulate cellular functions or treat metabolic disorders. Thus, probes for imaging bioactive small molecules allows us to understand their chemical mechanism and biomolecular events in living cells.

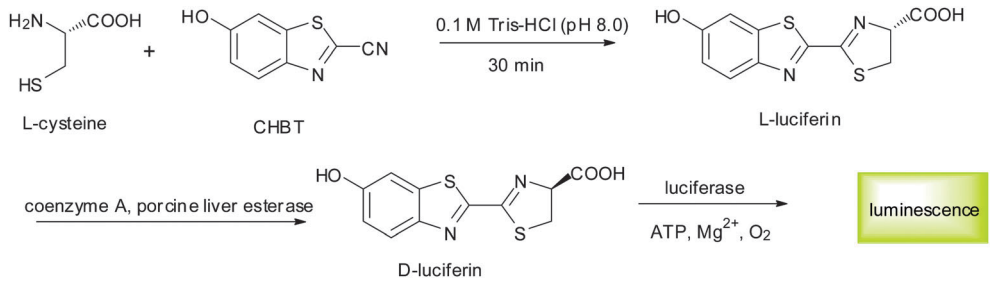
5.3.1 Bioluminescent imaging of hydrogen peroxide production in a murine tumor model. Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide (O^{2-}), hydroxyl radicals ($^{\bullet}OH$), and various peroxides (ROOR). H_2O_2 has been thought of as an unwanted by-product of an aerobic existence. It is assumed that the aberrant level of H_2O_2 mediates diverse pathological and biological processes of various diseases, such as angiogenesis, oxidative stress and aging, cancer, and neurodegenerative diseases. H_2O_2, as an inevitable signalling messenger, is also involved in cell proliferation, differentiation, and migration.[70] Real-time monitoring of H_2O_2 fluxes in living cells and animals is a powerful tool to reveal the chemical mechanisms underlying the development and progression of disease.

The Chang group recently disclosed a chemoselective bioluminescent probe for detecting H_2O_2, peroxy caged luciferin-1 (PCL-1) (Scheme 14).[71] This probe consists of three parts, a H_2O_2-sensitive aryl boronic acid, a luciferin group and a self-immolative linker. It was designed to image H_2O_2 production in a murine tumor model *in vivo*. Upon reaction with H_2O_2, PCL-1 can release the firefly luciferin, followed by reacting with firefly luciferase to emit light. The level of H_2O_2 is related to the total bioluminescent signal, both in the living cells and in the living mice. This probe provides technology for real-time imaging of the level of H_2O_2 in living mice, selectively and sensitively. Moreover, the contribution of the levels of H_2O_2 to health, aging, and disease may be clarified in the coming future by using such a bioluminescent imaging approach.

5.3.2 Bioluminescence assays for L-cysteine. Niwa *et al.* provided a fast and cost-effective assay method for L-cysteine and luciferase assay based on the biosynthetic pathway of firefly luciferin.[72] 2-Cyano-6-hydroxybenzothiazole (CHBT) can react with L-cysteine to yield L-luciferin in the absence of any enzymes. L-Luciferin is an enantiomer of D-luciferin and a luciferase competitive inhibitor that cannot produce bioluminescence when reacting with luciferase.[31] L-Luciferin can be converted into D-luciferin in the presence of coenzyme A (CoA)



Scheme 14 PCL-1 for H_2O_2 detection.



Scheme 15 Bioluminescence assays for L-cysteine.

and esterase, subsequently producing luminescence when reacted with luciferase. The quantity of L-cysteine can be measured by the output signal (Scheme 15). What is more, using L-luciferin and CHBT can replace D-luciferin for the luciferase assay, because the luciferase assay medium contains all the compounds for the biosynthesis of D-luciferin. Thus, this method is fast and cost-effective.

5.4 Photoactivable bioluminescent probes for imaging luciferase activity

In addition to detecting other enzymes, caged luciferin can be employed for determining luciferase activity. The caged luciferin substrates, which can be changed to uncaged luciferin by photolysis, might work as an efficient bioluminescent probe for tracking the dynamics of the luciferase expression in living animals. These caged substrates have virtuous cell membrane permeation and photo cleavable activities, and can be released after UV illumination, and then fluorescent and luminescent signals can be observed.[73] As a result, these probes can be facilitated *in vitro* and *in vivo* to monitor the dynamic activity of firefly luciferase (Scheme 16). This method does not use luciferin, but employs caged luciferin for imaging luciferase activity. Due to these probes being photoactivatable, the enzymes or small molecules can be imaged at a desired time and/or location in intact cells, tissues or living animals. The biggest advantage is that the imaging process can readily modulated by a beam of light with high spatial and temporal precision. This strategy is rarely seen in other research.

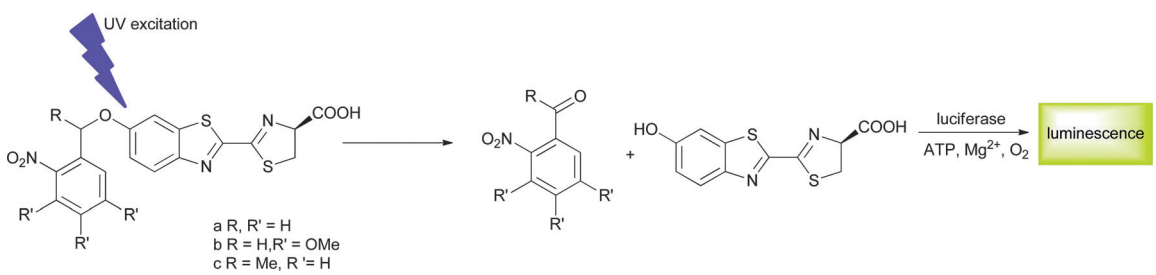
Photocaged luciferin makes high temporal and spatial regulation possible. Photoactive (or photocaged) compounds can be used for monitoring gene expression, mapping cellular function and cellular interaction *in vitro* and *in vivo*.

These photocaged compounds should be exposed to high-intensity UV or visible light in the uncaging process. However, the penetration of short-wavelength UV is very poor, and excessive exposure to UV light can cause acute tissue damage.

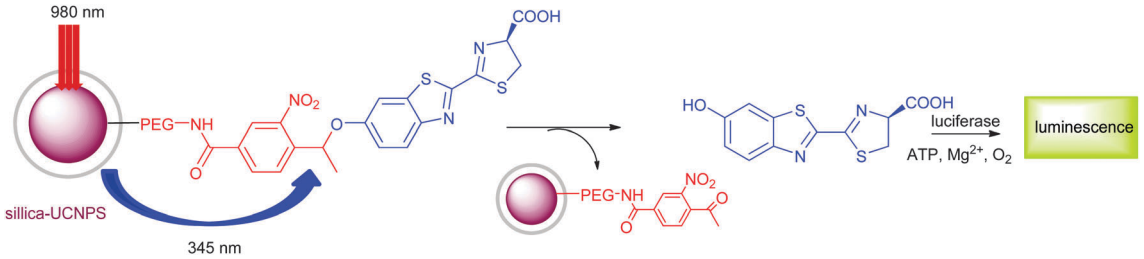
The immediate task for scientists is to find photoactivatable probes that can be activated by long-wavelength excitation. Thus, these probes can be used for deep tissue imaging with low damage. A photocaged upconversion nanoparticle can meet this requirement.[74] This probe consists of three parts: D-luciferin as the reporter which can react with luciferase and emit light, a photoactivatable linker that can be cleaved when excited with UV light, and silica-coated lanthanide-doped upconversion nanoparticles (UCNPs). UCNPs exhibit anti-Stokes emissions, which can convert excited continuous-wave 980 nm laser light into high-energy UV light. Therefore, the uncaging of D-luciferin can be triggered from D-luciferin-conjugated UCNPs after being excited by NIR light. A strong bioluminescent signal can be detected and then used for deep tissue imaging (Scheme 17). This method offers new possibilities for imaging target areas at specific times.

5.5 Imaging cellular uptake and release

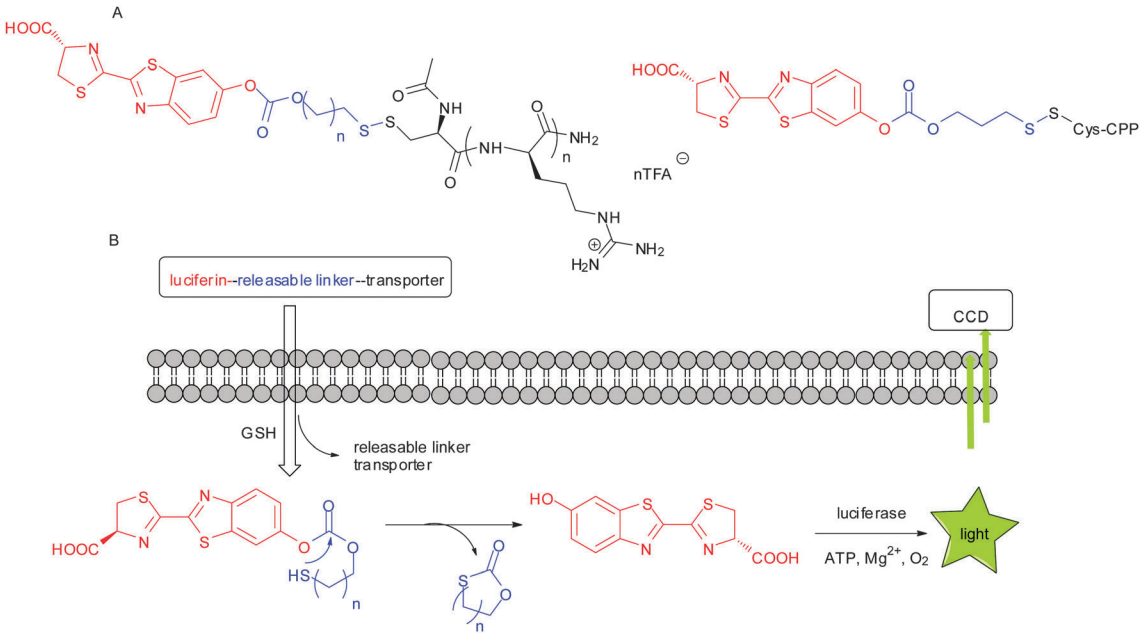
Good formulation and/or bioavailability may be important for promising leads to be advanced clinically. So, the sooner the physical–chemical properties of leads are predicted or the problems addressed, the better. The molecular transporters are designed to overcome various membrane biological barriers. They would be highly helpful for drug development. Molecular transporters are linked to or combined with a cargo to enable or enhance its ability to enter cells or tissues. In recent years, a number of molecular transporters have been well documented, such as cell-penetrating peptides (CPPs), oligocarbamates, peptoids, and so on.[75] However, there are two major challenges encountered during the development of this field, one is to create bioactivatable linkers that can release drugs/probes only in cells, and the other is to constitute probes



Scheme 16 Photoactivatable probes for monitoring luciferase activity.



Scheme 17 Bioluminescence imaging using photocaged D-luciferin-conjugated UCNPs.



Scheme 18 (A) The structures of two probes for cellular uptake and release. (B) Scheme of the luciferin-releasable-transporter conjugate for the cellular uptake assay.

that can evaluate internalization mechanisms and dynamic effectiveness of different transporters in real-time.

Hitherto, several luciferin release assays have been described for real-time quantification of uptake and release of molecular transporters through a bioactivatable disulfide linker.[76,77] The luciferin group, acting as surrogate drug, and the transporters are linked with a disulfide bond at the 6′-hydroxyl or 4-carboxyl position that would be cleaved after cell entry upon encountering a high glutathione concentration. After cyclization and self-cleavage of the resultant thiol linker, the free luciferin is released and a photon is detected by a luminometer to quantify the uptake and drug release (Scheme 18). Based on this mechanism, different types of molecular transporters can be evaluated by conjugating with luciferin.

6. Conclusions and prospective

Using the firefly luciferin derivatives as small molecular probes to detect biomacromolecules and bioactive small molecules in animals can greatly enhance our understanding of the pathogenesis and processes in the appropriate physiological settings. The caged luciferins are widely reported, and their applications can be extended to various fields according to the basic design principle reviewed above. These probes have many advantages, such as being rapid, sensitive, and low background. Due to bioluminescence being a common and naturally occurring phenomenon, and the probes being derived from biological sources, the toxicity is low to cells and living organisms.

There are also some disadvantages that appeared during the study of BLI. With regard to BLI for tumor imaging, BLI is less suited for the determination of absolute tumors mass in an animal because of quenching of the bioluminescence by tissue components, and the exact location of tumors because its spatial resolution is limited. On the other hand, the firefly luciferin–luciferase system needs many intracellular cofactors and the applications are genetically confined to engineered cells expressing luciferase. As a result, this method can only be used in cells or small animal models. It is a long way from being used in humans. This is an interesting field and great efforts are also needed to overcome the limit of BLI.

To date, the vast majority of efforts have focused on mutating luciferase enzymes. Mutation of enzyme structures leads to changes in the bioluminescent color. A wide variety of mutating luciferases with emission wavelengths red-shifted for bioluminescence reporting and imaging have emerged. These mutants enable a

smaller number of cells to be visualized in living subjects due to decreased attenuation by tissues. Split luciferase has proven useful for detecting protein–protein interactions.[16] In these assays, the N- and C-terminal fragments are translationally fused to unique proteins, respectively. When these two proteins interact with each other, split fragments can be completed and provide luciferase enzyme activity.

Compared with large amount of attention to luciferase mutation, only a handful of studies have focused on the modification of luciferins. Different insects with different luciferases all have a common substrate, D-luciferin and nearly all bioluminescent imaging is based on D-luciferin or aminoluciferin. The lack of diversity of luciferase substrates is a great restriction, limiting the applications of bioluminescence technology. Larger collections of light-emitting luciferins are required to expand the imaging toolkit. More attention should be paid to novel chemically modified luciferins, while rapid and reliable synthetic routes for these richly functionalized molecules are also required.

In addition to the lack of diversity of luciferins, the functional groups mainly link at the 6′-hydroxyl (or 6′-amino) position in the caged luciferin. Chemical modification at other positions may also affect the interaction between luciferin and luciferase, and quench the bioluminescence. However, caged luciferins at other positions are rarely reported.

Up to now, luciferin–luciferase bioluminescence imaging has been broadly utilized in a variety of fields in biomedical sciences. New applications of BLI are ever increasing as new caged luciferin modifications are described, as well as new models being developed. We can use them together to shed light on the unknown field of living organisms. Adapting the concept of caged firefly luciferin to new applications is a promising area of exploration.

Acknowledgements

The present work was supported by grants from the National Program on Key Basic Research Project (973 Program) (No. 2013CB734002), the Program of New Century Excellent Talents in University (No. NCET-11-0306), the Shandong Natural Science Foundation (No. JQ201019), the Independent Innovation Foundation of Shandong University, IIFSDU (No. 2010JQ005 and 2012JC002) and the Graduate Independent Innovation Foundation of Shandong University, GIIFSDU (No. yzc12096).

References

1 S. H. Haddock, M. A. Moline and J. F. Case, *Annu. Rev. Mar. Sci.*, 2010, **2**, 443–493.
2 E. A. Widder, *Science*, 2010, **328**, 704–708.
3 P. J. Herring, *Nature*, 1977, **267**, 788–793.
4 O. Shimomura, *Bioluminescence: chemical principles and methods*, World Scientific Pub. Co. Inc., Singapore, 2006.
5 S. Inouye, *Cell. Mol. Life Sci.*, 2010, **67**, 387–404.
6 J. E. Lloyd, *Annu. Rev. Entomol.*, 1983, **28**, 131–160.
7 Y. Ando, K. Niwa, N. Yamada, T. Enomoto, T. Irie, H. Kubota, Y. Ohmiya and H. Akiyama, *Nat. Photonics*, 2007, **2**, 44–47.
8 L. Pinto da Silva and J. C. G. Esteves da Silva, *ChemPhysChem*, 2011, **12**, 951–960.
9 P. Naumov, Y. Ozawa, K. Ohkubo and S. Fukuzumi, *J. Am. Chem. Soc.*, 2009, **131**, 11590–11605.
10 T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata and H. Kato, *Nature*, 2006, **440**, 372–376.
11 S. Hosseinkhani, *Cell. Mol. Life Sci.*, 2011, **68**, 1167–1182.
12 H. H. Seliger and W. D. McElroy, *Proc. Natl. Acad. Sci. U. S. A.*, 1964, **52**, 75–81.
13 C. d'Enfert, A. Vecchiarelli and A. J. Brown, *Virulence*, 2010, **1**, 174–176.
14 J. J. Hawes and K. M. Reilly, *Toxicol. Pathol.*, 2010, **38**, 123–130.
15 A. McCaffrey, M. A. Kay and C. H. Contag, *Mol. Imaging*, 2003, **2**, 75–86.
16 K. E. Luker, M. C. Smith, G. D. Luker, S. T. Gammon, H. Piwnica-Worms and D. Piwnica-Worms, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12288–12293.
17 J. A. Prescher and C. H. Contag, *Curr. Opin. Chem. Biol.*, 2010, **14**, 80–89.
18 C. Klerk, R. M. Overmeer, T. Niers, H. H. Versteeg, D. J. Richel, T. Buckle, C. Van Noorden and O. van Tellingen, *BioTechniques*, 2007, **43**, 7–13.
19 S. S. Chandran, S. A. Williams and S. R. Denmeade, *Luminescence*, 2009, **24**, 35–38.
20 K. E. Luker and G. D. Luker, *Antiviral Res.*, 2008, **78**, 179–187.
21 J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski and S. Subramani, *Mol. Cell. Biol.*, 1987, **7**, 725–737.
22 K. V. Wood, Y. A. Lam, H. H. Seliger and W. D. McElroy, *Science*, 1989, **244**, 700–702.
23 S. Bhaumik and S. S. Gambhir, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 377–382.
24 A. Pichler, J. L. Prior and D. Piwnica-Worms, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 1702–1707.
25 B. A. Tannous, D.-E. Kim, J. L. Fernandez, R. Weissleder and X. O. Breakefield, *Mol. Ther.*, 2005, **11**, 435–443.
26 E. A. Meighen, *Microbiol. Rev.*, 1991, **55**, 123–142.
27 C. H. Contag, P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson and D. A. Benaron, *Mol. Microbiol.*, 1995, **18**, 593–603.
28 S. Wu, E. Chang and Z. Cheng, *Curr. Org. Synth.*, 2011, **8**, 488–497.
29 E. Conti, N. P. Franks and P. Brick, *Structure*, 1996, **4**, 287–298.
30 B. Bitler and W. D. McElroy, *Arch. Biochem. Biophys.*, 1957, **72**, 358–368.
31 H. H. Seliger, W. D. McElroy, E. H. White and G. F. Field, *Proc. Natl. Acad. Sci. U. S. A.*, 1961, **47**, 1129–1134.
32 E. H. White, H. Wörther, H. H. Seliger and W. D. McElroy, *J. Am. Chem. Soc.*, 1966, **88**, 2015–2019.
33 C. C. Woodroofe, J. W. Shultz, M. G. Wood, J. Osterman, J. J. Cali, W. J. Daily, P. L. Meisenheimer and D. H. Klaubert, *Biochemistry*, 2008, **47**, 10383–10393.
34 G. R. Reddy, W. C. Thompson and S. C. Miller, *J. Am. Chem. Soc.*, 2010, **132**, 13586–13587.

35 H. Takakura, R. Kojima, Y. Urano, T. Terai, K. Hanaoka and T. Nagano, *Chem.–Asian J.*, 2011, **6**, 1800–1810.
36 N. R. Conley, A. Dragulescu-Andrasi, J. Rao and W. E. Moerner, *Angew. Chem., Int. Ed.*, 2012, **51**, 3350–3353.
37 D. C. McCutcheon, M. A. Paley, R. C. Steinhardt and J. A. Prescher, *J. Am. Chem. Soc.*, 2012, **134**, 7604–7607.
38 N. A. Thornberry, T. A. Rano, E. P. Peterson, D. M. Rasper, T. Timkey, M. Garcia-Calvo, V. M. Houtzager, P. A. Nordstrom, S. Roy, J. P. Vaillancourt, K. T. Chapman and D. W. Nicholson, *J. Biol. Chem.*, 1997, **272**, 17907–17911.
39 T. Monsees, W. Miska and R. Geiger, *Anal. Biochem.*, 1994, **221**, 329–334.
40 M. A. O'Brien, W. J. Daily, P. E. Hesselberth, R. A. Moravec, M. A. Scurria, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *J. Biomol. Screening*, 2005, **10**, 137–148.
41 M. Kindermann, H. Roschitzki-Voser, D. Caglic, U. Repnik, C. Miniejew, P. R. Mittl, G. Kosec, M. G. Grutter, B. Turk and K. U. Wendt, *Chem. Biol.*, 2010, **17**, 999–1007.
42 D. M. Leippe, D. Nguyen, M. Zhou, T. Good, T. A. Kirkland, M. Scurria, L. Bernad, T. Ugo, J. Vidugiriene, J. J. Cali, D. H. Klaubert and M. A. O'Brien, *Biotechniques*, 2011, **51**, 105–110.
43 R. A. Moravec, M. A. O'Brien, W. J. Daily, M. A. Scurria, L. Bernad and T. L. Riss, *Anal. Biochem.*, 2009, **387**, 294–302.
44 M. A. O'Brien, R. A. Moravec, T. L. Riss and R. F. Bulleit, *Methods Mol. Biol.*, 2008, **414**, 163–181.
45 D. E. Bassi, R. Lopez De Cicco, H. Mahloogi, S. Zucker, G. Thomas and A. J. Klein-Szanto, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 10326–10331.
46 A. Dragulescu-Andrasi, G. Liang and J. Rao, *Bioconjugate Chem.*, 2009, **20**, 1660–1666.
47 R. Geiger, E. Schneider, K. Wallenfels and W. Miska, *Biol. Chem. Hoppe-Seyler*, 1992, **373**, 1187–1191.
48 I. Masuda-Nishimura, S. Fukuda, A. Sano, K. Kasai and H. Tatsumi, *Lett. Appl. Microbiol.*, 2000, **30**, 130–135.
49 X. Yang, J. Janatova and J. D. Andrade, *Anal. Biochem.*, 2005, **336**, 102–107.
50 G. von Degenfeld, T. S. Wehrman and H. M. Blau, *Methods Mol. Biol.*, 2009, **574**, 249–259.
51 W. Zhou, J. W. Shultz, N. Murphy, E. M. Hawkins, L. Bernad, T. Good, L. Moothart, S. Frackman, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *Chem. Commun.*, 2006, 4620–4622.
52 D. E. Edmondson, A. Mattevi, C. Binda, M. Li and F. Hubalek, *Curr. Med. Chem.*, 2004, **11**, 1983–1993.
53 W. Zhou, M. P. Valley, J. Shultz, E. M. Hawkins, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert and K. V. Wood, *J. Am. Chem. Soc.*, 2006, **128**, 3122–3123.
54 M. P. Valley, W. Zhou, E. M. Hawkins, J. Shultz, J. J. Cali, T. Worzella, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert and K. V. Wood, *Anal. Biochem.*, 2006, **359**, 238–246.
55 R. B. Sykes and M. Matthew, *J. Antimicrob. Chemother.*, 1976, **2**, 115–157.
56 H. Yao, M. K. So and J. Rao, *Angew. Chem., Int. Ed.*, 2007, **46**, 7031–7034.
57 H. B. Fell and R. Robison, *Biochem. J.*, 1929, **23**, 765, 7677–784.
58 W. Zhou, C. Andrews, J. Liu, J. W. Shultz, M. P. Valley, J. J. Cali, E. M. Hawkins, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *ChemBioChem*, 2008, **9**, 714–718.
59 A. Kanno, Y. Yamanaka, H. Hirano, Y. Umezawa and T. Ozawa, *Angew. Chem.*, 2007, **119**, 7739–7743.
60 Y. C. Chang, P. W. Chao and C. H. Tung, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 3931–3934.
61 J. S. Rush, K. E. Beatty and C. R. Bertozzi, *ChemBioChem*, 2010, **11**, 2096–2099.
62 L. C. Wienkers and T. G. Heath, *Nat. Rev. Drug Discovery*, 2005, **4**, 825–833.
63 P. L. Meisenheimer, H. T. Uyeda, D. Ma, M. Sobol, M. G. McDougall, C. Corona, D. Simpson, D. H. Klaubert and J. J. Cali, *Drug Metab. Dispos.*, 2011, **39**, 2403–2410.
64 M. D. Sorensen, R. Martins and O. Hindsgaul, *Angew. Chem., Int. Ed.*, 2007, **46**, 2403–2407.
65 D. H. Dube and C. R. Bertozzi, *Nat. Rev. Drug Discovery*, 2005, **4**, 477–488.
66 A. S. Cohen, E. A. Dubikovskaya, J. S. Rush and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 8563–8565.
67 P. P. Minghetti and A. W. Norman, *FASEB J.*, 1988, **2**, 3043–3053.
68 L. Lamattina, C. García-Mata, M. Graziano and G. Pagnussat, *Annu. Rev. Plant Biol.*, 2003, **54**, 109–136.
69 R. C. O. Zanardo, V. Brancaleone, E. Distrutti, S. Fiorucci, G. Cirino and J. L. Wallace, *FASEB J.*, 2006, **20**, 2118–2120.
70 S. G. Rhee, *Science*, 2006, **312**, 1882–1883.
71 G. C. Van de Bittner, E. A. Dubikovskaya, C. R. Bertozzi and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 21316–21321.
72 K. Niwa, Y. Nakajima and Y. Ohmiya, *Anal. Biochem.*, 2010, **396**, 316–318.
73 Q. Shao, T. Jiang, G. Ren, Z. Cheng and B. Xing, *Chem. Commun.*, 2009, 4028–4030.
74 Y. Yang, Q. Shao, R. Deng, C. Wang, X. Teng, K. Cheng, Z. Cheng, L. Huang, Z. Liu, X. Liu and B. Xing, *Angew. Chem., Int. Ed.*, 2012, **51**, 3125–3129.
75 P. A. Wender, E. A. Goun, L. R. Jones, T. H. Pillow, J. B. Rothbard, R. Shinde and C. H. Contag, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 10340–10345.
76 L. R. Jones, E. A. Goun, R. Shinde, J. B. Rothbard, C. H. Contag and P. A. Wender, *J. Am. Chem. Soc.*, 2006, **128**, 6526–6527.
77 E. Eiriksdottir, I. Mager, T. Lehto, S. El Andaloussi and U. Langel, *Bioconjugate Chem.*, 2010, **21**, 1662–1672.

