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Review

Luminescence of imidazo[1,2-*a*]pyrazin-3(7*H*)-one compounds

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Abstract

In this review I will discuss chemical principles of the luminescence of imidazo[1,2-*a*]pyrazin-3(7*H*)-one compounds described to date. The review is composed of two main parts, the first dealing with the bioluminescence of coelenterate luciferin ''coelenterazine'' and Cypridina luciferin in marine organisms and the second with the chemiluminescence of these luciferins and their analogues. In the second section, possible applications of chemiluminescence and enhanced chemiluminescence in the area of bioassay are also discussed.

Keywords: Bioluminescence; Chemiluminescence; Coelenterazine; Aequorin; Imidazopyrazinone

1. Introduction

Bioluminescence has been widely observed in various organisms, such as insects, fish, cnidaria, crustacea, molluscs, bacteria, and mushrooms, and aroused scientific and nonscientific fascination throughout human history [1–5]. About a century ago, Dubois conducted a chemical investigation of bioluminescence in the luminous beetle *Pyrophorus* [6], and Harvey investigated bioluminescence in Japanese fireflies (*Luciola parva* and *Luciola vitticollis*), an ostracod crustacean (*Cypridina hilgendorfii*), a squid (*Watasenia scintillans*), a pennatulid (*Cavernularia haberi*), and a protozoan (*Noctiluca miliaris*) [7]. Since then,

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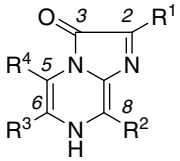


Fig. 1. Structure of imidazo[1,2-*a*]pyrazin-3(7*H*)-one.

the number of scientific studies on bioluminescence steadily increased. In the past half-century there has been a remarkable increase in scientific findings on bioluminescence in the fields of biochemistry, organic chemistry, physical chemistry, and biology.

Bioluminescence takes place when energy biochemically produced inside or outside an organism is converted to photon emission with high efficiency. Dubois thought that bioluminescence was the result of a reaction between the energy-source substance "luciferin" and the enzyme "luciferase". Studies on bioluminescence to date showed that a luciferin-luciferase system is involved in many kinds of luminous organisms [8,9], but an alternative bioluminescence system that does not involve a luciferin–luciferase system was found in some organisms, such as the jellyfish *Aequorea aequorea* [10], and it was called photoprotein system.

Bioluminescence is observed in a great variety of marine organisms; it is particularly common among the deep-sea organisms. Herring found bioluminescent species among 700 genera in 17 phyla [11]. The substances "imidazo[1,2-*a*]pyrazin-3(7*H*)-one (imidazopyrazinone) compounds" (Fig. 1) were found in organisms of many phyla, and "imidazopyrazinone" has been recognized to be a common structure in the luciferins involved in marine bioluminescence [12–18].

Some luciferins are capable of emitting light in the absence of luciferase or apophotoprotein; this phenomenon is called chemiluminescence [19–22]. Imidazopyrazinone luciferins and their analogues react with oxygen species resulting in the emission of bioluminescence and chemiluminescence. In the past, studies on the imidazopyrazinone chemiluminescence greatly contributed to our understanding of marine bioluminescence. Moreover, both bioluminescence and chemiluminescence are now widely used as a biochemical and diagnostic tool [23–32].

This review is focused on the imidazopyrazinone compounds capable of light emission. It is divided into two parts, the first part dealing with the bioluminescence of coelenterazine, aequorin, and Cypridina luciferin, and the second with the chemiluminescence of imidazopyrazinones. The latter section contains a discussion on the possible applications of chemiluminescence and enhanced chemiluminescence in the area of bioassays.

2. Imidazopyrazinone bioluminescence

2.1. Coelenterazine

Coelenterazine (Fig. 2) was first isolated from the liver of a squid, *Watasenia scintillans*; it was named *Watasenia* preluciferin and chemically synthesized by Inoue et al.[33]. In 1962, Shimomura et al. extracted and purified a Ca^{2+}-triggered bioluminescent photoprotein, aequorin, from the jellyfish *Aequorea* [10,34]. In 1974, the same group predicted the existence of coelenterazine in aequorin as its functional group [35], based on the charac-

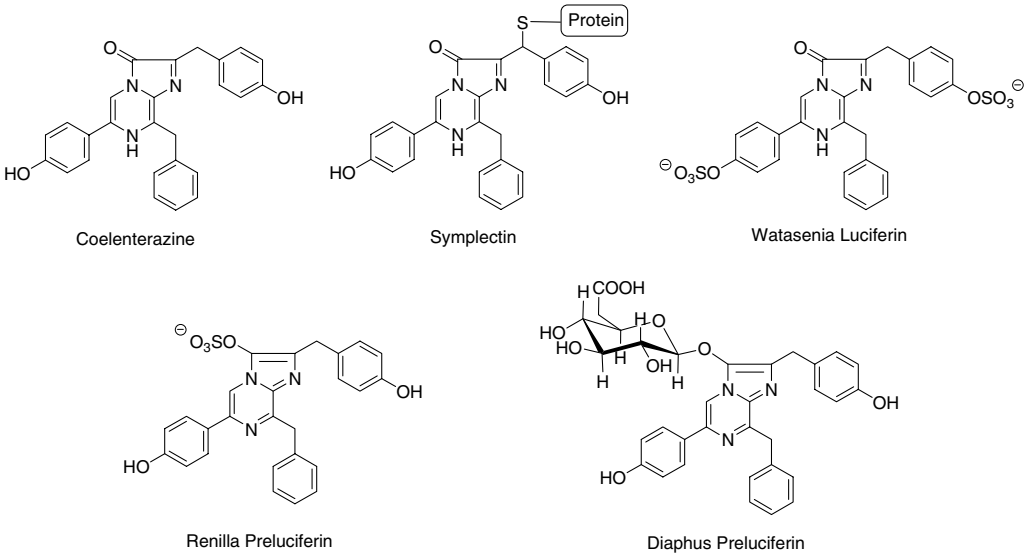


Fig. 2. The structures of coelenterazine and its derivatives found in bioluminescent organisms.

teristics of the Cypridina luciferin system [36,37] and the chemical structure of coelenteramide, the light emitter in the bioluminescence of aequorin [38]. Coelenterazine was found to be the luciferin in a wide range of bioluminescent marine organisms [14,16,18] such as coelenterates [12,39–52], squid [12,16], shrimp [12,16,53–56], fish [12,15,57–61], and copepods [16], and also in various non-bioluminescent marine organisms [14]. The derivatives of coelenterazine (Fig. 2) have also been found from several kinds of marine luminescent organisms, for example: in the photoprotein symplectin from the oceanic squid *Symplectoteuthis oualaniensis* [62–70], the disulfate as *Watasenia* luciferin from the deep-sea squid *Watasenia scintillans* (Hotaru-ika in Japanese) [71–75], the 3-enolsulfate as *Renilla* preluciferin from the sea pansy [43,76], and the 3-enol glucuronide as a preluciferin from a fish [59,60].

2.2. Aequorin

Aequorin emits blue light (λ_{max} about 465 nm) when a trace of Ca^{2+} is added, independently of the presence or absence of molecular oxygen [10]. Because the luminescence of aequorin is not a luciferin–luciferase reaction, its unique luminescence mechanism attracted attention of many researchers. Shimomura and coworkers have conducted a major investigation of aequorin, and the initial part of the investigation was described by Shimomura in "A Short Story of Aequorin" [77]. A review by Ohmiya and Hirano covers many of the studies on aequorin published up to 1995 [78]. In 2000, Shimomura's group has completely elucidated the structure and luminescence mechanism of aequorin, as summarized in Fig. 3 [79]. The photoprotein aequorin consists of apoprotein (apoaequorin) having three Ca^{2+}-binding sites and a coelenterazine–oxygen molecular complex having a hydroperoxide structure (upper right in Fig. 3). When the EF-hand Ca^{2+}-binding sites of aequorin are bound with two or more calcium ions [80,81], the conformation of the pro-

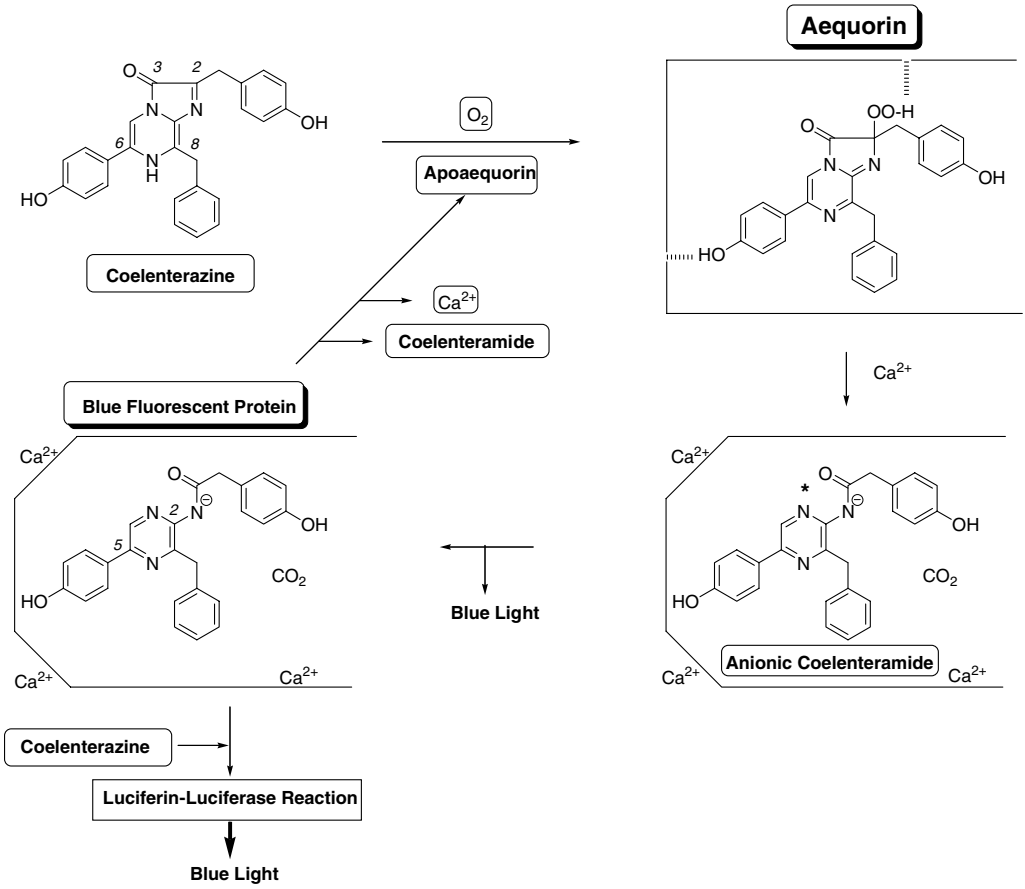


Fig. 3. Mechanism of the aequorin bioluminescence.

tein changes, triggering an intramolecular reaction that results in the formation of an unstable dioxetanone intermediate from the hydroperoxide moiety. Splitting of carbon dioxide from dioxetanone is accompanied by the formation of the singlet excited state of anionic coelenteramide, which relaxes to its ground state accompanied by the emission of light. Anionic coelenteramide remains bound to the protein after light emission. The coelenteramide–apoprotein–Ca^{2+} complex thus produced shows a blue fluorescence that spectrally corresponds to aequorin bioluminescence. This complex is known as blue fluorescent protein (BFP) [82,83]. Coelenteramide can be removed from BFP by gel filtration. An incubation of apoprotein and coelenterazine in the presence of molecular oxygen results in the regeneration of original aequorin [84].

Chemically interesting areas in the aequorin bioluminescence include the structure of the chromophore, the oxidation mechanism, and the structure of the emitter. In the studies of aequorin, Shimomura et al. first isolated a fluorescent compound, coelenteramine, as a possible light emitter of aequorin [85], and subsequently showed that the actual light emitter is not the excited state of coelenteramine nor that of coelenteramine bound to apoprotein, but it is the excited state of coelenteramide [86]. Later, their study on the yellow

compound produced by the treatment of aequorin with $NaHSO_3$ [87], and the ^{13}C NMR study of the aequorins prepared with ^{13}C-enriched coelenterazines and apoaequorin under $^{16}O_2$ and $^{18}O_2$ atmosphere [88], indicated that molecular oxygen is attached at the 2-position of coelenterazine, forming a peroxide or hydroperoxide group. Their proposed structure was proved to be correct by the X-ray crystallography of aequorin in 2000 [79].

A cDNA library for apoaequorin was constructed and the clone carrying the cDNA for the protein was isolated. The primary structure of the protein deduced from the nucleotide sequence showed that it is composed of 189 amino acid residues and has three EF-hand structures as the Ca^{2+}-binding sites [89,90]. Expression of apoaequorin in *Escherichia coli* was achieved, and the apoaequorin expressed was successfully regenerated into active aequorin [91]. Several different modified apoaequorins have also been produced by site-specific mutagenesis [92–98].

In 1975, Inoue et al. synthesized coelenterazine for the first time by the condensation of coelenteramine with *p*-acetoxybenzyl glyoxal (Fig. 4) [33], in which coelenteramine previously synthesized by Kishi et al. [99] was used. Recently, the synthesis method of coelenteramine was improved [100,101], and new methods for synthesizing 3,5-substituted 2-aminopyrazines were reported utilizing Pd-mediated Still coupling and Suzuki coupling (Fig. 5), respectively by Nakamura et al. and Johnes et al. [102–105]. Using these new methods, several coelenterazine analogues were synthesized [106,107]. With regard to the synthesis of the imidazopyrazinone ring system, Hirano et al. reported the preparation of α-ketoacetal using Grignard reagents and subsequent condensation of the α-ketoacetal with coelenteramine [108,109]. Several α-ketoacetal derivatives were prepared by this method [110]. Kakoi and Inoue reported an interesting method of synthesizing imidazo-

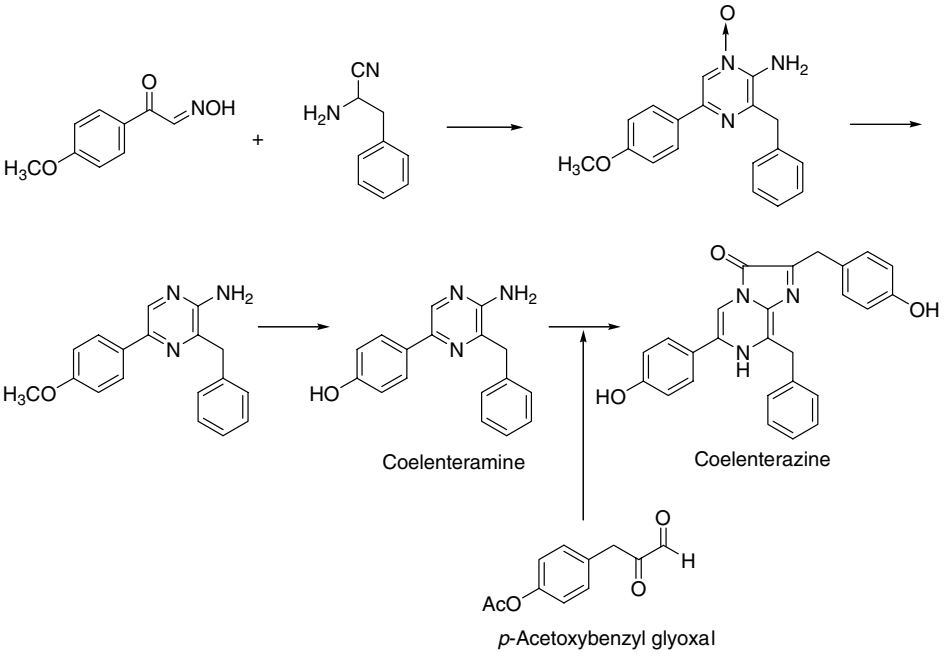


Fig. 4. Synthesis of coelenterazine.

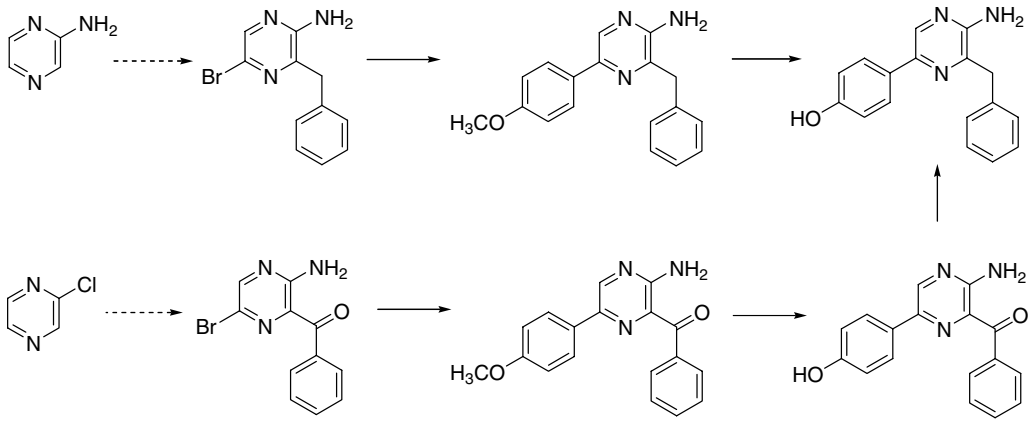


Fig. 5. Synthesis methods of coelenteramine by Nakamura et al. (upper scheme) and by Jones et al. (lower scheme).

pyrazinone ring using coelenteramine and *p*-hydroxyphenylpyruvic acid, in one batch process not involving a reduction step [111,112].

The synthesis of coelenterazine analogues made it possible to prepare various semi-synthetic aequorins, which consequently allowed researchers to investigate the functions of aequorin and also to develop novel aequorin derivatives as a scientific tool. In 1988, Shimomura et al. prepared four novel aequorin analogues for the first time, from four kinds of coelenterazine analogues and native apoaequorin, and named them semi-synthetic aequorins. One of the semi-synthetic aequorins obtained, *e*-aequorin (Fig. 6), was highly unique in its properties:(1) the rise time of luminescence in response to Ca^{2+} was only one-fourth in comparison with native aequorin; and (2) its luminescence spectrum showed two peaks, at 405 and 465 nm, and the ratio of heights was dependent on the concentration of Ca^{2+} in the range of 10^{-7} - 10^{-5} M, thus allowing the determination of Ca^{2+} concentrations [113]. Subsequently, 37 kinds of coelenterazine analogues were synthesized and then incorporated into native apoaequorin, giving 30 kinds semi-synthetic aequorins that possesses significant capabilities of light emission in the presence of Ca^{2+} [114]. These semi-synthetic aequorins showed widely different Ca^{2+}-sensitivities and light-emitting characteristics [114,115].

The preparations of recombinant aequorin and recombinant semi-synthetic aequorins were reported by Shimomura et al. [116,117]. The recombinant aequorin obtained, how-

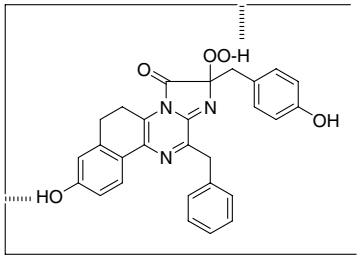


Fig. 6. *e*-Aequorin.

ever, did not match any of the ten kinds of natural isoaequorins in its chromatographic behavior, but it was very similar to aequorin J, although the Ca^{2+} sensitivity of the recombinant aequorin was greater than those of all isoaequorins except aequorin D [116,118]. It was also found that the rates of the regeneration of semi-synthetic aequorins varies widely: the regeneration of ordinary aequorin with normal coelenterazine is relatively fast, but the rates of regenerating semi-synthetic aequorins differed widely by the coelenterazine analogues used, and all are slower than the regeneration of ordinary aequorin, except *e*-aequorin [117]. Four kinds of recombinant semi-synthetic aequorins prepared from recombinant apoaequorin and synthetic coelenterazine analogues, *fch*-, *hcp*-, *e*- and *n*-types, showed their properties in parallel with those of the corresponding semi-synthetic aequorins prepared from natural apoaequorin. These recombinant semi-synthetic aequorins and natural semi-synthetic aequorins are highly suitable for monitoring cellular Ca^{2+}. In addition, Shimomura et al. prepared fluorescein-conjugated aequorin, which exhibits fluorescence in addition to Ca^{2+}-triggered greenish luminescence. Using this material, the diffusion and distribution of aequorin molecules can be visualized in cells [119–121]. Inouye biotinylated aequorin, targeting the lysine residues in the molecules [122].

In the hope of elucidating the three-dimensional structure of aequorin, the structural analysis of the crystals of recombinant aequorin was attempted, but the effort was unsuccessful probably due to the insufficient purity of the aequorin used [123]. Therefore, Shimomura's group obtained a sample of recombinant aequorin in a very high-purity by using the *in situ* regeneration method [124] and crystallized, then the crystal structure of aequorin was successfully determined in 2000 [79]. Aequorin was found to be a globular molecule containing a hydrophobic core cavity which accommodates coelenterazine-2-hydroperoxide. The peroxide is stabilized by the amino acid residues surrounding it. To investigate the reason why semi-synthetic aequorins show widely different Ca^{2+}-sensitivities, the crystal structures of four semi-synthetic aequorins (*cp*-, *i*-, *br*-, and *n*-aequorins) were determined in 2005 [125]. The results indicated that the protein structures of the four semi-synthetic aequorins were almost identical to native aequorin, with only some subtle differences. Of the four EF-hand domains, however, it was found that EF-hand II does not bind with Ca^{2+}, and the loop of EF-hand IV is deformed. Thus, it is likely that the binding of Ca^{2+} with EF-hands I and III triggers bioluminescence.

According to Shimomura, the light emitter in the bioluminescence of aequorin is normally the amide anion; the only exception is the un-ionized form, which is found in the luminescence of *e*-type aequorins [126,127]. On the other hand, Hirano et al. maintain their opinion that the phenolate anion of coelenteramide is the light emitter in aequorin bioluminescence, on the basis that *N*-methylcoelenteramide bound to apoaequorin shows blue fluorescence [128], and that the bioluminescence properties of the semi-synthetic aequorins prepared with the coelenterazine analogues having a fluoro atom on 6-(4-hydroxyphenyl) group match the fluorescence properties of the phenolate anions of the corresponding fluorinated coelenteramide [129,130].

BFP generates continuous luminescence when incubated with coelenterazine [84,131], in which apoaequorin functions as an enzyme in a manner similar to luciferase, and coelenterazine is oxidized with light emission. Inouye purified BFP and found that BFP is a heat-resistant complex and can catalyze the luminescent oxidation of coelenterazine in the presence of molecular oxygen, like a common luciferase [132]. Inouye further found that coelenteramide and apoaequorin are non-covalently bound in a molar ratio of 1:1 in BFP, that the removal of Ca^{2+} from BFP causes to change its fluorescence from blue to

greenish, and that the greenish fluorescent protein can be converted into aequorin without reducing reagents [132].

The use of aequorin as a bioscientific tool is increasing. In the SciFinder database, the term ''aequorin'' was found in more than 2800 studies in June 2006. Aequorin was shown to be useful in intracellular Ca^{2+} detection [133]; aequorin and aequorin generated *in situ* in living cells via the reaction of coelenterazine with the apoaequorin expressed, may be used for detecting the location of Ca^{2+} and measuring the concentration in real time [134–138]. The expression of apoaequorin is also used in monitoring gene expression [139]. In the generation of aequorin using apoaequorin and coelenterazine, the poor water-solubility of coelenterazine is often a problem. Teranishi and Shimomura improved its solubility using cyclodextrins that does not inhibit the generation of aequorin [140].

Although aequorin emits blue luminescence, the bioluminescence of *Aequorea* from which aequorin derived is green. The green luminescence of *Aequorea* is emitted by green fluorescent protein (GFP) that exists in the luminescent organs of this jellyfish [141–150]; GFP emits green light when excited by the excited state of the light emitter of aequorin through an intermolecular energy transfer.

2.3. *Cypridina luciferin*

Many kinds of crustacean show bioluminescence [151]. Certain Cypridinid ostracods such as *Cypridina hilgendorfii* squirt luciferin and luciferase into the seawater, which causes a luciferin–luciferase reaction when they are mixed, producing a blue light with a quantum yield of 0.3 [152,153]. Shimomura et al. isolated Cypridina luciferin in a crystalline state in 1957 [154]. In 1966 the compound was shown to be an imidazopyrazinone compound (Fig. 7) [155,156], and chemically synthesized [157,158]. The luciferase of Cypridina was purified [159–161], and Shimomura found that the fluorescence emission spectrum of the spent solution of Cypridina bioluminescence closely coincides with the bioluminescence spectrum, suggesting that the *Cypridina* oxyluciferin–luciferase complex is the light emitter in the Cypridina bioluminescence [162]. The chemiluminescence of Cypridina luciferin and its analogues was also investigated, contributing to the understanding of chemical mechanism of Cypridina luciferin–luciferase luminescence [8].

The gene of Cypridina luciferase was isolated [163], and it was suggested to be useful for monitoring gene expression by detecting the enzyme with Cypridina luciferin-luciferase luminescence reaction [164–167]. Nakamura et al. recently synthesized DL-Cypridina luciferin and its analogues using Pd-mediated cross coupling [168]. This method, which enables to synthesize Cypridina luciferin and its analogues in large quantities, is expected to make a significant contribution to the research in the field of life science.

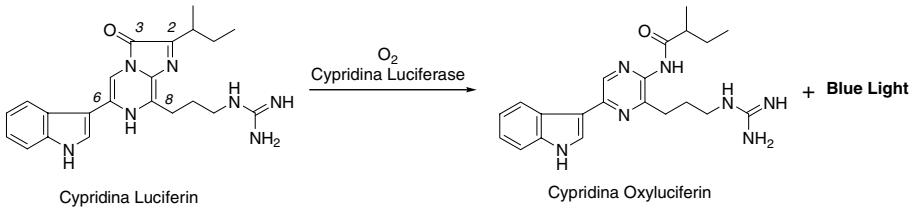


Fig. 7. Cypridina luciferin–luciferase luminescence.

Kishi et al. suggested the possibility that Cypridina luciferin is biosynthesized from three amino acids, L-arginine, L-isoleucine, and L-tryptophan (or tryptamine) or their equivalents [157]. Recently, Inouye et al. reported that L-arginine, L-isoleucine, and L-tryptophan may be the biosynthetic units of Cypridina luciferin based on the feeding experiments of isotope-labeled amino acids [169,170].

3. Imidazopyrazinone chemiluminescence

3.1. Chemiluminescence mechanism

Since the 1960s, several groups of chemists have studied the chemiluminescence of imidazopyrazinones with the aim of understanding the chemical mechanism of *Cypridina* bioluminescence. Johnson et al. found that Cypridina luciferin generates luminescence in dimethyl sulfoxide in the presence of air and a base without using luciferase, although its quantum yield was quite low [171]. McCapra and Chang reported that the oxidation in air of compound **1** (Fig. 8) in dimethyl sulfoxide in the presence of potassium *t*-butoxide gives the amidopyrazine **2** (Fig. 8) in 89% yield, accompanied by a light emission (λ_{max} 455 nm) that spectrally coincides with the fluorescence of the amidopyrazine in the same solvent. This experiment led to a conclusion that the amidopyrazine anion is the light emitter [172]. When the same experiment is conducted in the presence of triethylamine, a weak base, the chemiluminescence shows a maximum at 455 nm, but the fluorescence of the amidopyrazine in this medium is emitted from its neutral species (λ_{max} 380 nm), indicating that protonation occurs after the light emission [172]. Goto investigated the chemiluminescence of compound **3** (Fig. 8) in dimethyl sulfoxide in the presence of *t*-butoxide, and obtained the results similar to those of McCapra [173]. Although the quantum yield of Cypridina luciferin obtained in dimethyl sulfoxide with a base is very low [171], the light yield is greatly improved, nearly 100-fold, by using diethylene glycol dimethyl ether (diglyme) containing acetate buffer (pH 5.6) as the solvent; the quantum yield exceeds 10% of the quantum yield of Cypridina bioluminescence [173]. This result

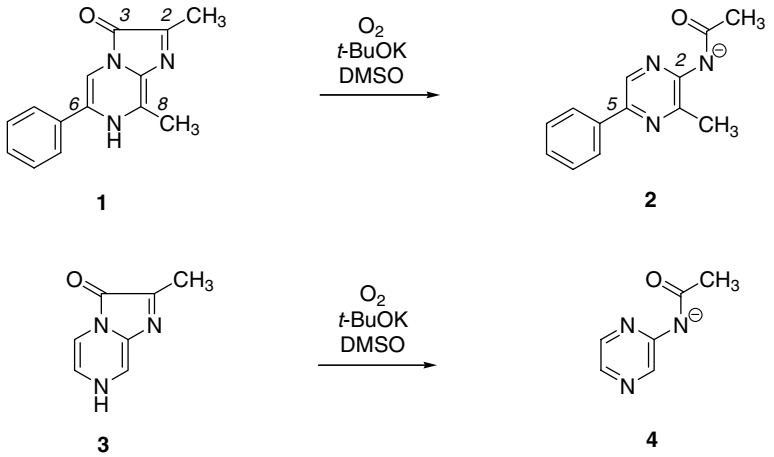


Fig. 8. Chemiluminescence of imidazopyrazinones.

may suggest that chemiluminescence and bioluminescence might involve a similar mechanism. The chemiluminescence spectrum of Cypridina luciferin in diglyme containing acetate buffer (pH 5.6) is similar to the fluorescence spectrum of oxyluciferin in the same solvent and is clearly different from that of oxyluciferin in alkaline solution. Thus, the emitting species involved must be neutral oxyluciferin, not anionic oxyluciferin [174].

Lophine [175] and indole derivatives [176,177] produce luminescence through the chemical reactions with air and bases. In these reactions, hydroperoxides [178–184] and dioxetanes [185] are the intermediates. On the basis of these reactions, McCapra proposed a pathway A for the chemiluminescence of Cypridina luciferin that involves a 2-hydroperoxide intermediate (Fig. 9) [172], and Goto presented another pathway B (Fig. 9) that involves a peroxoic acid intermediate for the same chemiluminescence reaction [8]. Goto preferred pathway B because the intermediate involved is likely to produce luminescence under very mild conditions such as a diglyme–acetate buffer (pH 5.6) system and an aqueous system containing hydrogen peroxide and ferric ions [8,186,187]. Shimomura and Johnson [188] and Cormier et al [189] persisted with the investigation of pathway A, ignoring pathway B. Fujimori et al. [190] and Teranishi et al. [191,192] suggested 5-hydroperoxide as another possible intermediate in the luminescence of imidazopyrazinone (pathway C in Fig. 9). Because the intermediates of luminescence reaction such as the hydroperoxides and dioxetanes should have very short lifetimes, the intermediates in the chemiluminescence reactions of imidazopyrazinones have not yet been established. When Hirano et al. investigated the chemiluminescence reactions of imidazopyrazinones by means of mass spectroscopy, only oxygenated ions $[M+OH]^-$ were obtained; these are believed to be mass spectrometric intermediates rather than the intermediates of luminescence reaction [193].

In order to investigate the characteristics of the hydroperoxide intermediates (Fig. 9) suggested by McCapra et al., Goto, and other groups, Teranishi et al. attempted to chemically synthesize these intermediates [192,194–197]. Three hydroperoxides, **1**–**3** (Fig. 10), were successfully synthesized and their properties investigated. Hydroperoxide **1** was prepared by photo-oxygenation in dichloromethane at −95 °C using polymer-bound rose bengal [198] as a sensitizer, and its structure was confirmed by ^{1}H NMR at −80 °C. The compound emits luminescence ($\lambda_{max} = 395$ nm) at a temperature above about −50 °C in both polar and non-polar solvents [194]. The luminescence spectrum matches the fluorescence spectrum of amidopyrazine **4** (Fig. 10) under the same conditions. Moreover, an addition of NaOH to a luminescing solution produces a new luminescence spectrum ($\lambda_{max} = 470$ nm) which is identical to the fluorescence spectrum of anionic amidopyrazine **5** (Fig. 10).

Hydroperoxide **2** was synthesized as shown in Fig. 10 [196]. This compound exhibits the luminescence of neutral amidopyrazine in non-polar solvents (λ_{max} 395 nm), and that of anionic amidopyrazine in polar solvents (λ_{max} 456 – 470 nm) differing from hydroperoxide **1**. Thus, it appears that the chemiluminescence emitter of hydroperoxide **2** is determined by the polarity of solvent. The difference between hydroperoxides **1** and **2** may be due to the acidity (pK_a) of the protons on these hydroperoxides; the pKa value of the proton on hydroperoxide **1** is much higher than that of the proton on hydroperoxide **2**, as shown by Goto [8]. The proton of hydroperoxide **2** easily dissociates in polar solvents, whereas the proton of hydroperoxide **1** does not dissociate in both polar and non-polar solvents. The difference of hydroperoxides **1** and **2** suggests that the selection of emitter species (neutral amide or anionic amide) depends on the structure of the hydroperoxide (hydroperoxide **1**

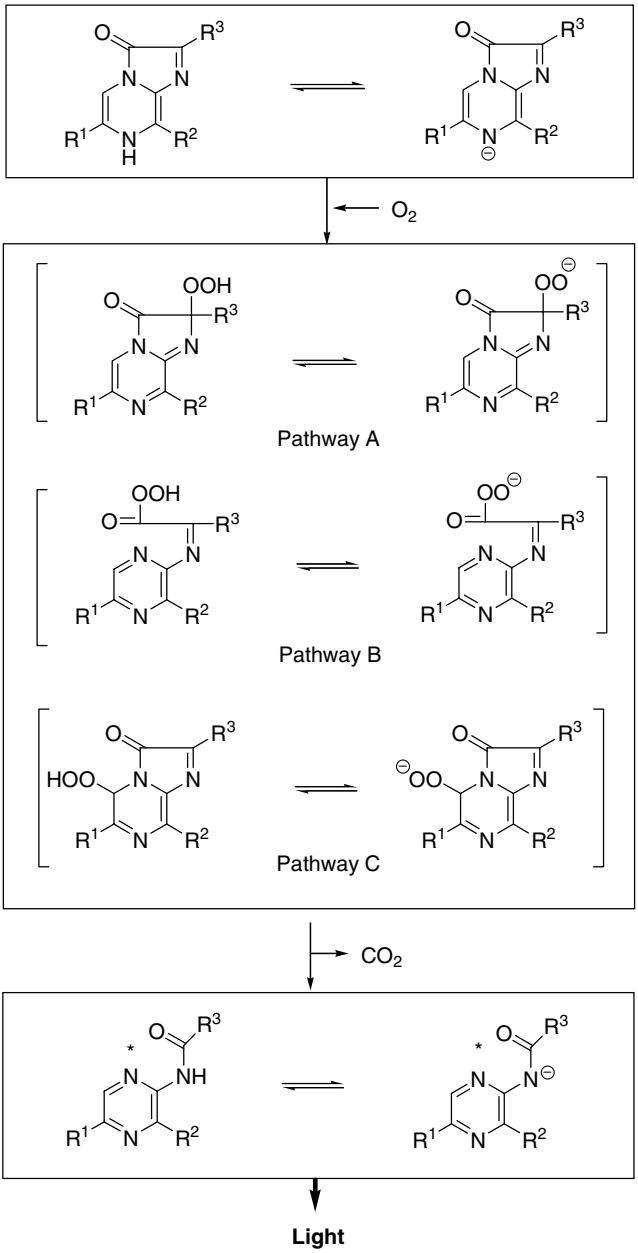


Fig. 9. Possible pathways involved in the imidazopyrazinone chemiluminescence.

or hydroperoxide **2**). In the case of aprotic solvents, however, neutral hydroperoxide **1** and neutral hydroperoxide **2** would produce neutral excited amidopyrazine, and anionic hydroperoxide **1** and anionic hydroperoxide **2** would produce anionic excited amidopyrazine, thus the pathway in the chemiluminescence reaction in dimethyl sulfoxide in the

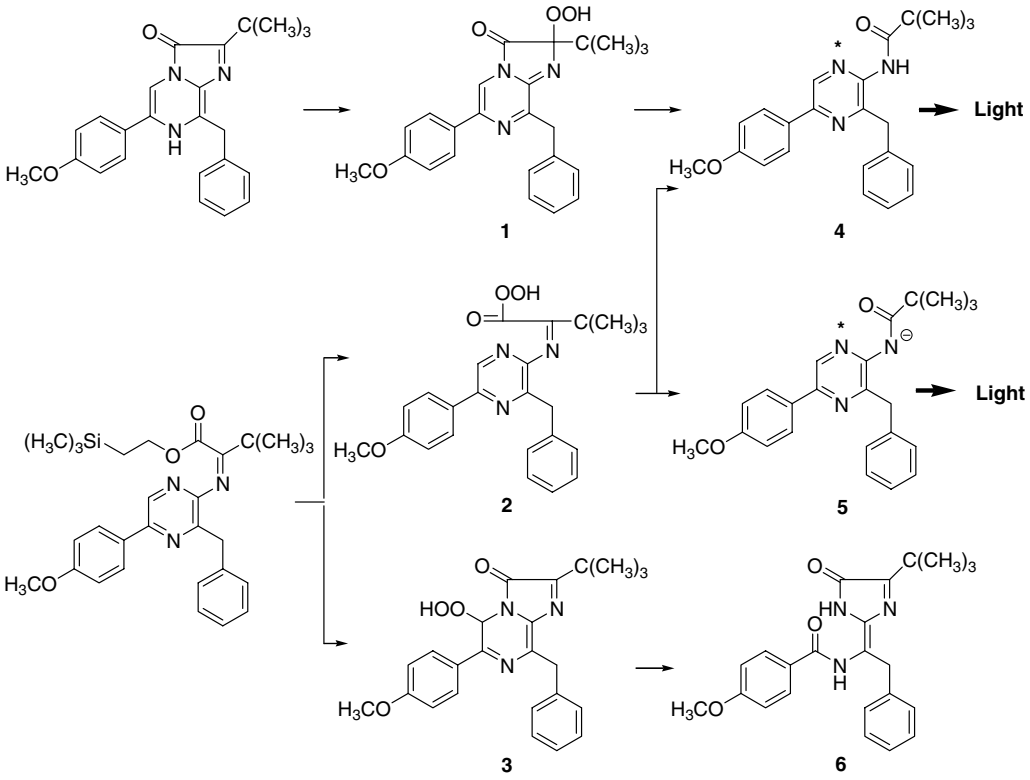


Fig. 10. Synthesis and luminescence reaction of the intermediary hydroperoxides postulated in the luminescence reaction of imidazopyrazinones.

presence or absence of strong base cannot be determined whether it involves A or B. In the case of the diglyme-acetate buffer system that contains water, the luminescence reaction media can protonate all of hydroperoxide **1**, hydroperoxide **2** and the excited amidopyrazine, thus it would be impossible to elucidate whether the intermediate is hydroperoxide **1** or hydroperoxide **2**.

Hydroperoxide **3** was synthesized and isolated; the half-lives of hydroperoxide **3** in methanol and tetrahydrofuran at 20 °C are 1.3 and 63 h, respectively [195]. Hydroperoxide **3** emits very weak luminescence in several solvents and produces compound **6**, indicating that hydroperoxide **3** is probably not an important intermediate in the chemiluminescence reactions of imidazopyrazinones.

If the chemiluminescence of imidazopyrazinones is emitted from the excited anionic amidopyrazine, protonation must be prevented in the pathway between the deprotonation of imidazopyrazinone and the light emission. However, when neutral amidopyrazine is the emitter, as in the reaction in the diglyme-acetate buffer system, the protonation must occur at the stages of hydroperoxides or excited state amidopyrazine. Goto suggested that protonation occurs on the excited state of amidopyrazine [174], and Ohashi et al. are in agreement with it [199].

3.2. Chemiluminescence efficiency

The efficiency of light emission is an important factor in chemiluminescence and bioluminescence. Simple, isolable dioxetanes such as tetramethyl-1,2-dioxetane are relatively stable and produce the excited state molecules of predominantly triplet state that emits little photons upon thermolysis [200], whereas bioluminescence systems produce the singlet excited state that efficiently emits photons. In bioluminescence reactions, the transient intermediate, 1,2-dioxetane, is extremely unstable, like the chemiluminescent compounds that produce highly efficient luminescence [201]. The high efficiency in producing singlet excited state and the instability of the dioxetane intermediates both in bioluminescence, and the highly efficient light emission from certain chemiluminescent compounds may be due to the conjugation between an electron-donating, highly fluorescent chromophore and the excited-state carbonyl group [202,203]. Koo et al. explained those effects in terms of a chemically initiated intramolecular electron-exchange luminescence (intramolecular CIEEL) mechanism [204,205]. In the case of firefly luciferin (Fig. 11), an excited state product is generated by the electron transfer from the easily oxidizable phenoxide anion to the highly energized dioxetanone moiety that causes heterolysis of the O–O bond, followed by a rapid decarboxylation and electron exchange. Thus, the formation of the phenoxide anion strongly accelerates the decomposition of dioxetane and the formation of singlet excited state [206]. The mechanism is supported by the observations reported by Schaap and Gagnon [207–209].

In order to investigate the effect of electron donation on the chemiluminescence of imidazopyrazinones, Goto et al. synthesized 5-(5-phenyl-2-pyrazinylamino)-1,2,4-trioxane derivatives (Fig. 12) having a substituent on the 4-position of the phenyl group, and then studied their chemiluminescence properties [210–212]. The 1,2,4-trioxanes exhibited chemiluminescence in dimethyl sulfoxide upon the addition of potassium *t*-butoxide. With these compounds, the yield of singlet excited state molecules is unaffected by various electron-donating substituent groups. The dioxetane is formed via cyclization of the intermediate hydroperoxide anion, with a negative charge on the nitrogen atom next to the dioxetane moiety which donates enough electrons to the dioxetane moiety to produce singlet excited state molecules; in this case, no other electron-donating moiety is needed.

Teranishi and Goto reported that coelenterazine analogues having an electron-donating group such as OCH_3 or $N(CH_3)_2$, instead of a hydroxyl group, at the 4-positon of the 6-phenyl group (structures at left in Fig. 13) do not increase chemiluminescence efficiencies

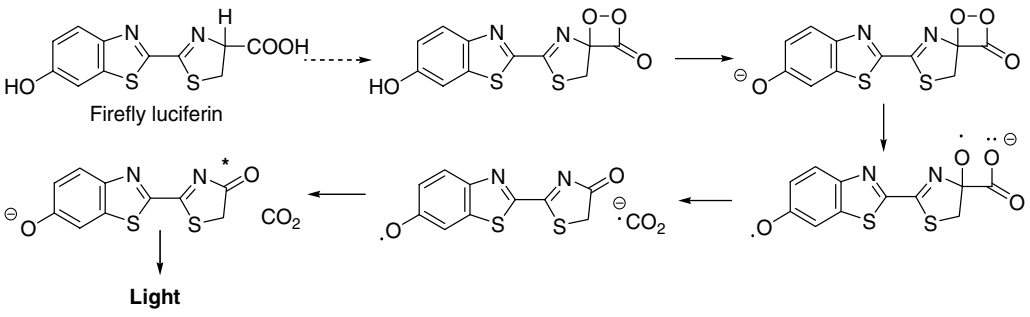


Fig. 11. CIEEL mechanism in the firefly bioluminescence.

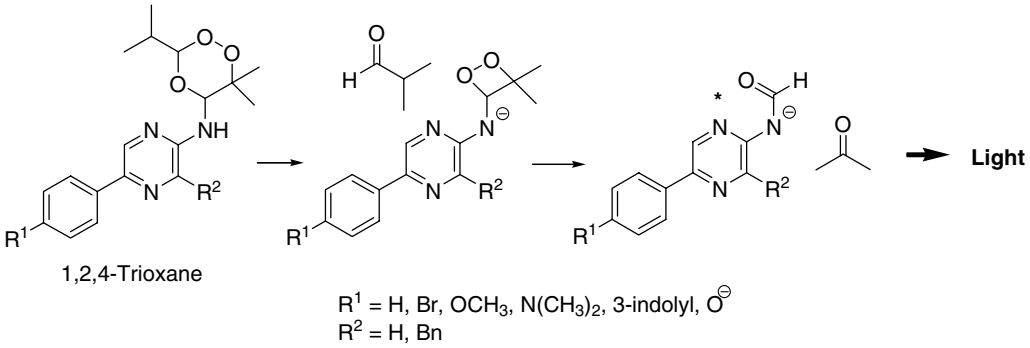


Fig. 12. Chemiluminescence of 1,2,4-trioxanes.

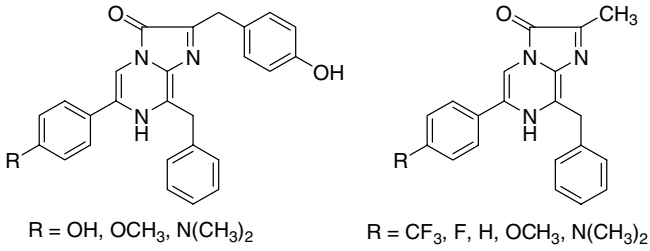


Fig. 13. Coelenterazine analogues used to investigate the effect of R group.

in dimethyl sulfoxide [213,214], suggesting that electron donation from the phenyl group is not necessary for the high chemiluminescence efficiency of coelenterazine. This result is similar to those obtained with 5-(5-phenyl-2-pyrazinylamino)-1,2,4-trioxanes mentioned above. Saito et al. investigated the effect of substituents in the chemiluminescence of the coelenterazine analogues possessing electron-donating (OCH_3 or $N(CH_3)_2$) and electron-withdrawing (CF_3 or F) groups on the 4-position of 6-phenyl moiety (structure at right in Fig. 13) [215]. Their results indicated that the substitution effect is small in the chemiluminescence in dimethyl sulfoxide; the chemiluminescence efficiencies of the analogues having electron-donating groups were only slightly lower than those of the analogues having electron-withdrawing groups. All these results support the hypothesis that "a negative charge on the N atom of amide group promotes the efficient production of singlet excited state from the dioxetane," which was developed in the investigations of the chemiluminescence of 5-(5-phenyl-2-pyrazinylamino)-1,2,4-trioxanes and coelenterazine analogues.

McCapra et al. [216], Cormier et al. [217], and Teranishi and Goto [213,214] investigated the oxygen-dependent chemiluminescence of coelenterazine and its analogues, and measured the quantum yields of coelenterazine in several organic solvents (Table 1). Although the chemiluminescence of Cypridina luciferin in the diglyme-acetate buffer system (best condition known) is highly efficient with a quantum yield of about 0.03 [173], the quantum yield of coelenterazine under the same conditions is only about 1×10^{-5} [214]. With coelenterazines, high quantum yields were obtained with dimethyl sulfoxide, *N*, *N*-dimethylformamide, and hexamethylphosphoric triamide without added base. In these

Table 1
Solvent effects on the quantum yield of coelenterazine

Solvent	Additive	Quantum yield
Dimethyl sulfoxide	None	0.0021
N, *N*-Dimethylformamide	None	0.0027
Hexamethylphosphoric triamide	None	0.005
Diglyme	None	0
Diglyme	Potassium *t*-butoxide	0.00013
Diglyme	Acetate buffer (pH5.6)	0.000013
Dimethyl sulfoxide	Potassium *t*-butoxide	0.00019
Dimethyl sulfoxide	Acetate buffer (pH5.6)	0.0019

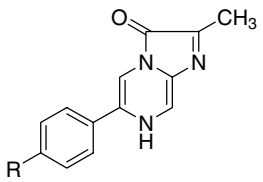
chemiluminescence reactions, the light emitter is the monoanion with its negative charge on the amide moiety. The addition of potassium *t*-butoxide to this chemiluminescence system dramatically decreases the quantum yield; the formation of dianion causes a decrease in the quantum yield.

3.3. Chemiluminescence in aqueous media

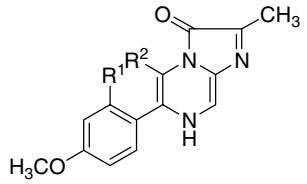
In general, bioluminescence efficiency is far greater than chemiluminescence efficiency [218]. For example, the quantum yield of *Cypridina* bioluminescence is 0.28–0.30 [152,153]. In the absence of luciferase, however, the chemiluminescence of Cypridina luciferin in aqueous media is extremely weak [8]. Shimomura discovered that the fluorescence of Cypridina oxyluciferin in water is very weak, but the fluorescence intensity is greatly increased by the addition of Cypridina luciferase, and the resulting fluorescence spectrally matches the bioluminescence of Cypridina luciferin [162]. It should be noted here that the active site of Cypridina luciferase [163] is highly hydrophobic. Goto and Fukatsu found that the fluorescence of oxyluciferin and the chemiluminescence of Cypridina luciferin are strongly enhanced in the micelle solutions of a cationic surfactant, hexadecyltrimethyl ammonium bromide, in aqueous solvent [219]. These results indicate that the hydrophobicity of the active site of Cypridina luciferase strongly contributes to the high fluorescence efficiency of Cypridina oxyluciferin and the high bioluminescence efficiency.

2-Methyl-6-phenylimidazo[1,2-*a*] pyrazin-3(7*H*)-one (CLA; Fig. 14) shows chemiluminescence in aqueous media in the presence of hydrogen peroxide and ferric ions which enhance the formation of hydroperoxy radicals as a promoter of oxidation reaction, although Cypridina luciferin does not show significant chemiluminescence under these conditions. 5-Phenyl-2-pyrazinylacetamide, the light emitter formed from CLA, shows fluorescence stronger than that of Cypridina oxyluciferin in water [219]. Thus, one difference between the chemiluminescence efficiencies of Cypridina luciferin and CLA in aqueous media appears to be their fluorescence efficiencies of the light emitters. 2-Methyl-6-(4-methoxyphenyl)imidazo[1, 2-*a*]pyrazin-3(7*H*)-one (MCLA; Fig. 14), a derivative of CLA, emits chemiluminescence in aqueous solutions in the presence of oxygen [220], and its quantum yield is three times that of CLA [221].

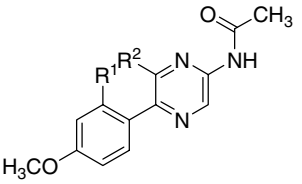
In the case of MCLA analogues, chemiluminescence in aqueous media is clearly related to the dihedral angle between the pyrazine ring and the 4-methoxyphenyl ring in their structures, and chemiluminescence efficiency is enhanced when the dihedral angle is decreased and vice versa [222]. Thus, the chemiluminescence efficiencies of compounds **2**



CLA: R = H
MCLA: R = OCH_3
FMCLA: R = OCF_3



Compound	R^1	R^2
1	H	CH_3
2	CH_2	
3	CH_2CH_2	
4	$CH_2CH_2CH_2$	



Compound	R^1	R^2
5	H	CH_3
6	CH_2	
7	CH_2CH_2	
8	$CH_2CH_2CH_2$	
9	H	H

Fig. 14. Various 2-methyl-6-phenyl-imidazopyrazinone analogues.

and **3** (Fig. 14) are higher than that of MCLA that has a larger dihedral angle, and compounds **1** and **4** (Fig. 14) that have large dihedral angles show very low chemiluminescence efficiencies. It appears that a change in dihedral angle causes a change in the fluorescence intensity of the light-emitter, consequently resulting in the change in the light intensity of a chemiluminescence reaction. Thus, in the chemiluminescence reactions of compounds **2** and **3**, the decreased dihedral angles resulted in an enhancement in the fluorescence of the light-emitters **6** and **7**, respectively (Fig. 14). The fluorescence quantum yields of light-emitters **5** and **8** (Fig. 14) formed from compounds **1** and **4**, respectively, are much less than that of **9**. Moreover, alkyl substitution at the 5-position could enhance the efficiency of singlet excited-state formation [222]. Thus, the high chemiluminescence efficiencies of compounds **2** and **3** may be explained by their high fluorescence efficiencies and high singlet excited-state formation efficiencies. It should be noted here, however, in the case of chemiluminescence in dimethyl sulfoxide, the chemiluminescence efficiency of coelenterazine analogues possessing a methyl group at the 5-position is almost the same as that of coelenterazine itself [213,214].

CLA, MCLA, and FMCLA (2-methyl-6-(4-trifluoromethoxyphenyl)imidazo[1, 2-*a*]-pyrazin-3(7*H*)-one; Fig. 14) show chemiluminescence in a mixture of water and *N*,*N*-dimethylformamide, and in several kinds of the mixtures of methanol and *N*, *N*-dimethylformamide [223]. Under these protic conditions, light is emitted predominantly from the neutral singlet excited-state of amidopyrazine, and chemiluminescence efficiency is much higher when the 6-phenyl group of imidazopyrazinone ring is substituted with a electron-donating 4-methoxy group, in comparison with no substitution or substitution with a 4-trifluoromethoxy group that has no electron-donating effect. In the water plus *N*,*N*-dimethylformamide mixture, the chemiluminescence spectrum of MCLA revealed the presence of two light emitters, showing the emission maxima at 410–420 nm and 460 nm; and it was also found that the high contents of water greatly decreases the chemiluminescence efficiencies. In the methanol plus *N*,*N*-dimethylformamide mixtures, the luminescence showed an emission maximum at 410–420 nm, giving almost the same chemiluminescence efficiencies in the mixtures of various proportions, and the efficiencies were much higher than those in the water-rich solvents. The relative chemiluminescence quantum yields of the imidazopyrazinone and the relative fluorescence quantum yields

of the corresponding amidopyrazine in the mixtures of water and *N*,*N*-dimethylformamide and in several mixtures of methanol and *N*,*N*-dimethylformamide do not vary significantly. These results seem to indicate that the neutral excited-state molecule that emits light at shorter wavelength (λ_{max} 410–420 nm) is the preferred form of excited state. The electron-donating effect of the methoxy group may contribute to the generation of the preferential neutral singlet excited-state molecules.

Cyclomaltooligosaccharides (cyclodextrins) have been reported to be effective in enhancing the chemiluminescence of lucigenin [224], peroxyoxalate [225], luminols [226], acridnium [227], and imidazopyrazinones [220], in which the hydrophobicity of the cyclodextrin cavity apparently contributes to the enhanced chemiluminescence. In the case of MCLA in an aqueous solution, Toya reported that the addition of β-cyclodextrin or heptakis(2,6-di-*O*-methyl)-β-cyclodextrin results in the emission of chemiluminescence with the quantum yields of 0.0034 and 0.010, respectively [220]. The study may indicate that the molecule of MCLA fits well in the hydrophobic cavity of β-cyclodextrin, although a very large amount of cyclodextrin would be needed for the purpose of chemiluminescence in aqueous medium. Teranishi et al. chemically attached MCLA to several different kinds of cyclodextrins (α-, β-, γ-, δ-cyclodextrins) through a single spacer via formation of an amide bond [221,228–230], and then investigated the oxygen-induced chemiluminescence of these compounds in a pH 8.0 aqueous phosphate buffer (Fig. 15) [231–233]. The results indicated that the chemiluminescence efficiency is strongly dependent on the type of cyclodextrin, the spacer length, and the cyclodextrin binding site to which the spacer is bound. The chemiluminescence efficiencies of γ-cyclodextrin-bound compounds are higher than those of α-cyclodextrin-, β-cyclodextrin-, and δ-cyclodextrin-bound compounds. In particular, compound **3** shows almost 44-fold enhancement compared with MCLA, with an efficiency of excited singlet state formation 23 times greater than that of MCLA. The wide entrance of the secondary site of γ-cyclodextrin and the short spacer permit the required interaction between the chromophore moiety and the hydrophobic cavity of cyclodextrin. Surprisingly, the quantum yield of compound **9** (Fig. 15) was found to be 0.082, which is 171 times greater than that of MCLA.

3.4. Chemiluminescence with reactive oxygen species

Chemiluminescence has been used for the analysis of reactive oxygen species. Because enzymes that catalyze luminescence reactions are not used, the reactions involved are simple. Various chemiluminescent compounds including luminols, lucigenins, acridinium esters, dioxetanes, oxalate derivatives, and imidazopyrazinones have been developed as chemiluminescent substances (probes) and used widely. Several reviews dealing with the chemiluminescence analysis of reactive oxygen species have been published [23–32].

CLA was developed as a chemiluminescent probe for analyzing superoxide anions by Goto and his collaborators in 1980 [234]. Since then, CLA was used as an immobilized enzyme indicator for superoxide anions [235] and also successfully adopted in the detection of superoxide anions in biological systems [236,237]. Subsequently developed MCLA emits 4.6 times higher intensity of chemiluminescence (λ_{max} 465 nm) compared with CLA. MCLA was developed for superoxide anions detection by Goto et al. [238,239], and its usefulness was confirmed by Nakano et al. [240–252]. Recently, CLA and MCLA became commercially available, resulting in the widespread use of these compounds in the chemiluminescence analysis of the superoxide anions in biological and chemical systems. In con-

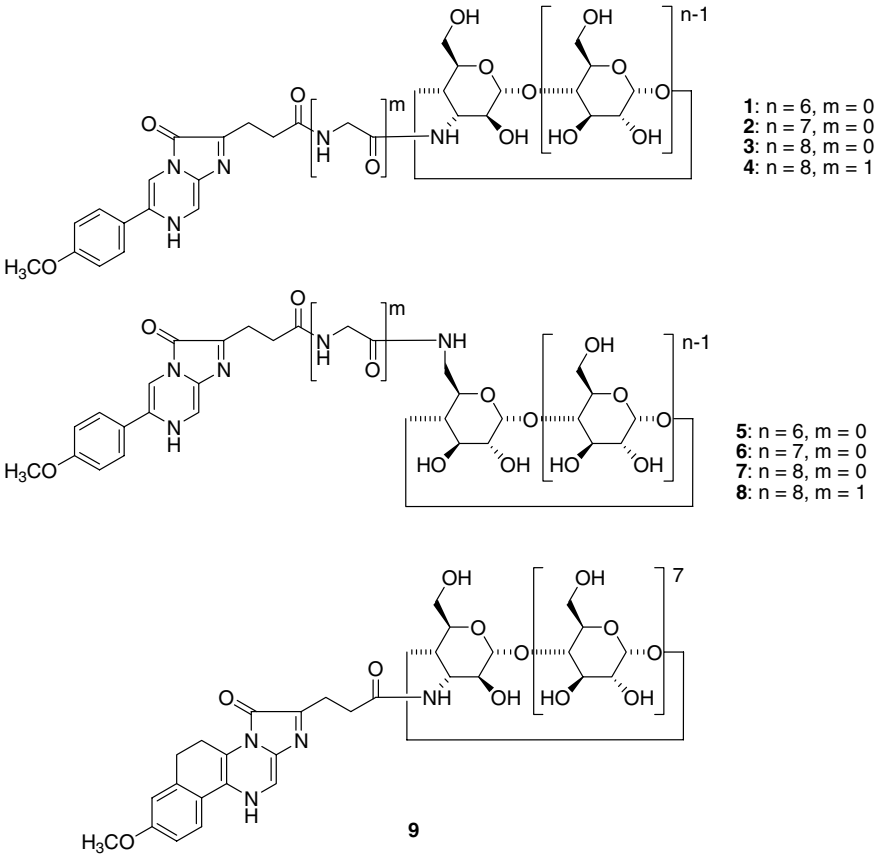


Fig. 15. Various cyclodextrin-bound MCLA compounds synthesized.

trast to the chemiluminescence of luminol, there is no necessity for the catalytic removal of hydrogen peroxide before analysis. Moreover, both CLA and MCLA react also with singlet oxygen, resulting in light emission [253–257]. Superoxide dismutase and azido ions can be used to differentiate the superoxide-dependent luminescence and the singlet-oxygen-dependent luminescence. In addition, a new analytical method using lipid hydroperoxide [258], and the determination of horseradish peroxidase concentration using MCLA have been reported [259].

Coelenterazine emits light in a reaction with superoxide anions [260]. Teranishi and Shimomura found, however, that the luminescence intensity is too weak for practical use in detecting superoxide anions due to the presence of a hydroxyl group on the 6-phenyl group in the structure. Thus, they synthesized several coelenterazine analogues having a methoxy group on the 6-phenyl group and investigated the superoxide-induced chemiluminescence of these analogues [261]. Alkyl substitution at position 5 of the imidazopyrazinone ring resulted in a decrease in the chemiluminescence intensity caused by superoxide anions, whereas a dimethylene bridge added between position 5 and the 6-phenyl group dramatically increased the luminescence intensity; the luminescence intensity of the bridged analogue is 33 times greater than that of MCLA.

Shimomura et al. synthesized five imidazopyrazinones **1**–**5** (Fig. 16) and investigated their use in superoxide anions detection using the hypoxanthine–xanthin oxidase system and the bacterium *Listeria monocytogenes* as the source of superoxide anions [262]. All five compounds appear to be superior to MCLA. Compound **2** (Fig. 16) shows the highest luminescence response and compound **3** (Fig. 16) shows a high signal-background ratio; the latter is an important criterion in measuring a wide range of superoxide anions concentrations with a high sensitivity.

Teranishi investigated the superoxide-induced chemiluminescence of cyclodextrin-bound MCLA (compounds **1**–**9** in Fig. 15 and Table 2) [263]. The luminescence intensities of compound **3** and **9** elicited by superoxide anions are 15 and 52 times greater, respectively, than that of MCLA. However, the signal-background ratio of **9** is by far the lowest of all the compounds studied, indicating that **9** would not be a useful probe. Differing from MCLA, the chemiluminescence intensity and emission wavelength of **3** are not significantly affected by environmental factors, including the presence of bovine serum albumin (BSA) and hexadecyltrimethyl ammonium bromide (CTAB), and also compound **3** has a desirable aqueous solubility that is important in practical use. Consequently, compound **3** is considered to be highly useful in the measurement of superoxide anions.

Lucigenin-enhanced chemiluminescence has been used for the assays of superoxide anions [264–271]. However, the ability of lucigenin to undergo redox cycling has raised questions concerning the use of this reagent. Lucigenin was found to undergo the cycles of univalent reduction that is followed by auto-oxidation accompanied by the generation of superoxide anions, which results in the acceleration of the rate of superoxide anions generation [272–276]. In contrast to lucigenin, coelenterazine has been shown to be a reliable probe [277–279]. In addition, it seems worthy to note that the superoxide-dependent luminescence intensities of MCLA and the coelenterazine analogues are higher compared with that of lucigenin.

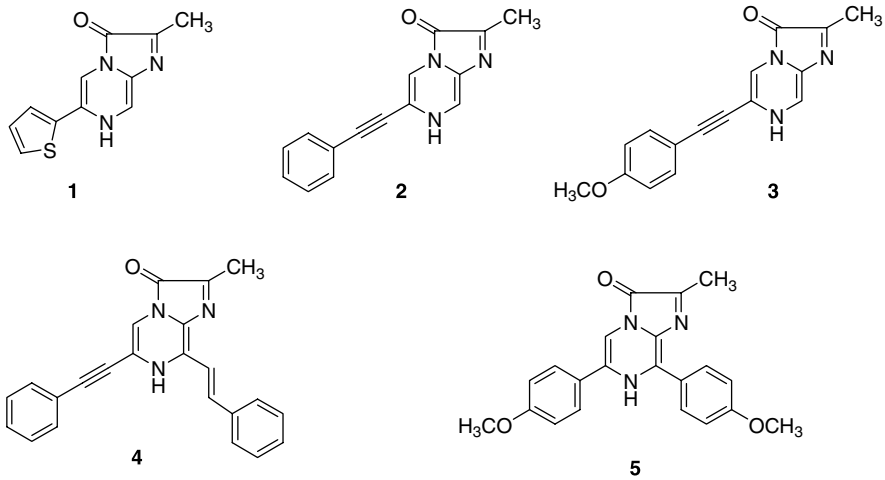


Fig. 16. Imidazopyrazinones for superoxide anion detection investigated by Shimomura et al.

Table 2
Superoxide-dependent chemiluminescence of cyclodextrin-bound MCLAs **1**–**9** in Fig. 15

Compound	Luminescence intensity (count/min)			
	Standard	S/B[b]	+CTAB	+ BSA
MCLA	120 [460 nm][a]	44	390 [420 nm][a]	75 [425 nm][a]
1	100	67	310	64
2	240	35	260	220
3	1770 [460 nm][a]	48	1520 [460 nm][a]	1610 [460 nm][a]
4	550	28	670	540
5	130	48	530	130
6	620	9.3	620	620
7	900	28	780	660
8	1020	20	580	1230
9	6180	12	6110	6160

[a] Wavelength maximum of luminescence spectrum.
[b] Signal-background ratio.

3.5. Green-chemiluminescent probes for superoxide anions detection

When measuring the luminescence of solutions containing pigments, luminescence may be absorbed by the pigments, reducing the optical transparency of the solutions, which sometimes renders the detection of superoxide anions impossible. To deal with this problem, one of the solutions is to use the luminescence of long-wavelength region. Among the commercially available chemiluminescent probes, only two kinds of probe emit green light under neutral conditions: FCLA (Fig. 17) [280] and green-chemiluminescent CD **1** (Fig. 18) [281]. In these probes, a chemiluminescence resonance energy transfer (CRET) mechanism operates on the basis of the Forster theory [282]; the energy of the singlet-excited state generated by the reaction of MCLA with superoxide anions is transferred to the fluorescein moiety, which in turn emits green light.

In the development of green-chemiluminescent CD, chemiluminescent probes **1**–**3** (Fig. 18) were synthesized, and their properties were investigated using the hypoxanthine-xanthine oxidase system as the source of superoxide anions (Table 3) [281]. In probe **1**, MCLA and fluorescein are covalently bound on the secondary and primary hydroxyl faces of γ-cyclodextrin, respectively, and in probes **2** and **3**, MCLA and fluorescein are covalently bound on the secondary hydroxyl faces of A and B glucose moieties, respectively,

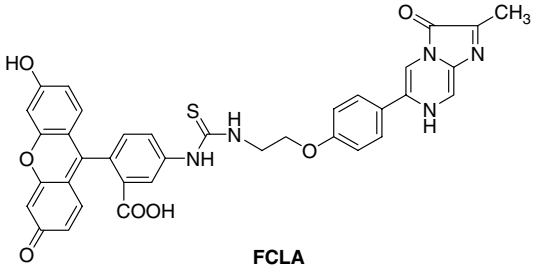


Fig. 17. Structure of FCLA

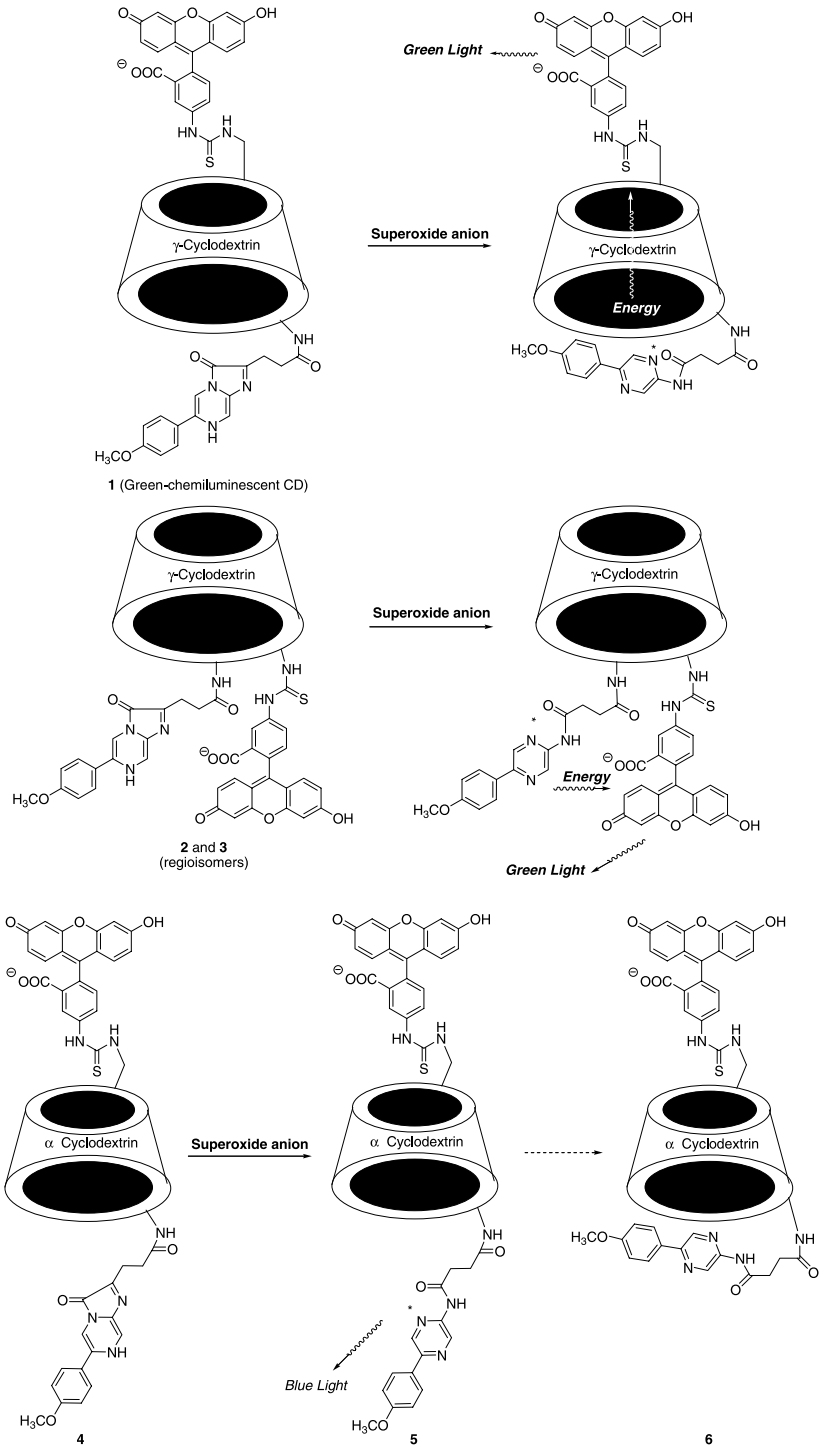


Fig. 18. Structures and luminescence mechanisms of green-chemiluminescent probes.

Table 3
Superoxide-dependent chemiluminescence intensity of compounds **1** – **3** in Fig. 18

Compound	Chemiluminescence intensity (counts/min)		S/B
	Superoxide-induced (S)	Background (B)	
FCLA	239	2.7	89
1	6120	70	87
2	743	11	68
3	731	10	73

or B and A glucose moieties, respectively, in γ-cyclodextrin. Although γ-Cyclodextrin-bound MCLA **3** in Fig. 15 shows superoxide-induced chemiluminescence with a peak at around 460 nm, the spectra of the superoxide-induced chemiluminescence of probes **1**–**3** show their luminescence maxima at around 515–527 nm. The absence of the luminescence from the MCLA moiety indicates that the energy of singlet-excited state generated from the MCLA moiety is efficiently transferred to the fluorescein moiety, even in the presence of the cyclodextrin molecule. These probes show higher superoxide-induced chemiluminescence intensity than FCLA. Probe **1** shows green-luminescence with its intensity 26 times that of FCLA. At the probe concentrations of less than 1.0 μmol/L, the signal-background ratio of probe **1** is greater than that of FCLA. The high chemiluminescence intensity and the superoxide-specificity at low probe concentrations suggest that probe **1** may be more useful than FCLA in the measurement of superoxide anions.

In spite of the shorter distances between the amidopyrazine and fluorescein moieties in probes **2** and **3** compared with probe **1**, the superoxide-induced chemiluminescence intensities of probes **2** and **3** are significantly lower than that of probe **1**. It might suggest that the excitation efficiency of the amidopyrazine moiety in probes **2** and **3** may be lower than that in probe **1**.

The chemiluminescence spectrum of compound **4** (Fig. 18) containing the MCLA moiety at the secondary face of α-cyclodextrin and the fluorescein moiety at the primary face of α-cyclodextrin shows a peak at 460 nm, similar to the chemiluminescence spectrum of α-cyclodextrin-bound MCLA. The chemiluminescence spectrum indicates that energy transfer from the excited state amidopyrazine moiety of compound **5** (Fig. 18) to the fluorescein moiety does not readily occur. In the chemiluminescence reaction of α-cyclodextrin-bound MCLA, **1** in Fig. 15, it has been shown that the singlet-excited amidopyrazine moiety is not situated near the entrance and/or cavity of α-cyclodextrin [221]. Therefore, the extremely low efficiency of the energy transfer in compound **5** may be attributed to the large distance between the amidopyrazine moiety and the fluorescein moiety.

The fluorescence spectrum of compound **6** (Fig. 18), excited at 330 nm, shows a fluorescence maximum at around 520 nm, but does not show a peak around 460 nm that is commonly seen in the fluorescence of amidopyrazine. This indicates that the excited state energy of the amidopyrazine moiety is transferred completely to the fluorescein moiety via the fluorescence resonance energy transfer (FRET) mechanism, differing from the superoxide-induced chemiluminescence of compound **4**. The different outcomes from compound **5** and compound **6** suggest that the amidopyrazine moiety of compound **5** cannot enter into the α-cyclodextrin cavity within the time scale of chemiluminescence reaction, but can do so after the chemiluminescence reaction.

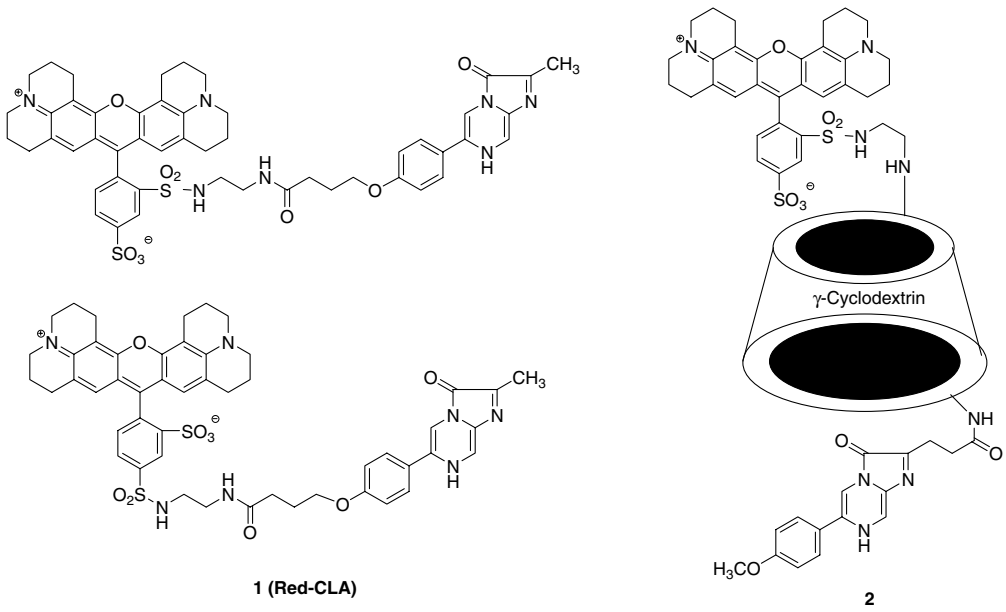


Fig. 19. Structures of red-chemiluminescent probes.

3.6. Red-chemiluminescent probes for superoxide anions detection

In an effort to develop useful probes for detecting superoxide anions with the luminescence of longer wavelengths beyond green, Teranishi synthesized two chemiluminescent probes (Fig. 19) [283,284]: probe **1** (a mixture of two regioisomers) called Red-CLA, and probe **2** consisting of the moieties of MCLA and sulforhodamine 101. These probes emit red chemiluminescence (λ_{max} 610 nm) and their intensities are more intense than those of MCLA and FCLA when tested with superoxide anions produced by the hypoxanthine-xanthine oxidase system. The signal-background ratio for **1** is comparable with those of MCLA and FCLA, whereas the ratio for **2** is lower than those for MCLA and FCLA. Considering the intense superoxide anion-induced chemiluminescence and the high specificity at low probe concentrations, chemiluminescent probe **1** is superior to MCLA and FCLA in the measurement of superoxide anions.

4. Summary

The study of bioluminescence and chemiluminescence, including imidazopyrazinone luminescence, has long been an interesting but formidable challenge for researchers. The structure and characteristics of the aequorin photoprotein and some imidazopyrazinone-luciferase bioluminescence systems have been elucidated through the efforts of Shimomura and other researchers; however, much remains to be studied in this area. Goto and other researchers have investigated the chemiluminescence of imidazopyrazinones in their efforts of understanding bioluminescence and developing analytical tools. Several studies in bioluminescence and chemiluminescence, including imidazopyrazinone luminescence, are in progress in both fields of the basic science and the applications. Furthermore, imidazopyr-

azinones have been shown to be potentially important as antioxidants, separately from their luminescence properties [285–292], which may contribute to the development of new drugs.

Acknowledgments

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