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Low-temperature Photooxygenation of Coelenterate Luciferin Analog Synthesis and Proof of 1,2-Dioxetanone as Luminescence Intermediate[1]

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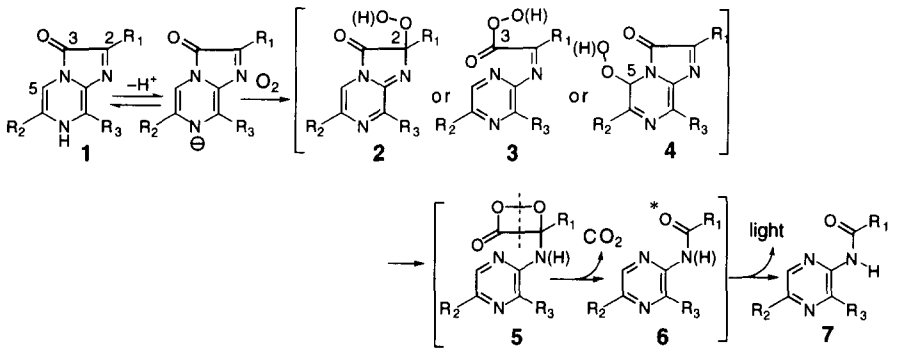
Abstract : Coelenterate luciferin analog having bulky tert-butyl group at the 2-position was suitable for studies on chemiluminescence under various conditions. Photooxygenation of the analog(s) at low temperature (-78°C) afforded luminous intermediates which were proved as peroxides by reduction with PPh_3 with resultant loss of luminescence ability. In order to clarify these structures of accumulated luminous intermediates by means of ^{13}C NMR, three ^{13}C enriched analogs were synthesized at the 2, 3 and 5 positions of 3,7-dihydroimidazo[1,2-a]pyrazin-3-one skeleton in 99% enrichment with site-specificity. These ^{13}C-enriched coelenterate luciferin analogs were photooxygenated at -78 °C to form two peroxidic products as luminescent intermediates. Structures of these unstable intermediates were deduced by means of ^{13}C NMR spectra at low temperatures using substrates enriched at three sites by ^{13}C. Photooxygenation in a mixture of CF_3CD_2OD and CD_3OD as highly protic solvents afforded the dioxetanone and 2-hydroperoxide. These two peroxides emitted light independently at different temperatures either at 400 nm (neutral species) and/or 475 nm (anionic species) after diluting to 10^{-5} M in diglyme (DGM) containing acid or base.

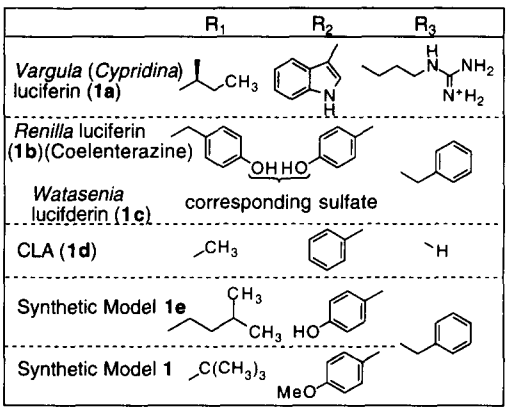
INTRODUCTION

Some kinds of marine organisms such as ostracod crustacean *Vargura* (formerly *Cypridina*) *hilgendorfii*, sea pansy *Renilla reniformis* or small squid *Watasenia scintillans* possess *Cypridina* luciferin[2] **1a**, *Renilla* luciferin[3] (coelenterazine) **1b** or *Watasenia* luciferin[4] **1c**, respectively. *Renilla* luciferin is designated generally as coelenterate luciferin.[5] They have a common skeleton, 3,7-dihydroimidazo[1,2-a]pyrazin-3-one as a partial structure. Each luciferin gives light by an action of luciferase (luciferin-luciferase reaction), which is called as bioluminescence. Luciferins receive spontaneous oxygenation in aprotic dipolar solvents such as DGM[6] or DMSO[7] at room temperatures, and they emit light without luciferase action, which is called as chemiluminescence. They change into oxyluciferin (the corresponding amide) and CO_2 after the luminescence, and the mechanism is considered to be identical with that of bioluminescence. The luminescent mechanisms so far assumed are summarized in **Scheme 1**. Luminescence reaction proceeds *via* short life-time intermediates, namely, a hydroperoxide (**2**, **3** or **4**) and/or a 1,2-dioxetanone anion **5**. Any direct proofs of these intermediates, however, had not been reported at the time when we started this study. The linkage position of peroxides is still uncertain. Cormier *et al.*,[8] McCapra *et al.*[9] and Johnson-Shimomura[10] persisted in the 2-peroxide **2** as the intermediate. Goto *et al.*,[11] on the other hand, emphasized the 3-peroxide (namely peracid) structure **3**. Coelenterazine chromophore is involved in the luminescence of some photoproteins.[5,12] Aequorin, a calcium-binding photoprotein isolated from jellyfish *Aequorea victoria*,[13] possesses a

chromophore derived from coelenterazine,[14] which links to apo-protein through a peroxidic bond at the 2-position.[15] Kishi and Shimomura *et al.*[16] demonstrated this structure by means of ^{13}C NMR spectroscopy of aequorin containing ^{13}C-enriched coelenterazine. Takahashi and Isobe has reported 3,7-dihydroimidazo[1,2-a]pyrazin-3-one chromophore is also included in the photoprotein of a luminous Okinawan squid, *Symplectoteuthis oualaniensis*.[17] Fujimori *et al.* and Teranishi *et al.*[18] suggested, respectively, that 5-peroxide of luciferin would also be a luminescent intermediate. In spite of skillful experiments on the luminescent intermediates by means of mass-spectroscopic method reported by Ohashi *et al.*,[19] the structures of peroxidic intermediates have not been clarified.



Scheme 1. Postulated luminescent mechanism and examples of coelenterate luciferin with imidazo[1,2-a]pyrazin-3-one system.



Scheme 1 suggests that the luminescence process includes two steps: the first formation of peroxide of a luciferin by reaction with oxygen, and the second conversion of the peroxide (**2**, **3** or **4**) into the dioxetanone **5** which rapidly collapses *via* excited **6** into oxyluciferin product **7**. The second step may be thermal process, suggesting slower process at low temperature. The first oxygenation step will be accelerated by photo-irradiation. From these speculations we anticipated that peroxidic luminescent intermediates would accumulate by photooxygenation of coelenterate luciferin at low temperature. Thus, it might be possible to collect chemical and structural information on luminescent intermediates accumulated at low temperature. Studies on low-temperature photooxygenation had been studied by Goto using CLA **1d**[11] as substrate in 1970's, and it was found that luminescent intermediate(s) accumulated by photooxygenation in MeOH at -78 °C.[20] However, information for luminescent intermediates was collected indirectly from trapping or reducing the reaction products. We here planned to do direct structural analysis by means of spectroscopic method of the intermediates, since the sensitivity of ^{13}C NMR today is high enough to re-study the structural analyses of luminescent intermediates accumulated by the low-temperature photooxygenation.

Candidates of the substrate for photooxygenation should be highly soluble in organic solvents even at low temperature (-78 °C). We first prepared and studied with a coelenterate luciferin analog **1e** having an isoamyl group at the 2-position which was soluble into MeOH even at -78 °C.[21] The structure of **1e** was rather similar to the natural luciferin than CLA **1d**, and **1e** afforded some peroxidic luminescent intermediates by photooxygenation in MeOH at -78 °C.[21] However, **1e** was not suitable for further studies, because many by-products were accompanied.[22] We have selected an analog **1** having *tert*-butyl and *p*-methoxyphenyl groups at the 2- and 6-positions, respectively, as a substrate for low-temperature photooxygenation.

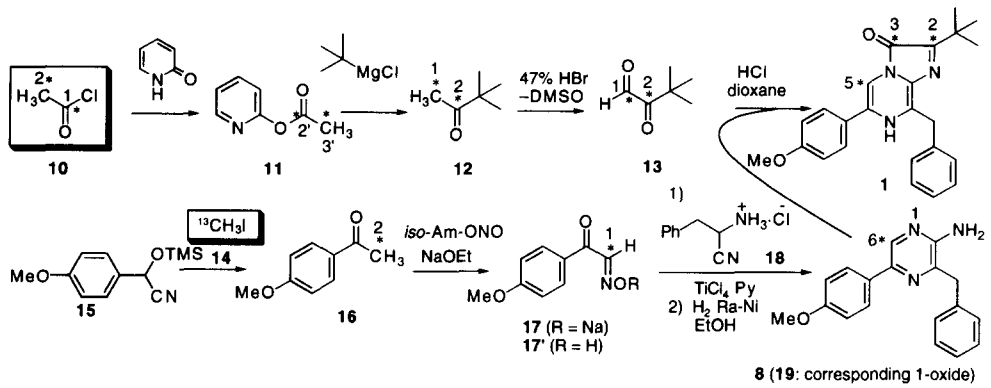
While our studies had been in progress, Teranishi *et al.*[23] independently reported that sensitized-photooxygenation of **1** in CH_2Cl_2 at -95 °C afforded the corresponding 2-hydroperoxide **2** as a luminescent intermediate. They reported its structural assignment from ^1H NMR spectra measured at -80 °C. We regarded that ^1H NMR would give only inconclusive information to differentiate those structures, e.g., **2**, **3** or **5**. Instead, ^{13}C NMR spectroscopy should provide enough information to conclude the structure of the intermediates. To enhance signal intensity of ^{13}C NMR spectroscopy, we prepared four isotopic isomers of **1** with 99% ^{13}C-enrichment at three different positions; thus, the 2-, 3- or 5-^{13}C singly enriched analogs [**1(2-^{13}C)**, **1(3-^{13}C)** or **1(5-^{13}C)**, respectively] and 2,3-$^{13}C_2$ doubly enriched analog [**1(2,3-$^{13}C_2$)**] have been prepared, and the detail of the synthesis is described in this paper.

The low-temperature photooxygenation of the site-specifically ^{13}C-enriched **1** as a substrate was established with a special equipment for the accumulation of luminescent intermediates. We herein describe photooxygenation of the analog **1** to estimate the final products (**7**, **8**, **9** in **Scheme 1** and **Table 1**) and maximize the peroxidic intermediates [either the 2-, 3- or 5-hydroperoxide (**2**,**3** or **4**, respectively) or the dioxetanone **5**]. Further, structural analyses of those accumulated intermediates at low temperatures are discussed by means of ^{13}C NMR spectroscopy. This paper also discusses the luminous species and mechanism based on the luminescence spectra under various conditions (e.g. temperature, pH, solvents etc.) of the intermediates.

RESULTS AND DISCUSSION

Synthesis of ^{13}C enriched analogs

Compound **1** with a natural abundant ^{13}C was prepared according to the reported procedure.[18,24] Each analog of **1** in ^{13}C-enriched at the three different carbons was synthesized essentially according to these reports except the procedure of ^{13}C-enrichment, which was improved to be most effective and summarized in **Scheme 2**. For the enrichment of ^{13}C at the 2- or 3-positions of **1**, we selected ^{13}C-acetyl chloride as ^{13}C-source, because of the following two reasons that 1-^{13}C- or 2-^{13}C-acetyl chloride **10(1-^{13}C)** or **10(2-^{13}C)** were commercially available and that two site-specifically ^{13}C-enriched analogs **1(2-^{13}C)** or **1(3-^{13}C)** could be prepared by the same synthetic procedure. Furthermore, 1,2-$^{13}C_2$-acetyl chloride **10(1,2-$^{13}C_2$)** was also commercially available, we could also prepare 2,3-$^{13}C_2$ doubly enriched analog **1(2,3-$^{13}C_2$)** by the same procedure. For ^{13}C-enrichment at the 5-position of **1**, that corresponds to 6-position of **8**, providing *p*-methoxyacetophenone ^{13}C-enriched at the methyl group of methylketone moiety should be achieved, and ^{13}C-iodomethane **14** would be most suitable as ^{13}C-source. Both ^{13}C-sources can be used in synthetic scale (gram quantity).



Scheme 2. Synthetic route of ^{13}C-enriched coelenterate luciferin analogs

The preparation of 1- or 2-^{13}C-glyoxal [**13(1-^{13}C)** or **13(2-^{13}C)**, respectively] and 1,2-$^{13}C_2$-glyoxal **13(1,2-$^{13}C_2$)** are shown in **Scheme 2**. Either 1- or 2-^{13}C-acetyl chlorides [**10(1-^{13}C)** or **10(2-^{13}C)**, respectively; 99% ^{13}C] or 1,2-$^{13}C_2$-acetyl chloride **10(1,2-$^{13}C_2$)** were treated with 2-hydroxypyridine to give 2-(3'-^{13}C)-acetoxypyridine **11(2'-^{13}C)**, 2-(2'-^{13}C)-acetoxypyridine **11(3'-^{13}C)** or 2-(2',3'-$^{13}C_2$)-acetoxypyridine **11(2',3'-$^{13}C_2$)** in around 70% yield.[25] The resulting ^{13}C-enriched pyridine derivatives **11(3'-^{13}C)**, **11(2'-^{13}C)** or **11(2',3'-$^{13}C_2$)** were treated with *t*-BuMgCl to afford ^{13}C-enriched pinacolones **12(1-^{13}C)**, **12(2-^{13}C)** or **12(1,2-$^{13}C_2$)**, respectively. Because of volatility, evaporation of extracted ^{13}C-enriched pinacolones **12(1-^{13}C)**, **12(2-^{13}C)** or **12(1,2-$^{13}C_2$)** in *n*-pentane solution was carefully performed below 0 °C. Furthermore, the scale did not allow to purify ^{13}C-enriched pinacolones **12(1-^{13}C)**, **12(2-^{13}C)** or **12(1,2-$^{13}C_2$)** by distillation; thus, they were obtained as dimethylsulfoxide solution for the next step in about 50% yield. Preparation of **13** with natural abundance of ^{13}C was started from commercially available pinacolone **12**, which was oxidized with selenium dioxide and distilled to glyoxal **13**.[26] This oxidation, however, could not be applied to the **12(1-^{13}C)**, **12(2-^{13}C)** or **12(1,2-$^{13}C_2$)** because the resultant selenium metal was not completely removable and the distillation resulted in a loss of significant amount of sample due to smaller scales in the isotope cases. The ^{13}C-glyoxals **13(1-^{13}C)**, **13(2-^{13}C)** or **12(1,2-$^{13}C_2$)** were, consequently, obtained by oxidation of ^{13}C-pinacolone **12(1-^{13}C)** and **12(2-^{13}C)** with HBr-DMSO,[27] and the resulting **13(1-^{13}C)**, **13(2-^{13}C)** or **13(1,2-$^{13}C_2$)** were directly used without further purification for the next step.

The ^{13}C enriched aminopyrazine **8(6-^{13}C)** was prepared through 2-^{13}C enriched ketone **16(2-^{13}C)**. Treatment of the cyanohydrin TMS-ether **15**[28] with LHMDS followed by addition of ^{13}C-methyl iodide **14** (99% ^{13}C) proceeded smoothly to give **16(2-^{13}C)** in 90% yield.[28] The ^{13}C enriched ketone **16(2-^{13}C)** was converted according to the reported procedure, as follows. At first, **16(2-^{13}C)** was treated with isoamyl nitrite in basic ethanol to afford the corresponding ketoaldoxime **17(1-^{13}C)** in 60% yield. The ketoaldoxime **17(1-^{13}C)** was treated with aminonitrile hydrochloride **18** to give the pyrazine-1-oxide **19(6-^{13}C)** in 47% yield. The pyrazine oxide **19(6-^{13}C)** was reduced under H_2 atmosphere in the presence of Raney Nickel to afford the corresponding aminopyrazine **7(6-^{13}C)** quantitatively.

Combination of different coupling partner between **13**, **13(1-^{13}C)**, **13(2-^{13}C)** or **13(1,2-$^{13}C_2$)**, and **7** or **7(6-^{13}C)** afforded the ^{13}C-enriched coelenterazine analogs **1**, **1(2-^{13}C)**, **1(3-^{13}C)**, **1(5-^{13}C)**

and **1(2,3-$^{13}C_2$)** in 20-80% yield. [The chemical yields with **13(1-^{13}C)**, **13(2-^{13}C)** or **13(1,2-$^{13}C_2$)** were assumed, because they were not purified at this stage. See Experimental section for details.]

Chemiluminescent characters of the coelenterate luciferin analog 1

Luciferins **1a-e** emit light when dissolved in dipolar aprotic solvent such as DGM[6], DMF or DMSO.[7] The analog **1** also emits light in these solvents. Quantum yields in the chemiluminescence which we measured according to the Seliger's method[29] are Φ_{CL} = 0.02 in DGM containing 0.5 vol% of 1 M *t*-BuOK/*t*-BuOH, Φ_{CL} = 0.01 in DMF or Φ_{CL} = 0.007 in DMSO. These values are similar to the reported values such as 0.028 on CLA **1d** in acidic (pH 5.6) DGM[6] or 0.0021 on coelenterazine **1b** in DMSO.[30]

Goto reported that CLA **1d** showed a different product distribution depending on concentration in chemiluminescence reaction in acidic (pH 5.6) DGM; thus, the higher concentration, the lower yield of oxyluciferin **7d** and the more by-products such as aminopyrazine **8d** and oxypyrazine **9d**.[31] We examined chemiluminescence of **1** under various concentrations in both of acidic and basic DGM. In case of **1**, however, such product distribution depending on the substrate concentration was not observed as clearly as that of **1d**: e.g., even at high concentration as 3.0 x 10^{-4} M, **1** afforded **7** as a predominant oxidative product in both of acidic and basic DGM (**Table 1**). Furthermore, in basic DGM the yield of **7** at 3.0 x 10^{-4} M concentration of **1** was as much as 70%.

Luciferins require molecular oxygen to emit light. This is easily confirmed by the chemiluminescent chart of the luciferin analog **1** in DGM at room temperature as shown in **Figure 2**. When molecular oxygen was removed from the reaction mixture, **1** immediately stopped emission of the light; while re-introduction of O_2 into the mixture showed recovery of the luminescence.

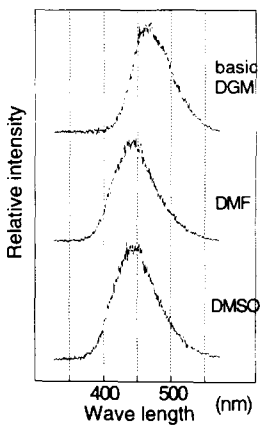
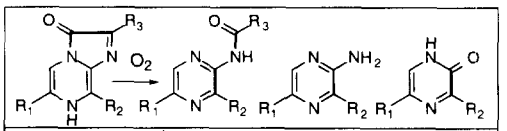


Fig. 1. Chemiluminescence spectra of **1**.
Concentration: 3 x 10^{-5} M. Basic DGM: see Fig. 15 caption.

Table 1. Products of chemiluminescence



Concentration (M)	Products (%)		
1	**7**	**8**	**9**
in basic DGM			
0.33 x 10^{-4}	88		
1.0 x 10^{-4}	78	+	
3.0 x 10^{-4}	70	3	+
in acidic DGM			
0.33 x 10^{-4}	88	4	
1.0 x 10^{-4}	75	6	+
3.0 x 10^{-4}	55	9	0.2
CLA **1d**[31]	**7d**	**8d**	**9d**
in acidic DGM			
0.33 x 10^{-4}	> 90		
1.0 x 10^{-4}	60	+	
3.0 x 10^{-4}	19	22	9

Oxydative products were determined by HPLC with detection at 350 nm.

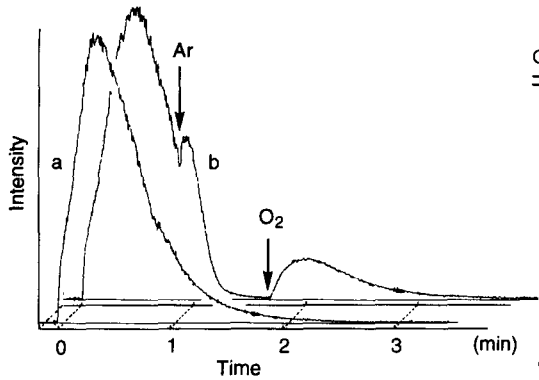


Fig. 2. Chemiluminescence chart of **1** in basic DGM. DGM was saturated with O_2 gas before **1** was added. a: spontaneous chemiluminescence initiated by adding **1** into DGM.
b: Chemiluminescence was initiated in O_2-sat. DGM. Ar or O_2 gas was passed at the points indicated by arrows in figure. Concentration: 2 x10^{-5} M in 1 mL DGM containing 0.5 vol% of 0.25 M *t*-BuOK/*t*-BuOH. Temperature: 20 °C.

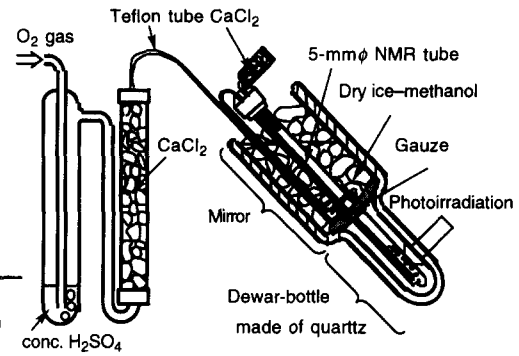


Fig. 3. An apparatus for low-temperature photooxygenation.

The ^{13}C-enriched analogs **1(2-^{13}C)**, **1(3-^{13}C)**, **1(5-^{13}C)** and **1(2,3-$^{13}C_2$)** showed all identical spectra in the chemiluminescence in basic or acidic (pH 5.6) DGM. They also gave almost the same luminescence patterns in basic DGM (data not shown). Thus, no isotope effect due to ^{13}C was observed in the oxidation reaction and light emission.

Analog **1** was superior as a model compound of coelenterate luciferin because of the high quantum yields, and high yields giving oxidation product amide **7** after the chemiluminescnece. The fact that ^{13}C enriched analogs gave the same luminescence profile indicates the essential characters in chemiluminescence reaction are identical to **1** without enrichment.

Accumulation of peroxidic luminescent intermediates in CF_3CH_2OH-CH_3OH

From our preliminary experiments, coelenterate luciferin analog **1** afforded the peroxidic luminescent intermediates by low-temperature photooxygenation in MeOH (2.1 mM). However, the yield of the oxyluciferin analog **6** obtained after warming the solution (containing the intermediate) was never higher than 20%, meaning that a concentration of the accumulated intermediates would be less than 0.4 mM. It was hardly possible to observe the signals from the accumulated intermediates by ^{13}C NMR even with 99% enrichment. In fact, no signal other than those of the luciferin analog **1** and the oxyluciferin analog **7** was observed in CD_3OD after this photooxygenation at -78 °C for 10 min. The luminescence from the cold mixture was clearly observed during the course of warming, which means the existence of the luminescent intermediates. Therefore we first improved the condition of photooxygenation by looking for more suitable solvent system. Conditions of the solvents required for low-temperature photooxygenation and NMR measurements were (1) lower freezing point below -78 °C, (2) commercial availability of the corresponding *deutrated* solvents, (3) to give high chemical yields of the oxyluciferin analog **7** (to confirm whether the photooxygenation reaction being identical with that in spontaneous chemiluminescence) and (4) to give high light yield by warming the photooxygenated solution (to estimate how much luminescent intermediates being accumulated).

Table 2. Solvent effect on low-temperature photooxygenation.

Solvent	Consumed 1 (%)	Produced 7 (%)	Relative L. Y.
CH_2Cl_2	49	1	(+)
Et_2O	96	46	–
THF	92	91	–
AcOEt	90	81	–
Acetone	93	3	–
[a]PhMe	74	19	+
CH_3OH	92	18	+++
CF_3CH_2OH –CH_3OH (7:3)	72	31	+++++++++ +++++++++

Comments for **Table 2.**

a: containing 1 vol% of MeOH. Relative L. Y. = Relative light yield (on warming). Solution of 2.1 mM of **1** was photooxygenated for 10 min at -78 °C, then was warmed in 80 °C bath. Consumed **1** and produced **7** are determined by HPLC. Light yields are measured with a lumi-photometer, and are indicated in relative values by using the mark +.

Table 2 summarizes the results. Tetrahydrofuran (THF) and ethyl acetate were found to be one of the good solvents because the yield of oxyluciferin was higher than 90%, but light yield after photoirradiated at -78 °C and warming was almost zero. The intermediate did not accumulate in these solvents. Methanol was efficient to accumulate the luminescent intermediates and some light yields. The best result was found with a mixture of 2,2,2-trifluoroethanol-methanol (7:3 v/v) which was reasonably good yield and extremely high light yield.

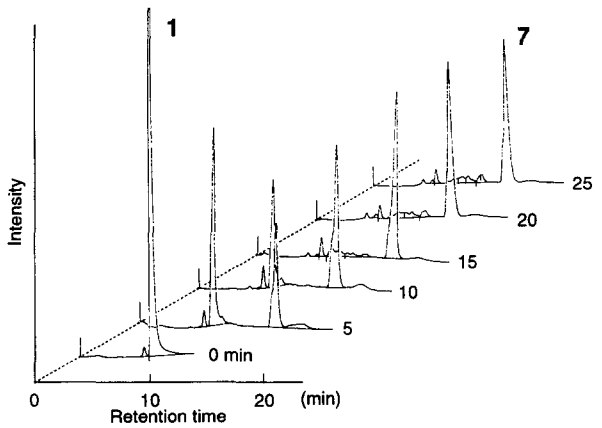


Fig. 4. HPLC chart of products in low-temperature photooxygenation of **1** in CF_3CH_2OH-MeOH (7:3). Mobile phase: MeCN-water (8:2) containing 0.1 vol% of TFA. Detection: UV 350 nm.

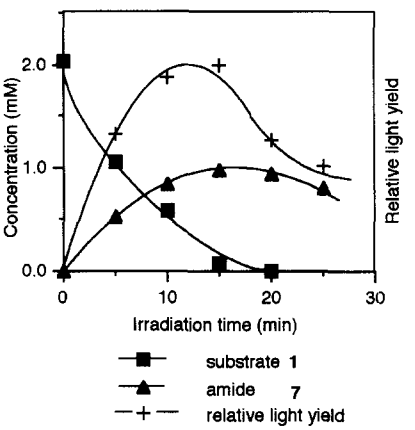
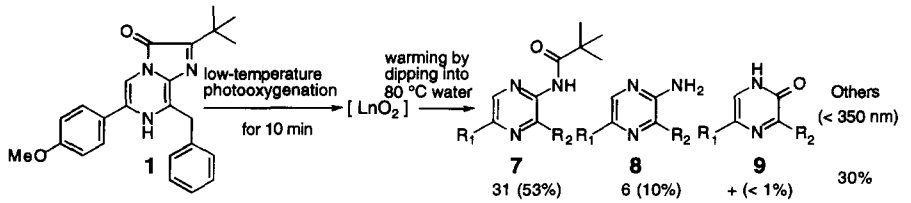


Fig. 5. Time course of photooxygenation of **1** in CF_3CH_2OH-CH_3OH at -78 °C.

The photoirradiated solution of coelenterate luciferin analog **1** (2.1 mM initial) in CF_3CH_2OH-CH_3OH (7:3) for 10 min at -78 °C emitted light on warming. In this case, the light yield was more than 6-fold of that in methanol. We measured the amount of the amide **7**, the aminopyrazine **8** and the oxypyrazine **9** in order to compare the reaction process between the low-temperature photooxygenation and the ordinal

chemiluminescence at room temperature with alkali. The results are shown in **Scheme 3**. It was noteworthy that very high concentration of 2.1 mM of **1** yielded the amide **7** in CF_3CH_2OH-CH_3OH.

Yields of the unreacted substrate **1** and the oxidative photo-products were determined by HPLC, and the chromatograms are shown in **Fig. 4**. Time course of the photooxygenation of **1** in CF_3CH_2OH-CH_3OH (7:3) is shown in **Fig. 5**. Substrate **1** disappeared in 20-min photo-irradiation. Relative light yield on the warming and the yield of **7** reached to the maximum in 10-15 min irradiation. Longer photooxygenation caused diminishing both of the relative light yield and the chemical yield of **7**. The curves of amide and light production are parallel in shorter irradiation time (<10 min). This fact means that the species that acted as



Scheme 3. Product determination in low-temperature photooxygenation. LnO_2 means the peroxidic luminescent intermediate. Yields in parentheses mean the corrected yield based on consumed **1**. Determination was carried out by HPLC with detection at 350 nm. (R_1= C_6H_4OMe, R_2= CH_2Ph)

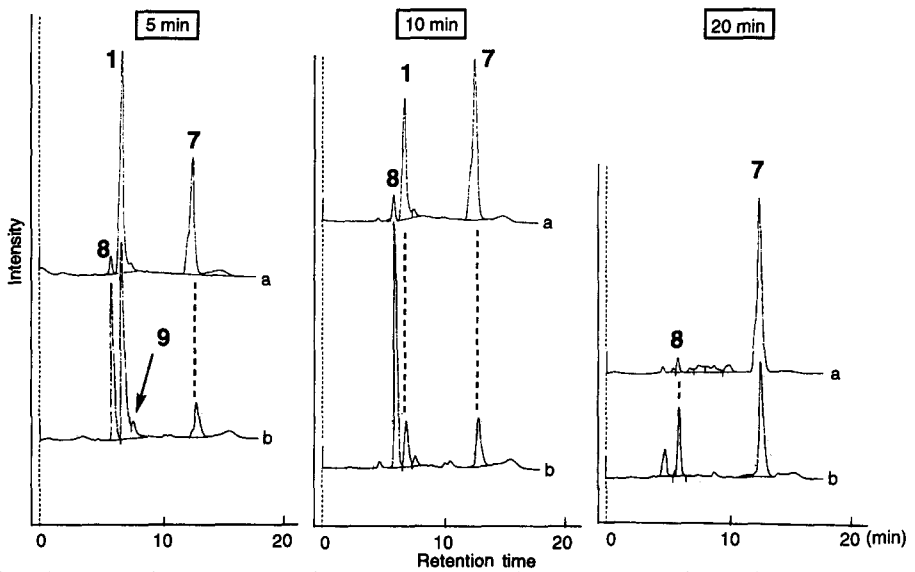


Fig. 6. HPLC chart of products in low-temperature photooxygenation in CF_3CH_2OH-CH_3OH (7:3). a: without reduction. b: after reduction with PPh_3.

light emitter is the amide **7**. An addition of triphenylphosphine into the photooxygenated mixture at -78 °C completely lost the luminescence activity upon the warming, and triphenylphosphine oxide was obtained.[32] The species destined to give light did receive reduction. We obtained following interesting results about the reduction of photooxygenated mixture of **1** in CF_3CH_2OH-CH_3OH (7:3). In **Fig. 6**, the HPLC before and after the phosphine reduction are illustrated with the 5 min, 10 min and 20 min photooxygenated solutions.

The final product **7** decreased, while aminopyrazine **8** increased by the phosphine reduction. Time course is shown in **Fig. 7**. The amount of the amide **7** after the reduction forms the straight line against the irradiation time. The difference of amide **7** before and after the reduction is plotted by broken line. This difference is almost identical with the production of the amine **8**, which was scarcely observed before the reduction. The difference (shown by a broken line in **Fig. 7**) and the amount of **8** (after reduction) was parallel with the light production. This fact indicates that the amine **8** is quantitatively produced by the reduction from the peroxides, that is discussed in **Scheme 5**. Estimation of the intermediates was about 0.6 mM by 10-min photo-irradiation. This value was supported from the result of titration experiment as shown in **Fig. 8**, in which each 10-min irradiated solution was mixed with the given amount of triphenylphosphine. Summarizing these facts, it became clear that the peroxidic luminescent intermediate would be reduced to give amine **8** or its precursors as shown in **Scheme 5**.

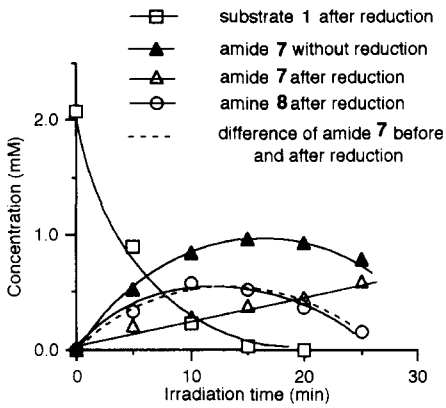


Fig. 7. Time course of low-temperature photooxygenation of in CF_3CH_2OH-CH_3OH followed by PPh_3 reduction.

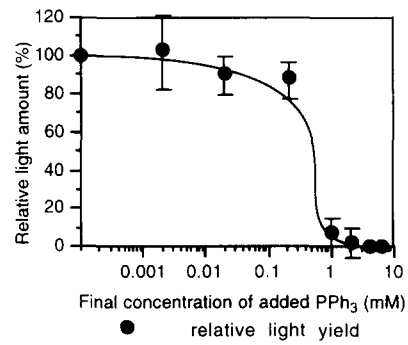


Fig. 8. Titration of the photooxyganated solution with PPh_3.
Photooxygenation time: 10 min.

NMR studies on the accumulated luminescent intermediates

According to the observation described above, the peroxidic luminescent intermediates accumulated as much as 0.6 mM (about 30% conversion yield, 53% corrected yield), and this concentration of the peroxide stimulated us to measure ^{13}C NMR spectra at -78 °C by using three site-specifically ^{13}C-enriched analogs **1(2-^{13}C)**, **1(3-^{13}C)** or **1(5-^{13}C)**. Since irradiation longer than 10 min rather diminished the amount of peroxidic intermediates and increased by-products, the NMR studies were monitored from even shorter time (3 min).

Firstly, a substrate **1(2-^{13}C)** was selected for the NMR studies, where all spectra (including the starting materials) were measured at -78 °C after pre-cooling the probe. Signal of C-2 before photooxygenation was at δ_C 157.9 (**Fig. 9a**). After 3 min-photooxygenation, intensity of the signal at δ_C 157.9 decreased and two new signals appeared at δ_C 108.1 and δ_C 108.7 (**Fig. 9b**). After additional 3 min (total 6 min) irradiation, signal intensity of δ_C 108.1 increased but that of δ_C 108.7 decreased, and a new

signal at δ_C 182.6 appeared (**Fig. 9c**). When the cold photooxygenated mixture was warmed by dipping in a hot water bath at 80 °C, brilliant bluish luminescence was observed in the beginning of the warming, and both signals of δ_C 108.1 and δ_C 108.7 completely disappeared, but that of δ_C 182.6 increased (**Fig. 9d**).

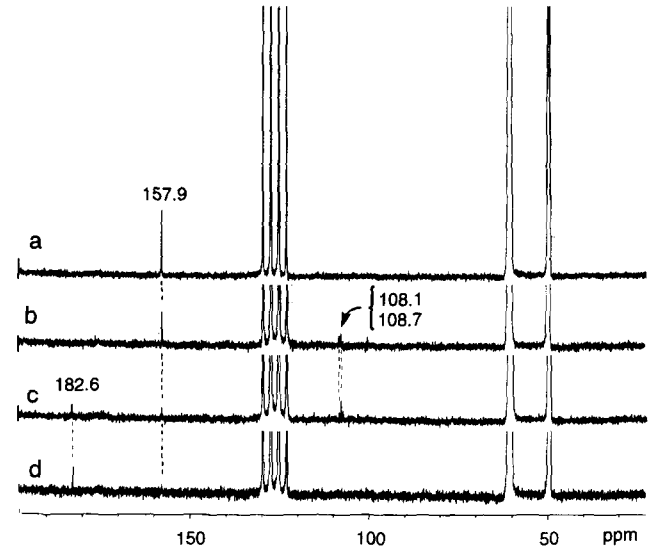


Fig. 9. ^{13}C NMR spectra of low-temperature photooxygenation of **1(2-^{13}C)**.

a: before photooxygenation.
b: after 3-min photooxygenation.
c: after (3+3)-min photo-oxygenation.
d: after warming.

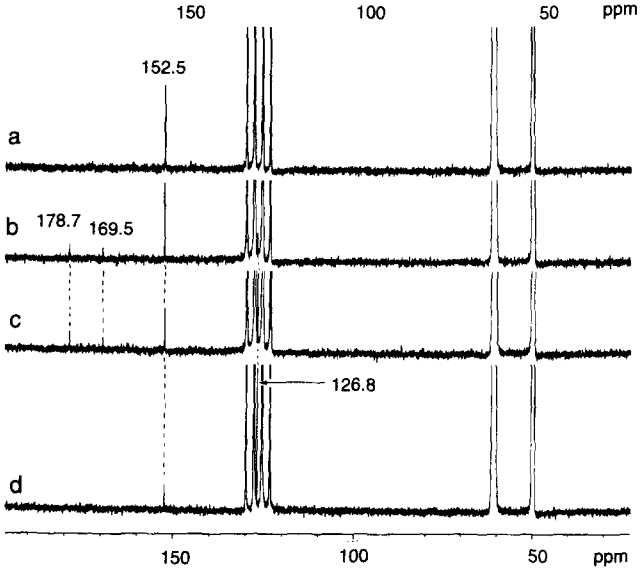


Fig. 10. ^{13}C NMR spectra of low-temperature photooxygenation of **1(3-^{13}C)**.

a: before photooxygenation.
b: after 3-min photooxygenation.
c: after (3+3)-min photo-oxygenation.
d: after warming.

Secondly, substrate **1(3-^{13}C)** was studied under the similar conditions; original signal of the C-3 appeared at δ_C 152.5 (**Fig. 10a**), and two new signals appeared at δ_C 169.5 and δ_C 178.7 with the signal of the final amide at δ_C 126.8 in 3-min and 6-min irradiation (**Fig. 10b**, **10c** and **10d**).

Fig. 11 shows the similar experiments with 5-^{13}C enriched substrate **1(5-^{13}C)**. Before photooxygenation the signal of C-5 appeared at δ_C 107.9 (**Fig. 11a**). Photo irradiation for 5 and 10 min (further 5 min) gave new signals at δ_C 109.8, δ_C 139.2 and δ_C 139.5; but signal at δ_C 107.9 ppm decreased

(**Fig. 11b, 11c**). After the warming the signals at δ_C 107.9 and δ_C 139.5 were observed (**Fig. 11d**). Signals at δ_C 109.8 and δ_C 139.2 are of the intermediates.

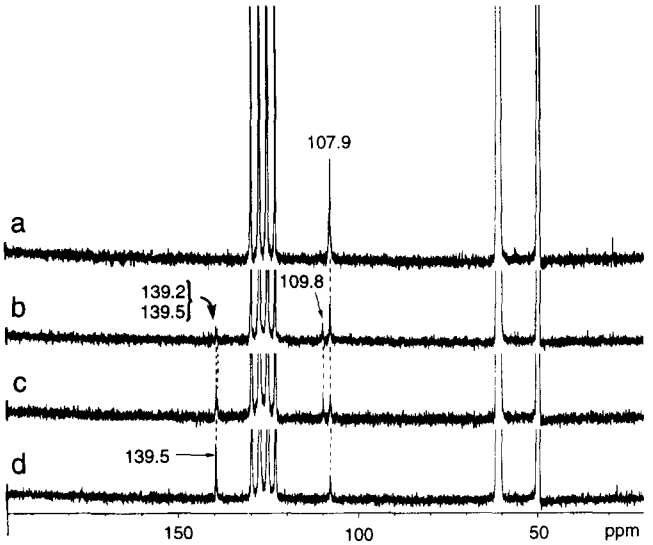


Figure 11. ^{13}C NMR spectra of low-temperature photooxygenation of **1(5-^{13}C)**.

a: before photooxygenation.
b: after 5-min photooxygenation.
c: after (5+5)-min photooxygenation.
d: after warming.

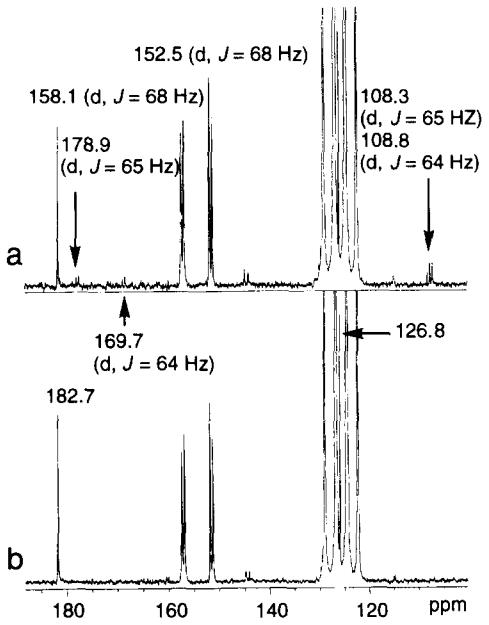


Fig. 12. ^{13}C NMR spectra of photo-products of **1(2,3-$^{13}C_2$)**. a: after 10-min photooxygenation. b: after warming.

Above results suggested the existence of two kinds of luminescent intermediates. Two sets of signals at low temperature ^{13}C NMR would correspond to two compounds **A** and **B**. This was confirmed with the doubly enriched substrate **1(2,3-$^{13}C_2$)**. Similar experiments with this substrate as above (2.1 mM initial) were unsuccessful due to the splitting by ^{13}C-^{13}C coupling. In this case, a 5.5 mM sample was photooxygenated for 10 min to give the spectrum as **Fig. 12a**. **Fig. 12b** shows that after the warming to give signals at δ_C 152.5 (d, J = 68 Hz) and δ_C 158.1 (d, J = 68 Hz) as substrate, and those at 126.9 (s) and 182.7 (s) as the final product(s). The two pairs of signals that observed only at -78 °C after photooxygenation are of intermediates [**A**, δ_C 108.8 (d, J = 64 Hz) and δ_C 169.7 (d, J = 64 Hz); and **B**, δ_C 108.3 (d, J = 65 Hz) and δ_C 178.9 (d, J = 65 Hz); and the results are summarized in **Table 3**].

The product obtained after luminescence by warming the luminescent intermediates was essentially a single compound **7** and CO_2. On the initial substrate **1**, ^{13}C chemical shifts of the 2-, 3- and 5-positions were assigned to δ_C 157.9, δ_C 152.5 and δ_C 107.9, respectively. After luminescence from the peroxidic intermediates on warming, signals at δ_C 182.6 and δ_C 139.5 (originated from the 2- and 5-^{13}C enriched substrate, respectively) were observed and respectively assigned to the amide-carbonyl and the 6-C in the pyrazine ring of the oxyluciferin analog **6**. On the 3-^{13}C enriched substrate **1(3-^{13}C)**, the signal at δ_C 126.8 was observed after luminescence, and it was assigned to CO_2.

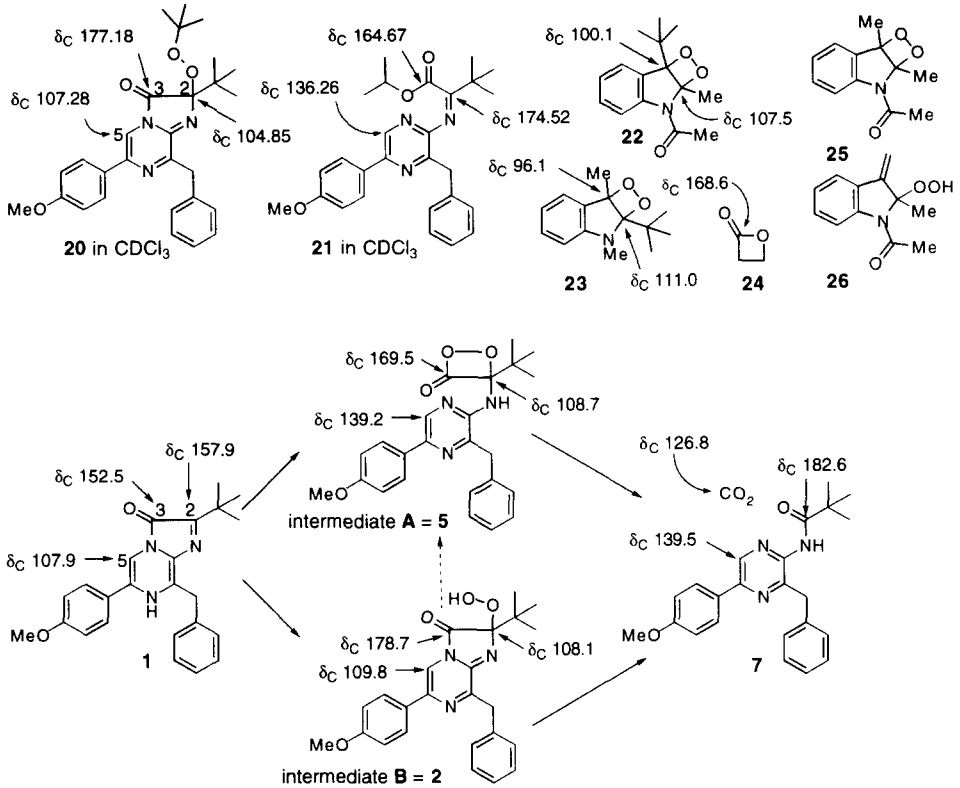
Table 3. ^{13}C Chemical shift of ^{13}C-enriched **1** in low-temperature photooxygenation.

Compound		2-^{13}C	3-^{13}C	5-^{13}C	2, 3-$^{13}C_2$
before photooxygenation		157.9	152.5	107.9	152.5 (d, J = 68 Hz) 158.1 (d, J = 68 Hz)
after photooxygenation	**A**	108.7	169.5	139.2	108.8 (d, J = 64 Hz) 169.7 (d, J = 64 Hz)
	B	108.1	178.7	109.8	108.3 (d, J = 65 Hz) 178.9 (d, J = 65 Hz)
after warming		182.6	126.8	139.5	126.9 (s) 182.7 (s)

Differences in chemical shift values within 0.2 ppm should be attributed to experimental errors.

This is the first time to demonstrate that coelenterate luciferin affords oxyluciferin and CO_2 in luminescence reaction by means of NMR spectroscopy. Intermediate **B** having δ_C 108.1-108.3 for 2-C, 178.7-178.9 for C-3 and 109.8 for C-5 was assigned to be 2-peroxide structure **2**, and the intermediate **A** having δ_C 108.7-108.8 for 2-C, 169.5-169.7 for C-3 and 139.2 for C-5 was assigned to be the dioxetanone **5**. One of the intermediates, the values for **B** are similar to the values of 2-*tert*-butylperoxide **20** reported by Teranishi, namely δ_C 104.85, δ_C 177.18 and δ_C 107.28, respectively, measured in $CDCl_3$ solvent.[33] On the other hand, intermediate **A** showed the signal at δ_C 139.2 from **1(5-^{13}C)**, which is a typical value of the 6-C in 2-aminopyrazines (between δ_C 135 and δ_C 140[34]). Thus, **A** is not assignable to the structure of 5-hydroperoxide **4**. We synthesized an isopropyl iminocarboxylate **21** as a model for the 3-hydroperoxide **3**, and have found that the ^{13}C chemical shifts of the carbons in **21** corresponded to the 2-, 3- or 5-carbons in the substrate **1** appeared at δ_C 174.52, δ_C 164.67 or δ_C 136.26, respectively (in $CDCl_3$).[35] We found that the signals at δ_C 169.5 and δ_C 139.2 ppm in the intermediate **A** showed similar to those at δ_C 164.67 and δ_C 136.26 in the model **21**, respectively. The signal at δ_C 108.7 of the intermediate **A** was rather similar to those carbons of partial structure -N-C(-C)(-C)-OO- as **22** and **23**, but far from δ_C 174.52 of the model **21**. The possibility of the 3-hydroperoxide **3** for intermediate **A** was excluded by the above reasons. The signal at δ_C 108.7 at the position originated from 2-C in the substrate **1**, which was almost the same chemical shift as that of the 2-peroxide **2**, but very different from the value δ_C 174.52 at the 2-position of **21** suggesting that the partial structure of the 2-C in intermediate **A** would have a peroxidic linkage. Furthermore, in case of dioxetanes of the indole derivatives **22** and **23**, the ^{13}C chemical shifts at the corresponding aminal carbon appeared at δ_C 107.5 and 111.0.[36-38] These facts suggested that intermediate **A** would also have dioxetane (or dioxetanone) structure. The signal at δ_C 169.5 at the position originated from 3-C in the substrate **1** must

be for carbonyl carbon of the dioxetanone **5**, which is close to the value of the 4-membered lactone (β-lactone) **24** appearing at δ_C 168.6.[39] Thus, we have assigned the dioxetanone **5** structure for intermediate **A**.

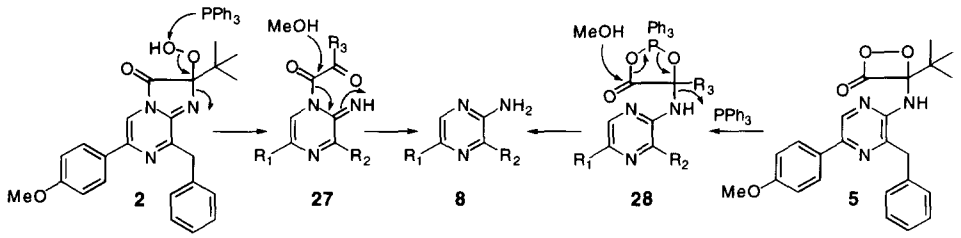


Scheme 4. Possible assignment of products in photooxygenation

Saito *et al.*[36] reported that photosensitized oxygenation of 2-*tert*-butyl-1,3-dimethylindole **23** at -78 °C gave the corresponding 1,2-dioxetane (in $CFCl_3$), and that the half-life of this 1,2-dioxetane was shortened in CD_3OD. Furthermore, they demonstrated that low-temperature photooxygenation in methanol did not produce the 1,2-dioxetane. Adam *et al.*[37] isolated the 1,2-dioxetane **25** and 2-hydroperoxide **26** of *N*-acylated indole instead of *N*-alkyl indole. Foote *et al.*[38] demonstrated in their studies on the photooxygenation of *N*-acetyl-2-methyl-3-*tert*-butylindole that methanol was a particularly favorable for dioxetane formation **22** due to the proticity of the solvents but not the polarity. The substrate **1** having bulky *tert*-butyl group on the 2-position of imidazopyrazinone would be suitable for stabilizing the 1,2-dioxetanone ring. Saito *et al.* reported that only 2-*tert*-butyl-1,3-dimethylindole afforded the corresponding 1,2-dioxetane **23**; on the other hand, 1,3-dimethyl-, 1,2,3-trimethyl and 2-cyclopropyl-1,3-dimethylindoles did not produce the corresponding 1,2-dioxetanes by low-temperature photooxygenation.[36] They also mentioned that the half-life of the dioxetane was shortened in polar solvents, and that 2-*tert*-butyl-1,3-dimethylindole did not produce the 1,2-dioxetanone in methanol by low-temperature photooxygenation. The opposite results were obtained by Foote *et al.* on photooxygenation of *N*-acylated indoles at low temperature.[38] They reported that

methanol was a particularly favorable for dioxetane formation, suspecting that the proticity played a major role in stabilizing the transition state leading to 1,2-dioxetane. Taking these reports into consideration, our photooxygenation of 2-*tert*-butyl-coelenterate luciferin analog **1** would give the corresponding 1,2-dioxetane (dioxetanone **5**) in more protic solvent CF_3CH_2OH-CH_3OH than CH_3OH. Thus, we believe that the 1,2-dioxetanone would accumulate in solvent with higher proticity as CF_3CH_2OH than CH_3OH by low-temperature photooxygenation.

Teranishi *et al.* reported that the 2-hydroperoxide **2** was obtained by sensitized-photooxygenation in methanol at -95 °C, and they assign the structure mainly from ^1H NMR.[23] They could not detect the 1,2-dioxetanone **5** and the decomposed amide **7** on the ^1H NMR measurement at -80 °C. In our experiments with a mixture CF_3CD_2OD-CD_3OD, we have detected the two intermediates **2** and **5** even at rather higher temperature at -78 °C. Solvent proticity rather than reaction temperature might make the dioxetanone formation possible, as Foote *et al.* mentioned. Photooxygenation time gave significant effects on accumulation of the 1,2-dioxetanone **5**. We also found that longer photooxygenation rather diminished the amount of the accumulated peroxidic species even at -78 °C, instead afforded many by-products. Such longer time photo-irradiation until the substrate **1** disappeared was reported by Teranishi *et al.* Thus, we have focused on the primary photooxidative products in a few minutes photooxygenation, while starting material still remained. The structures **5** and **2** as two luminescent intermediates **A** and **B** also satisfy the results of reduction with PPh_3 to afford **8**, through the hypothetical mechanisms indicated as **27** and **28**.



Scheme 5. Possible mechanism of the peroxides, **2** and **5** with triphenylphosphine to form aminopyrazine **8**.

Chemiluminescence of the accumulated peroxides

The photooxygenated solution emitted light simply by warming without molecular oxygen. This luminescence was due to thermal decomposition of the unstable peroxides accumulated during low-temperature photooxygenation. We next focused on this luminescence, which happened in the course of the cold solution to be allowed to come to room temperature. In order to reproduce these two peroxidic intermediates **A** and **B** in maximum amount, photooxygenation was repeated at -78 °C for 10 min under the same manner as optimized for NMR measurements; 2.1 mM of **1** in CF_3CH_2OH:CH_3OH (7:3) in the same NMR tubing.

The analog **1** emitted light in the presence of molecular oxygen, when dissolved in neutral, acidic (pH 5.6) or basic diglyme (DGM) solvent. Luminescence spectra of **1** and fluorescence spectra of the spent solution after luminescence under these conditions are shown in **Fig. 13** along with the fluorescence spectra of the amide **7** under the same conditions to give maxima at 400 nm in neutral and acidic DGM and 475 nm in basic media. The analog **1** emitted light of 475 nm through the excited anion amide **7** in basic, neutral and

even in acidic (pH 5.6) media. The intermediate hydroperoxide, regardless of mostly protonated form, gave the excited anionic amide species to emit light at 475 nm. Fluorescence spectrum of the spent solution after luminescence in basic DGM showed 400-nm maximum, indicating that the amide existed as protonated form in all media. Luminescent intensity changed differently under these conditions are shown in **Fig. 14**. We compared the maximum intensity under these conditions, to show relative values 1, 0.05 and 0.001 in basic, acidic (pH 5.6) and neutral DGM, respectively, and relative light yield integrated for 30 min also corresponded to these value.

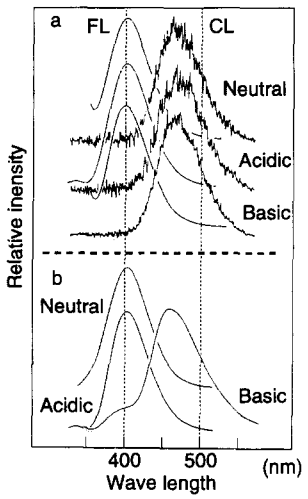


Fig. 13. Chemiluminescence and fluorescence spectra a: chemiluminescence spectra (CL) of **1** and fluorescence spectra of the spent solution after luminescence (FL) in neutral, acidic (pH 5.6) or basic DGM. b: fluorescence spectra of **7**.

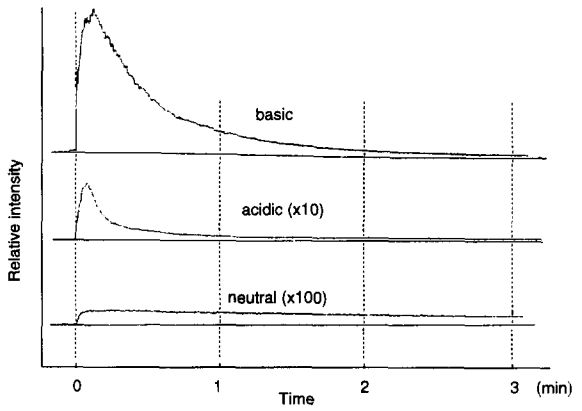
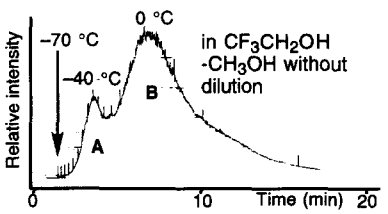


Fig. 14. Chemiluminescence chart of **1** in basic (containing 0.5 vol% of 0.25 M *t*-BuOK/*t*-BuOH), acidic (containing 0.5 vol% of 0.2 M acetate buffer pH 5.6) or neutral (no additive) DGM. Concentration: 1 x 10^{-6} M. Volume: 1.0 mL. Temperature: 23 °C.



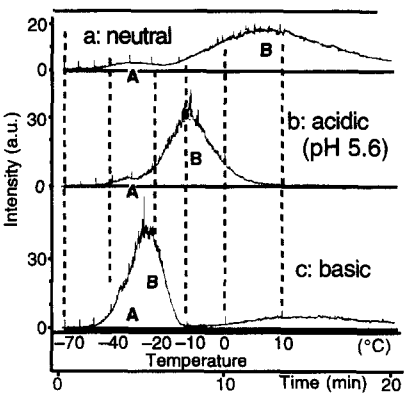


Fig. 15. Luminescence chart in cold DGM diluted solution while being allowed to come to room temperature. a: neutral DGM (no additives). b: acidic (pH 5.6) DGM (containing 0.5 vol% of 0.2 M acetate buffer of pH 5.6). c: basic DGM (containing 0.5 vol% of 1 M *t*-BuOK/*t*-BuOH).

The 10-min photooxygenated CF_3CH_2OH-CH_3OH solution, while warming to come to room temperature, gave two luminescence maxima against temperature; **A** at -40 °C and **B** at 0 °C as shown in **Fig. 15** left. These two peaks would correspond to the existence of two intermediates which were detected by ^{13}C NMR spectra. To measure luminescence spectra without sensitized luminescence at high concentration, the cold photooxygenated solution of 2.1 mM of **1** for 10 min was diluted with DGM to 3 x 10^{-5} M at -78 °C. The diluted solution with neutral DGM was allowed to come to room temperature, that gave the chart as **Fig. 15a**. The dilution with acidic (pH 5.6) or basic DGM made it possible to compare the luminescence spectra of the peroxides under different conditions. The temperature-luminescence intensity charts of the diluted solution [acidic (pH 5.6) and basic DGM] are shown in **Fig. 15b, c**. Two peaks **A** and **B** were observed in the neutral and acidic (pH 5.6) DGM media, but the intensity rate **A/B** were relatively smaller than that before dilution. This may be due to decomposition of **A** by dilution. In case of basic DGM dilution, both of **A** and **B** appeared in one peak due to overlapping luminescence **A** and **B** at around -30 °C.

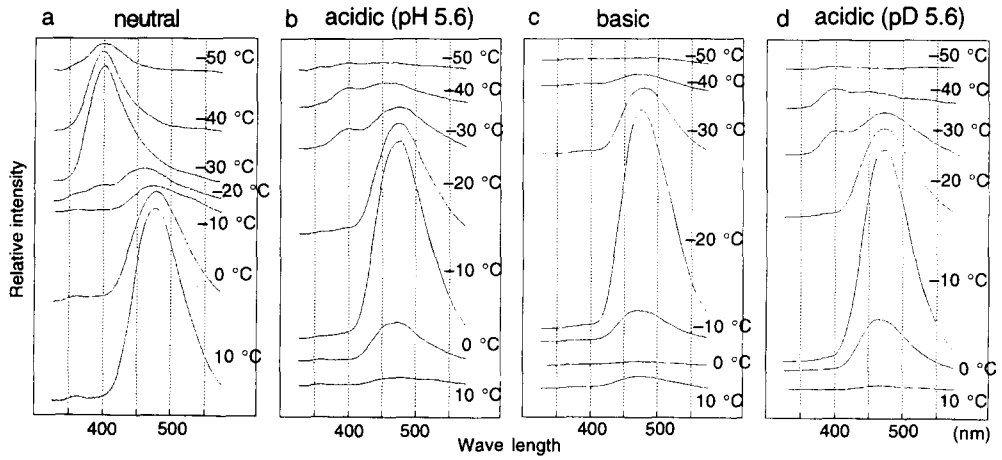
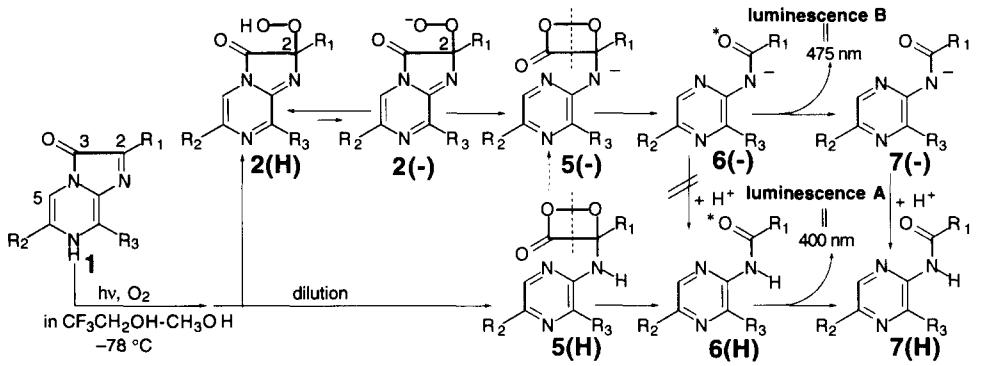


Fig. 16. Luminescence spectra of the diluted solution when allowed to come to room temperature. Neutral, acidic (pH 5.6) or basic: see **Fig. 15** caption.

The diluted solutions were placed in a spectrometer to record the luminescence spectra observed during rising temperature. First **Fig. 16a** indicates the spectra in neutral media. Luminescence of 400 nm was observed between -50 °C and -20 °C, and those of 475 nm were observed between -10 °C and 10 °C. Luminescence of 400 nm or 475 nm corresponds to luminescence **A** or **B** in **Fig. 15a**, respectively. In the case of acidic medium, both of 400- and 475-nm luminescence were observed (**Fig. 16b**) corresponding to luminescence **A** and **B** in **Fig. 15b**. Luminescence in the basic medium also emitted light at 400 nm at around -30 °C though the intensity was small (**Fig. 16c**) corresponding to luminescence **A**. The 475-nm luminescence was clearly recognized as luminescence **B** between -40 °C and -10 °C. As was discussed in **Fig. 13**, the 400 nm emitter is protonated amide **7**, and the 475 nm emitter is anionic amide **7**. Thus, the emitter of luminescence **A** (400 nm) is the protonated amide **7**, and that of luminescence **B** is the anionic amide **7**.

Goto *et al.* reported that luminescence of CLA [2-methyl-6-phenyl-dihydroimidazo[1,2a]pyrazin-3-one] in acidic (pH 5.6) DGM as neutral amide was due to competing protonation of the excited anionic amide

before or after going to its ground state. This was proven by the isotope effect that the luminescence of anionic amide increased in acidic DGM containing D_2O-buffer because protonation became slower.[40] We examined with the luminescent intermediates by diluting with the acidic DGM containing acetate buffer prepared with AcOD and AcONa in D_2O (pD 5.6). The luminescence pattern is shown in **Fig. 16d**, demonstrating that 400 nm luminescence appeared at around -30 °C was the same intensity as **Fig. 16b**. If 400 nm luminescence was originated from the protonation of excited anionic amide, the intensity should decrease as Goto *et al.* mentioned above.



Scheme 6. Luminescence mechanism of two peroxides in neutral, acidic (pH 5.6) or basic DGM. The dotted arrow (**5b** to **5a**) indicates a possible pathway in basic DGM. (R_1= *tert*-butyl, R_2= *p*-methoxyphenyl, and R3= benzyl).

Judging from these spectroscopic nature of the luminescent intermediates as above, the two kinds of peroxides are consistent to give luminescence **A** and **B** (in **Fig. 15**). And the precursor of luminescence **A** gave light always at around -30 °C under different conditions to afford the neutral amide (400 nm). No deuterium effect was observed as discussed above to exclude the protonation step from **6(-)** to **6(H)** in **Scheme 6**. Therefore, we concluded the dioxetanone **5(H)** as the precursor of luminescence **A**. The precursor of luminescence **B**, on the other hand, gave light at different temperatures depending on the pH conditions of DGM solvent. This was due to the mechanism that involved the dissociation step as equilibrium between **2(H)** and **2(-)**. The population of the peroxide anion **2(-)**, being very small, formed the anion **5(-)** under even pH 5.6 to give the anionic species **6(-)** and finally gave light at 475 nm. So, 2-hydroperoxide **2(H)** as precursor of luminescence **B** was concluded. Both of **2(H)** and **5(H)** were consistent with ^{13}C NMR data.

The dioxetanone **5(H)** having a proton on *N*-atom always decomposed at -40°C (luminescence **A**) regardless of the different acidity (of acid or base in DGM solvent). The fact that luminescence **B** shifted below -20 °C in basic DGM explained acceleration of the further reaction not only under the basic condition but also under slightly acidic condition in DGM solvent (**Fig. 15**). At the same time, the relative intensity of the luminescence at 475 nm in basic and acidic media increased (**Fig. 16**). This means that the sequence through the anionic species such as **2(-)**, **5(-)**, **6(-)** and **7(-)** increased than the case in neutral medium. The luminescence of 400 nm was observed in all media though the relative intensities were different.

The luciferin analog **1** usually does not give light in such special solvents as methanol or DGM containing acids (acetic acid, trifluoroacetic acid, etc.) or does only very weakly in CH_2Cl_2. The luminescent intermediates gave, however, light even in these solvents. The diluted solution by acid such as AcOH or TFA in DGM gave two luminescence maxima while warming allowed to come to room temperature though the intensity was very small (data not shown). In both of media, we recognized luminescence **A** and luminescence **B**. Luminescence **A** appeared at -30 °C in both of the media, but luminescence **B** gave maxima against different temperatures (-10 °C in DGM/TFA, and 0 °C in DGM/AcOH).Spectra corresponding to luminescence **A** and **B** gave maxima at 400 nm in both DGM/TFA and DGM/AcOH (**Fig. 17a**, **17b**). The 400 nm emission of luminescence **B** in this case was due to protonation of *N*-atom in 2-hydroperoxide **2a**, which was converted into the protonated dioxetanone **5c** followed by emission at 400 nm light in these acidic media.

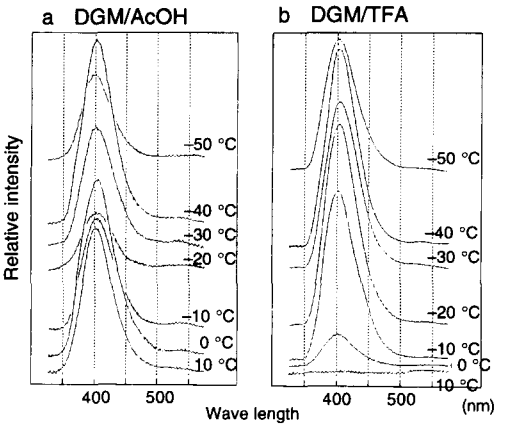


Fig. 17. Luminescence spectra in cold acidic DGM diluted solution while warming to room temperature.

The CH_2Cl_2- or MeOH-diluted solution gave seemingly one luminescence intensity maximum while being allowed to come to room temperature, as shown in **Fig. 18a** or **Fig. 18b**. The CH_2Cl_2 solution gave about 8-times intensity of MeOH solution. One peak appeared in luminescence spectra which corresponded to the fluorescence of neutral amide in both of CH_2Cl_2 (420 nm) and MeOH (440 nm) as shown in **Fig. 19a** or **19b**.

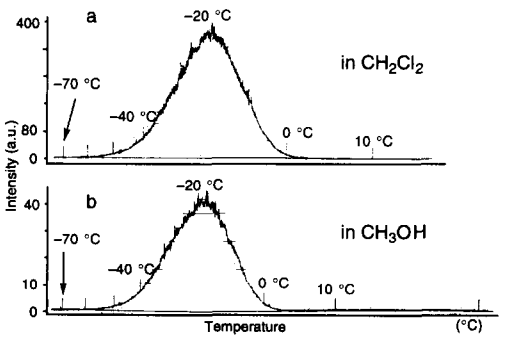


Fig. 18. Luminescence chart in cold solution while warming to room temperature.
a: diluted with CH_2Cl_2. b: diluted with MeOH. Photooxygenation: 10 min in CF_3CH_2OH-CH_3OH.

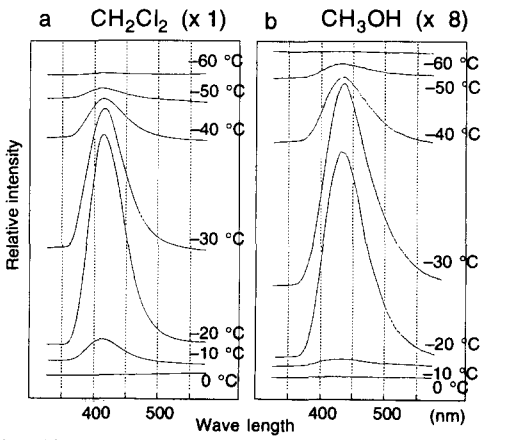


Fig. 19. Luminescence spectra in cold solution while warming to room temperature.
a: diluted with CH_2Cl_2. b: diluted with MeOH. Photooxygenation: 10 min in CF_3CH_2OH-CH_3OH.

Table 4 indicates the yield of amide **7** after luminescence and the relative light yield. Relative light yield was estimated on the light yield of **1** in basic DGM as 1.0. In the cases of neutral, acidic (pH 5.6) or

basic DGM, relative light yields are almost constant as 0.2. As described before, in the case of chemiluminescence of **1** at rt, relative light yields are 1, 0.05 or 0.001 in basic, acidic (pH 5.6) or neutral DGM. The difference between the light yields in chemiluminescence of **1** and that of the peroxides, therefore, should be attributed to the differences of conversion of **1** into peroxidic intermediate. After luminescence of peroxides, almost the same amount of amide **7** were obtained in the case of neutral and acidic (pH 5.6) DGM. In the basic DGM, relatively high yield of **7** was obtained. This may be due to the conversion of **1** into the amide **7** before HPLC analysis. These yields of the amide about 0.8 mM are the same as the case without dilution. Therefore, the possibility that remaining **1** gave light should be excluded. In the strongly acidic DGM, both the yield of **7** and relative light yield were smaller as compared with the other DGM cases. It indicates that in the strongly acidic DGM, some amount of the peroxides decomposed to the compounds other than the amide **7** even at -78 °C. The CH_2Cl_2 or MeOH cases, the yield of **7** were consistent to the neutral, acidic (pH 5.6) or basic DGM cases; relative light yields, however, were largely different from these cases. This would be due to the differences of fluorescence quantam yield of **7** in each medium.

Table 4. Amide **7** after warming and relative light yield.

Diluting solvent (additive)	Amide 7 (mM)	Relative L. Y.
DGM	0.8	1
DGM (0.5 vol% of 1 M *t*-BuOK/*t*-BuOH)	1.4	1[a]
DGM (0.5 vol% of 0.2 M Ac-buffer pH 5.6)	0.9	1
DGM (0.5 vol% of 0.2 M Ac-buffer pD 5.6)	0.7	–
DGM (0.5 vol% of AcOH)	0.4	0.3
DGM (0.5 vol% of TFA)	0.4	0.3
CH_2Cl_2	0.8	8.5
MeOH	0.7	1

Conc. of 2.1 mM was photooxygenated and diluted to 0.03mM. a: light yield between -78 °C and 0 °C. Relative L. Y. = Relative Light Yields

We have demonstrated that the direct photooxygenation of coelenterate luciferin analog **1** in CF_3CH_2OH-CH_3OH at -78 °C afforded two peroxidic species that emitted light on warming. These two peroxides are, consequently, assigned to the 2-hydroperoxide **2** and the 1,2-dioxetanone **5** of the coelenterate luciferin analog **1** by means of ^{13}C NMR spectroscopic method. These assignments are further supported by chemiluminescence characters of the peroxides on warming; thus, the 1,2-dioxetanone **5** decomposes to emit light (400 nm) through neutral amide at lower temperature than the 2-hydroperoxide **2** which decomposes to emit light (475 nm) through anionic amide. We could have observed the luminescence of **1** in strongly acidic DGM, CH_2Cl_2 or MeOH by conversion of **1** into the peroxides by low-temperature photooxygenation.

EXPERIMENTAL

General Procedures

All melting points were measured on a Yanaco MP-S3 and uncorrected. UV spectra were obtained on a JASCO U-best 50 spectrometer. IR spectra were determined on a JASCO FT/IR-7000S spectrophotometer. Proton NMR spectra were recorded on a JEOL EX 270 or GSX 270 for 270 MHz, a JEOL JNM*L*-500 for 500 MHz or a Bruker AMX-600 for 600 MHz. Chemical shifts (δ) are given in parts per million relative to

tetramethylsilane (δ 0.00) as internal standard and coupling constants (*J*) in Hz. Carbon NMR were recorded on a JEOL EX 270 or GSX 270 for 67.8 MHz or on a Bruker AMX-600 for 150.9 MHz. Chemical shifts are (δ) given in parts per million relative to $CDCl_3$ (δ 77.0) or CD_3OD (δ 49.0) as internal standard. Coupling constants (*J*) are given in Hz. The asterisks (*) represent signals of enriched carbon. Low-resolution EI mass spectra were measured with a JEOL D-100 or D-300, and FAB mass spectra were obtained with a JEOL DX-705L. High-resolution (HR) mass spectra were measured with a JEOL DX-705L. Elemental analyses and HRMS were performed by Analytical Laboratory of this school. Fluorescence spectra were measured with a JASCO FP-770 spectrometer. Chemiluminescence spectra were recorded on an Otsuka Electronics MCPD-110A spectrometer or a JASCO FP-770 spectrometer without excitation beam. Chemiluminescence profiles were recorded on a Labo Science Lumiphotometer TS-1000. Light yields in chemiluminescence were determined with a Labo Science TD-4000 lumiphotometer or a TS-1000 lumi-photometer by integrating total light emission.

Dichloromethane (CH_2Cl_2) was distilled from P_2O_5. Tetrahydrofuran (THF) or 1,4-dioxane were distilled from potassium metal in the presence of potassium benzophenone ketyl as an indicator. Diethyleneglycol dimethyl ether (DGM), dimethylformamide (DMF) or dimethylsulfoxide (DMSO) were distilled from calcium hydride under reduced pressure. Pyridine was dried over NaOH pellet and used without distillation. The other solvents were of reagent grade.

Analytical thin-layer chromatography (tlc) was conducted on precoated tlc plates: silica gel 60 F-254 [E. Merck (Art 5715) Darmstadt, Germany], layer thickness 0.25 mm. Silica gel columns for open-column chromatography utilized Fuji Devison (BW 820-MH).

The HPLC analyses were carried out using a JASCO PU-980 pump system equipped with a JASCO UV-970 UV/VIS detector and JASCO 807-IT integrator. An ODS column of 5φ × 250 mm (Develosil ODS-5, Nomura Chemical Co., LTD.) was eluted with MeOH:water (8:2) at a flow-rate of 0.5 mL/min at room temperature.

Cyano-(p-methoxyphenyl)methyl trimethylsilyl ether **15**

To a suspension of *p*-anisaldehyde (0.243 mL, 0.272 g) and ZnI_2 (catalytic amount) in CH_2Cl_2 (2.4 mL) was added TMSCN[28] (0.293 mL, 0.218 g) at 0 °C. After 5 min, the mixture was allowed to come to room temperature, and stirred under nitrogen atmosphere for 2 hr. The reaction mixture was partitioned between CH_2Cl_2 and water, and the water layer was extracted with CH_2Cl_2 for 4 times. The combined organic layer was washed with brine-water (1:1) twice, dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford the cyanohydrin TMS-ether **15** in almost pure state (0.453 g, 96%). ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 0.21 (9H, s), 3.82 (3H, s), 5.44 (1H,s), 6.93 (2H, dt, *J* = 8.9, 2.5 Hz), 7.39 (2H, dt, *J* = 8.9, 2.5 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ -0.5, 55.1, 63.2, 114.1, 119.2, 127.8, 128.1, 160.2 ppm.

For the next step, a solution of the cyanohydrin TMS-ether **15** in THF was prepared as follows; to a suspension of *p*-anisaldehyde (0.486 mL, 0.554 g) and ZnI_2 (catalytic amount) in CH_2Cl_2 was added TMSCN (0.586 mL, 0.436 g) at 0 °C. The mixture was allowed to come to room temperature after 5 min, stirred under nitrogen atmosphere for 2 hr, and evaporated under reduced pressure to remove CH_2Cl_2. To the resultant residue was added 4.5 mL of THF.

p-Methoxyacetophenone **16, 16(2-^{13}C)**

A solution of the cyanohydrin TMS-ether **15** (ca. 1.91 g, 8 mmol) in 9 mL of THF (prepared according to the procedure described above) was added dropwise over 20 min to a solution of 8.8 mL (1.0 M hexane solution) of LHMDS in THF (35 mL) under argon atmosphere at -78 °C. After stirring this solution for 1 hr, $^{13}CH_3I$ **14** (1.0 g, 7 mmol) was added dropwise over 30 min through a gas-tight syringe under argon atmosphere. After stirring for 40 min at -78 °C, the reaction mixture was allowed to come to 0 °C over 2 hr, and then poured into 1 N HCl (30 mL) slowly and stirred for 1 hr. The layers were separated, and the water layer was extracted with CH_2Cl_2 (x 3). The combined organic layer was washed firstly with 1/10 N HCl twice, secondly with 1N NaOH twice, and then dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave crude product (1.21 g), which was purified by chromatography on silica gel with ether:*n*-hexane (1:3) to afford **16(2-^{13}C)** (1.09 g) in 90% (3 steps). Mp 31-32 °C. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 2.55 (3H, d, J = 127.0 Hz), 3.87 (3H, s), 6.93 (2H, dt, J = 9.0, 2.5 Hz), 7.93 (2H, dt, J = 9.0, 2.5 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ *26.4 ppm. EIMS m/z 151 (M^+), 135 (M^+-CH_3), 121 (M^+-CH_3O), 107 (M^+-CH_3CO).

The compound **16** with natural abundant-^{13}C was synthesized according to the same procedure from ca. 0.94 g (4.0 mmol) of the TMSCN-adduct, 4.4 mL (1.0 M hexane solution) of LHMDS and 0.274 mL (0.625 g) of CH_3I in 80% yield (0.46 g). Mp 33-34 °C. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 2.55 (3H, s), 3.89 (3H, s), 6.93 (2H, dt, J = 9.0, 2.5 Hz), 7.94 (2H, dt, J = 9.0, 2.5Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 26.1, 55.2, 113.5, 130.1, 130.4, 163.3, 196.5 ppm. EIMS m/z 150 (M^+), 135 (M^+-CH_3), 107 (M^+ -CH_3CO).

2-(p-Methoxyphenyl)ethan-1,2-dion-1-oxime **17, 17(1-^{13}C)**

p-Methoxyacetophenone **16(2-^{13}C)** (1.00 g) was added to a solution of sodium ethoxide (prepared from 0.192 g of sodium metal and 5.0 mL of ethanol). Then to the resultant solution was added isoamylnitrite (1.2 mL) dropwise, and the mixture was allowed to stand in the dark for 4 days. The resultant mixture was filtered to obtain sodium salt **17(1-^{13}C)** (1.03 g) as red-brown powder. For the next step, the ketoaldoxime was used as sodium salt **17(1-^{13}C)**. The compound data were obtained as the free form after the following treatment. The sodium salt **17(1-^{13}C)** (0.121 g) was dissolved in 10 mL of water and washed with CH_2Cl_2. The water layer was acidified with 10% H_2SO_4 at 0 °C (the solution suspended), and extracted with CH_2Cl_2 for 3 times. The combined organic layer was washed with a mixture of brine and water (1:1), and dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave the ketoaldoxime **17'(1-^{13}C)** as white powder, which was crystallized from *n*-hexane [0.064 g (60%)]. White powder from *n*-hexane. Mp 115-117 °C. UV (EtOH) λ_{max} (log ε), 300 (4.06), 229 (4.07) nm. IR (KBr) ν_{max} 3277, 1663, 1601, 1453, 1323, 1246, 1188, 1055, 975, 864, 844 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 3.88 (3H, s), 6.96 (2H, dt, J = 8.9, 2.4 Hz), 8.02 (1H, d, J = 173.5 Hz), 8.10 (2H, dt, J = 8.9, 2.4 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 125.7 MHz), δ 55.5, 113.8, 132.4, 139.4, *148.6, 164.1, 186.4 (*C*=O, d, J = 61.6 Hz) ppm. EIMS m/z 180 (M^+), 136 (M^+-HCNOH).

The **17** with natural abundant-^{13}C was prepared from commercially available *p*-methoxyacetophenone **16** (10.0 g) according to the procedure described above in 60% yield. White powder from *n*-hexane. Mp 114-116 °C. UV (EtOH) λ_{max} (log ε), 300 (4.07), 229 (4.09) nm. IR (KBr) ν_{max} 3294, 1670, 1604, 1473, 1325, 1245, 1186, 1057, 982, 872, 846 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 3.88 (3H, s), 6.96 (2H, dt,

J = 8.8, 2.5 Hz), 8.04 (1H, s), 8.10 (2H, dt, J = 8.8, 2.5 Hz), 8.42 (1H, brs) ppm. ^{13}C-NMR ($CDCl_3$,125.7 MHz), δ 55.6, 113.8, 128.7, 132.4, 149.0, 164.1, 186.5 ppm. EIMS m/z 179 (M^+), 136 (M^+-HCNOH).

2-Amino-3-benzyl-5-(p-methoxyphenyl)pyrazine **8**, **8(6-^{13}C)**

A solution of the ketoaldoxime sodium salt **17(1-^{13}C)** (1.15 g) and the aminonitrile hydrochloric acid salt **18** (1.15 g) in pyridine (19 mL) was deoxygenated by passing argon gas. To this solution was added $TiCl_4$ (0.8 mL) dropwise at -20 °C. After being stirred for 30 min at -20 °C, the reaction mixture was allowed to come to room temperature and stirred at room temperature for 15 min, then at 80 °C for 2 hr. Then the reaction mixture was cooled at 0 °C and neutralized with sat. $NaHCO_3$. The resultant mixture was filtered through Celite and the filtrate was partitioned between ethyl acetate and water. The water layer was extracted with ethyl acetate (x3). The combined organic layer was washed successively with water (x2) and brine (x2), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to dryness. The resultant residue was crystallized from MeOH-ether to give 6-^{13}C-2-aminopyrazine-1-oxide **19(6-^{13}C)** (0.492 g). The filtrate was evaporated and chromatographed on silica gel with ethyl acetate to afford of **19(6-^{13}C)** (0.071 g) in total yield 47%. The compound **19(6-^{13}C)** was assigned by the comparison of the behavior on TLC with that of the natural abundant-^{13}C 2-aminopyrazine-1-oxide **19** and used for the next step in this state.

Raney nickel (W2) was added to a solution of the 1-oxide **19(6-^{13}C)** (0.595 g) in ethanol (35 mL). The resultant mixture was stirred under hydrogen atmosphere for 4 days. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to dryness to give crude **8(6-^{13}C)** (0.567 g). Slightly yellowish needles. Mp 154-155 °C. UV (EtOH) λ_{max} (log ε), 348 (3.98), 279 (4.34) nm. IR (KBr), ν_{max} 3470, 3294, 3124, 1637, 1459, 1247 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 3.94 (3H, s), 4.18 (2H, s), 4.35 (2H, brs), 7.00 (2H, dt, J = 8.8, 2.8 Hz), 7.25-7.35 (5H, m), 7.88 (2H, dt, J = 8.8, 2.8 Hz), 8.35 (1H, d, J = 177.5 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ *136.9 ppm. EIMS m/z 292 (M^+). HRMS (EI) calcd for $C_{17}{}^{13}C_1H_{17}N_3O$ 292.1405, found 292.1394.

The 1-oxide **19** with natural abundant-^{13}C was prepared for the comparison with **19(6-^{13}C)** under the same manner from the ketoaldoxime **17** (0.752 g) and the amino nitrile hydrochloric acid salt **18** (0.845 g) in 43% yield (0.51 g). **19**: White crystals. Mp 189-190 °C. UV (EtOH), λ_{max} (log ε), 362 (3.85), 286 (4.43), 252 sh (4.17), 205 (4.39) nm. IR (KBr) ν_{max} 3358, 3261, 3105, 1625, 1517, 1484, 1249, 1179, 1030, 832, 699 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 3.86 (3H, s), 4.25 (2H, s), 5.34 (2H, brs), 6.99 (2H, dt, J = 8.7, 2.5 Hz), 7.23-7.38 (5H, m), 7.82 (2H, dt, J = 8.7, 2.5 Hz), 8.39 (1H, s) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 41.1, 55.4, 114.4, 126.0, 127.2, 127.4, 128.0, 128.5, 129.2, 135.7, 142.5, 142.8, 143.3, 160.5 ppm. EIMS m/z 307 (M^+), 291 (M^+-O). Anal, calcd for $C_{18}H_{17}N_3O_2$: C, 70.36; H, 5.54; N, 13.68%. Found: C, 70.32; H, 5.33; N, 13.66%.

The compound **19** was converted into the corresponding 2-aminopyrazine **8** according to the same procedure. Compound **8** was crystallized from EtOH. **8**: Slightly yellowish needles. Mp 154-155 °C. UV (EtOH) λ_{max} (log ε), 349 (3.96), 281 (4.31), 206 (4.24) nm. IR (KBr) ν_{max} 3475, 3295, 3136, 1638, 1463, 1247 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 3.86 (3H, s), 4.18 (2H, s), 4.33 (2H, brs), 6.99 (2H, dt, J = 8.8, 2.5 Hz), 7.20-7.40 (5H, m), 7.88 (2H, dt, J = 8.8, 2.5 Hz), 8.34 (1H, s) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 41.3, 55.4, 114.2, 123.2, 127.0, 128.6, 128.9, 129.0, 136.8, 136.9, 140.5, 142.6, 151.2,

159.7 ppm. EIMS m/z 291 (M^+). Anal, calcd for $C_{18}H_{17}N_3O$; C, 74.39; H, 6.50; N, 10.84%. Found C, 74.33; H, 6.49; N, 10.46%.

2-Acetoxypyridine **11**, **11(2'-^{13}C)**, **11(3'-^{13}C)**, **11(2',3'-$^{13}C_2$)**

To a solution of 2-hydroxypyridine (24 mmol, 2.30 g) and pyridine (24 mmol, 1.94 mL, 1.90 g) in CH_2Cl_2 (130 mL) was added acetyl chloride **10** 2.00 g (23 mmol, 1.81 mL) in CH_2Cl_2 (4.0 mL) under argon atmosphere at 0 °C. After stirring at 0 °C for 30 min, the reaction mixture was allowed to come to room temperature and stirred at room temperature for 4 hr. The reaction mixture was evaporated under reduced pressure to remove half of CH_2Cl_2 and the resulting mixture was washed with 1/2 sat. NaCl aq. (x 1). The organic layer was dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave crude product (2.96 g, 94 %), and it was purified by column chromatography on silica gel with ether:*n*-hexane (1:1) to give of **11** (2.18 g) in pure state (69%). UV (*n*-hexane), λ_{max} (log ε) 300 (2.41), 257 (3.38), 210 (3.47) nm. IR (KBr), ν_{max} 1765, 1593, 1471, 1434, 1371, 1190, 918 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 2.35 (3H, s), 7.09 (1H, ddd, *J* = 8.2, 1.1, 0.7 Hz), 7.23 (1H, ddd, *J* = 7.6, 5.0, 1.1 Hz), 7.80 (1H, ddd, *J* = 8.2, 7.4, 2.2 Hz), 8.41 (1H, ddd, *J* = 5.0, 2.2, 0.7 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 21.2, 116.4, 122.1, 139.5, 148.5, 157.8, 169.0 ppm.

11(2'-^{13}C), **11(3'-^{13}C)** or **11(2',3'-$^{13}C_2$)** were prepared according to the procedure described above by using 1 g of **10(1-^{13}C)**, **10(2-^{13}C)** or **10(1,2-$^{13}C_2$)**, respectively. Each yield was 60, 66 or 75% (1.02, 1.10 or 1.29 g), respectively (these values were estimated on condition that the starting acetyl chlorides were exactly 1 g). **11(2'-^{13}C)**: UV (*n*-hexane), λ_{max} (log ε) 267 (sh) (3.38), 258 (3.43), 254 (sh) (3.33) nm. IR (KBr), ν_{max} 1728, 1593, 1469, 1434, 1371, 1164, 915 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 2.33 (3H, d, *J* = 7.0 Hz), 7.08 (1H, ddd, *J* = 8.2, 1.1, 0.7 Hz), 7.22 (1H, ddd, *J* = 7.4 , 5.0, 1.1 Hz), 7.78 (1H, ddd, *J* = 8.2, 7.4, 2.2 Hz), 8.39 (1H, ddd, *J* = 5.0, 2.2, 0.7 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 21.0 (3'-C, d, *J* = 60.0 Hz), 116.2, 121.9, 139.3, 148.4, 157.3 (2-C, d, *J* = 3 Hz), *169.0 (2'-C) ppm. **11(3'-^{13}C)**: UV (*n*-hexane), λ_{max} (log ε) 264 (sh) (3.41), 257 (3.45), 253 (sh) (3.36) nm. IR (KBr), ν_{max} 1764, 1593, 1470, 1434, 1360, 1188, 1004, 995, 914 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 600 MHz), δ 2.34 (3H, d, *J* = 130.3 Hz), 7.08 (1H, d, *J* = 8.2 Hz), 7.23 (1H, dd, *J* = 7.5, 4.8 Hz), 7.80 (1H, ddd, *J* = 8.2, 7.5, 2.2 Hz), 8.41 (1H, dd, *J* = 4.8, 2.2 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 150.9 MHz), δ *21.2 (3'-C), 116.4, 122.1, 139.5, 148.6, 157.8 (2-C), 169.0 (2'-C, d, *J* = 60.0 Hz) ppm. **11(2',3'-$^{13}C_2$)**: UV (*n*-hexane), λ_{max} (log ε) 265 (sh) (3.33), 257 (3.41), 253 (sh) (3.40) nm. IR (KBr), ν_{max} 1728, 1593, 1470, 1433, 1359, 1165, 912 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 2.33 (3H, dd, *J* = 129.9, 7.0 Hz), 7.09 (1H, d, *J* = 8.2 Hz), 7.23 (1H, dd, *J* = 7.8, 5.3 Hz), 7.80 (1H, ddd, *J* = 8.2, 7.0, 2.0 Hz), 8.41 (1H, dd, *J* = 5.3, 2.0 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ *21.1 (3'-C, d, *J* = 61.0 Hz), 116.5 (6-C), 122.1 (5-C), 139.6 (4-C), 148.5 (3-C), 157.8 (2-C), 169.0 (2'-C, d, *J* = 61.0 Hz) ppm.

3,3-Dimethylbutan-2-one **12**, **12(1-^{13}C)**, **12(2-^{13}C)**, **12(1,2-$^{13}C_2$)**

To a solution of 2-hydroxypyridine **11** (2.24g, 16 mmol) in THF (130 mL) was added dropwise a solution of 1.0 M *t*-BuMgCl in THF at 0 °C over a period of 1hr under argon atmosphere. After stirring at 0 °C for 30 min, the reaction mixture was poured into 100 mL of sat. NH_4Cl aq. at 0 °C. The resulting mixture was extracted with *n*-pentane (x3) and the combined organic layer was washed with water (x2) and sat. NaCl aq. (x1). The organic layer was dried over anhydrous Na_2SO_4 and evaporated at 0 °C under reduced pressure

(not dried up). Then DMSO (4.0 mL) was added to the resultant solution, which was further evaporated under reduced pressure at 20 °C. The product pinacolone **12** was obtained as DMSO solution and its chemical yield was estimated from the difference of the weight of DMSO solution to give ca. 0.8 g (ca. 50%). ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 1.15 (9H, s), 2.15 (3H, s) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ 24.4, 26.1, 44.1, 213.8 ppm. EIMS@ m/z 280 (M^+). HRMS@ (EI) calcd for $C_{12}H_{16}N_4O_4$ 280.1171, found 280.1158. [@: measured as the corresponding 2,4-dinitrophenylhydrazone]

12(1-^{13}C), **12(2-^{13}C)** and **12(1,2-$^{13}C_2$)** were prepared according to the procedure described above by using **11(3'-^{13}C)** or **11(2'-^{13}C)**, respectively. The chemical yield was ca. 50% in each case. **12(1-^{13}C)**: ^{1}H-NMR ($CDCl_3$, 600 MHz), δ 1.15 (9H, s), 2.14 (3H, d, J = 127.2 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 150.9 MHz), δ *24.6 ppm. EIMS@ m/z 281 (M^+). HRMS@ (EI) calcd for $C_{11}{}^{13}C_1H_{16}N_4O_4$ 281.1205, found 281.1187. **12(2-^{13}C)**: ^{1}H-NMR ($CDCl_3$, 600 MHz), δ 1.14 (9H, d, J = 4.1 Hz), 2.13 (3H, d, J = 5.6 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 150.9 MHz), δ *212.8 ppm. EIMS@ m/z 281 (M^+). HRMS@ (EI) calcd for $C_{11}{}^{13}C_1H_{16}N_4O_4$ 281.1205, found 281.1187. **12(1,2-$^{13}C_2$)**: ^{1}H-NMR ($CDCl_3$, 500 MHz), δ 1.15 (9H, dd, J = 4.3, 2.4 Hz), 2.15 (3H, ddd, J = 126.9, 5.5, 2.4 Hz) ppm. ^{13}C-NMR ($CDCl_3$, 150.9 MHz), δ *24.5 (d, J = 40.0 Hz), *214.1 (d, J = 40.0 Hz) ppm. EIMS@ m/z 282 (M^+). HRMS@ (EI) calcd for $C_{10}{}^{13}C_2H_{16}N_4O_4$ 282.1239, found 282.1230. [@: measured as the corresponding 2,4-dinitrophenylhydrazone]

3,3-Dimethylbutan-1,2-dione **13**, **13(1-^{13}C)**, **13(2-^{13}C)**, **13(1,2-$^{13}C_2$)**

A mixture of pinacolone **12** (ca. 0.8 g), DMSO (8 mL) and 47% HBr aq. (0.7 mL) was heated to reflux for 2 hr. The mixture was diluted with water (15 mL), and neutralized with sat. $NaHCO_3$ aq. then extracted with AcOEt (x5). The combined organic layer was washed with water (x3) and brine (x2). After drying over anhydrous Na_2SO_4, the organic layer was evaporated to dryness to afford crude **13** (0.32 g; 16% from the 2-acetoxypyridine **11**). **13** was used for the next step without further purification.

13(1-^{13}C), **13(2-^{13}C)** or **13(1,2-$^{13}C_2$)** were prepared under the same manner in 21, 16 or 15% yield (0.30, 0.36 or 0.24 g), respectively.

2-tert-Butyl-6-(p-methoxyphenyl)-8-benzyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one **1**, **1(2-^{13}C)**, **1(3-^{13}C)**, **1(5-^{13}C)** and **1(2,3-$^{13}C_2$)**

A mixture of crude state **13** (100 mg), aminopyrazine **7** (120 mg), 10% HCl (0.15 mL), water (2.0 mL) and dioxane (1.5 mL) was refluxed for 45 min. After keeping the mixture at 0 °C, yellow precipitates yielded, and were collected by filtration. The precipitates were dissolved into MeOH-CH_2Cl_2 and neutralized at 0 °C with sat. $NaHCO_3$ solution. This mixture was partitioned by water-CH_2Cl_2, and the water layer was extracted with CH_2Cl_2 for 3 times. The combined organic layer was washed with 1/2 sat. brine (x1), dried over anhydrous Na_2SO_4 and evaporated to dryness (0.114 g). The residue was crystallized from AcOEt-*n*-hexane to afford **1** as yellow powder (75 mg, 47%). Mp 119-122 °C (decomp). UV (EtOH), λ_{max} (log ε) 433 (3.91), 355 (3.72), 261 (4.32) nm. IR (KBr), ν_{max} 3059, 2958, 1736, 1636, 1560, 1513, 1456, 1361, 1250, 1178, 1147, 1028, 831 cm^{-1}. ^{1}H-NMR (CD_3OD, 600 MHz), δ 1.48 (9H, s), 3.82 (3H, s), 4.39 (2H, s), 7.01 (2H, d, J = 7.8 Hz), 7.22 (1H, t, J = 7.7 Hz), 7.30 (2H, t, J = 7.7 Hz), ca. 7.4 (1H, brs), 7.50 (2H, br) ppm. ^{13}C-NMR (CD_3OD, 125.7 MHz), δ 28.6, 33.9, 35.3, 55,8, 61.6, 106.4, 115.4, 125.3, 127.5, 128.2, 129.3, 129.8, 130.3, 137.3, 138.4, 153.1, 158.2, 162.3 ppm. MS (EI) m/z 387 (M^+). Anal, calcd for $C_{24}H_{25}N_2O_3$; C, 74.39; H, 6.50; N, 10.84%. Found; C, 74.40; H, 6.57; N, 10.50%.

1(5-^{13}C) was synthesized according to the same procedure described above from the crude **13** (90 mg) and 6-^{13}C-aminopyrazine **7(6-^{13}C)** (80 mg) in 84% yield (87 mg). Yellow powder. Mp 119-121 °C (decomp). UV (EtOH), λ_{max} (log ε) 429 (3.91), 353 (3.72), 261 (4.33) nm. IR (KBr), ν_{max} 3062, 2957, 1735, 1665, 1561, 1515, 1455, 1251, 1178, 1147, 1025, 833 cm^{-1}. ^{1}H-NMR (CD_3OD, 600 MHz), δ 1.48 (9H, s), 3.83 (3H, s), 4.39 (2H, s), 7.02 (2H, d, *J* = 7.4 Hz), 7.22 (1H, t, *J* = 7.4 Hz), 7.29 (2H, t, *J* = 7.7 Hz), 7.41 (2H, d, *J* = 7.4 Hz), ca. 7.40 (1H, brd, *J* = ca. 180 Hz) 7.53 (2H, br) ppm. ^{13}C-NMR (CD_3OD, 150.9 MHz), δ *107.5 ppm. MS (EI) m/z 388 (M^+). HRMS (EI), calcd for $C_{23}{}^{13}C_1H_{25}N_2O_3$ 388.1980, found 388.1976.

1(2-^{13}C), **1(3-^{13}C)** and **1(2,3-^{13}C$_2$)** were synthesized according to the same procedure described above, but the work-up was altered as follows; by being cooled at 0 °C, yellow precipitates were formed and filtered. These precipitates were dissolved into MeOH-CH_2Cl_2 and neutralized with sat. $NaHCO_3$ aq. at 0 °C. This mixture was partitioned by water-CH_2Cl_2, and the water layer was extracted with CH_2Cl_2 for 3 times. The combined organic layer was washed with 1/2 sat. NaCl aq. (x 1), dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was chromatographed on silica gel with 2% MeOH/CH_2Cl_2. The yellow-fluorescent eluents were collected and the solvent were evaporated under reduced pressure. The residue was crystallized from AcOEt-*n*-hexane. Because of crude state of the ^{13}C-**13**, the chemical yields was not accurate; the values were estimated on aminopyrazine **7**.

1(2-^{13}C) was prepared from the crude **13(2-^{13}C)** (299 mg) and the aminopyrazine **7** (441 mg) in 18% yield (105 mg). Mp 119-121 °C (decomp). UV (EtOH), λ_{max} (log ε) 428 (3.93), 353 (3.74), 259 (4.35) nm. IR (KBr), ν_{max} 3063, 2959, 1670, 1650, 1514, 1456, 1253, 1179, 1137, 1027, 834 cm^{-1}. ^{1}H-NMR (CD_3OD, 600 MHz), δ 1.48 (9H, d, *J* = 4.1 Hz), 3.83 (3H, s), 4.39 (2H, s), 7.02 (2H, d, *J* = 8.2 Hz), 7.22 (1H, dt, *J* = 7.8, 2.0 Hz), 7.30 (2H, dt, *J* = 7.8, 2.0 Hz), 7.41 (2H, d, *J* = 8.2 Hz), ca. 7.42 (1H, brs) 7.53 (2H, br) ppm. ^{13}C-NMR (CD_3OD, 150.9 MHz, measured at -78 °C), δ *157.2 ppm. MS (EI) m/z 388 (M^+). HRMS (EI), calcd for $C_{23}{}^{13}C_1H_{25}N_2O_3$ 388.1980, found 388.1970.

1(3-^{13}C) was prepared from the crude **12(1-^{13}C)** (254 mg) and the aminopyrazine **7** (402 mg) in 34% yield (180 mg). Yellow powder. Mp 119-122 °C (decomp). UV (EtOH), λ_{max} (log ε) 429 (3.94), 354 (3.75), 260 (4.36) nm. IR (KBr), ν_{max} 3064, 2959, 1671, 1610, 1509, 1456, 1253, 1178, 1147, 1025, 835 cm^{-1}. ^{1}H-NMR (CD_3OD, 600 MHz), δ 1.48 (9H, s), 3.83 (3H, s), 4.39 (2H, s), 7.02 (2H, d, *J* = 8.2 Hz), 7.22 (1H, t, *J* = 7.4 Hz), 7.29 (2H, t, *J* = 7.4 Hz), ca. 7.41 (1H, brd, *J* = ca. 12 Hz), 7.54 (2H, br) ppm. ^{13}C-NMR (CD_3OD, 150.9 MHz, measured at -78 °C), δ *153.2 ppm. MS (EI) m/z 388 (M^+). HRMS (EI), calcd for $C_{23}{}^{13}C_1H_{25}N_2O_3$ 388.1980, found 388.1963.

1(2, 3-^{13}C$_2$) was prepared from the crude **12(1,2-^{13}C$_2$)** (240 mg) and the aminopyrazine **7** (292 mg) in 32% yield (124 mg). Mp 119-121 °C (decomp). UV (EtOH), λ_{max} (log ε) 429 (3.94), 355 (3.76), 260 (4.36) nm. IR (KBr), ν_{max} 3060, 2956, 1670, 1637, 1610, 1511, 1456, 1253, 1177, 1135, 1024, 834 cm^{-1}. ^{1}H-NMR (CD_3OD, 600 MHz), δ 1.48 (9H, d, *J* = 4.2 Hz), 3.84 (3H, s), 4.39 (2H, s), 7.02 (2H, dd, *J* = 8.9, 2.8 Hz), 7.23 (1H, t, *J* = 7.3 Hz), 7.30 (2H, t, *J* = 7.6 Hz), 7.41 (2H, d, *J* = 7.6 HZ), 7.51 (1H, brs) ppm. ^{13}C-NMR (CD_3OD, 125.7 MHz; measured at -78 °C), δ *152.6 (3-C, d, *J* = 66 Hz), *157.4 (2-

C, d, J = 66 Hz) ppm. MS (EI) m/z 389 (M^+). HRMS (EI), calcd for $C_{22}{}^{13}C_2H_{25}N_2O_3$ 389.2014, found 389.2001.

2-(Trimethylacethylamino)-3-benzyl-5-(p-methoxyphenyl)pyrazine **7**

Pivaloyl chloride (0.31 g, 2.58 mmol) was added to a solution of **8** (0.5 g, 1.72 mmol), and pyridine (2.0 mL) in CH_2Cl_2 (6.7 mL), and the resulting mixture was stirred for 7 hr. After neutralization with 10% $NaHCO_3$ followed by dilution with water, the mixture was extracted with AcOEt (x4). The combined organic layer was washed with water (x3) and brine (x2), and evaporated to dryness. The residue was chromatographed on silica gel [AcOEt:*n*-hexane (2:1)] afforded **7** in 90% yield (0.53 g), which was recrystallized from AcOEt-*n*-hexane. White needles. Mp 123.0-123.5 °C. UV (MeOH), λ_{max} (log ε) 382 (4.10), 293 (4.11), 273 (4.08) nm. IR (KBr), ν_{max} 3361, 2963, 1683, 1607, 1495, 1251 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 1.22 (9H, s), 3.88 (3H, s), 4.31 (2H, s), 7.02 (2H, dt, J = 9.2, 2.2 Hz), 7.17-7.34 (5H, m), 7.38 (1H, brs), 7.97 (2H, dt, J = 9.2, 2.2 Hz), 8.68 (1H, s) ppm. ^{13}C NMR ($CDCl_3$, 67.8 MHz), δ 27.2, 39.2, 41.0, 55.1, 114.1, 126.4, 127.9, 128.1, 128.3, 128.6, 136.3, 137.8, 143.4, 148.9, 150.1, 160.7, 176.9 ppm.. MS (EI), m/z 375 (M^+). Anal, calcd for $C_{23}H_{25}N_3O_2$; C, 73.58; H, 6.71; N, 11.19%. Found; C, 73.59; H, 6.72; N, 11.15%.

2-Hydroxy-3-benzyl-5-(p-methoxyphenyl)pyrazine **9**

To a suspension of **8** (145 mg, 0.5 mmol) in 30% H_2SO_4 (8 mL) was added dropwise $NaNO_2$ (50 mg, 0.72 mmol) in water (1.5 mL) over 20 min at 3 °C. After evolution of N_2 gas ceased, the mixture was stirred at 3 °C for 1 hr. The reaction mixture was neutralized with sat. $NaHCO_3$, then extracted with AcOEt for 5 times. The combined organic layer was washed with H_2O (x5) and brine (x1), then evaporated to dryness. The residue was chromatographed on silica gel [*n*-hexane-AcOEt (3:1)] afforded **9** (27 mg, 19%) along with the starting **8** (101 mg). White crystals from AcOEt-*n*-Hexane. Mp 229-231 °C. UV (EtOH), λ_{max} (log ε) 357 (3.71), 278 (4.35), 202 (4.34) nm. IR (KBr), ν_{max} 2839, 1652, 1515, 1291, 1246, 1172, 1032, 832 cm^{-1}. ^{1}H NMR ($CDCl_3$+CD_3OD, 270 MHz), δ 3.85 (3H, s), 4.19 (2H, s), 6.96 (2H, dt, J = 8.8, 4.2 Hz), 7.17-7.45 (5H, m), 7.38 (1H, s), 7.69 (2H, dt, J = 8.8, 2.4 Hz) ppm. ^{13}C NMR ($CDCl_3$+CD_3OD, 125.7 MHz), δ 40.1, 55.7, 114.8, 120.5, 127.0, 127.1, 128.9, 129.4, 130.0, 134.6, 138.3, 156.0, 158.6, 160.5 ppm. MS (EI), m/z 292 (M^+), 264 (M^+-CO), 249 (M^+-CONH). HRMS (EI), calcd for $C_{18}H_{16}N_2O_2$ 292.1212, found 292.1200.

N-{1-(2'-Propoxy)-3,3-dimethyl-2-ene-1-one}-3-benzyl-5-(p-methoxyphenyl)-2-aminopyrazine **21**[41]

3,3-Dimethyl-2-butanone (12.5 mL) was added to a solution of $KMnO_4$ (30.0 g) and NaOH (10.0 g) in water (800 mL) and the mixture was stirred for 6 hr at 0 °C. The resulting precipitates were removed by filtration through Celite, and the filtrate was concentrated to ca. 300 mL. The concentrated mixture was acidified with conc. HCl at 0 °C, and extracted with CH_2Cl_2 for 3 times. The combined organic layer was dried over anhyd. Na_2SO_4, then evaporated to dryness. Distillation (95-100 °C) afforded of the product 3,3-dimethyl-2-oxo-butanoic acid[42] (9.47 g) in 73% yield.

To a solution 2-chloro-1-methylpyridinium iodide (6.39 g) in CH_2Cl_2 (8 mL) was added a mixture of dimethyl-2-oxo-butanoic acid (2.60 g), isopropanol (1.68 mL) and triethylamine (5.56 mL) in CH_2Cl_2 (52 mL) dropwise. After stirring for 5 hr at rt, water (20 mL) was added to the mixture, then partitioned. The water layer was extracted with CH_2Cl_2 for 3 times, and the combined organic layer was washed with 1/2 sat.

brine, then dried over anhyd. Na_2SO_4. Distillation under reduced pressure (19 Torr, 75-85 °C) of the residue afforded the product isopropyl 3,3-dimethyl-2-oxo-butanoate[43] (3.24 g) in 94%. ^1H NMR, (500 MHz, $CDCl_3$) δ 1.25 (9H, s), 1.34 (6H, d, J = 6.4 Hz), 5.19 (1H, sp, J = 6.4 Hz) ppm. ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 21.6, 25.7, 42.5, 69.9, 163.7, 202.4 ppm.

A mixture **7** (0.291 g), isopropyl 3,3-dimethyl-2-oxo-butanoate (0.52 g), CSA (0.01 g) and molecular sieves AW-300 (1.0 g) in DGM (1.0 mL) was heated at 120 °C overnight. One gram of MS AW-300 was added and heated another overnight. The resulting mixture was filtered through Celite, and the filtrate was evaporated to dryness. The residue was chromatographed on silica gel and further purified by plc to afforded **21** (0.03 g) in 6% along with **7** (0.215 g). The corrected yield of **21** was 23%. Slightly yellowish oil. UV ($CHCl_3$), λ_{max} (log ε) 343 (4.19), 291 (sh) (4.03), 275 (4.11), 256 (4.03) nm. IR (KBr), ν_{max} 2976, 1735, 1643, 1609, 1438, 1250, 1063 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) δ 0.86 (6H, d, J = 6.3 Hz), 1.26 (9H, s), 3.77 (3H, s), 4.21 (2H, s), 4.87 (1H, sp, J = 6.3 Hz), 6.91 (2H, d, J = 8.8 Hz), 7.09 (1H, t, J = 7.3 Hz), 7.17 (2H, t, J = 7.3 Hz), 7.26 (2H, d, J = 7.6 Hz), 7.87 (2H, d, J = 8.8.Hz), 8.42 (1H, s) ppm. ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 21.3, 27.5, 39.3, 40.1, 55.3, 68.7, 114.3, 126.1, 127.9, 128.1, 129.1, 129.2, 136.3, 138.6, 148.9, 149.1, 152.6, 160.7, 164.7, 174.5 ppm. EIMS (m/z) 445 (M^+). HRMS (EI), calcd for $C_{27}H_{31}N_3O_3$ 445.2365, found 445.2350.

Chemiluminescence

To a solution of DGM (1.00 mL) containing 0.5 vol% of additives was added a solution of (0.05 mL) in MeOH at final concentrations between 0.2 x 10^{-4} and 3.0 x 10^{-4} M. The resultant light emission was recorded with a lumiphotometer.

Low-temperature Photooxygenation

A solution of 0.49 mg of the luciferin analog **1** in 0.6 mL of 2,2,2-trifluoroethanol-methanol (7:3) was placed in a 5-mm NMR tube, and cooled at -78 °C. The sample was then photoirradiated with a 100-W high pressure Hg lamp under O_2 gas babbling condition in a special Dewar bottle as shown in the previous paper. The progress of the reaction was monitored by HPLC analysis with UV 350 nm detection.

Reduction of the photooxygenated mixture with PPh_3

Triphenylphosphine was added as a THF solution at a final concentration between 0.001 mM and 10 mM. The resulting mixture was stirred by passing pre-cooled argon gas at -78 °C. Then the mixture in NMR tubing was warmed by dipping into a water bath at 80 °C with monitoring light amount. Then products were analyzed by HPLC.

^{13}C NMR measurement by using ^{13}C-enriched analogs

Each solution of 0.49 mg of a luciferin analog **1(2-^{13}C)**, **1(3-^{13}C)** or **1(5-^{13}C)** in 0.6 mL of CF_3CD_2OD-CD_3OD (7:3) was placed in a 5 mm NMR tube, and cooled at -78 °C. ^{13}C-NMR spectra was measured at -78 °C as the initial stage (pulse angle was set at 30° and scan were accumulated by 10,000 times in all the NMR measurements at -78 °C). The sample was then irradiated with a 100-W high pressure Hg lamp under O_2 gas babbling condition for 3 or 5 min, then ^{13}C NMR spectra was measured at -78 °C as the first intermediary stage. After ^{13}C-NMR spectra measurement, this sample was again photoirradiated further 3 or 5 min, and ^{13}C NMR spectra was measured at -78 °C as the second intermediary stage. The sample was

warmed up at 80 °C (the solution showed luminescence) after this ^{13}C NMR measurement at -78 °C, and ^{13}C NMR spectra was measured at -78 °C as the stage after the intermediates decomposed along with luminescence.

In the case of **1(2,3-$^{13}C_2$)**, a solution of 1.3 mg of **1(2,3-$^{13}C_2$)** in 0.6 mL of CF_3CD_2OD-CD_3OD (7:3) was used. Scan were accumulated by 25,000 times in all the NMR measurements at -78 °C.

Chemiluminescence of photooxygenated mixture after dilution

A solution of the luciferin analog **1** (0.49 mg) in 2,2,2-trifluoroethanol-methanol (7:3) (0.6 mL) was placed in a 5-mm NMR tube, and cooled at -78 °C. The solution was photoirradiated with a 100-W high pressure Hg lamp for 10 min with O_2 gas babbling by using apparatus shown in the previous paper. The photooxygenated alcoholic solution (0.2 mL) was transferred through stainless tube to pre-cooled solvent (CH_2Cl_2, MeOH or DGM containing 0.5 vol% of additives) at -78 °C. The cold diluted solution in dry ice-methanol bath was taken out to ambient temperature (around 20 °C), and the resulting light emission induced by rising temperature was recorded with a lumiphotometer with monitoring temperature. Luminescence spectra of the light emission was measured at every 10 °C between -60 to 10 °C under the same way. Stirring of the mixture was performed manually with spiral thermosensor.

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21. Methanol had been temporarily used, because it does not freeze at -78 °C and **1e** dissolves enough in it.
22. We first had prepared an NMR sample at -78 °C using **1e** as substrate as follows; ether was evacuated *in vacuo* from the photooxygenated solution of **1e** (5 mg) in methanol:ether (1:199 mL) at -78 °C, then the resulting mixture was transferred to an NMR tubing. This procedure was too tedious, and, furthermore, the amount of accumulated luminescent intermediate would diminish by procedure as a result of comparison of light yield on warming before concentration with that after concentration.
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pyrrolidone was added 21.7 g (21.4 mL) of TMSCl (freshly distilled from CaH_2) and the resultant suspension was stirred under nitrogen atmosphere at room temperature for 3 days. Distillation of the reaction mixture (100 Torr, 52-61 °C) afforded 15.6 g of the cyanide (78%). ^{1}H-NMR ($CDCl_3$, 270 MHz), δ 0.34 (9H, s) ppm. ^{13}C-NMR ($CDCl_3$, 67.8 MHz), δ -1.9, 127.0 ppm.

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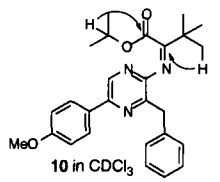
35. These chemical shifts are confirmed by the observation of long range ^{13}C-^{1}H correlation, as shown in the right figure:



10 in $CDCl_3$

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