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Theoretical study of the wavelength selection for the

2

photocleavage of coumarin-caged D-luciferin

3 Junko Usukura*[1], Miyabi Hiyama[2], Maki Kurata[1], Yuji Hazama[1,3], Xing Ping Qiu[4],

4 Francoise M. Winnik[5,6], Hidefumi Akiyama[1,3], and Nobuaki Koga[7]

5 [1]. Institute for Solid Physics, University of Tokyo, Chiba 277-8581, Japan

6 [2]. Graduate School of Science and Technology, Gunma University, Gunma 376-8515, Japan

7 [3]. AIST-UTokyo Advanced Operando-Measurement Technology Open Innovation Laboratory

8 (OPERANDO-OIL), Chiba 277-8589, Japan

9 [4]. Université de Montréal, Département de Chimie, Montréal, QC H3C 3J7, Canada

10 [5]. Laboratory of Polymer Science, PB 55, Department of Chemistry, University of Helsinki,

11 FI-00014 Helsinki, Finland

12 [6]. International Center for Materials Nanoarchitectonics (MANA), National Institute for

13 Materials Science (NIMS), Ibaraki 305-0044, Japan

14 [7]. Graduate School of Informatics, Nagoya University, Nagoya 464-8601, Japan

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17 *Corresponding author e-mail: usukura@issp.u-tokyo.ac.jp (Junko Usukura)

18

19 ## ABSTRACT

20 The equilibrium structures and optical properties of the photolabile caged luciferin, (7-
21 diethylaminocoumarin-4-yl)methyl caged D-luciferin (DEACM-caged D-luciferin), in
22 aqueous solution were investigated via quantum chemical calculations. The probable
23 conformers of DEACM-caged D-luciferin were determined by potential energy curve scans
24 and structural optimizations. We identified 40 possible conformers of DEACM-caged D-
25 luciferin in water by comparing the Gibbs free energy of the optimized structures. In spite of
26 the difference in their structures, the conformers were similar in terms of assignments,
27 oscillator strengths, and energies of the three low-lying excited states. From the
28 concentrations of the conformers and their oscillator strengths, we obtained a theoretical
29 UV/Vis spectrum of DEACM-caged D-luciferin that has two main bands of shape nearly
30 identical to the experimental UV/Vis spectrum. The absorption bands with maxima ~ 384 nm
31 and 339 nm were attributed to the electronic excitations of the caged group and the luciferin
32 moiety, respectively, by analysis of the theoretical UV/Vis spectrum. Furthermore, the
33 analysis showed that DEACM-caged D-luciferin is excited in the caged group only by light of
34 wavelength ranging from 400 to 430 nm. This wavelength range should be tested to lower
35 damage upon photocleavage.

36

37 ## INTRODUCTION

38 The firefly luciferin-luciferase systems are bioluminescence-imaging probes frequently used to
39 detect cellular adenosine triphosphate (ATP), to monitor transgene expression and tumor
40 growth, and to measure luciferase activity (1–6). The bioluminescent activating reaction of
41 photolabile caged luciferins, which releases luciferin by light irradiation, must be triggered at
42 the precise time and/or desired spectral location in order to track accurately the dynamic
43 properties of luciferase expression in living cells (7, 8). Photolabile caged luciferin may also
44 be applied in time-resolved spectroscopic studies of the catalytic reaction of the firefly
45 bioluminescence. This reaction is a two-step reaction of D-luciferin catalyzed by luciferase. In
46 the first step, D-luciferin reacts with ATP in the presence of Mg^{2+} to form luciferyl adenylate.
47 Next, the luciferyl adenylate oxidizes to form a dioxetanone intermediate, which is highly
48 unstable and is converted subsequently to the excited emitter, oxyluciferin (9-11). The
49 bioluminescent reaction has been extensively studied theoretically (12-24) and experimentally
50 (25-37). So far, there are no dynamics studies to prove the above scenario. Although time-
51 resolved spectroscopic measurements are in general effective to explore the elementary steps
52 of chemical reactions, their use for the firefly bioluminescent reaction has been hampered by
53 difficulty in identifying precisely the initial time of the reaction. By using photolabile caged
54 luciferin instead of D-luciferin, the triggering of the bioluminescence may be controlled by
55 light.

56 The 2-nitrobenzyl/nitrophenyl chromophore is used in a variety of photocleavable
57 caged compounds, (38) including D-luciferin 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (39),
58 1-(2-nitrophenyl)ethyl caged D-luciferin (40), and luciferin/upconversion nanoparticle
59 conjugates (41). Over recent decades, coumarin derivatives have attracted much attention in
60 view of their rapid and efficient photolysis (42–44). Photolyses of coumarin-caged cyclic
61 adenosine monophosphate (AMP) on the nanosecond timescale (45) and of coumarin-caged

62 glycine on the microsecond timescale (46) have been reported. We reported recently the
63 design and synthesis of a coumarin-based caged luciferin, namely (7-diethylaminocoumarin-4-
64 yl)methyl caged D-luciferin (DEACM-caged D-luciferin) (compound **a** in Fig. 1) (47).
65 DEACM-caged D-luciferin dissociates into 7-(diethylamino)-4-(hydroxymethyl)coumarin
66 (compound **b**) and D-luciferin (compound **c**) upon light irradiation. To this solution we added
67 luciferase, ATP, Mg^{2+}, and O_2 and measured the bioluminescence quantitatively as a function
68 of light-irradiation time. Moreover, we measured the absorption spectrum of the irradiated
69 DEACM-caged D-luciferin solution and characterized the photoproducts by chiral high-
70 performance liquid chromatography (HPLC). A maximum photocleavage yield of 16.2 % was
71 obtained after a 4-min irradiation with a 70 mW/cm^2 high-pressure mercury lamp (254–600
72 nm). The rather low yield was attributed to the degradation of caged-luciferin and to the
73 decomposition of luciferin, which are common problems associated with the photocleavage of
74 caged compound.

75 The degradation of DEACM-caged D-luciferin and the decomposition of D-luciferin
76 can be suppressed, or at least alleviated, by careful selection of the wavelength of the
77 irradiation light. It turns out that it is difficult to know in detail the electronic excitation
78 responsible for the cleavage of DEACM-caged D-luciferin, since, in aqueous solutions, it
79 exists in a large variety of conformational isomers. ~~This situation makes it problematic to~~
80 ~~select the most suitable wavelength range for DEACM-caged D-luciferin photolysis~~. Repeat!

81 <Figure 1>

82 The purpose of this study is to devise by a theoretical approach to select the optimal
83 spectral excitation band to photocleave DEACM-caged D-luciferin without damage. We
84 analyzed the experimental spectrum (47) via quantum chemical calculations, identified all the
85 conformational isomers of DEACM-caged D-luciferin, and calculated the molecular orbitals
86 (MOs), excitation energies, and oscillator strengths of each conformer. A detailed analysis of
87 the theoretical results led to the identification of the electronic excitation bands corresponding

88 to the experimental UV/Vis spectrum. Moreover, we predicted the optimal wavelength range
89 for the photolysis of DEACM-caged D-luciferin.

90 MATERIALS AND METHODS

91 The UV photolytic reaction of DEACM-caged D-luciferin (**a**) in neutral aqueous solution is
92 presented in Fig. 1. DEACM-caged D-luciferin (**a**) can exist in the *cis*- and the *trans*-forms; in the
93 *cis*-isomer form of **a**, the two sulfur atoms are on the same side of the bond between the thiazoline
94 ring and the benzothiazole group, whereas in the *trans*-form of **a**, they are on the opposite side of
95 this bond. By analogy with the firefly D-luciferin, known experimentally to adopt the *trans*-type
96 structure (48), we excluded the *cis*-form of **a** in the calculations. It was assumed that the DEACM-
97 caged D-luciferin (**a**) is in the anionic form via deprotonation at the carboxylic acid group since
98 the pK_a of carboxylic acids is usually ~ 4.3 to 4.7.

99 The Global Reaction Route Mapping (GRRM) program (49–51), which explores chemical
100 reaction pathways automatically, was used to estimate equilibrium structures. These GRRM
101 calculations gave several equilibrium structures of **a** that were used to identify all possible
102 conformers (see Fig. S1 and Table S1). Subsequently, to determine the structure of all possible
103 conformers, potential energy curve (PEC) scans were conducted over the relevant dihedral angles
104 of rotation around all single bonds. Rotations around some single bonds did not lead to new
105 potential energy minima or gave the same conformations as that obtained by a rotation around
106 another single bond. We took into consideration only the unique rotations around the bonds shown
107 in red, blue, and green in Fig. 1.

108 The structures of the conformers were optimized starting from the energy minima
109 structures in the PEC scan. CAM-B3LYP density functional theory (DFT) (52) with D3 version of
110 Grimme's dispersion (53) added and the cc-pVTZ basis set (54) were used for the scans and
111 optimizations.

112 At chemical equilibrium, the molar concentration of conformer i is given as the Boltzmann
113 distribution (55);

114 $$c_i \propto \exp(-\Delta G_i / RT), \qquad (1)$$

115 where ΔG_i is the Gibbs free energy of conformer i relative to the most stable conformer, R is the
116 gas constant, and T is the temperature. The free energies of conformers at temperature of 298.15 K
117 and pressure of 1 atm were estimated by using the standard equations for an ideal gas. In this
118 estimation, conformers present in concentrations lower than 1% of the most-stable conformer
119 concentration, i.e., conformers with ΔG_i larger than the free-energy-difference criterion of 2.73
120 kcal/mol (=ΔG_c), were neglected.

121 Energies and oscillator strengths of the electronic excitations to singlet excited states in the
122 conformers were calculated with time-dependent DFT by using CAM-B3LYP-D3/AUG-cc-pVTZ
123 (56). The UV/Vis spectrum at chemical equilibrium is given by the superposition of the oscillator
124 strengths of the conformers weighted according to their concentrations, c_i. To compare this
125 Boltzmann weighted UV/Vis spectrum with the experimental UV/Vis spectrum, the broadening
126 effect was taken into account with the assumption that the half-width at half maximum (HWHM)
127 of the Gaussian band shape is 0.22 eV. This value was estimated from the experimental spectrum
128 of D-luciferin (**c**) (47) by fitting two Gaussian functions to the peak at 329 nm as shown in Fig.
129 S2.

130 Throughout this study, GAUSSIAN09 (57) was used for the electronic structure
131 calculation and the polarizable continuum model (PCM) (58) was applied to the solvent effect in
132 aqueous solution.

133 RESULTS AND DISCUSSION

Classification of the conformers

The PEC was scanned thoroughly by rotation around the colored bonds in Fig. 1. We obtained ten *plane/twist structures*, six *diethylamino conformations*, and three *carboxylate conformations*. The structures were classified as described in the following paragraphs. Atoms related to conformation changes are labeled as indicated in Fig. 2, which shows the structure of the most-stable conformer mentioned later.

<Figure 2>

plane/twist structures. Rotations around the C1-O, O-C2, or C2-C3 bonds in the methyleneoxy linker induce conformational isomerism interconverting the planar and twist structures. These rotations form a plane/twist family of ten conformers as shown in Fig. 3. Two planar structures, denoted by plane 0 and plane 1, are interconverted with each other by rotation around the C1-O bond by about 180°. Eight twist structures are generated by rotation around the O-C2 or C2-C3 bonds in these two planar structures. The plane-*i* structure is converted to the twist *i*-1 (twist *i*-2) structure by rotating around the O-C2 bond by about –100° (+100°), and it is converted to the twist *i*-3 (twist *i*-4) structure by rotating around the C2-C3 bond by about –120° (+120°), where *i* = 0 or 1.

<Figure 3>

diethylamino conformations. Conformations of the diethylamino group are characterized by the directions of the two ethyl groups on the basis of the rotation angle around the N-C4 and N-C5 bonds. The six possible structures of the diethylamino group are shown in Fig. 4. The two types on the bottom of the figure are the *gauche-gauche* forms, in which the two ethyl groups project oppositely away from the coumarin plane. They are classified as diethylamino $g_{(-)}g_{(-)}$ or $g_{(+)}g_{(+)}$. The four structures in the top of the figure are the *gauche-trans* or *trans-gauche*

forms, in which one ethyl group projects out of the plane, and the other ethyl group is almost in the plane. They are classified as diethylamino $g_{(+)}t$, $g_{(-)}t$, $tg_{(-)}$, or $tg_{(+)}$.

<Figure 4>

carboxylate conformations. The thiazoline ring is puckered; thus, the directions of the two C6-O bonds and of the two C8-H bonds can change depending on the C6-C7-C8-S dihedral angle. Scanning the PEC by changing the dihedral angle gave three structure types, the carboxylates A, B, and C, as shown in Fig. 5.

<Figure 5>

Although the PEC scan calculations with the dispersion correction, which was added to describe the van der Waals interaction, gave the carboxylate-C structure, those without the correction demonstrated that this type of structure is not an equilibrium structure and that only carboxylates A and B are equilibrium structures (See Fig. S3). The stability of the carboxylate-C structure is highly dependent on the functional used in the DFT calculation. To obtain further insight into the stability of the carboxylate-C structure, a D-luciferin (**c**) molecule, without caged group, was examined for simplicity (see Supporting Information). The transition state connecting the carboxylate-B and -C structures for **c** was determined. The calculations showed that the potential energy of activation from the carboxylate-C structure is less than 1×10^{-2} kcal/mol as shown in Fig. S5. In consideration of the zero-point energy, this structure is expected to be unstable. It can be inferred that this conclusion is correct for **a**. Therefore, only the carboxylate-A and -B structures of **a** will be taken into account hereafter.

Gibbs free energies of conformers

As previously shown, a large number of conformers of **a** can be induced by combinations of rotations or torsions. Comparison of the Gibbs free energy of the conformers reveals that only 40 conformers among the numerous possible conformers are present at room temperature at a

181 concentration higher than 1% of the most stable conformer in aqueous solution. The Gibbs
182 free energies were calculated using vibrational frequencies obtained by the vibrational
183 analysis. The vibrational frequencies of the most stable conformer are listed in Table S2 as an
184 example. The relative Gibbs free energies and the relative molar concentrations (in
185 parentheses) of the 40 conformers are listed in Table 1. The most stable conformer, marked
186 with a dagger in Table 1, falls in the plane-0 type in the case of *plane/twist structures*,
187 diethylamino-$g_{(-)}g_{(-)}$ type in the case of *diethylamino conformations*, and carboxylate-B type in
188 the case of *carboxylate conformations*, as shown in Fig. 2. Hereafter, the above conformer is
189 called “plane-0 diethylamino-$g_{(-)}g_{(-)}$ carboxylate-B conformer”, and the other conformers are
190 named in the same manner. For example, the second most stable conformer, with a double
191 dagger, is called plane-0 diethylamino-$g_{(+)}g_{(+)}$ carboxylate-B conformer.

192 Conformers with a diethylamino group in the *gauche-trans* or *trans-gauche* form are
193 excluded from Table 1 owing to their high relative free energies. Relative free energies for
194 plane-0 carboxylate-B conformers with different diethylamino conformations are shown in
195 Fig. 4. According to the figure, the *gauche-gauche* forms are more stable than the *gauche-*
196 *trans* and *trans-gauche* forms by approximately 3 kcal/mol, which is higher than the free-
197 energy-difference criterion, ΔG_c. The free energies of the other conformers in *gauche-trans* or
198 *trans-gauche* forms relative to those in *gauche-gauche* form also exceed ΔG_c. Therefore, all
199 conformers in the *gauche-trans* or *trans-gauche* form can be neglected.

200 <Table 1>

201 UV/Vis spectrum

202 The oscillator strengths (vertical black lines) and the UV/Vis spectra (solid red lines) of the
203 carboxylate-A and -B conformers with the plane-0 diethylamino-$g_{(-)}g_{(-)}$ structure are shown in
204 Fig. 6 as typical examples. For both conformers, the oscillator strengths corresponding to the

205 first, second, and third excited states (S_1, S_2, and S_3) are predominant. The assignments of these
206 excited states are listed in Table 2. The two conformers are similar in their assignments; that
207 is, S_1, S_2, and S_3 are mainly composed of the electronic excitation from HOMO to LUMO+1,
208 from HOMO–1 to LUMO, and from HOMO–2 to LUMO, respectively, where HOMO
209 (LUMO) denotes the highest occupied (lowest unoccupied) molecular orbital. These MOs for
210 the two conformers are shown in Fig. 7. For both conformers, HOMO and LUMO+1 are
211 delocalized only in the caged group, while HOMO–1, HOMO–2, and LUMO are delocalized
212 only in the luciferin moiety. Therefore, S_1 is attributed to the electronic excitation of the caged
213 group, whereas S_2 and S_3 are attributed to the electronic excitation of the luciferin moiety.

214 <Figure 6>

215 <Table 2>

216 <Figure 7>

217 The shapes of the HOMO–1 and HOMO–2 in Fig. 7 differ according to the
218 conformation of the carboxylate group. Reflecting the difference in their MOs, the
219 carboxylates A and B are different in their oscillator strengths of S_2 and S_3. However, the
220 differences in the oscillator strengths are not observed in the UV/Vis spectra, due to the
221 broadening effect, with HWHM of 0.22 eV as shown by the solid red lines in Fig. 6.
222 Therefore, S_1 corresponds to the first band of the absorption spectrum with a maximum
223 wavelength at about 320 nm, and the superposition of S_2 and S_3 results in the second band
224 around 290 nm. The other conformers are also similar to each other in terms of assignments,
225 oscillator strengths, and energies of the three low-lying excited states.

226 A theoretical UV/Vis spectrum was obtained by the Boltzmann-weighted sum over the
227 40 conformers. It has two bands (Fig. S4) and its shape agrees well with that of the
228 experimental spectrum(47) (Fig. 8, solid black spectrum). The bands are centered at 384 nm
229 and 339 nm in the experimental spectrum, versus 323 nm and 292 nm, respectively, in the

230 theoretical spectrum. Such discrepancy of band position between experiment and TD-DFT
231 calculation has been reported in studies of a molecule (59) and on solid state (60). The relative
232 values of excitation energies are known to be more reliable than the excitation energies
233 themselves. Accordingly, the theoretical UV/Vis spectrum is often corrected with an offset to
234 facilitate the comparison of the theoretical and experimental spectra (60, 61). In our work, the
235 relative band positions in the spectra of D-luciferin (See Fig. 7) as well as DEACM-caged
236 luciferin are actually in good agreement with the experimental spectra. Therefore, we used an
237 offset of 0.6 eV for the theoretical UV/Vis spectrum so that the peak position of the first band
238 of the theoretical spectrum matches to that of the experimental spectrum. The offset of 0.6 eV
239 around a 300 nm spectral region is not exceptionally large. This theoretical UV/Vis spectrum
240 with the offset is shown in Fig. 8 (solid red line). The energies of the two peaks in the
241 theoretical UV/Vis spectrum are consistent with those in the experimental spectrum. The
242 difference between the theoretical and experimental spectra with regard to the second peak is
243 partially due to the fact that the two-electron excitation was not taken into account.

244 <Figure 8>

245 The theoretical absorption bands (with offset) for the three low-lying excitations are
246 shown by the dotted red line in Fig. 8. The first band at 384 nm is attributed to the first excited
247 state, S_1, corresponding to the electronic excitation of the caged group, while the second band
248 at 339 nm is attributed to the second and third excited states, S_2 and S_3, corresponding to the
249 electronic excitation of the luciferin moiety. Each absorption band of the three low-lying
250 excitations is relatively compact although the band consists of oscillator strengths of various
251 conformers. Therefore, only the caged group or only the luciferin moiety of DEACM-caged
252 D-luciferin is excited by selecting wavelength. This property is advantageous to study
253 experimentally the light suitable to the photocleavage of DEACM-caged D-luciferin. We
254 predict the wavelength range suitable to photocleavage on the basis of the theoretical analysis

255 of the electronic excitation of DEACM-caged D-luciferin and inference made from reaction
256 rates obtained in the previous experimental work (47) in the following.

257 Irradiation induces the photocleavage of DEACM-caged D-luciferin and the single
258 bond between the caged group and the luciferin moiety is broken, leading to the formation of
259 the D-luciferin dianion or the D-luciferin anion following a proton transfer from the
260 environment. Such a cleavage reaction activates the bioluminescent reaction. At the same time,
261 irradiation causes the degradation of DEACM-cage D-luciferin, which occurs by all the other
262 possible reactions, such that the cleavage of bonds other than the target bond and the
263 formation of side products. Irradiation also causes the decomposition of D-luciferin even after
264 the successful photocleavage of DEACM-caged D-luciferin. In a previous experimental study
265 (47), we measured the rates of the following three reactions: DEACM-caged D-luciferin
266 cleavage to luciferin (rate $\gamma_1 = 0.20$ /min), DEACM-caged D-luciferin degradation that does
267 not produce luciferin (rate $\gamma_2 = 0.28$ /min), and luciferin decomposition (rate $\gamma_3 = 0.20$ /min).

268 The degradation of DEACM-caged D-luciferin is thought to be due to the degradation
269 of its luciferin moiety, with a rate denoted by γ_{2L}, and due to the degradation of the other parts,
270 with a rate denoted by γ_{2O}, that is, $\gamma_2 = \gamma_{2L} + \gamma_{2O}$. Given the similarity between D-luciferin and the
271 luciferin moiety of DECAM-caged D-luciferin as mentioned later, the rate of degradation of
272 the luciferin moiety was estimated to be approximately $\gamma_{2L} = 0.20$ /min like luciferin
273 decomposition rate $\gamma_3 = 0.20$ /min. Thus, the rate of degradation of the other parts is
274 approximately $\gamma_{2O} = 0.08$ /min. Since the rate γ_{2L} is more than twice as large as γ_{2O}, the
275 degradation of the luciferin moiety can be a major factor in the degradation of DEACM-caged
276 D-luciferin.

277 The similarity between D-luciferin and the luciferin moiety of DEACM-caged D-
278 luciferin was confirmed by conducting quantum chemical calculations for D-luciferin. The
279 calculation showed that D-luciferin is similar to the luciferin moiety of DEACM-caged D-

280 luciferin in terms of structure, molecular orbital, and electronic excitation (See Figs. S6 and S7
281 and Tables S3 and S4).

282 Based on these considerations, the light for activating DEACM-caged luciferin should
283 be selected outside the wavelength range of the S_2 and S_3 absorption bands in order to avoid the
284 degradation of the luciferin moiety of DEACM-caged D-luciferin. In addition, the light should
285 be out of the luciferin UV/Vis spectrum to avoid decomposition of D-luciferin. The S_2
286 absorption band extends to nearly 400 nm. The experimental UV/Vis spectrum of D-luciferin,
287 shown by the dotted black line in Fig. 8 (47), lies in a wavelength range shorter than 400 nm;
288 accordingly, irradiation at wavelength lower than 400 nm can damage the luciferin moiety of
289 DEACM-caged D-luciferin and D-luciferin. Given that the light should be within the
290 wavelength range in which a certain amount of absorbance is observed, the best wavelength
291 range for photolysis of DEACM-caged D-luciferin is expected to be from 400 to 430 nm.

292 CONCLUSION

293 The structures and optical properties of DEACM-caged D-luciferin in neutral aqueous solution
294 were investigated theoretically. All probable conformers of the DEACM-caged D-luciferin
295 were revealed by PEC scans and structural optimizations. Moreover, comparison of the Gibbs
296 free energies of optimized structures identified 40 conformers present in aqueous solution. The
297 40 DEACM-caged D-luciferin conformers were classified according to the structures of their
298 methyleneoxy linker group, diethylamino group, and carboxylate group.

299 The analysis of the excited states assignments and MOs showed that for every
300 conformer, S_1 is attributed to the electronic excitation of the caged group, and S_2 and S_3 are
301 attributed to the electronic excitation of the luciferin moiety. The UV/Vis spectrum was
302 recovered from the relative concentrations and oscillator strengths of the conformers. The
303 theoretical UV/Vis spectrum with a Gaussian width has two prominent peaks, whose shape

304 agrees well with the experimental UV/Vis spectrum. The absorption bands of the three low-
305 lying excitations in the experimental UV/Vis spectrum were determined by comparison with
306 the theoretical UV/Vis spectrum. This result indicates that the absorption band at 384 nm in
307 the experimental UV/Vis spectrum is due to electronic excitation of the caged group, whereas
308 the band at 339 nm is due to electronic excitations of the luciferin moiety.

309 It is essential to reduce the degradation of DEACM-caged D-luciferin and the
310 decomposition of D-luciferin for successful photocleavage of DEACM-caged D-luciferin. We
311 inferred from experimental reaction rates (47) that the degradation of DEACM-caged D-
312 luciferin is mainly due to the degradation of its luciferin moiety. As a result, by selective
313 electronic excitation of the caged-group, it should be possible to reduce considerably the
314 degradation of DEACM-caged D-luciferin and to avoid the decomposition of D-luciferin.
315 Atheoretical analysis of the UV/Vis spectrum showed that it is possible to excite selectively
316 the caged group by light within the 400 to 430 nm wavelength range, wavelength window
317 outside the UV/Vis spectrum of D-luciferin. Therefore, for high yields of pure luciferin, the
318 wavelength of the light used to photocleave DEACM-caged D-luciferin is expected within the
319 400 to 430 nm spectral range,.This light selection will be tested experimentally in a future
320 study.

321 **ACKNOWLEDGMENTS**: M. H. acknowledges useful discussion with Prof. Koichi
322 Ohno and Prof. Takeaki Iwamoto. M. H. thanks KAKENHI No.17KT0094 from the Japan
323 Society for the Promotion of Science (JSPS). N. K. thanks JST CREST Grant Number
324 JPMJCR13L5, Japan. This work was partly supported by KAKENHI No.18H01469 from
325 JSPS, OPERANDO-OIL, and NEDO in Japan. The computations were carried out at the
326 Information Technology Center of Nagoya University and the ISSP Supercomputer Center of
327 the University of Tokyo.

328 SUPPORTING INFORMATION

329 Figures S1-S7 and Tables S1-S4 can be found at DOI: xxxx-xxxxxx.s1.

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TABLES

Table 1. Relative Gibbs free energies in kcal/mol of the DEACM-caged D-luciferin conformers present in aqueous solution. Values in parentheses are ratios of concentrations.

	diethylamino $g_{(-)}g_{(-)}$		diethylamino $g_{(+)}g_{(+)}$

	carboxylate A		carboxylate B	
plane 0	0.52	(0.41)	0.00	(1)†
twist 0-1	0.34	(0.56)	0.39	(0.52)
twist 0-2	0.37	(0.54)	0.37	(0.53)
twist 0-3	0.82	(0.25)	0.89	(0.22)
twist 0-4	0.80	(0.26)	0.99	(0.19)
plane 1	1.14	(0.15)	0.96	(0.20)
twist 1-1	0.79	(0.26)	1.49	(0.08)
twist 1-2	0.86	(0.23)	0.50	(0.43)
twist 1-3	1.61	(0.07)	1.13	(0.15)
twist 1-4	1.90	(0.04)	1.58	(0.07)

	carboxylate A		carboxylate B	
plane 0	0.51	(0.42)	0.09	(0.86)‡
twist 0-1	0.46	(0.46)	0.70	(0.30)
twist 0-2	0.78	(0.27)	0.67	(0.32)
twist 0-3	0.93	(0.21)	0.82	(0.25)
twist 0-4	0.77	(0.27)	0.73	(0.29)
plane 1	1.17	(0.14)	0.80	(0.26)
twist 1-1	0.77	(0.27)	1.12	(0.15)
twist 1-2	0.78	(0.27)	1.02	(0.18)
twist 1-3	1.62	(0.07)	1.26	(0.12)
twist 1-4	1.50	(0.08)	1.71	(0.06)

Table 2. Assignments of the three low-lying excited states of conformers of DEACM-caged D-luciferin. The assignments are given for the carboxylate-A and -B conformers with the plane-0 diethylamino-$g_{(-)}g_{(-)}$ structure as typical examples.

carboxylate A	Assignments	
S_1	HOMO→LUMO+1	94.0 %
S_2	HOMO–1→LUMO	82.5 %
	HOMO–2→LUMO	8.4 %
S_3	HOMO–2→LUMO	79.7 %
	HOMO–1→LUMO	7.5 %
carboxylate B	**Assignments**	
S_1	HOMO→LUMO+1	94.0 %
S_2	HOMO–1→LUMO	80.3 %
	HOMO–2→LUMO	9.1 %
S_3	HOMO–2→LUMO	73.0 %
	HOMO–1→LUMO	9.4 %

FIGURE CAPTIONS

Figure 1. UV photolytic reaction of DEACM-caged D-luciferin (**a**) to 7-(diethylamino)-4-(hydroxymethyl)coumarin (**b**) and D-luciferin (**c**). Conformers of DEACM-caged D-luciferin are interconverted by rotation around red, blue and green bonds.

Figure 2. Structure of the most-stable conformer of DEACM-caged D-luciferin. It is classified as plane-0 diethylamino-$g_{(-)}g_{(-)}$ carboxylate-B conformer. The atoms related to the conformation changes are labeled.

520 **Figure 3.** Plane/twist family for DEACM-caged D-luciferin conformers. All conformers
521 shown here have diethylamino-$g_{(-)}g_{(-)}$ carboxylate-B structure. A similar family exists for each
522 diethylamino-carboxylate structure.

523 **Figure 4.** Structures and relative Gibbs free energies for DEACM-caged D-luciferin isomers
524 with the plane-0 and carboxylate-B structure and different conformations in the diethylamino
525 group.

526 **Figure 5.** Classification of DEACM-caged D-luciferin with respect to the carboxylate
527 conformation. The left figure in each panel shows full view of structures of carboxylate-A, -B,
528 and -C conformers with plane-0 diethylamino-$g_{(-)}g_{(-)}$ structure. The right figure shows the
529 corresponding partial structural formula around the carboxylate group.

530 **Figure 6.** Oscillator strength of the conformers of DEACM-caged D-luciferin. Those of
531 carboxylate-A and -B conformers with plane-0 diethylamino-$g_{(-)}g_{(-)}$ structure are shown as
532 typical examples. The vertical black lines show oscillator strengths, and the solid red lines
533 show the UV/Vis spectra calculated by superposition of the oscillator strengths to account for
534 the broadening effect.

535 **Figure 7.** MOs of conformers of DEACM-caged D-luciferin, exemplified in the case of the
536 carboxylate-A and -B conformers with plane-0 diethylamino-$g_{(-)}g_{(-)}$ structure as typical
537 examples.

538 **Figure 8.** UV/Vis spectra of DEACM-caged D-luciferin. The solid black line and the dotted
539 line shows the experimental UV/Vis spectrum of DEACM-caged D-luciferin and D-luciferin,
540 respectively (47). The solid red line shows an adjusted theoretical UV/Vis spectrum of
541 DEACM-caged D-luciferin, which is shifted lower by 0.6 eV in energy from the original
542 theoretical spectrum. The dotted red lines show S_1, S_2, and S_3 absorption bands from the ground
543 state (S_0).

544