# Design, Synthesis, and Evaluation of the Transition-State Inhibitors of Coelenterazine Bioluminescence: Probing the Chiral Environment of Active Site

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*Received October 3, 2000*

Bioluminescence is one of the most attractive natural phenomena, displayed by various different types of organisms from bacteria to fishes.[1] Recently, considerable attention has been given to bioluminescence reactions for use as highly sensitive, nondestructive analytical tools, particularly in monitoring gene expressions and studying protein−protein interactions by luminescence energy transfer.[2] Coelenterazine (**1a**) is well-known to be involved in the luminescence reactions of various organisms, such as jellyfishes,[3] sea pansies,[4] and deep-sea shrimps.[5] It is widely distributed among both bioluminescent and nonbioluminescent marine organisms.[6] The oxidation of coelenterazine results in light emission either in the presence or absence of a protein factor, providing a commonly used distinction between bioluminescence and chemiluminescence. The quantum yield is much greater in the bioluminescence that involves an enzyme than in the chemiluminescence.[7] The luminescence reaction is an oxidation process that takes place at the C-2 and C-3 positions of coelenterazine, and it appears to involve several key peroxy intermediates or transition states (**3**−**4**)[7,8] (Figure 1).

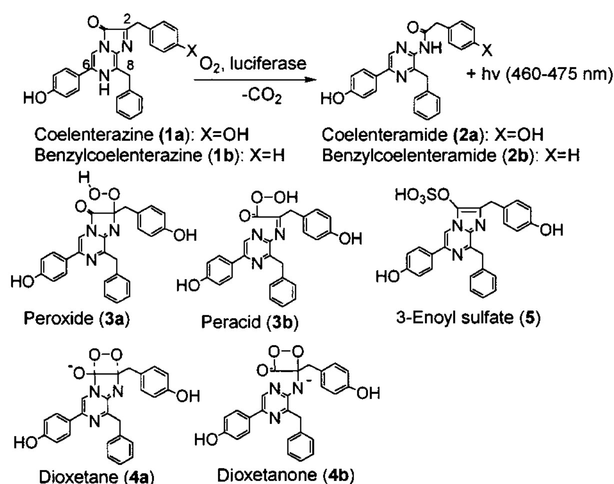


**Figure 1.** Bioluminescence reaction of coelenterazine (**1a**) and its possible intermediates and transition states (**3**, **4**) and 3-enoyl sulfate of coelenterazine (**5**).

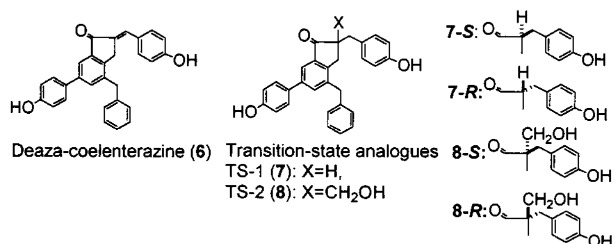


**Figure 2.** Stable deazacoelenterazine analogue (**6**) and its transition-state analogues (**7**, **8**).

Among the bioluminescence systems of coelenterazine, *Renilla* luciferase has attracted much attention recently because of its simplicity in the components required for luminescence reaction and of usefulness as a reporter protein.[2,9] The structure of coelenterazine peroxide (**3a**) in aequorin was found to have 2*S* configuration by $^{13}$C NMR experiments and X-ray crystallography.[10,11] Model studies on the peroxy intermediates of coelenterazine analogues showed that peroxides decompose with light emission at −50 °C and above, with lower efficiencies than those found in bioluminescence.[12] None of the peroxy intermediates or transition states of luciferase reactions have been characterized, except for a $^{18}$O-labeling study of the luminescence reaction of a shrimp luciferase.[5a] The inhibitory effects of several coelenterazine analogues were investigated by Cormier et al.,[13] revealing that the 3-enoyl sulfate of coelenterazine (**5**) was a strong inhibitor ($K_i = 9.1 \times 10^{-9}$ M). To investigate the enzymatic reaction of coelenterazine bioluminescence that involves a highly efficient chemical excitation step, we designed some inhibitors that are stable and not altered by luciferases (**6**−**8**) (Figure 2).

The transition-state analogues synthesized inhibited the luminescence reaction of recombinant *Renilla* luciferase,[9,14] apparently reflecting the differences in the chirality of these inhibitors. The results suggested for the first time that the mechanism-based inhibitors of bioluminescence might be useful as the tools for investigating the chiral environment of the active sites of enzyme where the excited species is formed at a high efficiency.

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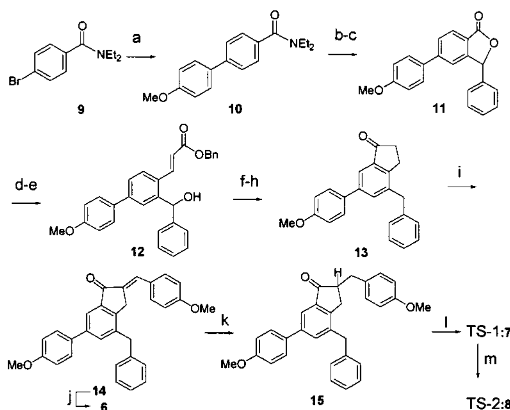
(1) (a) Harvey, E. N. *Living Light*; Princeton University Press: Princeton, 1940. (b) Campbell, A. K. *Chemiluminescence*; VCH Publishers: New York, 1988.
(2) (a) Wilson, T.; Hastings, J. W. *Annu. Rev. Cell Dev. Biol.* **1998**, *14*, 197−230. (b) Naylor, L. H. *Biochem. Pharmacol.* **1999**, *58*, 749−757.
(3) (a) Shimomura, O.; Johnson, F. H. *Nature* **1970**, *227*, 1356−1357. (b) Shimomura, O.; Johnson, F. H. *Tetrahedron Lett.* **1973**, *31*, 2963−2966. (c) Shimomura, O.; Johnson, F. H. *Nature* **1975**, 236−238. (d) Shimomura, O.; Johnson, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2611−2615.
(4) Matthews, J. C.; Hori, K.; Comier, M. J. *Biochemistry* **1977**, *16*, 5217−5220.
(5) (a)Shimomura, O.; Masugi, T.; Johnson, F. H.; Haneda, Y. *Biochemistry* **1978**, *17*, 994−998. (b) Inouye, S.; Watanabe, K.; Nakamura, H.; Shimomura, O. *FEBS Lett.* **2000**, *481*, 19−25.
(6) (a) Shimomura, O.; Inoue, S.; Johnson, F. H.; Haneda, Y. *Comp. Biochem. Physiol.* **1980**, *65B*, 435−437. (b) Shimomura, O. *Comp. Biochem. Physiol.* **1987**, *86B*, 361−363. (c) Campbell, A. K.; Herring, P. *J. Mar. Biol.* **1990**, *104*, 219−225.
(7) (a) Goto, T. In *Marine Natural Products, Chemical and Biological Perspective*; Scheuer, P. J., Ed.; Academic Press: New York, 1980; Vol. 3, pp 179−222. (b) Hori, K.; Wampler, J. E.; Matthew, J. C.; Cormier, M. J. *Biochemistry* **1973**, *12*, 4463−4468.
(8) (a) MaCapra, F. *Endeavour* **1973**, *32*, 139−145. (b) Shimomura, O.; Johnson, F. H. *Biochem. Biophys. Res. Commun.* **1971**, *44,* 340−346. (c) Fujimori, K.; Nakajima, H.; Akutu, K.; Mitani, M.; Sawada, K.; Nakayama, M. *J. Chem. Soc., Perkin Trans. 2* **1993**, 2405−2409.
(9) Inouye, S.; Shimomura, O. *Biochem. Biophys. Res. Commun.* **1997**, *233*, 349−353.
(10) A photoprotein from a jellyfish contains a peroxide moiety and the luminescence is triggered by calcium ion to emit blue light of 465 nm with a high quantum efficiency of 0.23.[3a]
(11) (a) Musicki, B.; Kishi, Y.; Shimomura, O. *J. Chem. Soc., Chem. Commun.* **1986**, 1566. (b) Head, J. F.; Inouye, S.; Teranishi, K.; Shimomura, O. *Nature* **2000**, *405*, 372−376.
(12) (a) Teranishi, K.; Ueda, K.; Nakao, H.; Hisamatsu, M.; Yamada, T. *Tetrahedron Lett.* **1994**, *35*, 8181−8184. (b) Usami, K.; Isobe, M. *Tetrahedron Lett.* **1995**, *36*, 8613−8617, *Chem. Lett.* **1996**, 215−216, and *Tetrahedron* **1996**, *52*, 12061−12090. (c) Teranishi, K.; Hidamatsu, M.; Yamada, T. *Tetrahedron Lett.* **1997**, *38*, 2689−2692.
(13) Matthews, J. C.; Hori, K.; Cormier, M. J. *Biochemistry* **1977**, *16*, 5217−5220.
(14) Lorenz, W. W.; MaCann, R. O.; Longiaru, M.; Cormier, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4438−4442.

10.1021/ja005663v CCC: $20.00 © 2001 American Chemical Society
Published on Web 01/25/2001

**Scheme 1**[a]




[a] (a) 4-MeO-$C_6H_4B(OH)_2$, 2 M $Na_2CO_3$, $Pd(PPh_3)_4$, benzene−MeOH, 80 °C, 24 h, 99%; (b) *sec*-BuLi, TMEDA, −78 °C, then s.m, THF, 1 h, benzaldehyde, −78 °C, 30 min; (c) toluene, 110 °C, 24 h, 80% for 2 steps; (d) DIBAL, −78 °C, $CH_2Cl_2$, 90%.; (e) $Ph_3P$−CHCOOBn, benzene, 80 °C, 1 h, 95%; (f) $H_2$, Pd/C, EtOH-EtOAc, 23 °C, 36 h, 94%; (g) $SOCl_2$ 80 °C, 3 h; (h) $TiCl_4$, $CH_2Cl_2$, 12 h, 0 °C to 5 °C, 50% for 2 steps; (i) 4-methoxybenzaldehyde, 2.5 M NaOH, EtOH, 24 °C, 20 h, 74%; (j) $BBr_3$ , −78 °C to rt, 24 h, 52%; (k) $PtO_2$, $H_2$, EtOH, 23 °C, 48 h, 70%; (l) $BBr_3$ , −78 °C to rt, 48 h, 50%; (m) NaOH, HCHO, THF−$H_2O$, 23 °C, 2 h, 60%.

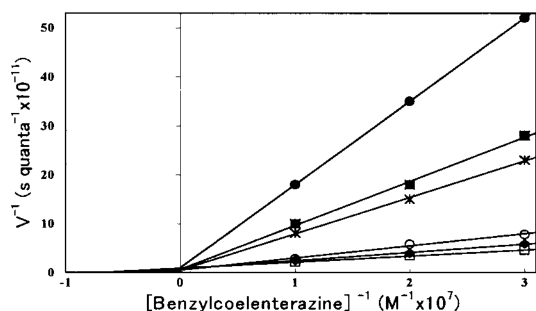



**Figure 3.** Lineweaver−Burk plot of the results of *Renilla* luciferase inhibition assay for the synthesized inhibitors, deazacoelenterazine analogue (**6**, ◆) and the transition-state analogues, *R*-TS-1 (*), *S*-TS-1 (■), *R*-TS-2 (●), and *S*-TS-2 (○) at a concentration of $1.1 \times 10^{-7}$ M. The initial rate of luminescence of a substrate, benzylcoelenterazine (**1b**, X = H), with the recombinant enzyme (0.5 $\mu$g/mL) was measured in 25 mM Tris buffer pH 7.5 containing 0.1 M NaCl at 23 °C with or without inhibitors (□).

Our first object in this study was the synthesis of a stable core structure of coelenterazine, for which we selected a deaza-analogue of coelenterazine (Scheme 1). The synthesis was started with 3-bromobenzamide (**9**), and a *p*-hydroxyphenyl group was introduced by Suzuki coupling[15] to yield a biphenyl compound (**10**). A benzyl group was added by the coupling reaction of an ortho-metalated carbanion of **10** with benzaldehyde.[16] After lactonization, a two-step transformation provided an acid which was converted to an indanone (**13**) by standard procedure.

A second *p*-hydroxyphenyl moiety was introduced to the indanone by aldol condensation with *p*-methoxybenzaldehyde to form the olefin (**14**) with an *E*-exo configuration. After deprotection, a stable deazacoelenterazine analogue (**6**) with an isomeric double bond was obtained. The compound showed a moderate inhibition of recombinant *Renilla* luciferase in a competitive manner (Figure 3).

For the synthesis of transition-state models having a chiral $sp^3$ carbon at position 2, the olefin **14** was subjected to hydrogenation with $PtO_2$ followed by deprotection and separation on a chiral column (DAICEL OC), affording two enantiomers, (*S*)-TS-1 (**7-*S***) and (*R*)-TS-1 (**7-*R***).[17,18] As expected, these compounds showed

(15) Miyaura, N.; Suzuki, A. *Chem. Rev.* **1995**, *95*, 2457−2483.
(16) Beak, P.; Brown R. A. *J. Org. Chem.* **1982**, *47*, 34−46.

**Table 1.** Inhibitory Effects of the Transition-State Analogues on the Bioluminescence Reaction of Recombinant *Renilla* Luciferase and Benzylcoelenterazine (**1b**)

| cmpd | $K_i$/M | cmpd | $K_i$/M |
|---|---|---|---|
| deaza (**6**) | $1.3 \times 10^{-7}$ | benzylcoelenteramide (**2b**) | $2.3 \times 10^{-8a}$ |
| *S*-TS-1 (**7-*S***) | $4.0 \times 10^{-8}$ | *R*-TS-1 (**7-*R***) | $5.0 \times 10^{-8}$ |
| *S*-TS-2 (**8-*S***) | $2.1 \times 10^{-7}$ | *R*-TS-2 (**8-*R***) | $9.3 \times 10^{-9}$ |

[a] Data from ref 13.

stronger inhibitory effects than the $sp^2$ analogue, however with little difference between the two in the extents of inhibition. The results suggest that the *p*-hydroxybenzyl group of the bound enantiomers might be located at a similar position in the active site of the enzyme, in resemblance to the finding in the acetylcholine esterase inhibition.[19] To examine the effect of the peroxide moiety which might enhance selective binding, a hydroxymethyl group was introduced at position 2 by an aldol reaction. The enantiomers (TS-2) were separated with a chiral column, and their configurations were determined by comparing their CD spectra with those of model compounds.[18,20,21] An enantiomer (**8-*R***) with an *R*-configuration gave the strongest inhibition with a large enantioselectivity (about 22) (Table 1).

The mechanism of imidazopyrazinone bioluminescence involves several transition states and intermediates, despite the apparent simplicity of the reaction system that requires only luciferin and luciferase in addition to oxygen. Although the 3D structures of imidazopyrazinone luciferases are still unknown except for the photoprotein aequorin, it seems most likely that the luciferases serve to stabilize the structure of coelenterazine substrate while catalyzing the oxidation reaction that results in a highly efficient luminescence. The structure of the enantiomer (*R*)-TS-2 (**8-*R***) might represent the structure of one of the transition states involved in the luminescence reaction. Interestingly, opposite enantioselectivity was observed in the inhibition of aequorin regeneration with benzylcoelenterazine (**1b**) and the enantiomer (*S*)-TS-2 (**8-*S***).

In summary, we have established a novel and effective route to synthesize stable, chiral analogues of coelenterazine for studying transition states and unstable intermediates involved in the bioluminescence of coelenterazine. Among the analogues studied, the hydroxymethyl model of the hydroperoxide structure, (*R*)-TS-2, showed the most potent inhibition of recombinant *Renilla* luciferase. Efforts to elucidate the key structure required for efficient luminescence and the identification of catalytic site are in progress.

**Acknowledgment.** A Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan is gratefully acknowledged. We thank financial support from the Naito Foundation and a JSPS fellowship to C.W.

**Supporting Information Available:** Syntheses and characterization of the compounds (PDF). This material is available free of charge via the Internet at http://acs.pubs.org.

JA005663V

(17) The *S* [CD (EtOH): 270 ($\Delta\epsilon$ +7.4), 330 (0.0), and 350 nm (−1.1)] and *R* [CD (EtOH) 270 ($\Delta\epsilon$ −7.4), 330 (0), and 350 nm (+1.1)] of **7** were eluted from an OC column (4.6 × 250 mm) at 20 and 24 min, respectively, with a mobile phase of hexane−ethanol (2:1, 0.5 mL/min).
(18) These absolute configurations were assigned by comparing their CD spectra and $[\alpha]_D$ with those of similar indanone compounds, which were consistent with the sector rule for indanones: Lucke, M. I.; Marguet A.; G. Snatzke, *Tetrahedron* **1972**, *28*, 1677.
(19) Kawakami, Y.; Inoue, A.; Kawai, T.; Wakita, M.; Sugimoto, H.; Hopfinger, A. J. *Bioorg. Med. Chem.* **1996**, *4*, 1429−1446.
(20) The *S* $[\alpha]^{25}_D$ +108 (*c* 0.02, EtOH), CD (EtOH) 263 ($\Delta\epsilon$ +10), 330 (0.0), 350 nm (−1.1) and *R* $[\alpha]^{25}_D$ −108(*c* 0.02, EtOH)CD (EtOH) 263 ($\Delta\epsilon$ −10) 330 (0.0) 350 nm (+1.1). Enantiomers of **8** were separated by the same OC column with a mobile phase of hexane−ethanol (6:1, 0.5 mL/min) at 32 and 39 min, respectively.
(21) Kuroyanagi, M.; Fukuoka, M.; Yoshida, K.; Natori, S. *Chem Pharm. Bull.* **1979**, *27*, 731−741.