Supplementary Information

Fabrication of a New Lineage of Artificial Luciferases from Natural Luciferase Pools

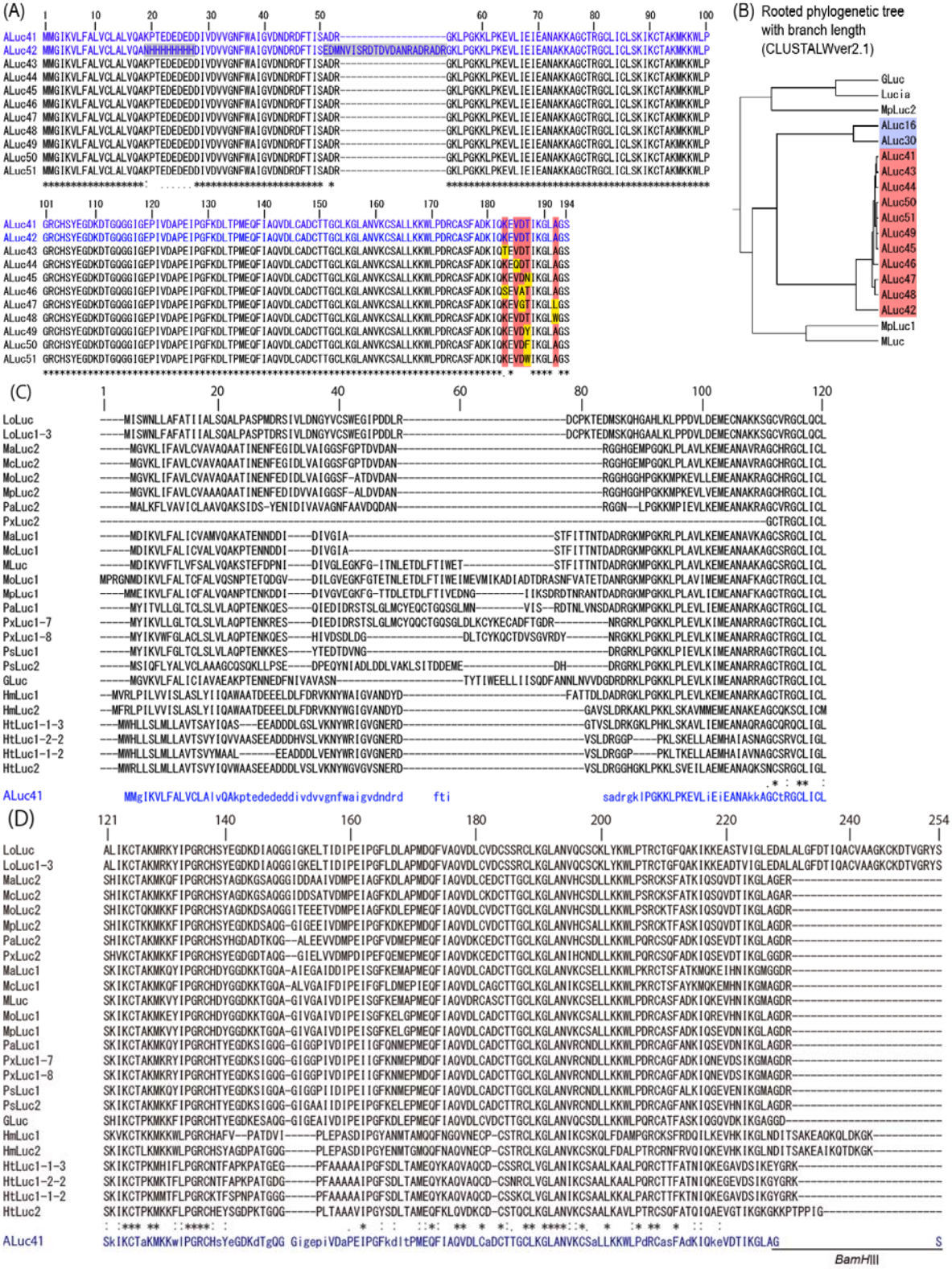
Sung Bae Kim*[,1], Ryo Nishihara[2], Daniel Citterio[2], Koji Suzuki[2]

1. Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba 305-8569, Japan
2. Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan

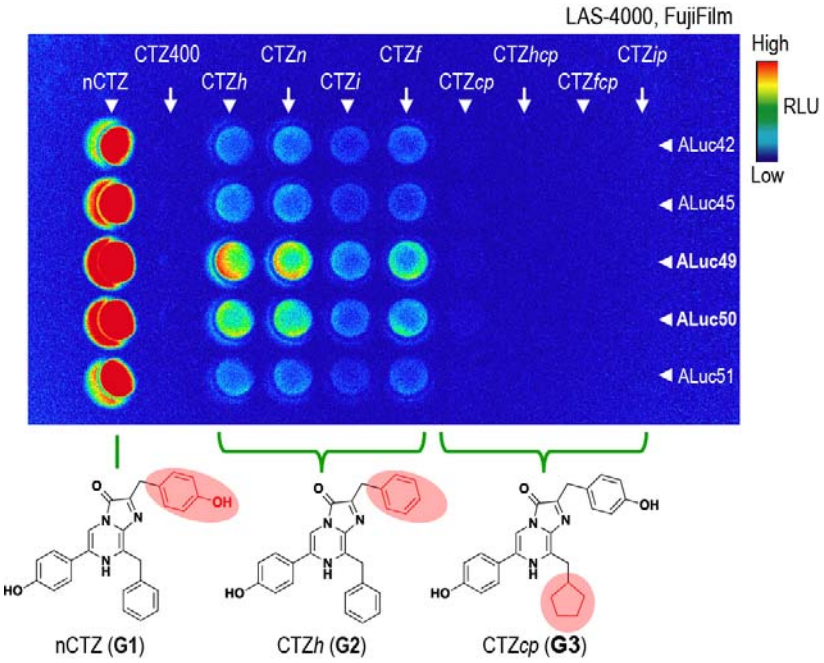
*** Corresponding author:** kimu-sb@aist.go.jp

Supplementary Figures

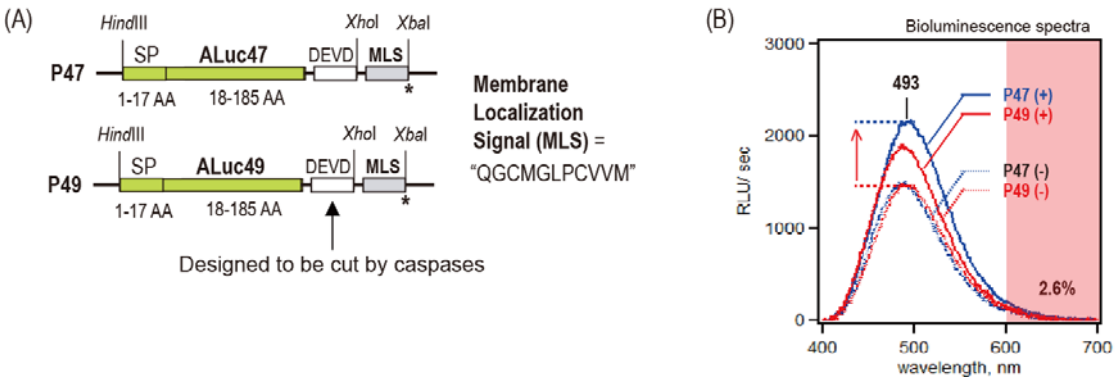
Supplementary Fig. 1.



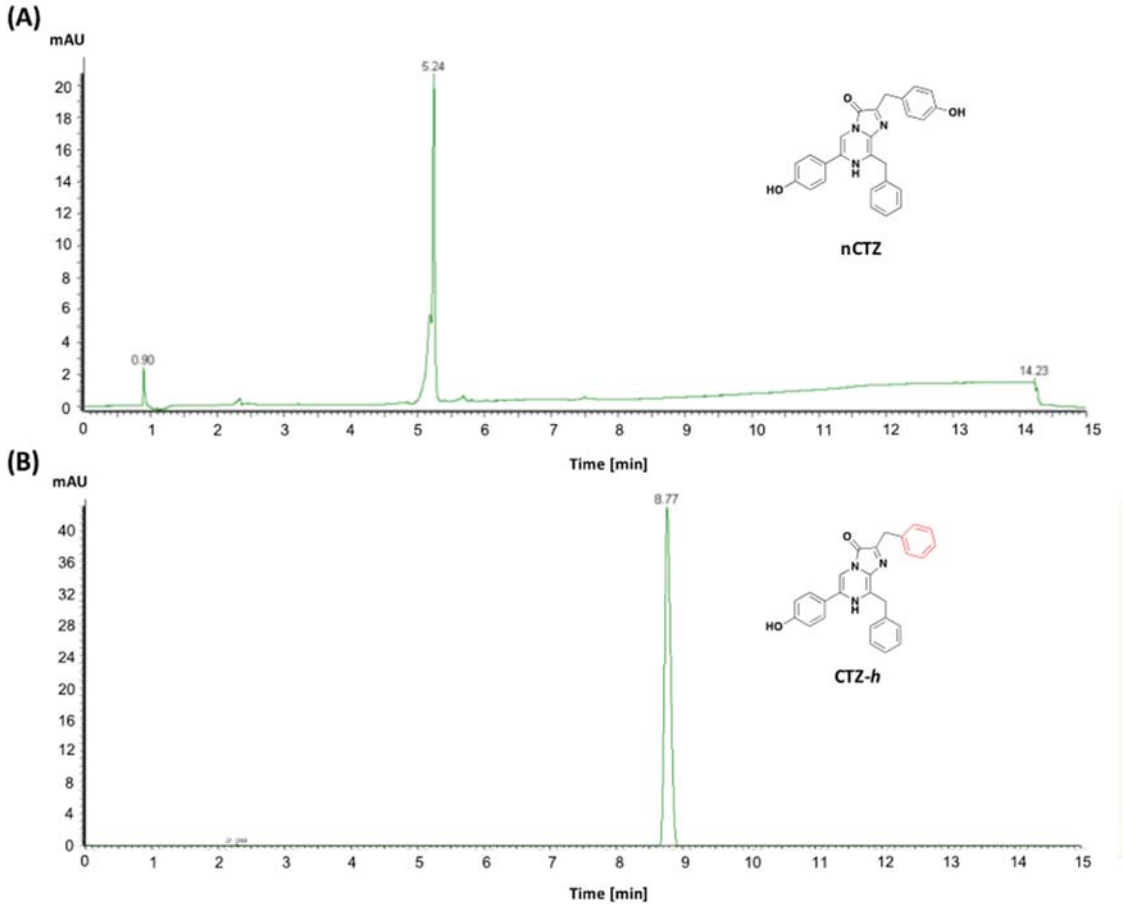
(A) A specified alignment of the sequences of the newly created ALucs. ALuc41 (blue) is extracted from an alignment of copepod luciferases in the public database NCBI with a sequence logo generator (WebLogo version 2.8.2). Its sequence is further modified by the addition of a His-tag epitope and an extended sequence in the N-terminal region. The sequences of ALuc43–51 were created by altering the C-terminal amino acids (red and yellow). (B) A rooted phylogenetic tree of the newly created ALucs and existing marine luciferases according to CLUSTALW version 2.1. The red shaded area indicates the newly created ALucs; the blue shaded area denotes the previous ALucs. (C-D) 25 copepod luciferases in alignment. The sequence generated by the sequence logo generator was slightly modified in the view of the single sequence alignment (SSA) and increase of consensus amino acids between the two-consecutive domains as specified in Figure 1(A). The C-terminal end was modified for making an artificial *BamH*I site. The consequent ALuc41 sequence in blue was attached at the bottom for a reference view.



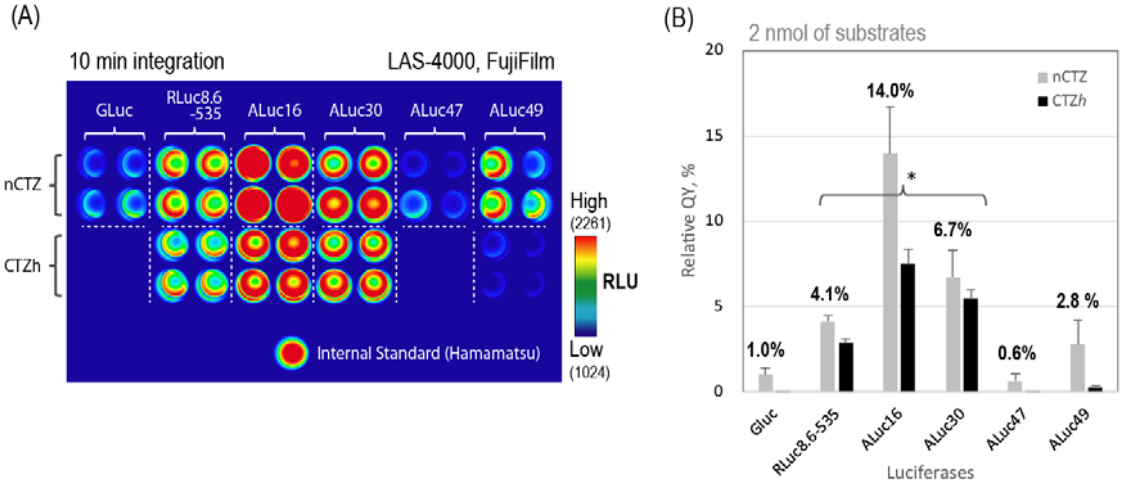
Supplementary Fig. 2. Substrate-specific bioluminescence intensities of the new lineage of ALucs. The substrate-driven optical intensities were determined with the use of various coelenterazine analogues. The new ALucs showed a greatly biased optical intensity with nCTZ (Group 1). The CTZ*h* and CTZ*cp* represent Groups 2 and 3, respectively. The examined coelenterazine analogues may be categorized into three groups: the first group (Group 1; G1) has hydroxyl groups (OH) at both the C2 and C6 positions of the imidazopyradinone backbone; the second group (G2) has no OH group or has a modification at the C2 position; and the third group (G3) commonly has a cyclopentyl (CP) or an isopropyl (IP) group at the C8 position. The maximal optical intensities were found with the nCTZ in Group 1, whereas no considerable optical intensities were observed with Group 3, which carried CP or IP. Very poor optical intensities were observed with Group 2.



Supplementary Fig. 3. Illumination of caspase activities with ALuc47- and ALuc49-based bioluminescent probes, referred to as "bioluminescent capsules," in living mammalian cells. (A) The cDNA constructs encoding the created bioluminescent capsules, **P47** and **P49**. The capsules were made by linking the newly fabricated ALuc47 or ALuc49 with the membrane localization signal (MLS) through flexible linkers. (B) The optical spectra of the capsules with and without an apoptosis inducer staurosporine (STS). The capsules are designed to be initially anchored in the PM. The (+) and (-) signs indicate the addition of the apoptosis inducer staurosporine (STS) and the vehicle, respectively.



Supplementary Fig. 4. HPLC spectra showing the purity of the substrates nCTZ (A) and CTZ*h* (B). Unique single peaks were found at 5.24 and 8.77 minutes without any other peaks showing impurity.



Supplementary Figure 5. Determination of the relative optical intensities. (A) The optical image after long-term integration (10 minutes) of the emitted photons. The diluted substrates (2 nmole) were set for complete consumption by overexpressed luciferases (n=4). (B) The relative optical intensities of the luciferases in percentages according to the substrates. The specific values are shown in Supplementary Table 1. The percentages on the bars denote the apparent sum of photons over the applied amount of nCTZ. The asterisk "*" indicates a group of luciferases that emit relatively unbiased bioluminescence with CTZ analogues.

With respect to the substrate-binding chemistry, the old lineage of ALucs may be close to RLuc8.6-535, whereas the new lineage of ALucs (ALuc47 and ALuc49) should be categorized into a different group, considering the greatly biased optical preference only for nCTZ. The overall trend of the optical intensities indicates that the new lineage of ALuc47 and ALuc49 may have poorer optical efficiency compared with the previously reported ALuc16 and ALuc30, but a greatly biased optical preference only for nCTZ as a unique optical property. Considering the percentages of photon amounts of RLuc8.6-535 is slightly smaller than the previously reported QYs for RLuc and RLuc8 (5.3% and 6.9%, respectively, with nCTZ) [1], the percentages may be close to the absolute QYs and reflect the trend of the QYs of the luciferases. The detailed photon counts and experimental procedure were specified in Supplementary Table 1 and Supplementary Experimental Procedure 2, respectively.

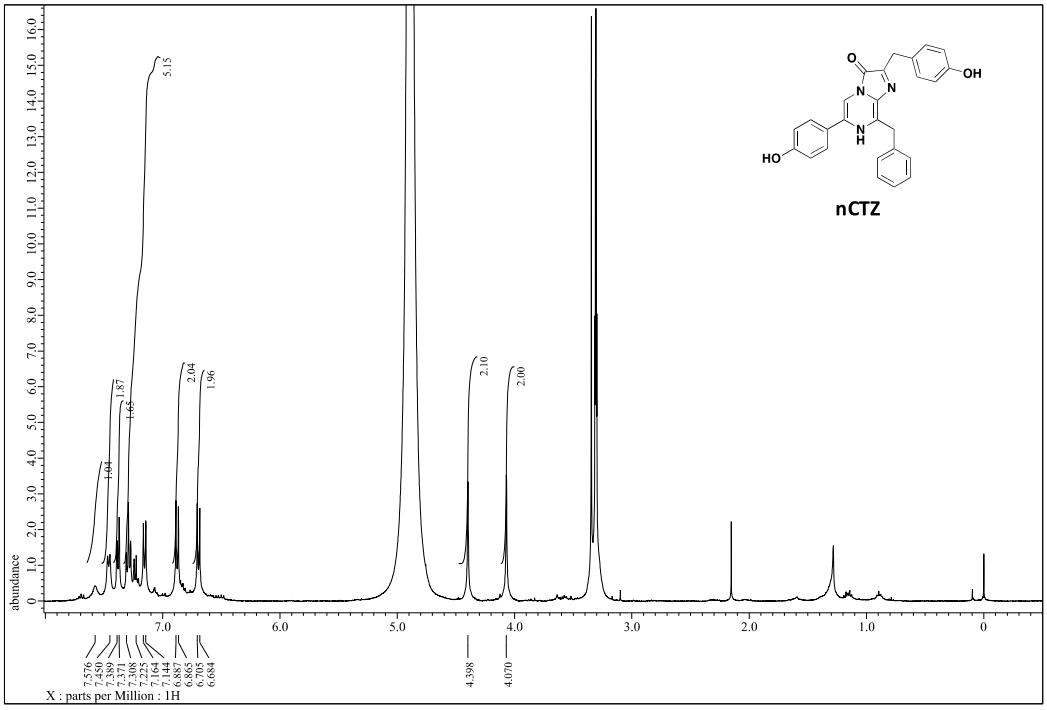
Supplementary Table 1. The total photon counts of the marine luciferases with nCTZ and CTZ*h*. The values are presented in the bar graphs in Suppl. Fig. 5. Regarding the experimental procedure, refer to Supplementary Experimental Procedure 2.

(Photons/mm^2/sec)

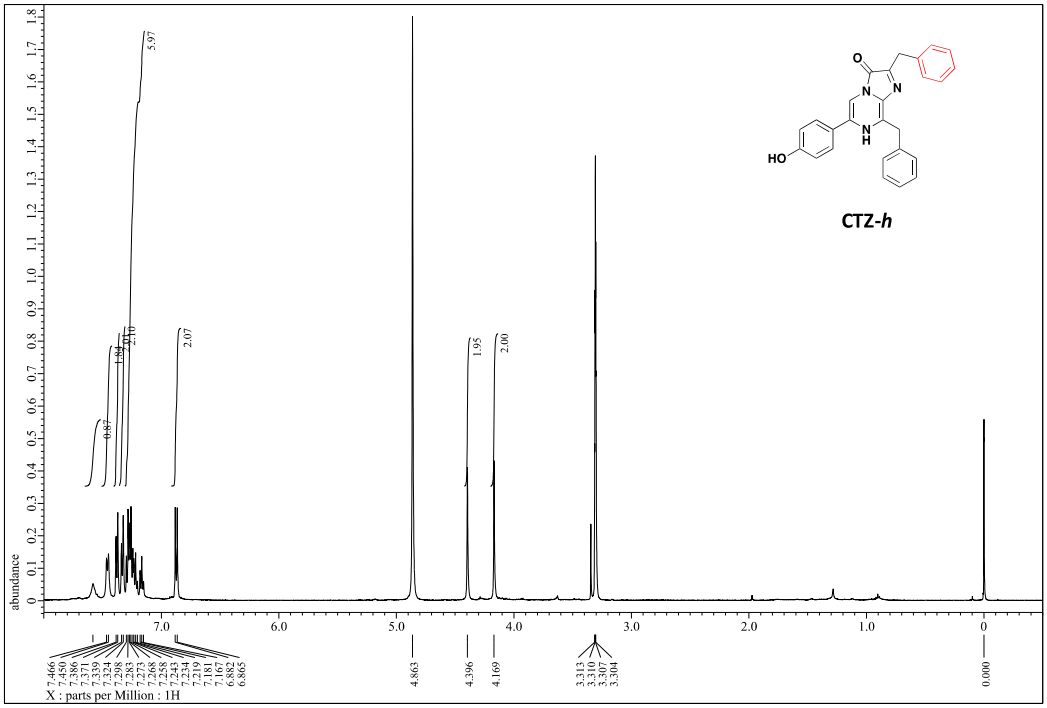
	nCTZ			CTZ*h*		
	Ave		SD	Ave		SD
Gluc	10,840	±	3,874	353	±	58
RLuc8.6-565	44,499	±	3,864	31,298	±	2,350
ALuc16	151,312	±	29,485	81,471	±	8,802
ALuc30	72,429	±	17,696	59,087	±	5,879
ALuc47	6,772	±	4,811	250	±	87
ALuc49	29,956	±	15,357	2,987	±	1,286

Supplementary Experimental Procedure 1. Detailed procedure of the organic synthesis of nCTZ and CTZ*h*, which were synthesized as described in a previous research[18]. The purity of each compound was confirmed by the ^{1}H-NMR and HPLC spectra. The ^{1}H-NMR spectra were recorded on an ECA-500 or ECS-400 spectrometer (JEOL Ltd.). The chemical shifts were determined with the use of tetramethylsilane as an internal standard material. HPLC measurements were carried out with an Acquity UPLC system (Waters Ltd.) under the following conditions: eluent A (H_2O + 0.1% CH_2O_2) and eluent B (CH_3CN + 0.1% CH_2O_2) (A/B = 1/0 → 0/1). The absorbance at 254 nm was monitored.

^{1}H-NMR spectra



^{1}H-NMR (400 MHz, CD_3OD): (ppm) = 7.57 (s, 1H), 7.46 (d, *J* = 7.8 Hz, 2H), 7.38 (d, *J* = 7.3 Hz, 2H), 7.30-7.14 (m, 5H), 6.87 (d, *J* = 9.0 Hz, 2H), 6.69 (d, *J* = 8.7 Hz, 2H), 4.39 (s, 2H), 4.07 (s, 2H).



^{1}H-NMR (500 MHz, CD_3OD): (ppm) = 7.58 (s, 1H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.37 (d, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.29-7.15 (m, 6H), 6.87 (d, *J* = 8.5 Hz, 2H), 4.39 (s, 2H), 4.16 (s, 2H).

Supplementary Experimental Procedure 2. Determination of relative optical intensities of new ALucs and conventional luciferases. The relative optical intensities of the existing marine luciferases and newly fabricated ALucs were determined with the use of the substrates nCTZ and CTZ*h* (Supplementary Fig. 5; Supplementary Table 1).

For the measurement, we referred to a previous methodology for the quantum yields (QYs) [2]. In brief, in the presence of an excess level of enzymes, the quantum yields (QYs) are defined by N_{tot}/S_{tot}, where N_{tot} and S_{tot} denote the total amounts of emitted photons and consumed substrate, respectively. If the luciferase level is in excess compared with luciferins (E >> S), all the luciferins should be consumed. In this case, the initial amount of luciferin (S_{ini}) is equivalent to the total consumed luciferin (S_{tot}).

For exact measurement of the apparent amount of the photons, we custom-synthesized nCTZ and CTZ*h* and carefully determined the purity beforehand based on the NMR peaks and HPLC spectra (Supplementary Fig. 4). The detailed procedure for the synthesis is described in Supplementary Experimental Procedure 1.

For this measurement, the COS-7 cells grown in a 6-well microplate were transiently transfected with the mammalian expression plasmid pcDNA3.1(+) encoding one of the following luciferases: GLuc, RLuc8.6-535, ALuc16, ALuc30, ALuc47, and ALuc49, by using the lipofection reagent TransIT-LT1 (Mirus). The cells were incubated for 2 days for overexpression of the luciferases, followed by lysing with a lysis buffer (Promega).

An aliquot of the lysates (20 μL) was set in each well of a 96-well optical bottom microplate and simultaneously mixed with 60 μL of low-concentration substrates (2 nmole) by using a 12-channel micropipette (Gilson). The microplate was immediately set in the dark chamber of the LAS-4000 image analyzer (Fujifilm), and the total light emission was integrated until the reaction approached completion (about 10 min) at room temperature (22.5 ℃); a few seconds of delay occurred between the substrate injection and measurement. In parallel, the photons from a standard LED light source (L11494-525; Hamamatsu) (1 pW) were measured as an internal standard. Thus, the photon counts were easily calculated from the optical intensities.

References

1. Loening, A. M.; Fenn, T. D.; Wu, A. M.; Gambhir, S. S., Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output. *Protein Eng. Des. Sel.* **2006,** *19* (9), 391-400.

2. Seliger, H. H.; Mc, E. W., Spectral emission and quantum yield of firefly bioluminescence. *Arch. Biochem. Biophy.* **1960,** *88*, 136-141.