Supplementary Note 1

The Designing and Synthesis of CTZ Analogs

Several CTZ analogs have previously been prepared for red-shifted bioluminescence[1], although they typically give low emission with their tested luciferases[2-4]. Most of these analogs have not been directly tested with NanoLuc, and their impact on the bioluminescence of NanoLuc is still unknown because the spectral shift observed for one CTZ-utilizing luciferase is not necessarily transferable to another[1]. For example, although the extended conjugation at the C-6 position of 6-pi-OH-CTZ (**Supplementary Fig. 1**) was shown to red-shift the bioluminescence of an RLuc mutant, Rluc8, by 47 nm, there was hardly any bioluminescence observed when using NanoLuc[5]. We therefore reasoned that NanoLuc might have limited tolerance to structural changes at the C-6 position, and subsequently, focused our efforts on the derivatization of CTZ at the C-8 position. Introducing heteroatoms, such as selenium, into fluorescent dyes or D-luciferin is a proven strategy to red-shift fluorescence or bioluminescence emission[6-8]. Oxygen and sulfur atoms have also been introduced to the C-8 position of CTZ to shift the bioluminescence of RLuc to longer wavelengths[3,4]. On the basis of these results, we hypothesized that introducing selenium to the C-8 position of CTZ could be effective to red-shift the bioluminescence of NanoLuc. We therefore designed selenoterazine (STZ) and developed a synthetic route for this molecule from inexpensive, commercially available chemicals in six steps with 5.2% overall yield (**Supplementary Note 2**, **Scheme 1**). We also serendipitously prepared another CTZ analog, diphenylterazine (DTZ), which extends the conjugation at C-8 through an aromatic ring. The precursor of DTZ was initially derived as a side-product during the synthesis of STZ. Later on, we tested DTZ with NanoLuc and fortunately found it to be one of most useful CTZ analogs to enhance and red-shift the bioluminescence of NanoLuc. We revised a reported procedure[9] and prepare diphenylterazine in large quantities from commercially available chemicals in two steps with 23.1% overall yield (**Supplementary Note 2**, **Scheme 2**).

References:

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2. Inouye, S. & Shimomura, O. *Biochem. Biophys. Res. Commun.* **233**, 349-353 (1997).
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4. Yuan, M.-L., Jiang, T.-Y., Du, L.-P. & Li, M.-Y. *Chin. Chem. Lett.* **27**, 550-554 (2016).
5. Nishihara, R. et al. *Chem. Commun.* **51**, 391-394 (2015).
6. Sun, Y.Q. et al. *Angew. Chem. Int. Ed. Engl.* **51**, 7634-7636 (2012).
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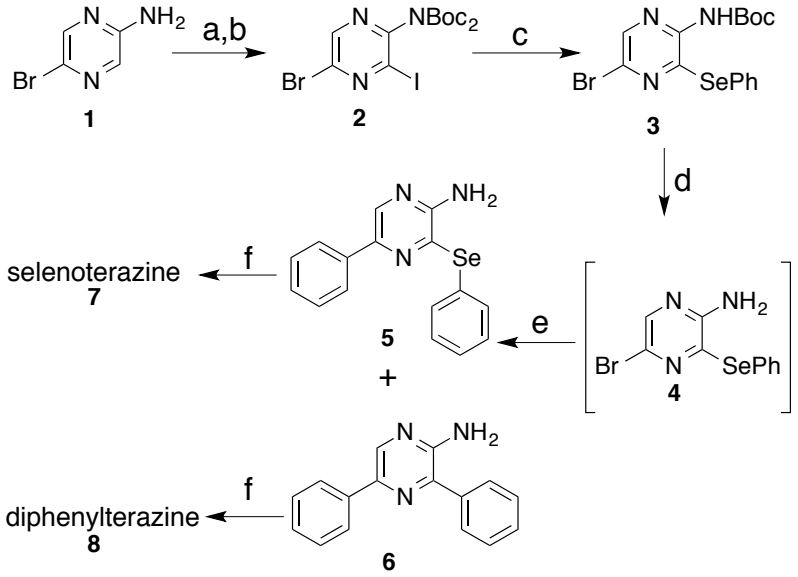
Supplementary Note 2

Methods for Chemical Synthesis

1) Method to synthesize selenoterazine (STZ)



Scheme 1| Synthetic route for preparing selenoterazine (and diphenylterazine as a minor product): (a) TFA, NIS, ACN, Reflux; **(b)** $(Boc)_2O$, NEt_3, THF, RT, 85% from **1**; (**c**) $PhSe_2$, Cu_2O, bpy, Mg, DMF, 85°C, 51%; (**d**) TFA, CH_2Cl_2, RT; (**e**) $(C_6H_5CN)_2PdCl_2$, $(C_6H_5)_2P(CH_2)_4P(C_6H_5)_2$, $PhB(OH)_2$, Na_2CO_3, toluene, EtOH, Reflux, 55% for **5** and 13% for **6** from **3**; (**f**) $PhCH_2COC(OEt)_2$, 6N HCl, EtOH, Reflux, 22% for **7** and 35% for **8**.

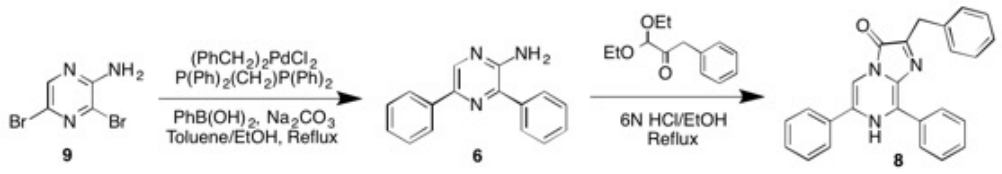
di-*tert*-butyl (5-bromo-3-iodopyrazin-2-yl)carbamate (**2**): Trifluoroacetic acid (1.1 mL, 14.3 mmol) and *N*-iodosuccinimide (NIS, 7.76 g, 34.5 mmol) were added to a solution of 2-amino-5-bromopyrazine (**1**, 5 g, 28.7 mmol) in acetonitrile (50 mL) at 0°C. The mixture was stirred at reflux for 18 h under N_2. After cooling to room temperature, the solvent was removed under reduced pressure. The residue was extracted with 50 mL ethyl acetate, neutralized and washed twice with saturated aq. $NaHCO_3$ (50 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated to give a black residue. The crude was next dissolved in dry THF (50 mL), to which was added Boc_2O (13.78g, 63.1 mmol) and triethylamine (12.1 mL, 86.2 mmol). The mixture was stirred under N_2 for additional 5 h. The progress of the reaction was monitored with TLC (hexane/ethyl acetate = 3:1). After completion of the transformation, MeOH (10 mL) was added to quench the reaction. The solvent was removed under reduced pressure. The residue was purified using column chromatography (silica gel; gradient elution with hexane/ethyl acetate from 20:1 to 5:1) to give compound **2** as white solid (9.7 g, 85% over two steps). *^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.46 (s, 1H), 1.41 (s, 18H); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ 151.3, 149.0, 144.6, 136.8, 119.0, 84.7, 28.0; ESI-MS ($C_{14}H_{19}BrIN_3O_4$): $[M+Na]^+$ calcd: 521.96, found: 521.95.*

***tert*-butyl (5-bromo-3-(phenylselanyl)pyrazin-2-yl)carbamate** (**3**): Compound **2** (2.7g, 5.4 mmol) and diphenyl diselenide (0.9 g, 2.9 mmol) were added to a mixture of Cu_2O (155 mg, 1.08 mmol), magnesium granule (196 mg, 8.16 mmol) and 2,2′-bipyridine (0.34 g, 2.2 mmol) in dry DMF (20 mL). The mixture was stirred at 85 °C under N_2 for 6 h. After removing the solvent *in vacuo*, the residue was dissolved in minimum CH_2Cl_2 and next purified with column chromatography (silica gel; hexane/ethyl acetate = 4:1) to give compound **3** (1.18 g, 51%) as yellow oil. *^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.95 (s, 1H), 7.49 (dd, 2H, J = 8.0, 1.2 Hz), 7.26 (t, 3H, J = 8.0 Hz), 1.47 (s, 9H); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ 151.6, 148.6, 145.0, 141.5, 135.8, 135.1, 129.7, 127.7, 127.2, 84.2, 28.3; ESI-MS ($C_{15}H_{16}BrN_3O_2Se$): $[M+Na]^+$ calcd: 451.96, found: 451.91.*

5-phenyl-3-(phenylselanyl)pyrazin-2-amine (**5**): To a solution of compound **3** (500 mg, 1.17 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (5 mL). After stirring at RT for 30 min, the reaction was diluted with 50 mL CH_2Cl_2, and neutralized with saturated aq. $NaHCO_3$. The organic layer was isolated, washed twice with saturated aq. $NaHCO_3$ (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to give the crude compound **4**, which was next dissolved in 2 mL EtOH for later use without further purification. In another round-bottom flask, 1,4-bis(diphenylphosphino)butane (BDPB, 30 mg, 0.07 mmol) was added to a suspension of bis(benzonitrile)dichloro palladium (23 mg, 0.06 mmol) in toluene (3 mL), and the mixture was stirred at RT under N_2 for 30 min. Next, to the solution of compound **4** in EtOH, phenylboronic acid (172 mg, 1.4 mmol), 1.0 M aq. Na_2CO_3 (1 mL), toluene (8 mL), and the mixture of BDPB and bis(benzonitrile)dichloro palladium in toluene were added sequentially. The mixture was maintained at reflux under N_2 for 12 h. The progress of the reaction was monitored by TLC (hexane/ethyl acetate = 2:1). Next, the mixture was cooled down to RT, and the solvent was removed *in vacuo*. The residue was extracted with ethyl acetate (30 mL), which was washed twice with water (30 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was next purified with column chromatography (silica gel; gradient elution with hexane/ethyl acetate from 4:1 to 2:1) to give compound **5** (209 mg, 55%). Compound **6** (37 mg, 13%) was also isolated as a by-product. *^{1}H-NMR ($CDCl_3$, 500 MHz) δ 8.21 (s, 1H), 7.71 (dd, 2H, J = 8.5, 1.5 Hz), 7.61 (dd, 2H, J = 7.5, 1.5 Hz), 7.38-7.33 (m, 6H), 5.77 (bs, 2H); ^{13}C-NMR (CDCl3, 125 MHz) δ 151.2, 143.3, 139.2, 135.7, 134.8, 134.7, 132.61, 129.9, 129.8, 129.0, 126.8, 125.7 ppm; ESI-MS ($C_{16}H_{13}N_3Se$): $[M+H]^+$ calcd: 328.02; found: 328.05.*

selenoterazine (**7**): To a solution of compound **5** (50 mg, 0.153 mmol) and 1,1-diethoxy-3-phenylacetone (51 mg, 0.23 mmol) in degassed EtOH (2 mL) was added 6 N HCl (0.3 mL) under continuous N_2 flow. The reaction flask was wrapped in aluminum foil and heated to 80 °C while stirring for 12 h. The mixture was then cooled down to RT. Solvent was removed *in vacuo*, and the residue was re-dissolved in 1 mL acetonitrile, which was next purified using preparative RP-HPLC (acetonitrile/water = 30:70 to 98:2, 2 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give selenoterazine (15.5 mg, 22%), which has to be stored as solid at -80 °C for long-term stability. *^{1}H-NMR (DMSO-d_6, 500 MHz) δ 7.98 (s, 1H), 7.57 (d, 2H, J = 8.5 Hz), 7.41 (d, 2H, J = 7.5), 7.35-7.26 (m, 11H), 4.05 (s, 2H); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 139.7, 136.0, 134.2, 132.8, 131.7, 129.3, 129.1, 128.8, 128.5, 128.3, 127.7, 126.0, 114.9, 31.9; ESI-MS ($C_{25}H_{19}N_3OSe$): $[M+H]^+$ calcd: 458.07, found: 458.08.*

2) Method to synthesize diphenylterazine (DTZ)



Scheme 2| Synthetic route for preparing diphenylterazine as the major product.

2-amino-3,5-diphenylpyrazine (**6**): 1,4-Bis(diphenylphosphino)butane (BDPB, 30 mg, 0.07 mmol) was added to a suspension of bis(benzonitrile)dichloro palladium (23 mg, 0.06 mmol) in toluene (3 mL) and the mixture was stirred at RT for 30 min under N_2. To this mixture were sequentially added compound **9** (303 mg, 1.2 mmol) in EtOH (2 mL), phenylboronic acid (318 mg, 2.6 mmol), 1.0 M aq. Na_2CO_3 (1 mL) and toluene (8 mL). The mixture was heated under reflux for 8 h. After cooling down to RT, the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate (30 mL), which was washed twice with water (30 mL), dried over Na_2SO_4, and concentrated on a rotary evaporator. The residue was purified with column chromatography (silica gel; gradient elution with hexane/ethyl acetate from 4:1 to 2:1) to give compound **6** (193mg, 66%). *^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H), 7.95 (d, 2H, J = 10.0 Hz), 7.78 (m, 2H), 7.47-7.24 (m, 6H), 5.21 (s, 2H) ppm; HRMS (ESI-TOF) calcd for $C_{16}H_{13}N_3$ [M + H]$^+$: 248.11, found: m/z 248.12.*

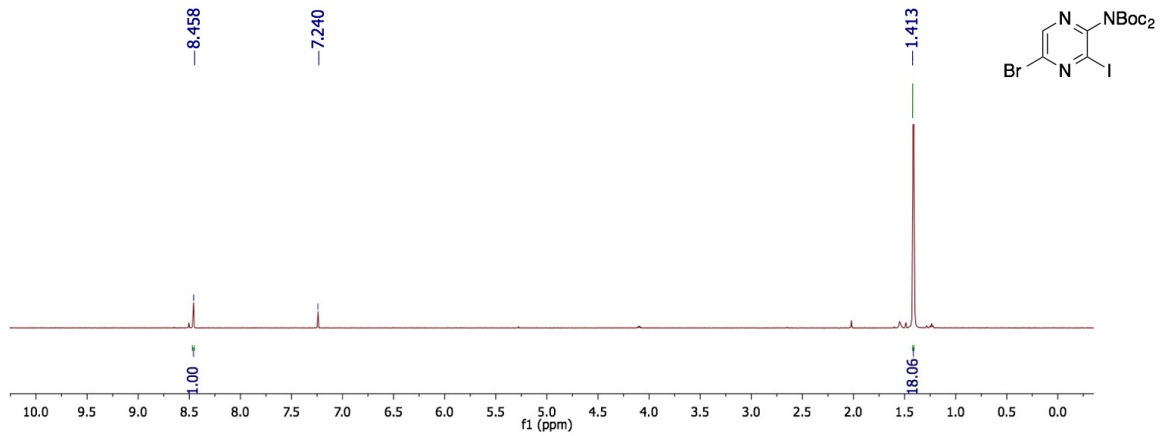
diphenylterazine (**8**): To a solution of compound **6** (50 mg, 0.2 mmol) and 1,1-diethoxy-3-phenylacetone (67mg, 0.3 mmol) in degassed EtOH (3 mL) was added 6 N HCl (0.3 mL) under continuous N_2 flow. The reaction flask was wrapped with aluminum foil and heated at 80 °C with stirring for 6 h. The mixture was cooled down to room temperature, before the solvent was removed *in vacuo*. The residue was re-dissolved in 1 mL acetonitrile, which was next purified with preparative RP-HPLC (acetonitrile/water = 30:70 to 98:2, 2 mL/min, UV 254 nm). Product fractions were combined and lyophilized to give diphenylterazine (26.7mg, 35%), which has to be stored as solid at -80 °C for long-term stability. *^{1}H-NMR (DMSO-d6, 500 MHz) δ 9.54 (s, 1H), 8.16 (d, 2H, J = 7.0 Hz), 7.57 (d, 2H, J = 7.5 Hz), 7.53 (d, 2H, J = 7.0 Hz), 7.33-7.24 (m, 9H), 4.17 (s, 2H) ppm; ^{13}C-NMR (DMSO-d6, 125 MHz) δ 139.9, 136.0, 134.0, 130.5, 130.2, 129.8, 129.3, 128.8, 128.5, 128.3, 128.2, 127.8, 127.3, 126.3, 125.9, 32.0 ppm; HRMS (ESI-TOF) calcd for $C_{25}H_{19}N_3O$ [M - H]$^-$: 376.15, found: m/z 376.14.*

Supplementary Note 3

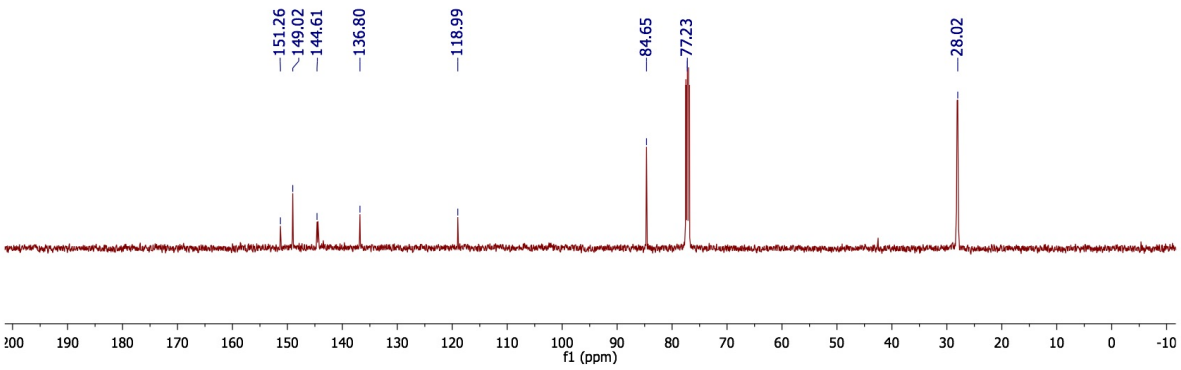
NMR and MS Characterization of Synthesized Compounds

di-*tert*-butyl (5-bromo-3-iodopyrazin-2-yl)carbamate (2)

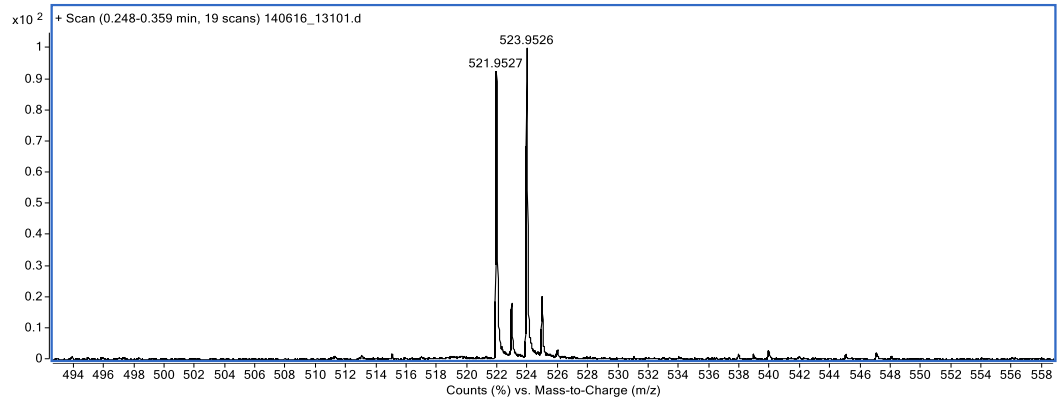
^{1}H NMR



^{13}C NMR

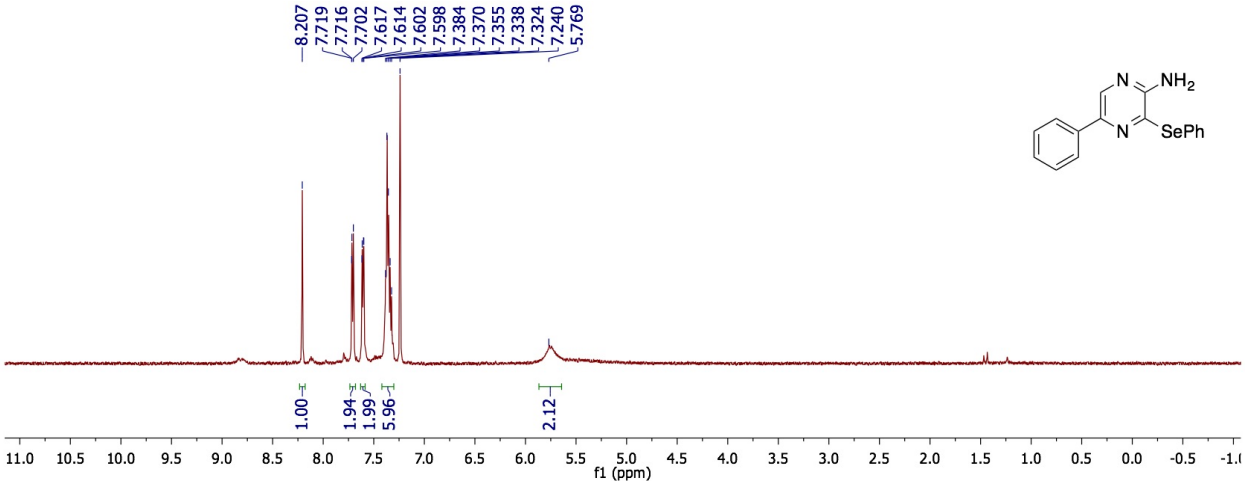


ESI-MS

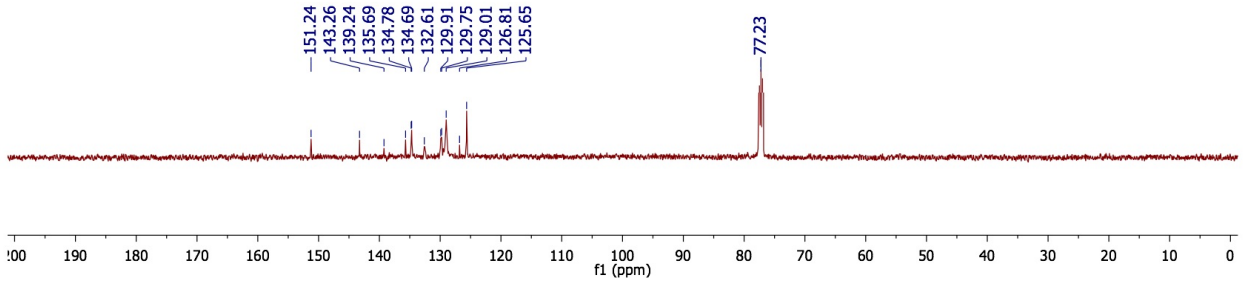


5-phenyl-3-(phenylselanyl)pyrazin-2-amine (5)

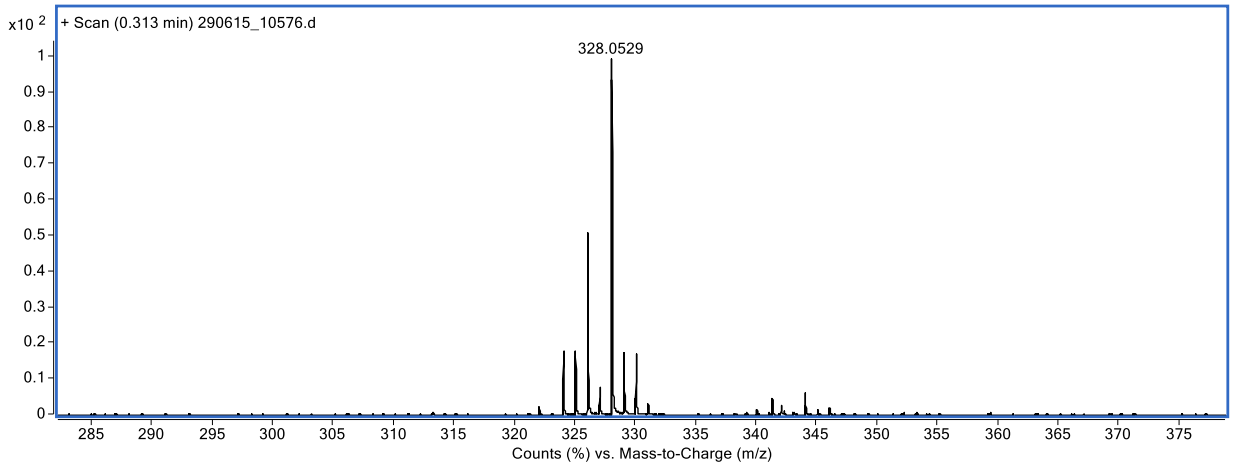
^{1}H NMR



^{13}C NMR

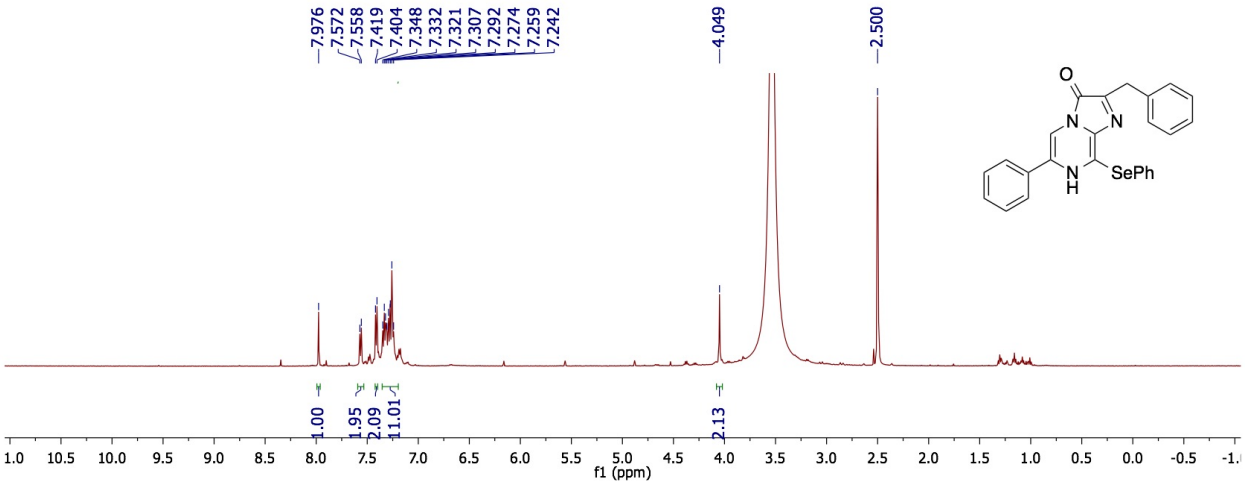


ESI-MS

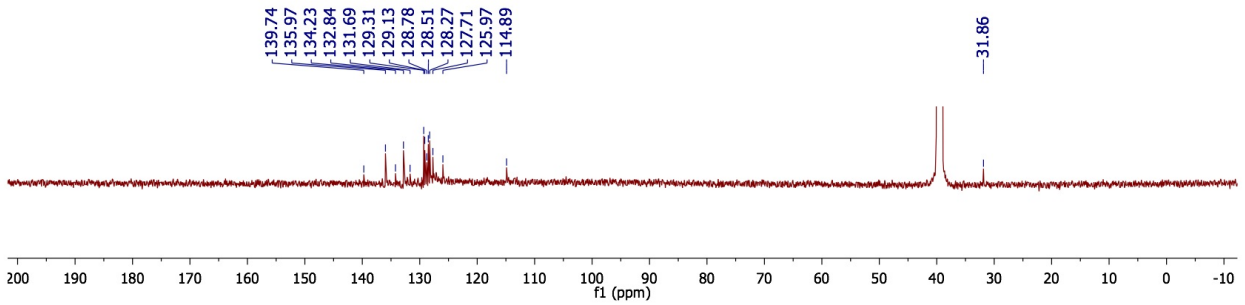


selenoterazine (**7**)

^{1}H NMR



^{13}C NMR



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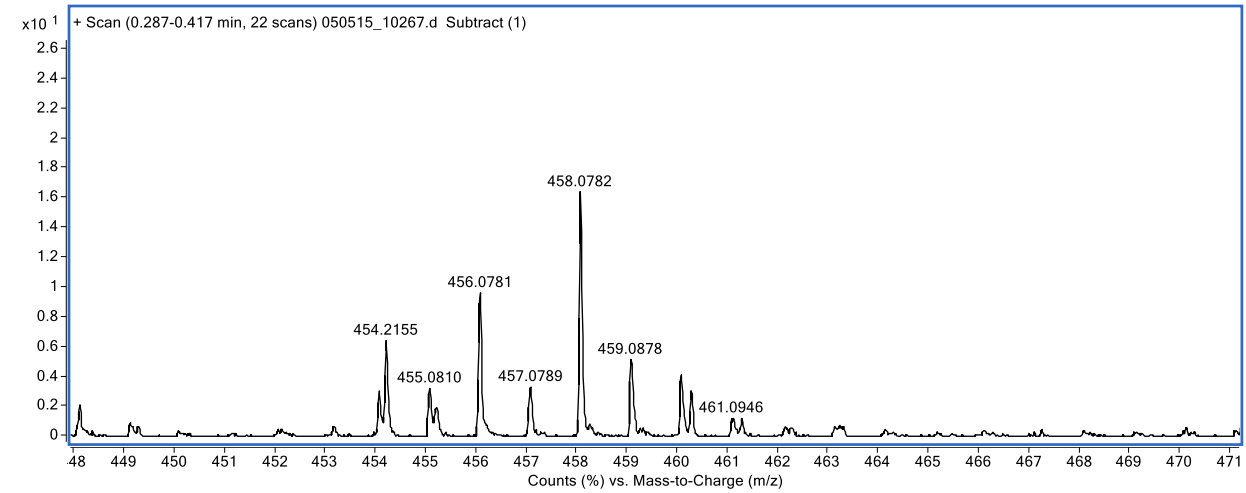
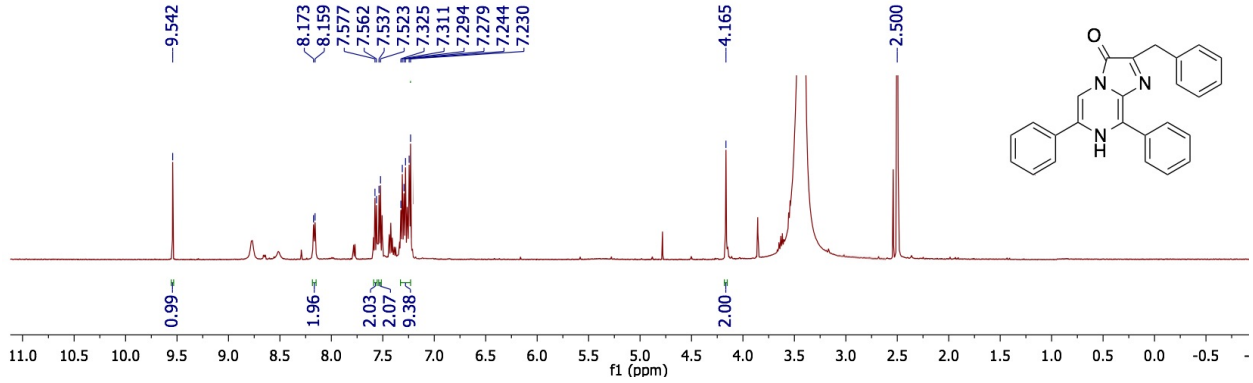
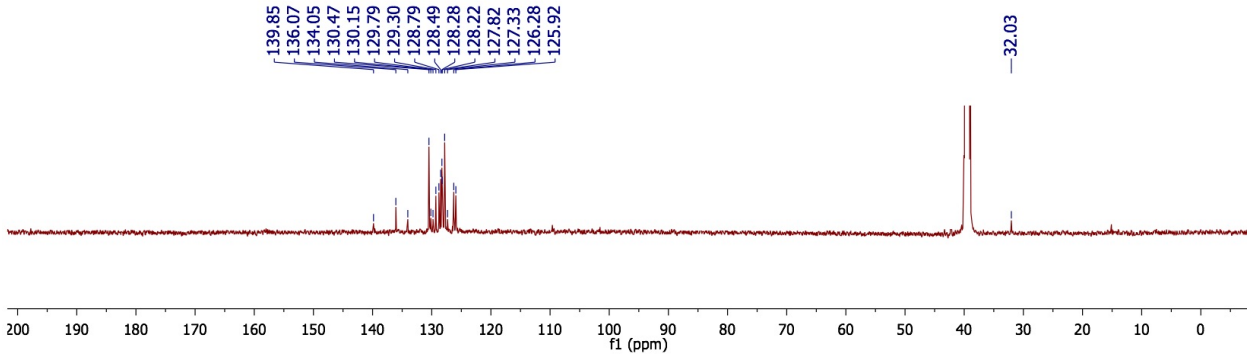


diphenylterazine (8)

^{1}H NMR



^{13}C NMR



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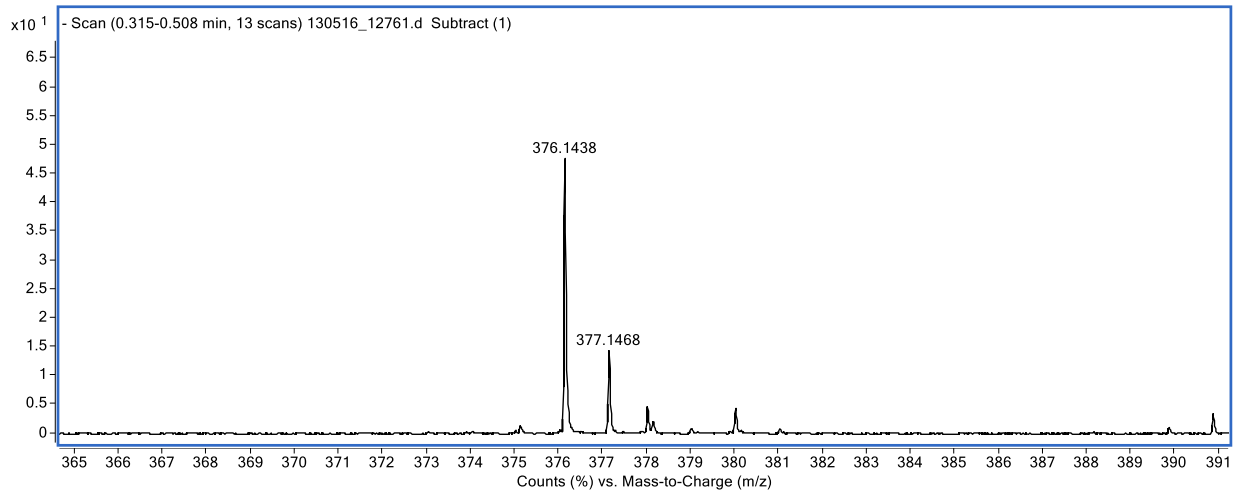

Supplementary Tables

Supplementary Table 1. **Photoluminescence properties of luciferase/luciferin pairs as determined by protein assays.**

	λ_{max} (nm)	K_m (μM)[a]	k_{cat} (s^{-1})[a]	QY (%)[a]	Relative overall emission [b]
NanoLuc/CTZ	458	ND[c]	ND[c]	ND[c]	3.3[d] [3.2][e]
NanoLuc/furimazine	456	11.5±0.6	88.2±1.7	5±0.2	45.5[d] [43.5][e]
NanoLuc/DTZ	500	5.3±0.2	ND[c]	ND[c]	20[d] [22.2][e]
NanoLuc/STZ	527	17.8±1.0	ND[c]	ND[c]	1.5[d] [1.57][e]
teLuc/DTZ	502	10.2±0.4	88.1±0.1	10.8±0.5	114[d] [113][e]
yeLuc0.8/STZ	527	6.3±0.5	ND[c]	ND[c]	11.4[d] [7.4][e]
yeLuc/STZ	527	11.9±0.9	175±5	0.8±0.1	17.3[d] [12.6][e]
FLuc/D-luciferin	563	6.3±0.2	0.4±0.005	44±1.2	1[d] [1][e]
FLuc/AkaLumine-HCl	677	2.4±0.2	ND[c]	ND[c]	2.8[d] [3.4][e]
ReNL/furimazine	459, 583	14.5±1.3	104±4	2.6±0.2	28.4[d] [25.7][e]
Antares/furimazine	456, 583	11.1±0.5	112±2	2.7±0.3	32.7[d] [29.6][e]
Antares2/DTZ	501, 583	9.7±0.2	86.1±0.7	8.6±0.3	82.5[d] [79.4][e]

a. mean±s.d., n=3 (see **Online Methods** for details); **b.** Intensity values normalized to FLuc/D-luciferin under comparable experimental conditions with purified recombinant proteins (30 μM substrates and 100 pM proteins), respectively; **c**. Not determined; **d**. Maximal intensity; **e**. Integrated intensity over the first 10 min.

Supplementary Table 2. Oligonucleotides used in this study.

Oligo name	Nucleotide sequence
XhoI-NL-F	GCAACTCGAGCATGGTCTTCACACTCGAAGATTTCGTTGG
NL-R-HindIII	TTGCCAAGCTTACGCCAGAATGCGTTCGCACAGCCGC
I44I54NNK-F	CCGATCCAAAGGNNKGTCCTGAGCGGTGAAAATGGGCTGAAGNNKGACATCCATGTC
I44I54NNK-R	GACATGGATGTCMNNCTTCAGCCCATTTTCACCGCTCAGGACMNNCCTTTGGATCGG
I138NNK-F	TGGAACGGCAACAAAATTNNKGACGAGCGCCTGATCAAC
I138NNK-R	GTTGATCAGGCGCTCGTCMNNAATTTTGTTGCCGTTCCA
L18D19NNK-F	CAGACAGCCGGCTACAACNNKNNKCAAGTC CTTGAACAGGGAGGTGTG
L18D19NNK-R	CACACCTCCCTGTTCAAG GACTTGMNNMNNGTTGTAGCCGGCTGTCTG
R162C164NNK-R	TTGCCAAGCTTACGCCAGAATGCGTTCMNNCAGMNN CCAGCCGGTCACTCCGTT
HindIII-NL-F-Koz	AATAAAGCTTGCCGCCACCATGGTCTTCACACTCGAAGATTTCGTTGG
NL-R-XhoI	TAATCTCGAGTTACGCCAGAATGCGTTCGCA
NL-R-164H	TAATCTCGAGTTACGCCAGAATGCGTTCATG
NL-R-164S	TAATCTCGAGTTACGCCAGAATGCGTTCACT
FLuc-F	ATTATAAAGCTTGCCGCCACCATGGAAGACGCCAAAAACATAAAGAAAG
FLuc-R	TTATTCTCGAGTTACAATTTGGACTTTCCGCCCTTCTTGG
Ant-HindIII-F-koz	ATTATAAAGCTTGCCGCCACCATGGTGAGCAAGGGCGAGGAG
Ant-XhoI-R	TTATTCTCGAGTTACTTGTACAGCTCGTCCAT
Te19DtoS_F	GGCTACAACTTGAGTCAAGTCCTTGAA
Te19DtoS_R	TTCAAGGACTTGACTCAAGTTGTAGCC
Te85DtoN_F	TACCCTGTGGATAATCATCACTTTA
Te85DtoN_R	TAAAGTGATGATTATCCACAGGGTA
Te164CtoH_F	TGACCGGCTGGCGTCTGCATGAACGCATTCTGG
Te164CtoH_R	CCAGAATGCGTTCATGCAGACGCCAGCCGGTCA
Antares_F_XhoI	ATAACTCGAGCATGGTGAGCAAGGGCGAGGAG
Antares_R_HindIII	TTGCCAAGCTTACTTGTACAGCTCGTCCAT
NL-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCGCCAGAATGCGTTCGCACAGCCGC
teLuc-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCGCCAGAATGCGTTCATGCAGACGC
yeLuc-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCGCCAGAATGCGTTCACTCAGACGC
FLuc-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCAATTTGGACTTTCCGCCCTTCTTGG
Antares-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCTTGTACAGCTCGTCCATGCCTCCG
Myc-R-XhoI	TAATTCTCGAGTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCCGGAGCC
P2A-FLuc-F	GCAGGCTGGAGACGTGGAGGAGAACCCTGGACCTATGGAAGACGCCAAAAACATAAAGAA
P2A-ext-R	CCAGGGTTCTCCTCCACGTCTCCAGCCTGCTTCAGCAGGCTGAAGTTAGTAGCTCCGCTT
teLuc-P2A-R	AGGCTGAAGTTAGTAGCTCCGCTTCCCGCCAGAATGCGTTCATGCAGACGC
NLuc-P2A-R	AGGCTGAAGTTAGTAGCTCCGCTTCCCGCCAGAATGCGTTCGCACAGCCGC
Luc2-F-HindIII-Kozak	ATTATAAAGCTTGCCGCCACCATGGAAGATGCCAAAAACATTAAGA
Luc2-Myc-R	TGAGTTTTTGTTCGCCGGAGCCCACGGCGATCTTGCCGCCCTTCTT