N-Alkylated 6′-Aminoluciferins Are Bioluminescent Substrates for Ultra-Glo and QuantiLum Luciferase: New Potential Scaffolds for Bioluminescent Assays

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ABSTRACT: A set of 6′-alkylated aminoluciferins are shown to be bioluminescent substrates for Ultra-Glo and QuantiLum luciferases. These studies demonstrate that both the engineered and wild-type firefly luciferases tolerate much greater steric bulk at the 6′ position of luciferin than has been previously reported. The nature of the alkyl substituent strongly affects the strength of the bioluminescent signal, which varies widely based on size, shape, and charge. Several compounds were observed to generate more light than the corresponding unsubstituted 6′-aminoluciferin. Determination of Michaelis−Menten constants for the substrates with Ultra-Glo indicated that the variation arises primarily from differences in V_{max}, ranging from 1.33×10^4 to 332×10^4 relative light units, but in some cases K_m (0.73−10.8 μM) also plays a role. Molecular modeling results suggest that interactions of the side chain with a hydrogen-bonding network at the base of the luciferin binding pocket may influence substrate−enzyme binding.

Bioluminescence offers a remarkably sensitive platform for probing biological systems. The absence of an excitation source eliminates many problems commonly associated with fluorescent techniques, including background, autofluorescence, and photobleaching, and assays based on bioluminescence typically enjoy a dynamic range covering several orders of magnitude. The firefly luciferase enzyme has been studied for over 50 years (*1*). This system catalyzes the O_2-, Mg^{2+}-, and ATP-dependent oxidative decarboxylation of luciferin (**1**) to an excited-state oxoluciferin, which then emits a photon of light (Scheme 1) (*2*).

Light output from bioluminescent assays can be correlated to a variety of limiting reagents: concentrations of luciferase enzyme, luciferin substrate, ATP (*3*), oxygen, or magnesium ion. In the luciferin-limited assay, enzymes of interest can be assayed by monitoring the enzymatic conversion of a nonbioluminescent compound to a bioluminescent luciferase substrate. Such assays have been reported for proteases (*4*), glycosidases (*5*), cytochrome P450s (*6*), monoamine oxidases (*7*), and glutathione *S*-transferases (*8*). To date, only a few bioluminescent substrates are known (*9, 10*), likely because of the presumed extreme substrate specificity of luciferase (*10*−*13*). The ability of luciferase to hold the excited oxoluciferin species in a fixed emissive conformation, reducing nonradiative relaxation processes, is believed to be essential to the relatively high quantum yield (0.41) observed for bioluminescence (*14*). In addition, structure−activity studies of luciferase substrates have implied a requirement for a strongly electron-donating substituent at the 6′ position (*15*). Acylation of luciferin or aminoluciferin at the 6′-substituent or alkylation of luciferin at the 6′-phenol, for example, abolishes bioluminescence (*15*). Nearly all reported bioluminogenic luciferase-based assays rely on releasing either **1** or **2** (*15*) as the luciferase substrate. The corresponding N-alkylated aminoluciferins, however, had not been reported. We now describe a structure−bioluminescence relationship study designed to explore the flexibility and limitations of the bioluminescent substrate scope. The results of our study outline a new series of luciferase substrates which could serve as scaffolds for the next generation of bioluminogenic luciferase-based assays.

EXPERIMENTAL PROCEDURES

P450-Glo buffer and luciferin detection reagent (LDR)[1] were purchased from Promega Corp. as part of the CYP450 1A1 assay kit. Ultra-Glo luciferase and QuantiLum luciferase were obtained from Promega Corp. Prionex was purchased from PentaPharm. Synthetic routes to 6-amino-2-cyanobenzothiazole (*15*), *tert*-butyl 6-bromo-1-hexanoate (*16*), and aminoluciferin (*15*) have been previously disclosed. 4-Bromo-1-(*tert*-butyldimethylsilyloxy)butane was synthesized as previously described (*17*), and 5-bromo-1-(*tert*-butyldimethylsilyloxy)pentane was prepared using the same procedure. *tert*-Butyl 4-bromo-1-butyrate was prepared in a manner analogous to the hexanoate compound. 1-Bromo-8-(tetrahydropyranyloxy)octane was purchased from Lancaster Synthesis. All other compounds were purchased from Sigma Aldrich and used as received. Analytical HPLC was performed on an

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[1] Abbreviations: LDR, luciferin detection reagent; TFA, trifluoroacetic acid; TREAT, triethylamine trishydrofluoride; DIAD, diisopropyl azodicarboxylate; TLC, thin-layer chromatography; CoA, coenzyme A; DLSA, 5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine.

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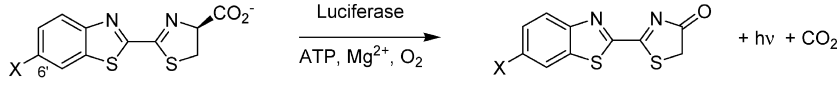
Scheme 1: Bioluminescent Transformation of Luciferins



1 X = OH
2 X = NH_2

Agilent 1100-1 using a Synergi 250 mm column with a gradient over 24 min of 5%−100% acetonitrile in water containing 0.1% TFA. Under these conditions aminoluciferin elutes at 9.7 min; identity was confirmed by coinjection with an authentic sample of aminoluciferin. Preparative HPLC was performed on a Waters HPLC system with dual-wavelength detection at 254 and 354 nm, equipped with Varian Dynamax 25 cm Microsorb 60-8 C18 columns of 21.4 mm (20 mL/min flow rate) or 41.4 mm (40 mL/min flow rate) inner diameter. A linear gradient from 0% to 100% acetonitrile in water (0.1% TFA) over 35 min was used unless otherwise specified. ^{1}H NMR spectra were acquired on a Varian Mercury 300 MHz instrument and were referenced to internal solvent peaks. *J* values are reported in hertz. Mass spectral data were acquired on an Esquire 4000 in ESI mode. Calculated masses are for M + H unless otherwise specified.

Light Output Studies. Stock solutions were prepared at 25 mM in DMSO, DMF, or DMF−Tris using ∼1 mg of compound. Random tests indicated that the solvent used for stock solution preparation had no effect on light output (data not shown). Sample stock solutions were diluted serially to working concentrations of 50 and 1 or 0.4 μM with 400 mM potassium phosphate buffer (pH 7.5), and aminoluciferin stock solution was diluted serially to a regression concentration series that included 1 and 0.4 μM for direct comparison with the samples. A blank control solution of 0.1% DMSO in phosphate buffer was also included. A vial of luciferin detection reagent was reconstituted with P450-Glo buffer. A 50 μL aliquot of each stock solution was combined with a 50 μL aliquot of LDR in an Eppendorf tube, the reaction was mixed and incubated for 20 min at room temperature, and bioluminescence was measured in a Turner 20/20 luminometer. Background as determined from a blank control containing 0.1% DMSO was subtracted, and the resulting luminescence readings were normalized to the theoretical aminoluciferin light output determined from a standard curve. Light output measurements were performed in triplicate.

Computational Details. Protein modeling, simulations, and analyses were carried out using Discovery Studio 1.6 from Accelrys (*18*).

Enzyme−Substrate Initial Structures. A homology model of Ultra-Glo luciferase (a thermostable mutant of *Photuris pennsylvanica* firefly luciferase, accession number AAB60897) was built using the X-ray crystallographic structure of a related luciferase as a template (*Luciola cruciata*, PDB code 2D1S (*13*), 59% amino acid sequence identity). The luciferyl adenylate intermediate analogue (DLSA) bound in the active site of the template structure was included in the model. N-Substituted aminoluciferin adducts of adenosine monophosphate were manually docked into the model based on the position of DLSA.

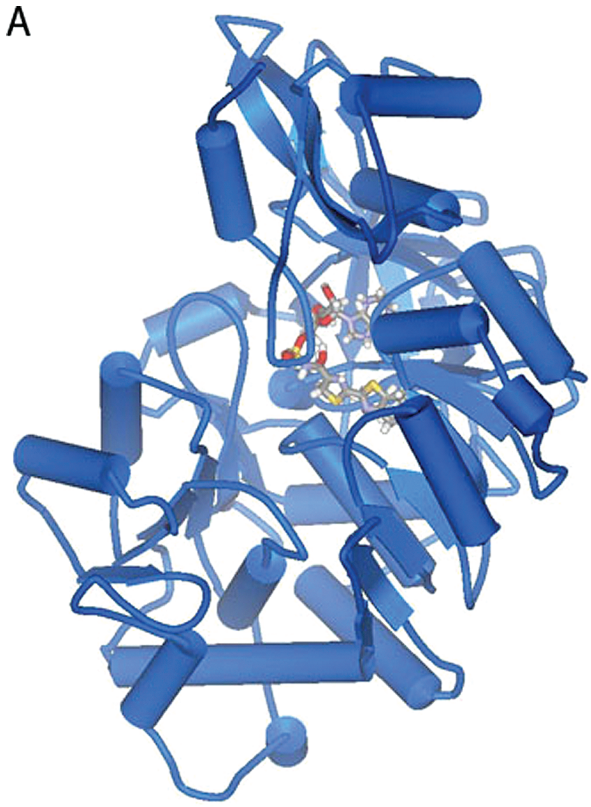
Molecular Dynamics Simulations. Fixed atom restraints were specified to allow movement of only the substrate benzene ring plus substituents and protein residues within 7 Å of the longest N-substituted aminoluciferin, **10c**. A harmonic restraint was applied to the peptide backbone. Molecular dynamics simulations were performed using the Standard Dynamics Cascade protocol employing CHARMm all atom force field parametrization and an implicit solvent model with distance-dependent dielectrics to calculate electrostatic interactions. Results shown in Figure 1 were generated with an equilibration phase of 4 ps and a production phase of 5 ps. The following hydrogen-bonding criteria were used: distance cutoff about 3.2 Å (donor atom to acceptor atom) and angle cutoff about 120−180° (donor atom−hydrogen−acceptor atom).

Kinetic Constant Measurements. All components were added as solutions in Tricine buffer (100 mM, pH 8.3) containing 0.1% Prionex. Luciferase substrate concentration was determined for a stock solution in methanol based on an estimated extinction coefficient of 20000 M^{-1} cm^{-1} and then diluted serially to the desired concentration with the aqueous buffer. Enzyme solutions contained 100 ng/mL Ultra-Glo luciferase or 17 ng/mL QuantiLum luciferase, and the ATP solution contained 1 mM ATP and 10 mM $MgSO_4$. To each well of a 96-well luminometer plate was dispensed 25 μL of substrate solution. A 25 μL aliquot of enzyme solution was added to each well using a multichannel pipet, and the wells were mixed. The plate was immediately placed into a Veritas plate luminometer (Turner BioSystems) programmed to inject 50 μL of ATP solution. After injection, the light output over 3 s (Ultra-Glo) or 1.5 s (QuantiLum) was measured. Light integration times were based on the time required to approach steady state (see Supporting Information). The total light output at each concentration was then fit to the Michaelis−Menten equation using Kaleidagraph v.4.0. Measurements were performed in triplicate, and the data shown represent the averages of three runs.

Synthesis. General Alkylation Procedure. **3** (350 mg, 2 mmol) was combined with K_2CO_3 (1.39 g, 10 mmol, 5 equiv) and alkyl bromide (3 mmol, 1.5 equiv) in 6 mL of anhydrous DMF. The reaction was stirred vigorously in a 100 °C oil bath and monitored by TLC. Upon disappearance of the starting material, the reaction was cooled and diluted with H_2O and CH_2Cl_2, and the layers were separated. The aqueous layer was extracted with 2 × 50 mL of CH_2Cl_2; the combined organic layers were dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by silica gel chromatography using ethyl acetate−heptanes. Where appropriate, the material was further purified by a second silica gel column eluting with ethyl acetate gradients in CH_2Cl_2 or by preparative HPLC to remove remaining traces of starting material.

*6-(2-Hydroxyethylamino)-2-cyanobenzothiazole (**4a**).* Purified by preparative HPLC. ^{1}H NMR (CD_2Cl_2) δ 7.93 (d, 1 H, J = 9.1), 7.04 (d, 1 H, J = 2.3), 6.98 (dd, 1 H, J = 2.3, 9.1), 3.90 (dd, 2 H, J = 5.1, 5.4), 3.38 (dd, 2 H, J = 5.1, 5.4). MS: calcd for $C_{10}H_{10}N_3OS$ 220.0, found 220.1.

*6-(3-tert-Butyldimethylsiloxypropylamino)-2-cyanobenzothiazole (**5a**).* Purified by two silica gel columns, the first eluting with 3:1 heptanes−ethyl acetate, the second with



E

Hydrogen bond	1	5c	6c
R336:NH1 – G227:0	X	X	
R336:NH2 – G227:0	X	X	X
R336:NH2 – N228:OD1	X	X	
N228:ND – E310:OE1	X	X	
R336:NH2 – E310:OE1	X		
R336:NH2 – E310:OE2	X	X	
R336:NE – E310:OE2	X	X	
K353:NZ – E310:OE1	X	X	X
K353:NZ – E310:OE2	X	X	X
R336:NE – Compound:O		X	
R336:NH2 – Compound:O		X	X
N228:OD1 – Compound:O		X	X
K353:NZ – N228:OD1			X
K353:NZ – G227:O			X

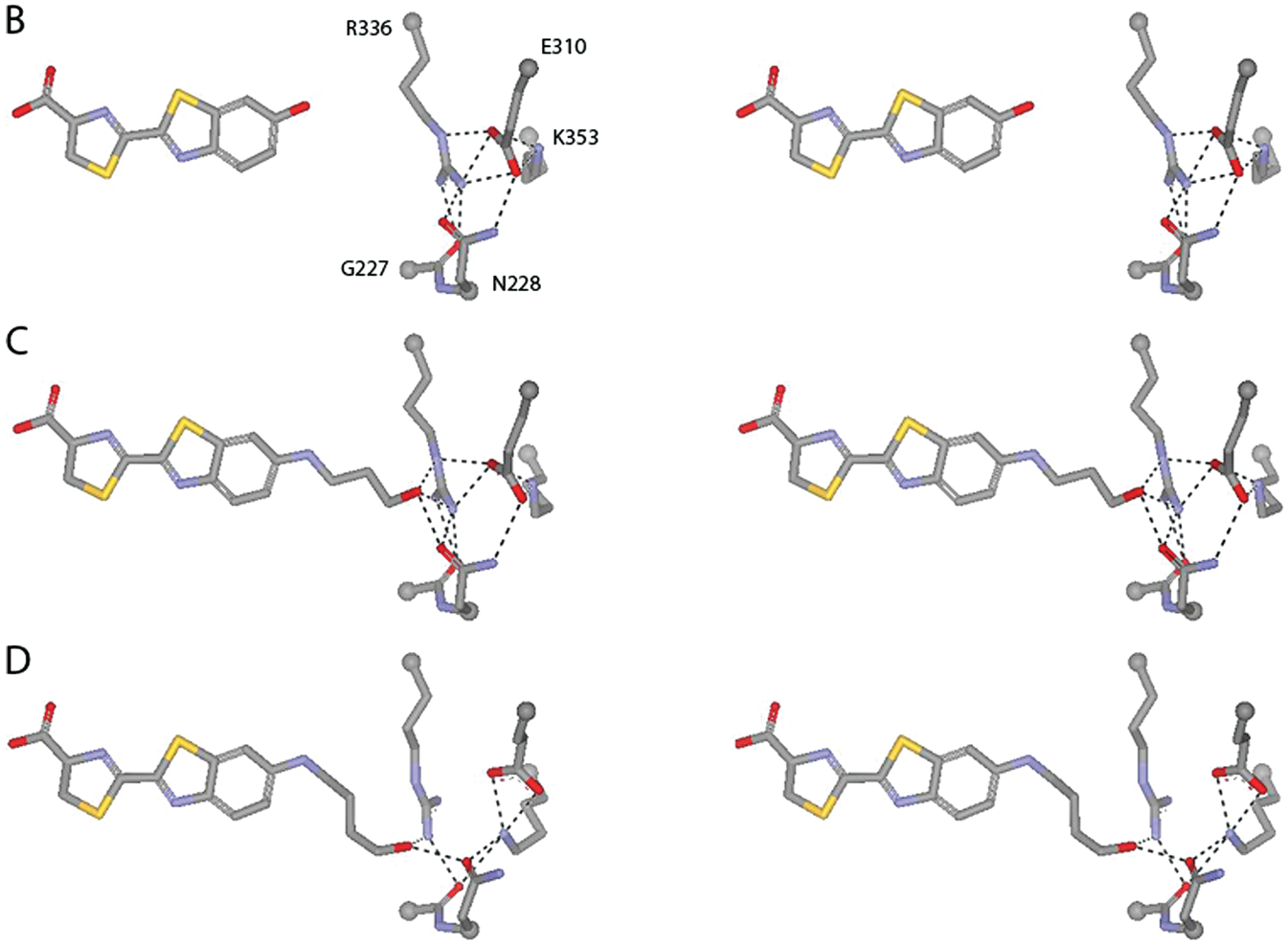
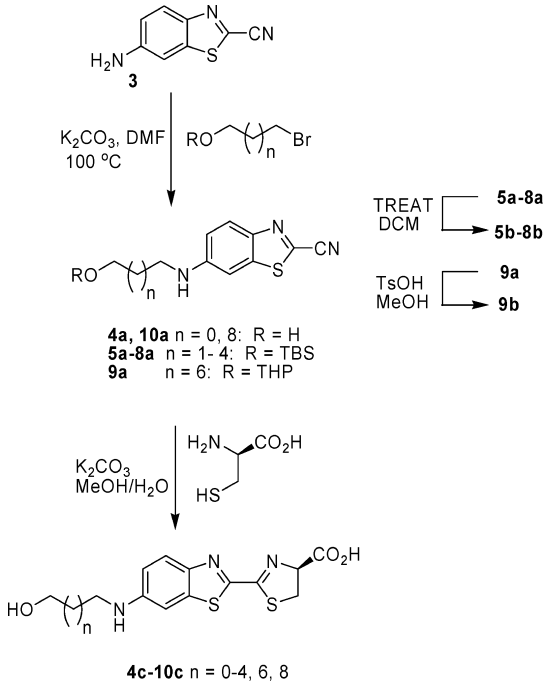


FIGURE 1: Models of Ultra-Glo luciferase. (A) Full structure homology model with DLSA in the active site. (B−D) Active site close-ups showing side chains of residues N228, E310, R336, and K353 with the native substrate **1** (B), **5c** (C), or **6c** (D). C_α atoms are rendered as gray spheres. Hydrogen bonds are indicated as dashed lines and listed with their respective donor and acceptor atoms (E).

Scheme 2: Synthesis of Hydroxyalkyl Aminoluciferins



CH_2Cl_2. ^{1}H NMR (CD_2Cl_2) δ 7.79 (d, 1 H, J = 9.1), 6.87 (d, 1 H, J = 2.3), 6.79 (dd, 1 H, J = 2.3, 9.1), 4.81 (br s, 1 H), 3.72 (t, 2 H, J = 5.6), 3.22 (t, 2 H, J = 6.5), 1.76−1.84 (m, 2 H), 0.85 (s, 9 H), 0.0 (s, 6 H). MS: calcd for $C_{17}H_{26}N_3OSSi$ 348.2, found 348.3.

*6-(4-tert-Butyldimethylsiloxybutylamino)-2-cyanobenzothiazole (**6a**).* Purified by two silica gel columns, the first eluting with 3:1 heptanes−ethyl acetate, the second with CH_2Cl_2. ^{1}H NMR (CD_2Cl_2) δ 7.82 (d, 1 H, J = 9.0), 6.82−6.89 (m, 2 H), 4.38 (br s, 1 H), 3.62 (t, 2 H, J = 6.0), 3.15 (br m, 2 H), 1.64−1.74 (m, 2 H), 1.52−1.62 (m, 2 H), 0.83 (s, 9 H), 0.0 (s, 6 H). MS: calcd for $C_{18}H_{28}N_3OSSi$ 362.2, found 362.3.

*6-(10-Hydroxydecylamino)-2-cyanobenzothiazole (**10a**).* ^{1}H NMR (CD_2Cl_2) δ 7.90 (dd, 1 H, J = 8.9), 6.96 (d, 1 H, J = 2.3), 6.91 (dd, 1 H, J = 2.3, 8.9), 3.60 (t, 2 H, J = 6.6), 3.19 (t, 2 H, J = 7.0), 1.28−1.81 (m, 16 H). MS: calcd for $C_{18}H_{26}N_3OS$ 332.2, found 332.3.

*6-(Isobutylamino)-2-cyanobenzothiazole (**11a**).* ^{1}H NMR (CD_2Cl_2) δ 7.90 (d, 1 H, J = 8.9), 6.96 (d, 1 H, J = 2.3), 6.92 (ddd, 1 H, J = 0.6, 2.3, 8.9), 4.39 (br s, 1 H), 3.01−3.05 (m, 2 H), 1.96 (septuplet, 1 H, J = 6.7), 1.03 (d, 6 H, J = 6.7). MS: calcd for $C_{12}H_{14}N_3S$ 232.1, found 232.1.

*6-(N-Benzylamino)-2-cyanobenzothiazole (**12a**).* The general procedure for alkylation was carried out at room temperature for 6 h rather than at 100 °C. After extraction, the material was purified by two flash chromatography columns, the first in isocratic CH_2Cl_2 and the second using 1:1 CH_2Cl_2−heptanes; 214 mg (40% yield) of **12a** was isolated. ^{1}H NMR (CD_2Cl_2) δ 7.93 (d, 1 H, J = 9.7), 7.28−7.42 (m, 5 H), 6.97−7.01 (m, 2 H), 4.79 (br s, 1 H), 4.45 (s, 2 H). MS: calcd for $C_{15}H_{12}N_3S$ 266.1, found 266.1.

*6-(tert-Butylcarboxymethyl-1-amino)-2-cyanobenzothiazole (**13a**).* ^{1}H NMR (CD_2Cl_2) δ 7.95 (d, 1 H, J = 8.9), 6.98 (dd, 1 H, J = 2.3, 8.9), 6.94 (d, 1 H, J = 2.3), 4.9 (br t, 1 H), 3.89 (d, 2 H, J = 5.1), 1.5 (s, 9 H). MS: calcd for $C_{14}H_{16}N_3O_2S$ 290.1, found 290.3.

*6-(tert-Butyl-5-carboxypentyl-1-amino)-2-cyanobenzothiazole (**15a**).* The general procedure was followed using *tert*-butyl 6-bromo-1-hexanoate (620 mg, 1.25 equiv) and heating at 85 °C for 24 h. After extraction, the residue was purified by flash chromatography eluting with 3:1 heptanes−ethyl acetate to yield 80 mg (11% yield) of yellow crystalline material. ^{1}H NMR (CD_2Cl_2) δ 7.90 (d, 1 H, J = 8.9), 6.97 (d, 1 H, J = 2.3), 6.91 (dd, 1 H, J = 2.3, 8.9), 4.35 (br s, 1 H), 3.18−3.24 (m, 2 H), 2.24 (t, 2 H, J = 7.2), 1.59−1.75 (m, 4 H), 1.41−1.51 (m, 2 H), 1.44 (s, 9 H). MS: calcd for $C_{18}H_{24}N_3O_2S$ 362.2, found 362.3.

*6-(Methylcarboxymethyl-1-amino)-2-cyanobenzothiazole (**16a**).* The general alkylation procedure was followed using a reaction temperature of 50 °C; 33 mg of pure **16a** was isolated after flash chromatography in CH_2Cl_2. ^{1}H NMR (CD_2Cl_2) δ 7.97 (d, 1 H, J = 8.9), 7.00 (dd, 1 H, J = 2.3, 8.9), 6.96 (d, 1 H, J = 2.3), 4.91 (br t, 1 H), 4.02 (d, 2 H, J = 5.4), 3.81 (s, 3 H). MS: calcd for $C_{11}H_{10}N_3O_2S$ 248.1, found 248.1.

*6-(6-(tert-Butoxycarbonylamino)hexylamino)-2-cyanobenzothiazole (**19a**).* The general procedure was followed using 1-(*tert*-butoxycarbonylamino)hexyl bromide (840 mg, 3 mmol, 1.5 equiv). After extraction and flash chromatography on silica eluting with CH_2Cl_2 followed by 10% EtOAc in CH_2Cl_2, 102 mg (13.6% yield) of a yellow residue was obtained. ^{1}H NMR (CD_2Cl_2) δ 7.80 (d, 1 H, J = 8.9), 6.87 (d, 1 H, J = 2.3), 6.83 (dd, 1 H, J = 2.3, 8.9), 4.49 (br s, 1 H), 4.32 (br s, 1 H), 3.10 (t, 2 H, J = 7.2), 2.95−3.04 (m, 2 H), 1.51−1.64 (m, 2 H), 1.23−1.45 (m, 6 H), 1.33 (s, 9 H).

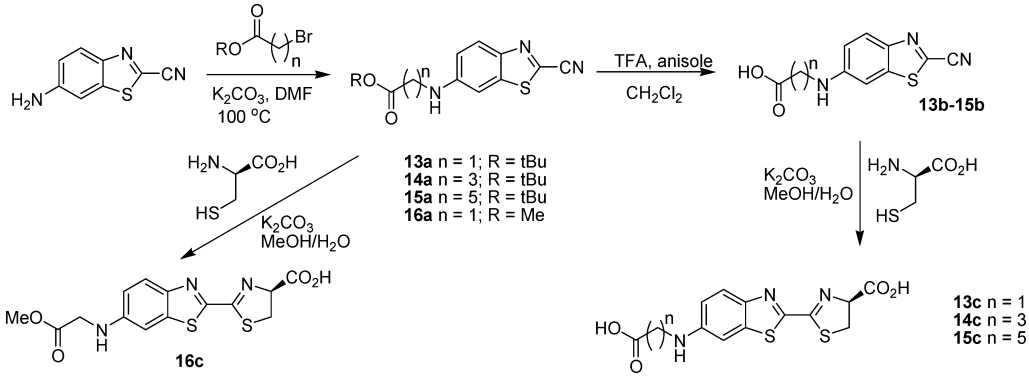
*General Silyl Deprotection Procedure. 6-(3-Hydroxypropylamino)-2-cyanobenzothiazole (**5b**).* **5a** was taken up in 5 mL of CH_2Cl_2 and treated with 1.5 mL of triethylamine trishydrogen fluoride (TREAT) for 4 h at ambient temperature, at which time the reaction was quenched by addition of aqueous 0.5 M NH_4Cl, extracted with 3 × 50 mL of CH_2Cl_2, dried over Na_2SO_4, and evaporated. The desired product was purified on a silica gel column by eluting exhaustively with CH_2Cl_2 to remove any traces of aminocyanobenzothiazole and then eluting with 95:5 CH_2Cl_2−MeOH. 34 mg (7.3% yield, 2 steps) of a bright yellow residue was obtained. ^{1}H NMR (CD_2Cl_2) δ 7.91 (d, 1 H, J = 8.9), 6.99 (s, 1 H), 6.93 (d, 1 H, J = 8.9), 4.71 (br s, 1 H), 3.83 (t, 2 H, J = 5.6), 3.35 (t, 2 H, J = 6.1), 1.89−1.97 (m, 2 H). MS: calcd for $C_{11}H_{12}N_3OS$ 234.1, found 234.2.

*6-(4-Hydroxybutylamino)-2-cyanobenzothiazole (**6b**).* **6a** was subjected to the same procedure described for **5a** to give 27 mg (5.5% yield, 2 steps) of **6b**. ^{1}H NMR (CD_2Cl_2) δ 7.90 (d, 1 H, J = 8.9), 6.98 (d, 1 H, J = 2.3), 6.92 (dd, 1 H, J = 2.3, 8.9), 4.45 (br s, 1 H), 3.70 (t, 2 H, J = 6.1), 3.24 (t, 2 H, J = 7.0), 1.64−1.83 (m, 4 H). MS: calcd for $C_{12}H_{14}N_3OS$ 248.1, found 248.2.

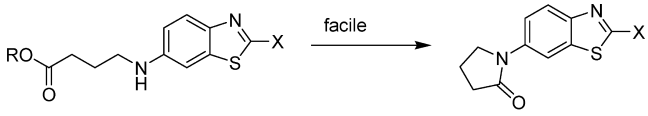
*6-(5-Hydroxypentylamino)-2-cyanobenzothiazole (**7b**).* **7a** was prepared by the general alkylation procedure and subjected to deprotection as in **5a** without further purification to afford 42 mg (8% yield over two steps) of **7b**. ^{1}H NMR (CD_2Cl_2) δ 7.90 (d, 1 H, J = 9.0), 6.97 (d, 1 H, J = 2.3), 6.92 (dd, 1 H, J = 2.3, 9.0), 3.65 (t, 2 H, J = 6.3), 3.22 (t, 2 H, J = 7.1), 1.72 (tt, 2 H, J = 7.1, 7.5), 1.46−1.67 (m, 4 H). MS: calcd for $C_{13}H_{16}N_3OS$ 262.1, found 262.2.

*6-(6-Hydroxyhexylamino)-2-cyanobenzothiazole (**8b**).* **8a** was prepared by the general alkylation procedure and subjected to deprotection as in **5a** without further purification to afford **8b**. ^{1}H NMR (CD_2Cl_2) δ 7.92 (d, 1 H, J = 9.1),

Scheme 3: Synthesis of Carboxyalkyl Aminoluciferins



Scheme 4



14a-c
R = H, tBu
X = CN, 3-carboxythiazolidin-2-yl

7.03 (s, 1 H), 6.96 (d, 1 H, $J = 9.1$), 3.62 (t, 2 H, $J = 6.4$), 3.21 (t, 2 H, $J = 7.2$), 1.39−1.74 (m, 8 H). MS: calcd for $C_{14}H_{18}N_3OS$ 276.1, found 276.2.

*6-(8-Hydroxyoctylamino)-2-cyanobenzothiazole (**9b**).* The general procedure was followed using 1-bromo-8-(tetrahydropyranyloxy)octane (840 μL, 3 mmol, 1.5 equiv) and heating at 100 °C for 7 h. After extraction and evaporation, the residue was purified by silica gel chromatography using 1:4 ethyl acetate−heptanes to afford 121 mg of a yellow oil. MS: calcd for $C_{21}H_{30}N_3O_2S$ 388.2, found 388.2 (M − THP + H 304.1, found 304.3, observed as major peak). This material was stirred in 5 mL of MeOH with 60 mg (1 equiv) of TsOH for 1 h. The reaction was partitioned between CH_2Cl_2 and saturated $NaHCO_3$; the organic layer was dried over Na_2SO_4 and evaporated onto silica gel. Purification by flash chromatography eluting with 1:1 heptanes−ethyl acetate was followed by preparative HPLC to yield 50 mg (8.2%, two steps) of a fluffy yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.83 (d, 1 H, $J = 8.9$), 6.84 (d, 1 H, $J = 2.3$), 6.79 (dd, 1 H, $J = 2.3, 8.9$), 4.10 (br s, 1 H), 3.58 (t, 2 H, $J = 6.4$), 3.11 (t, 2 H, $J = 7.1$), 1.46−1.66 (m, 6 H), 1.25−1.39 (m, 6 H). MS: calcd for $C_{16}H_{22}N_3OS$ 304.1, found 304.2.

*6-(Carboxymethyl-1-amino)-2-cyanobenzothiazole (**13b**).* **13a** was treated with 5% (v/v) anisole in TFA for 2 h in an ice bath. The solvents were removed in vacuo, and the product was isolated by preparative HPLC. ^{1}H NMR (CD_3OD) δ 7.87 (d, 1 H, $J = 9.8$), 7.04−7.08 (m, 2 H), 3.99 (s, 2 H). MS: calcd for $C_{10}H_8N_3O_2S$ 234.0, found 234.2.

*6-(3-Carboxypropyl-1-amino)-2-cyanobenzothiazole (**14b**).* **14a** (43 mg of crude material) was prepared according to the general alkylation procedure and treated with anisole (75 μL) and TFA (1.5 mL) in CH_2Cl_2 (1.5 mL) in an ice bath for 4 h. The reaction was stripped, and the desired compound was obtained by preparative HPLC (0%−>50% MeCN gradient over 35 min) as a mixture containing 30% cyclized byproduct. ^{1}H NMR (CD_3OD) δ 7.90 (d, 1 H, $J = 9.1$), 7.07 (d, 1 H, $J = 2.3$), 6.99 (dd, 1 H, $J = 2.3, 9.1$), 3.19 (t, 2 H, $J = 7.2$), 2.29 (t, 2 H, $J = 7.3$), 1.88−1.98 (m, 2 H). Residual resonances from contaminant were visible at 8.44 (d, 1 H), 8.07 (d, 1 H), 7.60 (dd, 1 H), 4.20 (t, 2 H), 2.30 (t, 2 H), and 1.9 (m, 2 H).

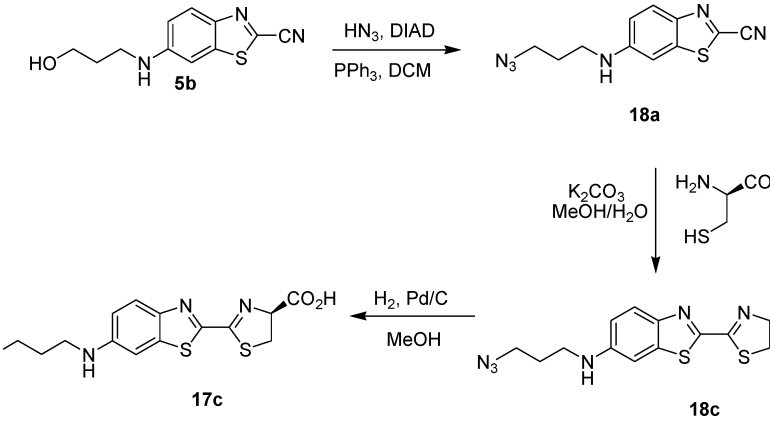
*6-(5-Carboxypentyl-1-amino)-2-cyanobenzothiazole (**15b**).* **15a** (42 mg, 0.12 mmol) was combined with anisole (50 μL) and TFA (1 mL) in CH_2Cl_2 (1 mL) in an ice bath, and the reaction was stirred for 3 h. The reaction was then evaporated to dryness, and the residue was purified on a silica gel column eluting with CH_2Cl_2 and then 95:5 CH_2Cl_2−MeOH to afford 31 mg (89%) of a light yellow solid. ^{1}H NMR (CD_2Cl_2) δ 7.90 (d, 1 H, $J = 8.9$), 6.96 (s, 1 H), 6.91 (d, 1 H, $J = 8.9$), 3.2 (t, 2 H, $J = 6.8$), 2.40 (t, 2 H, $J = 6.9$), 1.46−1.74 (m, 6 H). MS: calcd for $C_{14}H_{16}N_3O_2S$ 290.1, found 290.2.

*6-(3-Azidopropylamino)-2-cyanobenzothiazole (**18a**).* **5b** (120 mg, 0.529 mmol) was stirred in 5 mL of CH_2Cl_2 with PPh_3 (277 mg, 2 equiv) and HN_3 (530 μL of a 2 M solution in toluene, 2 equiv) in an ice bath. Diisopropyl azodicarboxylate (DIAD, 103 μL, 2 equiv) was added, and the reaction was stirred for 2 h at 0 °C. A small amount of silica gel was added to quench the reaction, the solvents were removed under reduced pressure, and the residue was purified by silica gel chromatography eluting with 3:1 heptanes−ethyl acetate. The resulting crystalline yellow solid contained approximately 2 equiv of the DIAD byproduct by NMR and was carried on crude. ^{1}H NMR (CD_2Cl_2) δ 7.93 (dd, 1 H, $J = 0.4, 8.9$), 7.00 (d, 1 H, $J = 2.3$), 6.94 (dd, 1 H, $J = 2.3, 8.9$), 4.4 (br s, <1 H), 3.49 (t, 2 H, $J = 6.4$), 3.34 (t, 2 H, $J = 6.7$), 1.91−1.99 (m, 2 H). MS: calcd for $C_{11}H_{11}N_6S$ 259.1, found 259.1. DIAD byproduct 6.38 (br s, 2 H), 4.94 (septuplet, 2 H, $J = 6.4$), 1.26 (d, 12 H, $J = 6.3$).

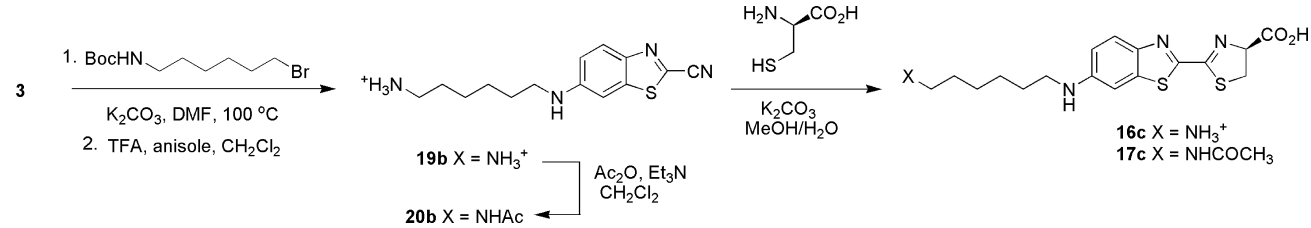
*6-(6-Aminohexylamino)-2-cyanobenzothiazole (**19b**).* 100 mg of **19a** was dissolved in 5 mL of CH_2Cl_2, stirred in an ice bath, and treated with 0.1 mL of anisole and 2.0 mL of TFA. After 1.5 h, the volatile components were evaporated, and the residue was triturated with Et_2O. The orange solid that formed on standing was isolated by centrifugation to afford 77 mg (103% yield) of the desired product. ^{1}H NMR (CD_3OD) δ 7.82 (d, 1 H, $J = 8.9$), 7.05 (d, 1 H, $J = 2.3$), 6.99 (dd, 1 H, $J = 2.3, 8.9$), 3.19 (t, 2 H, $J = 6.9$), 2.89−2.94 (m, 2 H), 1.62−1.75 (m, 4 H), 1.43−1.56 (m, 4 H). MS: calcd for $C_{14}H_{19}N_4S$ 275.1, found 275.2.

*6-(6-Acetamidohexylamino)-2-cyanobenzothiazole (**20b**).* 50 mg (0.13 mmol, TFA salt) of **19b** was dissolved in 5 mL of CH_2Cl_2 at ambient temperature, and the stirred solution was treated with acetic anhydride (14.5 μL, 1.25 equiv) and Et_3N (50 μL, 3 equiv). After 2 h, the reaction was concentrated under reduced pressure and purified on silica gel eluting with first CH_2Cl_2 and then 20−30% EtOAc in CH_2Cl_2 to give 35

Scheme 5



Scheme 6



mg (85%) of a crystalline yellow solid. ^{1}H NMR (CD_2Cl_2) δ 7.90 (dd, 1 H, J = 2.5, 9.1), 6.91−6.98 (m, 2 H), 5.48 (br s, 1 H), 3.17−3.26 (m, 4 H), 1.92 (s, 3 H), 1.64−1.74 (m, 2 H), 1.36−1.59 (m, 8 H). MS: calcd for $C_{16}H_{21}N_4OS$ 317.1, found 317.2.

General Cyclization Procedure. A methanolic solution of the appropriately substituted 6-amino-2-cyanobenzothiazole under nitrogen was treated with an aqueous solution of D-cysteine hydrochloride salt monohydrate (2 equiv) that had first been adjusted to pH 8 with solid K_2CO_3. The reaction mixture was stirred for 20 min at ambient temperature, at which time the MeOH was evaporated, and the reaction was acidified by the addition of TFA. The resulting suspension was dissolved by the addition of MeCN, filtered, and purified by preparative HPLC. The solvents were removed by rotary evaporation and subsequent lyophilization.

*6′-(2-Hydroxyethyl)aminoluciferin (**4c**).* ^{1}H NMR (DMSO-d_6) δ 7.78 (d, 1 H, J = 8.9), 7.10 (d, 1 H, J = 2.3), 6.93 (dd, 1 H, J = 2.3, 8.9), 5.35 (dd, 1 H, J = 8.2, 9.7), 3.56−3.74 (m, 4 H), 3.17 (t, 2 H, J = 5.9). MS: calcd for $C_{13}H_{14}N_3O_3S_2$ 324.0, found 324.2. HPLC: 92.6% pure at 295 nm; R_t = 10.9 min.

*6′-(3-Hydroxypropyl)aminoluciferin (**5c**).* ^{1}H NMR (DMF-d_7) δ 7.90 (d, 1 H, J = 8.9), 7.18 (d, 1 H, J = 2.3), 7.01 (dd, 1 H, J = 2.3, 8.9), 5.45 (dd, 1 H, J = 8.4, 9.5), 3.71−3.85 (m, 2 H), 3.66 (t, 2 H, J = 6.2), 3.27 (t, 2 H, J = 6.7), 1.81−1.89 (m, 2 H). MS: calcd for $C_{14}H_{16}N_3O_3S_2$ 338.1, found 338.1. HPLC: 100.0% pure at 295 nm; R_t = 10.8 min.

*6′-(4-Hydroxybutyl)aminoluciferin (**6c**).* ^{1}H NMR (DMSO-d_6) δ 7.72 (d, 1 H, J = 8.9), 7.01 (d, 1 H, J = 2.3), 6.84 (dd, 1 H, J = 2.3, 8.9), 5.29 (dd, 1 H, J = 8.1, 9.4), 3.52−3.69 (m, 2 H), 3.37 (t, 2 H, J = 6.0), 3.02 (t, 2 H, J = 6.9), 1.41−1.61 (m, 4 H). MS: calcd for $C_{15}H_{18}N_3O_3S_2$ 352.1, found 352.2. HPLC: 88.2% pure at 295 nm; R_t = 11.1 min.

*6′-(5-Hydroxypentyl)aminoluciferin (**7c**).* ^{1}H NMR (DMSO-d_6) δ 7.77 (d, 1 H, J = 8.9), 7.50 (d, 1 H, J = 2.2), 6.89 (dd, 1 H, J = 2.2, 8.9), 5.34 (dd, 1 H, J = 8.2, 9.5), 3.57−3.74 (m, 2 H), 3.39 (t, 2 H, J = 6.4), 3.06 (t, 2 H, J = 7.0), 1.53−1.62 (m, 2 H), 1.35−1.50 (m, 4 H). MS: calcd for $C_{16}H_{20}N_3O_3S_2$ 366.1, found 366.5. HPLC: 95% pure at 295 nm; R_t = 11.9 min. 0.2% aminoluciferin at 295 nm.

*6′-(6-Hydroxyhexyl)aminoluciferin (**8c**).* ^{1}H NMR (CD_3-OD) δ 7.74 (d, 1 H, J = 9.1), 7.01 (d, 1 H, J = 2.3), 6.89 (dd, 1 H, J = 2.3, 9.1), 5.25 (t, 1 H, J = 8.8), 3.67−3.78 (m, 2 H), 3.56 (t, 2 H, J = 6.4), 3.16 (t, 2 H, J = 7.0), 1.42−1.73 (m, 8 H). MS: calcd for $C_{17}H_{22}N_3O_3S_2$ 380.1, found 380.3. HPLC: 96.0% pure at 295 nm; R_t = 13.1 min.

*6′-(8-Hydroxyoctyl)aminoluciferin (**9c**).* ^{1}H NMR (CD_3OD) δ 7.76 (d, 1 H, J = 8.8 Hz), 7.04 (d, 1 H, J = 2.3), 6.92 (dd, 1 H, J = 2.3, 8.8), 5.35 (t, 1 H, J = 9.2), 3.67−3.79 (m, 2 H), 3.53 (t, 2 H, J = 6.4), 3.16 (t, 2 H, 7.2), 1.62−1.71 (m, 2 H), 1.33−1.57 (m, 10 H). MS: calcd for $C_{19}H_{26}N_3O_3S_2$ 408.1, found 408.3. HPLC: 95% pure at 295 nm; R_t = 15.6 min. 0.06% aminoluciferin at 295 nm.

*6′-(10-Hydroxydecyl)aminoluciferin (**10c**).* ^{1}H NMR (CD_3-OD) δ 7.74 (d, 1 H, J = 9.2), 7.00 (d, 1 H, J = 2.3), 6.89 (dd, 1 H, J = 2.3, 9.2), 5.34 (t, 1 H, 9.2), 3.66−3.78 (m, 2 H), 3.52 (t, 2 H, J = 6.6), 3.14 (t, 2 H, J = 7.0), 1.61−1.71 (m, 2 H), 1.31−1.58 (m, 14 H). MS: calcd for $C_{21}H_{30}N_3O_3S_2$ 436.2, found 436.4. HPLC: 99.0% pure at 295 nm; R_t = 18.04 min. 0.17% aminoluciferin at 295 nm.

*6′-Isobutylaminoluciferin (**11c**).* ^{1}H NMR (CD_3OD) δ 7.76 (d, 1 H, J = 8.9), 7.05 (d, 1 H, J = 2.3), 6.93 (dd, 1 H, J = 2.3, 8.9), 5.35 (t, 1 H, J = 8.9), 3.68−3.79 (m, 2 H), 2.99 (d, 2 H, J = 6.9), 1.95 (septuplet, 1 H, J = 6.7), 1.01 (d, 6 H, J = 6.7). MS: calcd for $C_{15}H_{18}N_3O_2S_2$ 336.1, found 336.2. HPLC: 97.0% pure at 295 nm; R_t = 18.02 min.

*6′-Benzylaminoluciferin (**12c**).* ^{1}H NMR (DMSO-d_6) δ 7.79 (d, 1 H, J = 8.9), 7.29−7.39 (m, 4 H), 7.20−7.26 (m, 1 H), 7.08 (d, 1 H, J = 2.2), 6.96 (dd, 1 H, J = 2.2, 8.9), 5.34

Table 1: Comparative Light Output of N-Substituted Aminoluciferins

Compound	Side Chain Structure	% **2** contamination[a,b]	Bioluminescence[c,d]
4c	HO	0	23
5c	HO	0	120
6c	HO	0	80
11c		0	64
7c	HO	0.2	71
12c		1.1	4.6
8c	HO	0	10
9c	HO	0.06	1.0
10c	HO	0.17	0.2
13c	$^{-}$O O		21
16c	MeO O	1.2	5.5
14c	$^{-}$O O	<1	24.5[e]
15c	$^{-}$O O	0.14	2.6
17c	$^{+}H_3N$	0	0.4
18c	N_3	0.6	15
19c	$^{+}H_3N$	0	0.5
20c	H N O	0.3	21

[a] Measured at 295 nm. [b] Under HPLC conditions, 295 nm is the absorption maximum for **2**, whereas substrates **4c**−**20c** typically have absorption maxima in the range 350−380 nm. Thus, this column should be regarded as an overestimate. [c] Percentage of **2** light output at the same concentration (typically 0.5−2 μM). [d] Low light output (<5%) results were confirmed by measuring at 50 μM. [e] Contains 15−20% of a dark contaminant.

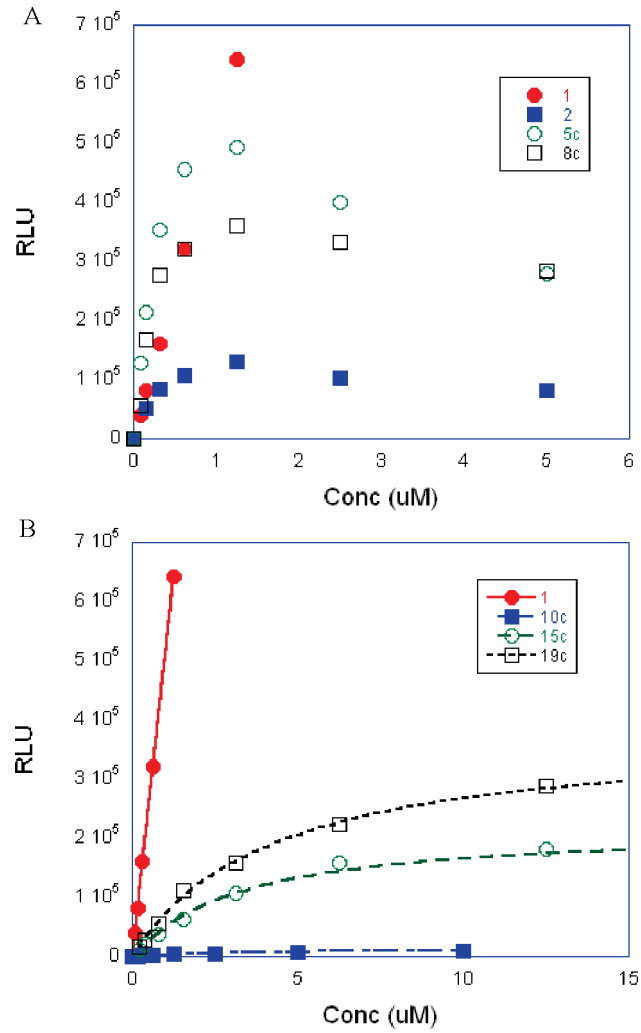


FIGURE 2: Light output as a function of concentration with QuantiLum luciferase. (A) Inhibiting substrates: **1**, red solid circles; **2**, blue solid squares; **5c**, green open circles; **8c**, black open squares. (B) Noninhibiting substrates: **1**, red solid circles; **10c**, blue solid squares; **15c**, green open circles; **19c**, black open squares.

(dd, 1 H, J = 8.3, 9.6), 4.35 (s, 2 H), 3.57−3.74 (m, 2 H). MS: calcd for $C_{18}H_{16}N_3O_2S_2$ 370.1, found 370.2. HPLC: 96.4% pure at 295 nm; 1.1% aminoluciferin contamination at 295 nm.

*6′-(Carboxymethyl)aminoluciferin (**13c**).* ^{1}H NMR (CD_3OD) δ 7.80 (d, 1 H, J = 9.1), 7.03 (d, 1 H, J = 2.3), 6.96 (dd, 1 H, J = 2.3, 9.1), 5.35 (t, 1 H, J = 8.9), 3.98 (s, 2 H), 3.73 (m, 2 H). HPLC: 86.7% pure at 395 nm; R_t = 11.9 min. MS: no signal observed.

*6′-(3-Carboxypropyl)aminoluciferin (**14c**).* ^{1}H NMR (CD_3OD) δ 7.78 (d, 1 H, J = 8.9), 7.12 (d, 1 H, J = 2.3), 6.95 (dd, 1 H, J = 2.3, 8.9), 5.36 (t, 1 H, J = 9.0), 3.68−3.78 (m, 2 H), 3.24 (t, 2 H, J = 6.9), 2.44 (t, 2 H, J = 7.2), 1.90−1.99 (m, 2 H). Contaminant (15 mol % by NMR, HPLC): ^{1}H NMR (CD_3OD) δ 8.35 (d, 1 H, J = 1.9), 8.08 (d, 1 H, J = 8.9), 7.91 (dd, 1 H, J = 1.9, 8.9), 5.41 (t, 1 H, J = 9.2), 4.02 (t, 2 H, J = 7.1), 3.74−3.84 (m, 2 H), 2.65 (t, 2 H, J = 7.9), 2.17−2.29 (m, 2 H). LCMS: calcd for $C_{15}H_{16}N_3O_4S_2$ 366.1, found 366.3 for major peak; contaminant shows M + H − 18. HPLC: 77.2% pure at 295 nm; R_t = 12.2 min. Major contaminant: 14.1% at 13.4 min.

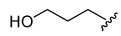
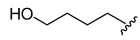
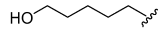
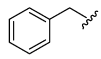
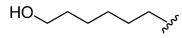
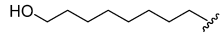
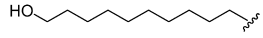
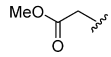
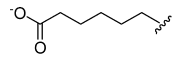
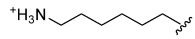
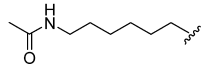
*6′-(5-Carboxypentyl)aminoluciferin (**15c**).* ^{1}H NMR (CD_3OD) δ 7.81 (d, 1 H, J = 9.0), 7.14 (d, 1 H, J = 2.2), 6.98 (dd, 1 H, J = 2.2, 9.0), 5.37 (t, 1 H, J = 9.1), 3.69−3.80 (m, 2 H), 3.21 (t, 2 H, J = 6.9), 2.33 (t, 2 H, J = 7.2), 1.63−1.76 (m, 4 H), 1.44−1.54 (m, 2 H). MS: calcd for $C_{17}H_{19}N_3O_4S_2$ 394.1, found 394.3. HPLC: 98.3% pure at 295 nm; R_t = 13.1 min. 0.14% aminoluciferin at 295 nm.

*Methyl 6′-(Carboxymethyl)aminoluciferin (**16c**).* ^{1}H NMR (CD_3OD) δ 7.82 (d, 1 H, J = 9.0), 6.99 (d, 1 H, J = 2.3), 6.93 (dd, 1 H, J = 2.3, 9.0), 5.34 (t, 1 H, J = 9.2), 4.02 (s, 2 H), 3.75 (s, 3 H), 3.73 (d, 2 H, J = 9.2). MS: calcd for $C_{14}H_{14}N_3O_4S_2$ 352.0, found 352.2. HPLC: 98.8% pure at 295 nm; R_t = 14.0 min. 1.2% aminoluciferin at 295 nm.

*6′-(3-Azidopropyl)aminoluciferin (**18c**).* ^{1}H NMR (DMSO-d_6) δ 7.79 (d, 1 H, J = 9.0), 7.09 (d, 1 H, J = 2.3), 6.89 (dd, 1 H, J = 2.3, 9.0), 5.35 (dd, 1 H, J = 8.4, 9.6), 3.58−3.75 (m, 2 H), 3.46 (t, 2 H, J = 6.9), 3.16 (t, 2 H, J = 6.9), 1.78−1.87 (m, 2 H). MS: calcd for $C_{14}H_{15}N_6O_2S_2$ 363.1, found 363.3. HPLC: 94.4% pure at 295 nm; R_t = 18.1 min. 0.6% aminoluciferin at 295 nm.

*6′-(6-Aminohexyl)aminoluciferin (**19c**).* ^{1}H NMR (CD_3OD, D_2O, DMSO-d_6, referenced to CD_3OD peak) δ 7.81 (d, 1 H, J = 9.0), 7.15 (d, 1 H, J = 2.3), 6.98 (dd, 1 H, J = 2.3, 9.0), 5.18 (dd, 1 H, J = 8.5, 9.7), 3.63−3.82 (m, 2 H), 3.19 (t, 2 H, J = 6.7), 2.94−2.99 (m, 2 H), 1.63−1.75 (m, 4 H),

Table 2: K_m and V_{max} Values for N-Substituted Aminoluciferins and Ultra-Glo or QuantiLum Luciferase

Compound	Side Chain Structure	Ultra-Glo K_m (μM)	Ultra-Glo V_{max} (RLU, x 10^4)	QuantiLum K_m (μM)	QuantiLum V_{max} (RLU, x 10^4)
2		1.1	21.9		
5c		10.8	332		
6c		1.17	56.1		
11c[b]		2.76	122		
7c		1.97	67.0		
12c[b]		0.727	50.0		
8c		6.56	53.3		
9c		3.3	13.5	1.3	6.37
10c		2.5	1.33	3.5	1.35
13c		3.98	30.9		
16c		8.93	15.4		
15c		9.58	11.4	3.3	22
19c		50	5.5		
20c[b]		0.253	8.66	4.2	37.9

[a] RLU = relative light units. [b] Some substrate inhibition was observed; data obtained above the substrate inhibition point were not included in the fitting process.

1.43−1.50 (m, 4 H). MS: calcd for $C_{17}H_{23}N_4O_2S_2$ 379.1, found 379.3. HPLC: 100.0% pure at 295 nm; R_t = 8.8 min. No aminoluciferin at 295 nm; 0.19% at 330 nm.

*6′-(6-Acetamidohexyl)aminoluciferin (**20c**).* ^{1}H NMR (CD_3OD) δ 7.86 (d, 1 H, J = 9.1), 7.28 (d, 1 H, J = 2.2), 7.06 (dd, 1 H, J = 2.2, 9.1), 5.38 (t, 1 H, J = 8.9), 3.70−3.81 (m, 2 H), 3.23 (t, 2 H, J = 7.0), 3.16 (t, 2 H, J = 6.9), 1.91 (s, 3 H), 1.65−1.74 (m, 2 H), 1.36−1.57 (m, 6 H). MS: calcd for $C_{19}H_{25}N_4O_3S_2$ 421.1, found 421.3. HPLC: 97.0% pure at 295 nm; R_t = 12.8 min. 0.3% aminoluciferin at 295 nm.

*6′-(3-Aminopropyl)aminoluciferin (**17c**).* Twenty-three milligrams (0.064 mmol) of 3-azidopropyl-6′-aminoluciferin was dissolved in 2 mL of MeOH and hydrogenated over Pd/C (7.5 mg) at atmospheric pressure overnight. The reaction was filtered through Celite, and the desired compound (10 mg, 47% yield) was isolated by preparative HPLC. ^{1}H NMR (DMSO-d_6) δ 7.81 (d, 1 H, J = 9.1), 7.69 (br s, 2 H), 7.11 (d, 1 H, J = 2.3), 6.90 (dd, 1 H, J = 2.3, 9.1), 5.35 (dd, 1 H, J = 8.3, 9.6), 3.58−3.75 (m, 2 H), 3.18 (t, 2 H, J = 7.0), 2.88 (br s, 1 H), 1.78−1.88 (m, 2 H). MS: calcd for $C_{14}H_{17}N_4O_2S_2$ 337.1, found 337.2. HPLC: 94.4% pure at 330 nm; R_t = 9.5 min. No aminoluciferin was detected.

RESULTS AND DISCUSSION

The primary project goal was to examine luciferase substrate tolerance with respect to size, shape, and charge. The determination of a rough cutoff for 6′-substituent size was undertaken first. A series of aminoluciferins bearing straight-chain alkyl substituents of increasing length was prepared (Scheme 2). Terminal hydroxyl groups were incorporated in the interest of avoiding solubility and aggregation problems. In general, N-substituted aminoluciferins were prepared in three steps: alkylation of 6-amino-2-cyanobenzothiazole **3**, deprotection as appropriate, and cyclization of the cyano group with D-cysteine to afford the target compounds. Chiral HPLC analysis established the retention of optical activity in the isolated compounds. The alkylation step was performed in DMF at 100 °C in the presence of excess potassium carbonate, with typical yields ranging from 5−30% presumably owing to the poor nucleophilicity of the amine. The incorporation of nonpolar protecting groups aided in the rigorous removal of the aminocyanobenzothiazole starting material, a vital consideration because contamination with traces of this material leads to aminoluciferin contamination in the final product. Subsequent deprotection as required for **5a**−**9a** was followed by cyclization with D-cysteine under mildly basic conditions to furnish the desired aminoluciferins **4c**−**10c**. In addition, the effect of altered distribution of steric bulk on bioluminescence was investigated via the analogous preparation of isobutyl- and benzyl-substituted aminoluciferins (**11c** and **12c**, respectively).

Existing structural information suggested (*13*) that the active site pocket accommodating these substituents was likely hydrophobic. We investigated this dogma by testing a series of aminoluciferins bearing amine- or carboxylate-substituted side chains. The carboxylate-substituted compounds **13c**−**15c** were prepared using *tert*-butyl ester protecting groups as shown in Scheme 3. The carboxypropyl intermediates and substrate **14a**−**c** underwent a facile lactamization, and **14c** was obtained as a mixture containing ~15% of the corresponding lactam. *N*-Acyl aminoluciferins have been generally observed to be dark (*15*), and this lactam was not examined further. The methyl ester **16c** was prepared for comparison with **13c**, in order to more fully examine the effect of charge.

Synthesis of 3-aminopropyl aminoluciferin **17c** was problematic in that alkylation of **3** with 3-(*tert*-butoxycarbonyl)aminopropyl bromide did not proceed detectably. Thus, **17c** was prepared via Mitsunobu conversion of hydroxypropyl **5b** to azidopropyl **18a**, cyclization to the 3-azidopropyl aminoluciferin **18c**, and subsequent reduction to **17c** (Scheme 5). The 6-aminohexyl aminoluciferin **19c** and its acetylated analogue **20c** were prepared as shown in Scheme 6.

Once synthesized, all substrates were tested for bioluminescence with Ultra-Glo luciferase (*19*) suspended in P450-Glo buffer. The Ultra-Glo enzyme is a thermostable luciferase mutant that, when used with the P450-Glo reconstitution buffer, replaces the characteristic flash of bioluminescence with a stable signal that is constant over 3 h and that is proportional to substrate concentration. Ultra-Glo luciferase was chosen as the primary enzyme in this study owing in part to ease of handling and reliable reproducibility. Light output for each compound was measured against an aminoluciferin standard curve and normalized to the aminoluciferin signal observed or extrapolated for the same concentration. These results are reported as percentages in Table 1. The photophysical properties of compounds **4c**−**20c** relative to aminoluciferin **2** were measured in methanol and in water (see Supporting Information for a full table). Typically, N-substitution resulted in a slight (5−21 nm) red shift of the absorption and emission maxima compared to aminoluciferin **2** (λ_{abs} = 368 nm; λ_{em} = 493 in MeOH). Methanolic quantum yields of fluorescence were generally found to be identical to aminoluciferin **2** within experimental error (±8%), while the quantum yield in water was decreased by 6−30%. Methanol as a solvent is likely the better mimic of the luciferase active site (*1*), and therefore these numbers are more relevant to bioluminescence. Any contribution to bioluminescent light output variation from differences in intrinsic quenching or enhancement of fluorescence properties should thus be trivial. Based on the correlation of light output with size, substituents with more than nine atoms in the ω-hydroxyalkyl chain are generally poorly tolerated by the luciferase enzyme. The determination of a maximum for side chain size was one of the original goals of the project; however, an optimum size was also indicated. Unexpectedly, the 3-hydroxypropyl compound **5c** gave a light output 20% greater than that of the parent aminoluciferin **2**. The 4-hydroxybutyl **6c** and 5-hydroxypentyl **7c** exhibited a similar but slightly decreased light output, while side chains one methylene shorter (**4c**) or longer (**8c**) effected a significant decrease. This pattern suggests a potential hydrogen bond between substrate and enzyme that can be accessed by **5c**, **6c**, or **7c** but not by substrates **4c** and **8c**. The *N*-isobutyl aminoluciferin **11c** emitted an intermediate amount of light, and the benzyl compound **12c** gave very little light. The decrease in light output with increasing size was gradual, possibly reflecting the flexibility of the side chains examined. In addition, **20c** was 20-fold brighter than the slightly smaller 8-hydroxyoctyl aminoluciferin **9c**, revealing that size is not the only factor involved. The accommodation of relatively long side chains is consistent with the reports of Inouye and co-workers (*20*) that linolenic and arachidonic acids are converted to the fatty acyl-CoA esters by luciferase, presumably via the corresponding adenosyl monophosphate adducts.

In order to examine the effect of charge, a series of ω-carboxy-substituted alkyl side chains one, three, and five methylenes in length (**13c**−**15c**) were compared. Somewhat unexpectedly, light emission of the carboxy series followed the trend set by the ω-hydroxy-substituted series (**4c**, **6c**, **8c**) with a comparative decrease of only 1−6-fold between each hydroxyl compound and its similarly sized carboxylate counterpart. Carboxymethyl aminoluciferin **13c** generated an almost identical amount of light as the similarly sized 2-hydroxyethyl aminoluciferin **4c**. The carboxypropyl aminoluciferin **14c** is decreased by 4−6-fold compared to **6c**, and carboxypentyl aminoluciferin **15c** light output is decreased by only 4-fold compared to **8c**. Perhaps the most puzzling aspect of the carboxy series is the significantly decreased light output for the methyl ester of carboxymethyl aminoluciferin **16c** as compared to the corresponding acid **13c**.

In sharp contrast to the results observed for the carboxylate series, the presence of a positive charge in **17c** and **19c** appears to nearly eliminate bioluminescence. Minimal light output was observed for either substrate, a particularly impressive contrast when comparing aminopropyl aminoluciferin **17c** with hydroxypropyl aminoluciferin **5c**. Even the azidopropyl substrate **18**c emitted 15% as much light as aminoluciferin, compared to 0.4% observed for **17c**.

A structural homology model of Ultra-Glo luciferase, generated from the available crystal structure of *Luciola cruciata* (PDB code 2D1S (*13*)), was used for docking studies with selected N-substituted aminoluciferin adducts of adenosine monophosphate. The model suggests the presence of a stabilizing hydrogen bond network at the base of the luciferin binding pocket, involving residues G227, N228, E310, R336, and the solvent-exposed K353. The brightest compound, **5c**, seems to fit well into the binding pocket and to increase the net number of putative hydrogen bonds in the network by two. It appears able to contribute three new hydrogen bonds through its terminal hydroxyl group, while one original hydrogen bond is lost due to minor repositioning of the hydrogen-bonding residues. The slightly longer **6c** does not seem as easily accommodated in the binding pocket and appears to effect a significant repositioning of the hydrogen-bonding residues, reducing the net number of putative hydrogen bonds by two. These data are shown in Figure 1. The presence of R336 and K353 is consistent with the enzyme's good recognition of negatively charged substituents and near intolerance of positively charged substituents.

Apparent Michaelis−Menten kinetic constants were measured for a subset of the substrates with Ultra-Glo enzyme in a minimal reaction system (Tricine buffer, pH 8.3,

containing 1 mM ATP and 10 mM $MgSO_4$). The new substrates were also tested as substrates for QuantiLum, the native *Photinus pyralis* firefly luciferase (*21*). The protein sequences of QuantiLum and Ultra-Glo are 62% identical overall and about 86% identical in the binding region for the modified aminoluciferins examined in this study. Burst kinetics and moderate to severe substrate inhibition precluded the determination of K_m and V_{max} values for most of the substrates with QuantiLum.

The primary difference in light output appears to arise from V_{max} values, with a few notable exceptions. Among these exceptions are **19c** and **20c**, where acetylation of **19c** to afford **20c** decreases the K_m constant by a factor of approximately 200-fold, whereas the change in V_{max} value is relatively insignificant. The high enzyme affinity of **20c** also clarifies somewhat the discrepancy between the similarly sized **20c** and **9c** in Table 1, which likewise have relatively similar V_{max} values. Interestingly, **5c** possesses a maximum light output more than 10 times brighter than **2** at saturating concentrations, but the approximately 10-fold weaker affinity nearly cancels this effect under the conditions in Table 1. The difference between the carboxylate **13c** and the corresponding methyl ester **16c**, in contrast, arises from the cumulative effect of a higher K_m and lower V_{max} for **16c** compared to **13c**. Finally, the increase in V_{max} for **11c** and **12c** suggests that the relatively low light output observed in Table 1 is a result of interaction with components of the p450 reconstitution buffer. **11c** in particular is significantly brighter than aminoluciferin **2** in the minimal system, and many of the substrates examined here have a maximum brightness equal to or greater than **2**.

Substrate specificity for QuantiLum luciferase appears to vary significantly from Ultra-Glo luciferase. Whereas **1** is by far the brightest substrate, several of the new substrates (e.g., **5c**, **8c**, **13c**) are brighter than the parent **2** as shown in Figure 2. The size limitation remains, as **10c** is a relatively poor substrate for both enzymes. In sharp contrast to the Ultra-Glo results, positive charges are tolerated well by QuantiLum, as **19c** affords a brightness similar to the carboxylate-substituted aminoluciferins **13c** and **15c**. This is consistent with the presence of a glutamic acid in QuantiLum at the position corresponding to K353 in Ultra-Glo. Perhaps the most salient aspect of QuantiLum is the generally low threshold for substrate inhibition, often below 5 μM. Table 2 includes apparent Michaelis−Menten constants for the substrates that did not display substrate inhibition.

CONCLUSIONS

In conclusion, we have discovered that N-monosubstituted 6′-aminoluciferins can be bioluminescent substrates of Ultra-Glo and QuantiLum luciferase enzymes and have examined the substrate scope and limitation through a structure−activity relationship study. In doing so, we have found that substantial steric bulk up to approximately nine linear atoms can be tolerated at the 6′ position of **2** and that aminoluciferins bearing neutral or negatively charged side chains are well tolerated by Ultra-Glo while those bearing positively charged side chains are not. Several of the new substrates are significantly brighter under saturating conditions than 6′-aminoluciferin itself, and most but not all of the variation among the substrates appears to arise from changes in the apparent V_{max}. Molecular modeling suggests that interactions with a putative hydrogen-bonding network at the base of the luciferin binding pocket of the Ultra-Glo enzyme could play a role in the level of light output among the new substrates. Our findings represent a direct contradiction of several long-held beliefs about the luciferin−luciferase system, an important expansion of the known bioluminescent substrates, and an open door to many new possibilities for bioluminescent assay design.

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SUPPORTING INFORMATION AVAILABLE

^{1}H NMR spectra, photophysical procedures and data, and chiral analysis of new luciferase substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Baldwin, T. O. (1996) Firefly luciferase: the structure is known, but the mystery remains. *Structure 4*, 223–228.
2. Lundin, A. (2000) Use of firefly luciferase in ATP-related assay of biomass, enzymes and metabolites. *Methods Enzymol. 305*, 346–370.
3. Kumar, M., Hsiao, K., Vidugiriene, J., and Goueli, S. A. (2007) A Bioluminescent-Based, HTS-Compatible Assay to Monitor G-Protein-Coupled Receptor Modulation of Cellular Cyclic AMP. *Assay Drug Dev. Technol. 5*, 237–246.
4. O'Brien, M. A., Daily, W. J., Hesselberth, P. E., Moravec, R. A., Scurria, M. A., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2005) Homogeneous, Bioluminescent Protease Assays: Caspase-3 as a Model. *J. Biomol. Screening,* 137–148.
5. Zhang, Y., and Pardridge, W. M. (2005) Delivery of Beta-Galactosidase to Mouse Brain via the Blood-Brain Barrier Transferrin Receptor. *J. Pharmacol. Exp. Ther. 313*, 1075–1081.
6. (a) Cali, J. J., Ma, D., Sobol, M., Simpson, D. J., Frackman, S., Good, T. D., Daily, W. J., and Liu, D. (2006) Luminogenic Cytochrome P450 Assays. *Exp. Opin. Drug Metab. Toxicol. 2*, 629–645. (b) Cali, J. J., Ho, S., Ma, D., Simpson, D. J., Frackman, S., Daily, W. J., Hesselberth, P. E., Good, T. D., Bulleit, R. F., and Klaubert, D. H. (2005) Bioluminescent P450 Assays That Use D-Luciferin Derivatives As Substrates For CYP1A1, 1A2, 1B1, 2C8, 2C9, 2J2, 3A4, 3A7, 4A11, 4F3B, 4F12, and 19, *Proceedings of the 14th International Conference on Cytochrome P450s. Medimod S.r.l. International Proceedings*, pp 77−84.
7. Zhou, W., Valley, M. P., Shultz, J., Hawkins, E. M., Bernad, L., Good, T., Good, D., Riss, T. L., Klaubert, D. H., and Wood, K. V. (2006) New Bioluminogenic Substrates for Monoamine Oxidase Assays. *J. Am. Chem. Soc. 128*, 3122–3123.
8. Zhou, W., Shultz, J. W., Murphy, N., Hawkins, E. M., Bernad, L., Good, T., Moothart, L., Frackman, S., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2006) Electrophilic aromatic substituted luciferins as bioluminescent probes for glutathione S-transferase assays. *Chem. Commun.,* 4620–4622.
9. Branchini, B. R. (2000) Chemical synthesis of firefly luciferin analogs and inhibitors. *Methods Enzymol. 305*, 188–195.
10. Branchini, B. R., Hayward, M. M., Bamford, S., Brennan, P. M., and Lajiness, E. J. (1989) Naphthyl- And Quinolylluciferin: Green and Red Light Emitting Firefly Luciferin Analogues. *Photochem. Photobiol. 49*, 689–695.
11. Seliger, H. H., McElroy, W. D., White, E. H., and Field, G. F. (1961) Stereospecificity and firefly bioluminescence, a comparison of natural and synthetic luciferins. *Proc. Natl. Acad. Sci. U.S.A. 47*, 1129–1134.
12. White, E. H., Wörther, H., Field, G. F., and McElroy, W. D. (1965) Analogs of Firefly Luciferin. *J. Org. Chem. 30*, 2344–2348.

13. Nakatsu, T., Ichiyama, S., Hiratake, J., Saldanha, A., Kobashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence. *Nature 440*, 372–376.
14. (a) Seliger, H. H., and McElroy, W. D. (1960) Spectral Emission and Quantum Yield of Firefly Bioluminescence. *Arch. Biochem. Biophys. 88*, 136–141. (b) Ando, Y., Niwa, K., Nobuyuki, Y., Enomoto, T., Irie, T., Kubota, H., Ohmiya, Y., and Akiyama, H. (2007) Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nat. Photonics 2*, 44–47.
15. White, E. H., Wörther, H., Seliger, H. H., and McElroy, W. D. (1966) Amino Analogs of Firefly Luciferin and Biological Activity Thereof. *J. Am. Chem. Soc. 88*, 2015–2019.
16. Agatsuma, T., Ogawa, H., Akasaka, K., Asai, A., Yamashita, Y., Mizukami, T., Akinaga, S., and Saitoh, Y. (2002) Halohydrin and Oxime Derivatives of Radicicol: Synthesis and Antitumor Activities. *Bioorg. Med. Chem. 10*, 3445–3454.
17. Fürstner, A., Mlynarski, J., and Albert, M. (2002) Total Synthesis of the Antiviral Glycolipid Cycloviracin B_1. *J. Am. Chem. Soc. 124*, 10274–10275.
18. Accelrys Software Inc (2006)San Diego, CA.
19. Hall, M. P., Gruber, M. G., Hannah, R. R., Jennens-Clough, M. L., and Wood, K. V. (1998) Stabilization of firefly luciferase using directed evolution, in *Bioluminescence and Chemiluminescence Perspectives for the 21st Century* (Roda, A., Pazzagli, M., Kricka, L. J., and Stanley, P. E., Eds.) pp 392−395, John Wiley & Sons, Chichester, U.K.
20. Oba, Y., Ojika, M., and Inouye, S. (2003) Firefly luciferase is a bifunctional enzyme: ATP-dependent monooxygenase and a long chain fatty acyl-CoA synthetase. *FEBS Lett. 540*, 251–254.
21. de Wet, J. R., Wood, K. V., Deluca, M., Helinski, D. R., and Subramani, S. (1987) Firefly Luciferase Gene: Structure and Expression in Mammalian Cells. *Mol. Cell. Biol. 7*, 725–737.

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