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Synthesis of an *N*-acyl sulfamate analog of luciferyl-AMP: A stable and potent inhibitor of firefly luciferase☆

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Abstract—In the first of two half-reactions resulting in the emission of visible light, firefly luciferase forms luciferyl-adenylate from its natural substrates beetle luciferin and Mg-ATP. The acyl-adenylate is subsequently oxidized producing the light emitter oxyluciferin in an electronically excited state. In vitro, under mild conditions of temperature and pH, the acyl-adenylate intermediate is readily hydrolyzed and susceptible to oxidation. We report here the multi-step synthesis and physical and enzymatic characterization of an *N*-acyl sulfamate analog of luciferyl-adenylate, 5′-*O*-[(*N*-dehydroluciferyl)-sulfamoyl]-adenosine (compound **5**). This represents the first example of a stable and potent (K_i = 340 nM) reversible inhibitor of firefly luciferase activity based on the structure of the natural acyl-adenylate intermediate. Additionally, we present the results of limited proteolysis studies that demonstrate that the binding of the novel acyl-adenylate analog protects luciferase from proteolysis. The findings presented here are interpreted in the context of the hypothesis that luciferase and the other enzymes in a large superfamily of adenylate-forming proteins adopt two conformations to catalyze two different partial reactions. We anticipate that the novel *N*-acyl sulfamate analog will be a valuable reagent in future studies designed to elucidate the role of conformational changes in firefly luciferase catalyzed bioluminescence.

1. Introduction

Firefly luciferase (Luc) catalyzes the formation of luciferyl-adenylate (LH_2-AMP) from substrates luciferin (LH_2) and ATP (Eq. 1). Through a multi-step oxidative process, LH_2-AMP is converted to excited state oxyluciferin, the emitter of the bioluminescent reaction (Eqs. 2 and 3)[1–6]

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \overset{\mathrm{Mg^{2+}}}{\leftrightarrows} \mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{PP_i}, \quad (1)$$

$$\mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc \cdot AMP \cdot oxyluciferin^*} + \mathrm{CO_2}, \quad (2)$$

$$\mathrm{Luc \cdot AMP \cdot oxyluciferin^*} \rightarrow \mathrm{Luc} + \mathrm{oxyluciferin} + \mathrm{AMP} + h\nu. \quad (3)$$

Luc binds the highly reactive, labile intermediate LH_2-AMP (Fig. 1) tightly (K_m = 4.7 μM).[7] We considered that a hydrolytically stable analog of the adenylate should be a strong inhibitor of Luc and, thus, a useful reagent for providing structural and conformational information from limited proteolysis and crystallographic studies.

The beetle luciferases are members of a large superfamily[8–10] of enzymes that includes the non-ribosomal peptide synthetases (NRPS) and the aryl- and acyl-CoA synthetases or ligases. These enzymes, like Luc, activate carboxylic acid substrates to form acyl-adenylate intermediates. In the NRPSs, acyl-adenylates are then transferred to the thiol groups of protein-bound cofactors; while Luc oxidizes LH_2-AMP to oxyluciferin and light (Eqs. 2 and 3). Crystal structure studies with several superfamily enzymes complexed with substrates and adenylates have depicted the proteins in two distinct conformations.[11–15] Gulick and colleagues[13,15] proposed that these enzymes are capable of restructuring the

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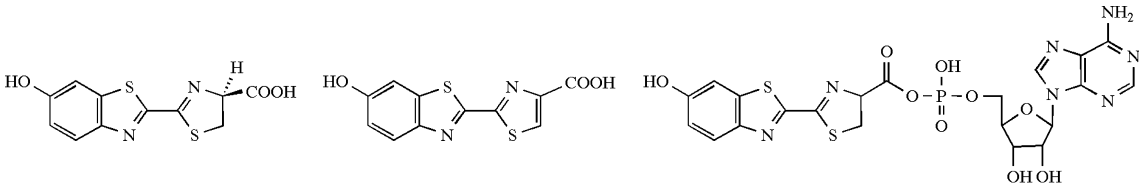


Figure 1. Chemical structures of substrate firefly luciferin (LH_2), dehydroluciferin, and luciferyl-adenylate (LH_2-AMP).

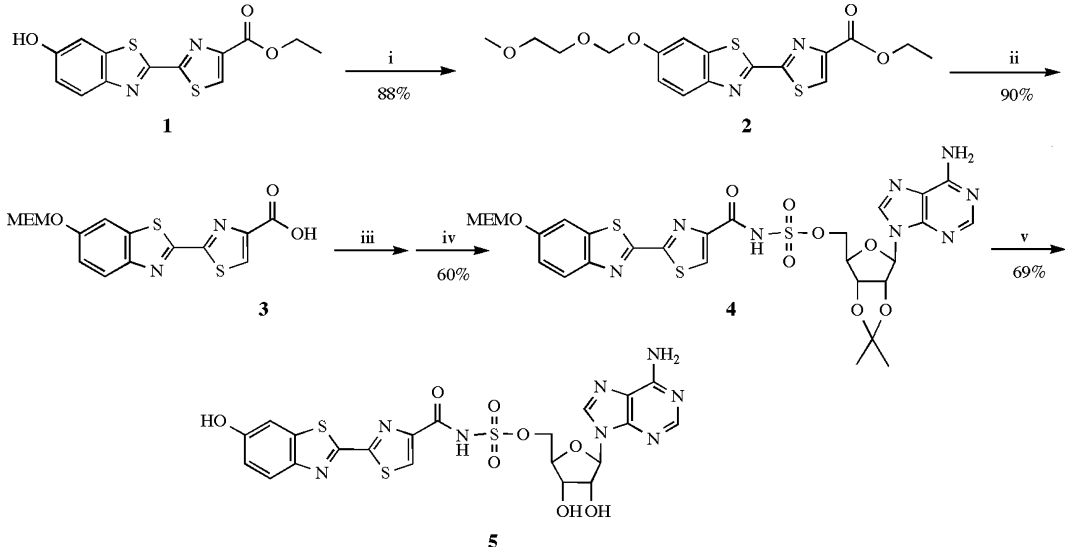
active site by rotation of the C-domain, and thus, catalyzing each half-reaction from a distinct conformation.

Several laboratories have reported the preparation of *N*-acyl sulfamate analogs of acyl-adenylates.[16–21] These novel reagents inactivated the adenylation domains of NRPSs and aminoacyl tRNA synthetases with inhibitory constants in the nanomolar range.[16–21] Previously, we identified two Luc amino acids conserved in the superfamily, one (Lys^{529}) essential for adenylation,[7] the other (Lys^{443}) required for the oxidative chemistry.[22] These results supported the hypothesis that, through conformational change(s), Luc positions specific amino acids to catalyze the individual partial reactions of the bioluminescent process. To date, Luc has been crystallized only in the absence of bound substrates or ligands.[23] Trapping Luc in a complex using a stable analog of LH_2-AMP, with the enzyme poised to undergo the oxidative half-reaction, may provide structural information related to the role of domain rotation in Luc catalysis.

We report here the synthesis of a novel *N*-acyl sulfamate analog, 5′-*O*-[(*N*-dehydroluciferyl)-sulfamoyl]-adenosine (**5**), and show that it is a stable, specific, and potent reversible inhibitor of Luc. Additionally, evidence is presented that supports the notion that Luc binds compound **5** in a different conformation than its natural substrates.

2. Chemistry

We designed and prepared an *N*-acyl sulfamate analog of LH_2-AMP based on the structure of dehydroluciferin (Fig. 1). Luc can catalyze the in vitro formation of dehydroluciferyl-adenylate,[24,25] which lacks a C-4 proton and cannot react further, and thus, is a reversible Luc inhibitor. Further, unlike LH_2, dehydroluciferin is resistant to oxidative decomposition in vitro. The synthetic scheme used to prepare milligram quantities of 5′-*O*-[(*N*-dehydroluciferyl)-sulfamoyl]-adenosine (**5**) (Scheme 1) in 33% overall yield from the ethyl ester of dehydroluciferin (**1**)[1] was based generally on published procedures for the preparation of *N*-acyl sulfamate analogs of amino acyl-adenylates.[16–21] The multi-step synthesis (see 'Supplementary materials') was undertaken with commercially available starting materials 2′,3′-*O*-isopropylideneadenosine, which was converted into 2′,3′-*O*-isopropylidene-5′-sulfamoyladenosine by reaction with sulfamoyl chloride,[17] and 2-cyano-6-hydroxybenzothiazole, which was transformed in two steps into compound **1**.[1] The key step in the synthetic pathway was the 1,1′-carbonyldiimidazole mediated condensation of derivatives of dehydroluciferin (**3**) and 5′-sulfamoyladenosine, each containing acid-sensitive protecting groups, which produced the doubly protected compound **4** in 60% yield. Protection of the phenol group as an MEM ether greatly improved the solubility of



Scheme 1. Synthesis of 5′-*O*-[(*N*-dehydroluciferyl)-sulfamoyl]-adenosine (**5**). Reagents and conditions: (i) THF, NaH, 0 °C to rt, 1.5 h; MEM-chloride, 0 °C to rt, 20 h; (ii) THF/MeOH/H_2O (2:1:1), LiOH · H_2O, 75 °C, 1.5 h; (iii) DMF, 1,1′-carbonyldiimidazole, 40 °C, 1.5 h; (iv) 2′,3′-isopropylidene-5′-sulfamoyladenosine in DMF, DBU, 40 °C, 90 min; (v) TFA, rt, 2 h; H_2O, rt, 15 min.

dehydroluciferin in the required solvents and was essential to the success of the condensation step. *N*-Acyl sulfamate analog **5** was obtained as the TFA salt, confirmed by the ^{19}F NMR signal observed at −74.86 ppm, in good yield after removal of both protecting groups with TFA.

The structure of **5** was confirmed by ^{1}H and ^{13}C NMR, IR, UV–vis, and HRMS. The UV–vis spectrum of **5** contains two maxima of nearly equal intensity at 263 and 354 nm, a general characteristic of adenylates containing LH_2 and its analogs. The *N*-acyl sulfamate in pH 8.0 sodium phosphate buffer at 20 °C was completely resistant to hydrolysis, as assessed by LC–MS (data not shown), for at least 8 weeks. Additionally, compound **5** was stable to hydrolysis in buffers over the pH range 6–9 at 37 °C for at least 2 weeks, showing no evidence of decomposition.

3. Inhibition studies

Compound **5** was a reversible, non-competitive inhibitor of Luc with respect to the natural substrates LH_2 and Mg-ATP with inhibition constants (K_i) of 34 ± 5 and 41 ± 3 nM, respectively (Figs. 2A and B). The finding that the adenosine sulfamate derivative **5** was a non-competitive inhibitor of Luc with respect to both LH_2 and Mg-ATP suggests that this inhibitor binds at a site that differs from that populated by either of these substrates. In contrast, the inhibition of Luc by compound **5** with respect to synthetic LH_2-AMP (as the sole substrate) was reversible and competitive with K_i = 340 ± 50 nM (Fig. 2C), indicating that this novel compound functions by binding to a unique site on Luc available to LH_2-AMP. Possibly, **5** binds to Luc in a conformation that restricts access of the natural substrates to their respective binding sites.

Limited proteolysis methods can be used to probe tertiary structure and dynamics of proteins in solution.[26,27] Under mild conditions, cleavage sites generally represent exposed, mobile, and flexible regions of native proteins that can conform (local unfolding) to the active sites of the proteases. The pattern of cleavage of Luc by chymotrypsin under limiting conditions is shown in Figure 3A. Peptides of estimated masses of approximately 52, 42, 38, 27, 25, 23, and 14 kDa were detected. When either LH_2 or Mg-ATP was present at concentrations 100-fold greater than their respective K_m values, there

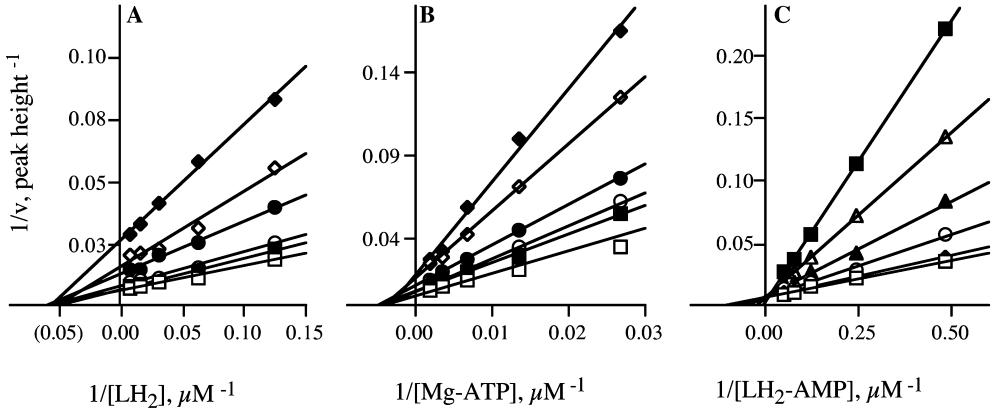


Figure 2. Lineweaver–Burk plots of inhibition of Luc by 5′-*O*-[(*N*-dehydroluciferyl)-sulfamoyl]-adenosine (**5**) with respect to LH_2 (A), Mg-ATP (B), and LH_2-AMP (C). Luc activity was measured as described in Supplementary materials. For panels A and B, the concentration of one substrate was maintained at saturation while the other was varied: 5.0–128.0 μM LH_2 (A) and 22.0–522.0 μM Mg-ATP (B). In studies with LH_2-AMP (C), the concentration of the single substrate ranged from 0.8 to 21.0 μM. Each point represents mean (standard deviation of ⩽10%) of triplicate measurements. In panels A and B, the concentrations of compound **5** were: 0 (□); 8.8 nM (■); 17.5 nM (○); 35.4 nM (●); 42.0 (◊); 70.0 nM (♦); and in panel C: 0 (□); 3.5 nM (♦); 305 nM (○); 510 nM(▲); 910 nM (△); 3.5 μM(■).

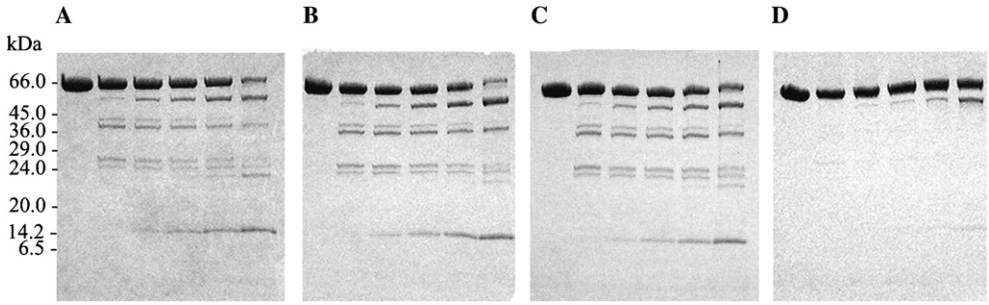


Figure 3. Limited proteolysis of Luc by chymotrypsin. Proteolysis of Luc was performed at 20 °C with a protease/enzyme ratio of 1:125, w:w, as described in Supplementary materials, in the presence of: no additions (A); 1.5 mM LH_2 (B); 16 mM Mg-ATP (C); 50 μM compound **5** (D). All SDS–PAGE gels (10–20% gradient) show the chymotryptic fragmentation observed in each study at 0, 0.5, 5, 10, 20, and 60 min. The migration of molecular weight standards (kDa) is indicated.

Table 1. Chymotryptic fragments from Luc digestion

Band on SDS–PAGE gel	SDS–PAGE gel band mass (Da)[a]	LC–ESMS analysis mass (Da)[b]	Putative identity of peptide	Calculated mass $[M + H]^{+}$[c]
1	61,704 ± 560	61,159 ± 6.1	Gly^{-5}–Leu^{550}	61,157
2	52,398 ± 2620	49,883 ± 4.9	Gly^{-5}–Tyr^{444}/Lys^{445}	49,883
3	41,693 ± 1540	nd[d]	—	—
4	38,387 ± 960	37,470 ± 3.7	Gly^{-5}–Phe^{331}/His^{332}	37,467
5	26,934 ± 2183	24,915 ± 2.5	Gly^{-5}–Phe^{219}/Ser^{220}	24,914
6	25,162 ± 1636	23,708 ± 2.4	Phe^{331}/His^{332}–Leu^{550}	23,707
7	23,013 ± 1495	22,332 ± 2.2	Gly^{-5}–Leu^{194}/Ile^{195}	22,332
8	14,200 ± 3550	11,291 ± 1.1	Tyr^{444}/Lys^{445}–Leu^{550}	11,291

[a] Masses assigned with Kodak ID Image Analysis software from 10 to 20% gradient gels by comparison to the relative migration of Sigma low molecular weight standards.

[b] Mass ($[M+H]^{+}$) determined from ThermoFinnigan Bioworks Browser 3.0 deconvolution software following LC–ESMS under conditions described in Supplementary materials.

[c] Using the Protein Analysis Work Sheet (PAWS) software (ProteoMetrics), masses obtained from LC–ESMS were correlated to unique Luc chymotryptic peptides. Gly^{-5} indicates the initial glycine of GPLGS–, which remains after the cleavage of Luc from the fusion protein. The symbol '/' indicates an internal chymotrypsin cleavage site.

[d] Not detected.

were no significant differences in the rates of protease digestion, or in the patterns of peptides generated (Figs. 3B and C). In the presence of compound **5** (50 μM, $\sim 150 \times K_i$ value), however, a marked reduction in the digestion of Luc by chymotrypsin was evident (Fig. 3D). Apparently, Luc binding of **5** (and presumably LH_2-AMP) is accompanied by a significant conformational change(s) that protects susceptible regions from chymotryptic proteolysis. Similarly, limited proteolysis studies of the NRPS tyrocidine synthetase 1 (TY1) by Dieckmann et al.[27,28] demonstrated that trypsin cleavage of the TY1 adenylation domain was appreciably reduced when substrates Phe and ATP were present together, but not when only one was included. These results[27,28] were taken as evidence that the adenylation domain had undergone a conformational change 'during or upon adenylate formation.'

Chymotrypsin digests of Luc were analyzed by LC–ESMS enabling us to assign accurate masses to the SDS–gel bands (Table 1). Making use of the Protein Analysis Work Sheet (PAWS) software (ProteoMetrics), the masses obtained by ESMS were used to tentatively determine the identities of the Luc peptides in the SDS–gel bands. Chymotrypsin cleavages at Phe^{331} and Tyr^{444} of Luc (Figs. 3A–C) were clearly evident as the complementary peptide fragments for each cut corresponded to the masses assigned to SDS–gel bands 4 and 6, and 2 and 8, respectively (Table 1). The results of additional limited digestion trials performed at 0 °C demonstrated that Phe^{331} and Tyr^{444} were the initial cleavage sites (data not shown). Chymotrypsin cleavage at Leu^{194} and Phe^{219}, indicated by SDS bands 7 and 5, respectively, likely resulted from subsequent digestion of band 2 and/or 4. Notably, the presence of compound **5** initially reduced the proteolysis of Luc at Phe^{331} and Tyr^{444} to barely detectable levels (Fig. 3D). The eventual appearance of bands 2 and 8, however, indicated the limited accessibility of the Tyr^{444} cleavage site. Remarkably, one of the first sites of trypsin cleavage of TY1 was at Arg^{424}. The equivalent Luc residue is Lys^{445}, which is adjacent to the Luc chymotrypsin cleavage site at Tyr^{444}. These Luc amino acids are part of the A8 β-hairpin motif (residues 442–449), which were shown to be involved in catalysis of the oxidative half-reaction.[22] Based on modeling [22,29] and X-ray studies, [11–14,23] this motif is ~30 Å from the active site for adenylate formation. Possibly, domain rotation to move the β-hairpin motif into place to form a new active site, and local conformational changes within the motif, account for the protection against proteolysis observed in the presence of compound **5**.

Taken together, the kinetic and limited proteolysis results reported here support the hypothesis that Luc forms LH_2-AMP and then undergoes a conformational change(s) to carry out the subsequent oxidative reaction. 5′-*O*-[(*N*-Dehydroluciferyl)-sulfamoyl]-adenosine (**5**) should be a valuable reagent for use in planned structural studies designed to identify the Luc residues that participate in LH_2-AMP binding. Current research is in progress to further evaluate this new inhibitor in an investigation of the role of conformational changes in Luc catalysis.

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Supplementary data

Details of materials, general methods, chemical syntheses, and characterizations are provided in a separate file, available online. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.05.115.

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