False Positives in a Reporter Gene Assay: Identification and Synthesis of Substituted *N*-Pyridin-2-ylbenzamides as Competitive Inhibitors of Firefly Luciferase

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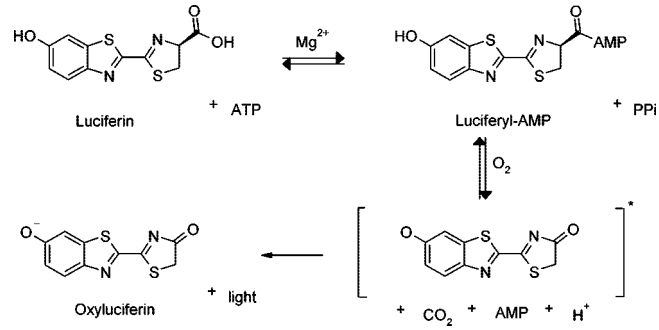
Luciferase reporter-gene assays are a commonly used technique in high-throughput screening campaigns. In this study, we report on a luciferase inhibitor (**1**), which emerged from an antagonistic G protein-coupled receptor luciferase reporter-gene assay screen. Instead of displaying receptor activity, compound **1** was shown to potently inhibit luciferase in an in vitro enzymatic assay with an IC_{50} value of 1.7 ± 0.1 μM. In addition, **1** was a competitive inhibitor with respect to the substrate luciferin. A database search yielded another inhibitor (**3**) with a similar *N*-pyridin-2-ylbenzamide core. Subsequently, several analogues were prepared to investigate the structure−activity relationships of these luciferase inhibitors. This yielded the most potent inhibitor of this series (**6**) with an IC_{50} value of 0.069 ± 0.01 μM. Further molecular modeling studies suggested that **6** can be accommodated in the luciferin binding site. This paper is meant to alert users of luciferase reporter-gene assays for possible false positive hits including highly "druglike" molecules due to direct luciferase inhibition.

Introduction

Firefly luciferase has a long history of use in biology. This enzyme catalyzes the formation of luciferyl adenylate from the substrates luciferin and ATP[a] (Scheme 1).[1] The luciferyl adenylate is oxidized and converted to an electronically excited state of oxyluciferin. Return to the ground state results in the emission of visible light with a wavelength of approximately 562 nm. The cloning of firefly luciferase in 1985 generated a great deal of interest in possible applications of the gene as a tool in scientific research.[2,3] For example, luciferase has been proposed as a model for the μ-opioid receptor, as there are structural similarities between the catalytic site of the enzyme and the opioid binding site of the receptor.[4] Nowadays, luciferase is applied widely as a reporter gene in high throughput screening processes because of its high sensitivity and ease of use.[5] Reporter assays couple the biological activity of a target to the expression of a variety of readily detected enzymes and thereby provide a highly amplified signal. It should be noted that the luciferin−luciferase reaction has been shown to be inhibited strongly by the products oxyluciferin and AMP.[6,7] In addition, many substrate-like compounds such as luciferin[8] and ATP analogues,[7] but also dissimilar compounds such as pifithrin-α,[9] lipoic acid,[10] and *N*-tosylphenylalanine chloromethyl ketone (TPCK),[11] have been shown to inhibit luciferase activity.

An in-house antagonistic luciferase reporter-gene assay screen resulted in a high amount of false positive hits. In the present study, we report that compound **1**, which emerged from that screen, is a highly potent competitive luciferase inhibitor with respect to one of the substrates, luciferin. Very recently, luciferase inhibitors in the Pubchem database were described in a paper by Auld and co-workers.[12,13] That library contained several structural analogues of **1** of which one compound was a highly potent luciferase inhibitor (**3**). Therefore, different *N*-pyridin-2-ylbenzamide analogues (**4**−**12**, **22**−**39**) were prepared to shed light on the molecular requirements for luciferase inhibition. In addition, the most potent inhibitor (**6**) was docked into the crystal structure of luciferase at the luciferin binding site, suggestive of its competitive nature. Since these compounds are druglike molecules, it should be taken into account that "false positives" can easily emerge when luciferase activity is used as a readout in high-throughput screens.

Scheme 1. Luciferase-Catalyzed Reaction[a]



[a] In the presence of ATP, luciferin is activated to luciferyl-AMP, which is oxidized by O_2 to produce an excited state of oxyluciferin (∗). On return to the ground state, light is emitted.

Results and Discussion

Chemistry. The initial active compound, *N*-quinolin-2-ylbenzamide (**1**), was obtained from the reaction of 2-aminoquinoline with benzoyl chloride in pyridine.[14] On the basis of the interesting behavior of quinoline **1** and the more active

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[a] Abbreviations: AID, assay identifier number in PubChem data repository; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin, CID, compound identifier number in PubChem data repository; DCM, dichloromethane; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; K_M, substrate concentration at half-maximal reaction rate; IC_{50}, inhibitory concentration (50%); rmsd, root-mean-square deviation; SEM, standard error of the mean; TLC, thin-layer chromatography; V_{max}, maximal reaction rate.

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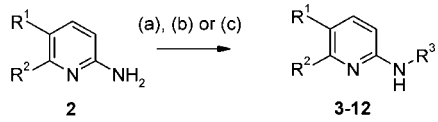
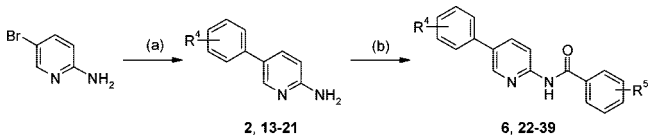
Scheme 2. Synthetic Route to the Compounds[a]



2a. R^1 = Ph R^2 = H
2b. R^1 = H R^2 = Ph
2c. R^1 = CH_3 R^2 = H
2d. R^1 = H R^2 = H

[a] (a) **3**−**7**, **9**, **11**, and **12**: acid chlorides, pyridine. (b) **8**: benzyl chloride, pyridine, MW, 180 °C, 2 h. (c) **10**: phenyl isocyanate, dioxane.

Scheme 3. Synthetic Route to the *N*-(5-Phenylpyridin-2-yl)benzamides **22**−**39**[a]



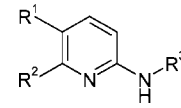
[a] (a) (subst) phenylboronic acid, Na_2CO_3, $Pd(OAc)_2$, DMF, H_2O, 110°C; (b) (subst) benzoyl chloride, pyridine.

pyridine **3**[13] in the luciferase assay, *N*-pyridin-2-ylbenzamide analogues (**3**−**12**, **22**−**39**) were synthesized (Schemes 2 and 3). A Suzuki coupling between phenylboronic acid and 2-amino-5-bromopyridine gave the 2-amino-5-phenylpyridine (**2a**).[15] The 2-amino-5-phenylpyridine (**2a**), the commercially available 2-amino-6-phenylpyridine (**2b**), 2-amino-5-methylpyridine (**2c**), and 2-aminopyridine (**2d**) were benzoylated in pyridine at room temperature to the desired *N*-pyridin-2-ylbenzamides (**3**−**7**). A small library was designed around the privileged *N*-(5-phenylpyridin-2-yl)benzamide (**6**). The benzamide function of **6** was replaced by a benzylamine (**8**), phenylurea (**10**),[16] phenyl carbamate (**9**), butanamide (**11**), and cyclohexanecarboxamide (**12**). The benzylamine **8** was obtained from the reaction of 2-aminopyridine **2a** and benzyl chloride in the microwave reactor at 180 °C. A reaction with phenyl isocyanate and **2a** yielded the phenylurea analogue (**10**). Compounds **9**, **11**, and **12** were obtained by the reaction of **2a** with the appropriate acid chlorides. Introduction of substituents on the phenyl rings of **6** according to the Topliss system of substitution[17] was done by Suzuki coupling and acylation as described above, resulting in compounds **22**−**39** (Table 2).

Structure−**Activity Relationships.** In an initial screen, several compounds were tested for activity at a certain GPCR using the firefly luciferase reporter-gene system (data not shown). This resulted in a high amount of apparent receptor antagonists. We wondered whether some of these compounds were luciferase inhibitors rather than receptor antagonists, yielding false positive hits, an observation that has also been reported in a high-throughput screen for antibacterials with the same reporter gene.[18] Therefore, we tested the "active" compounds in an in vitro enzymatic luciferase assay as described in the Experimental Section. This led to the discovery of **1** that displayed significant enzyme inhibition with an IC_{50} value of 1.7 ± 0.1 μM (Figures 1 and 2).

Because the luciferase assay is based on a readout of light production at 562 nm, quenching could cause false positive hits. Therefore, the absorbance spectra (240−600 nm) were determined for D-luciferin, **1**, and "Nile red", which was identified as a luciferase inhibitor in the antibacterial screen mentioned above.[18] As expected from its structure (and naming), the latter compound was a quencher of the luciferase signal with an absorption peak around 550 nm. On the other hand, both the endogenous substrate and compound **1** did not show any absorption at 350 nm and higher (data not shown).

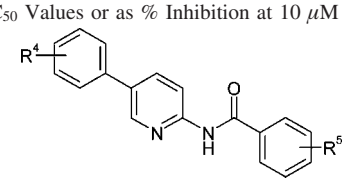
Table 1. Inhibition of Luciferase Activity by Compounds **4**−**12**, Expressed as IC_{50} Values or as % Inhibition at 10 μM



Compound	R^1	R^2	R^3	IC_{50} (μM) or % inh.[a]
4	H	H		27 % (26-28)
5	H	Ph		30 % (29-31)
6	Ph	H		0.069 ± 0.01
7	Me	H		6.4 ± 0.4
8	Ph	H		20 % (19-21)
9	Ph	H		0 % (-3-3)
10	Ph	H		20 % (16-24)
11	Ph	H		22 % (22-23)
12	Ph	H		2.4 ± 0.1

[a] Inhibition of luciferase activity ($IC_{50} \pm$ SEM (μM), $n = 3$, duplicate) or % inhibition at 10 μM concentrations ($n = 2$, duplicate).

Table 2. Inhibition of Luciferase Activity by Compounds **6**, **22**−**39**, Expressed as IC_{50} Values or as % Inhibition at 10 μM



compd	R^4	R^5	IC_{50} (μM) or % inh[a]
6	H	H	0.069 ± 0.01
22	4-Cl	H	0.56 ± 0.02
23	4-OMe	H	0.31 ± 0.02
24	4-Me	H	51% (47−55)
25	3-Cl	H	38% (34−42)
26	4-$N(Me)_2$	H	14% (12−16)
27	4-O^{i}Pr	H	38% (27−48)
28	4-^{t}Bu	H	49% (46−53)
29	4-CF_3	H	39% (35−42)
30	3,4-diCl	H	4% (0−9)
31	H	4-Cl	0% (0−0)
32	H	4-OMe	28% (27−28)
33	H	4-Me	18% (17−19)
34	H	3-Cl	0.16 ± 0.01
35	H	3,4-diCl	13% (11−16)
36	H	2,4-diOMe	26% (22−30)
37	H	4-NH_2	1.2 ± 0.05
38	H	4-O^{i}Pr	12% (8−17)
39	H	4-$N(Me)_2$	35% (29−42)

[a] Inhibition of luciferase activity ($IC_{50} \pm$ SEM (μM), $n = 3$, duplicate) or % inhibition at 10 μM concentrations ($n = 2$, duplicate).

Further studies were undertaken to investigate the pharmacological characteristics of compound **1**. Therefore, the Michaelis−Menten kinetics of luciferin in the absence and presence of two concentrations of **1** were examined (Figure 3a). Saturation of luciferase activity by increasing concentrations of luciferin resulted in a K_M value of 12 ± 2 μM in the absence

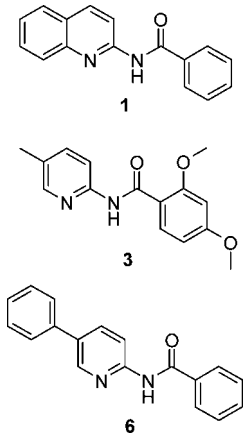


Figure 1. Chemical structures of compounds **1**, **3**, and **6**.

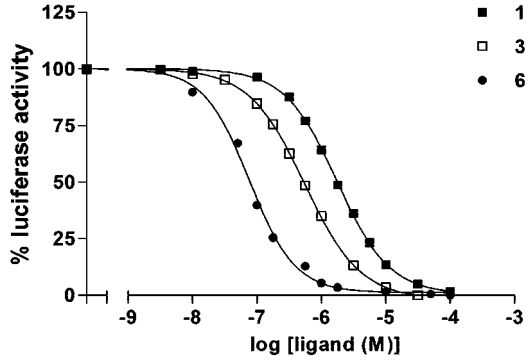


Figure 2. Inhibition of luciferase activity by compounds **1**, **3**, and **6**. The IC_{50} values of **1**, **3**, and **6** were 1.7 ± 0.1, 0.61 ± 0.09, and 0.069 ± 0.01 μM, respectively. Representative graphs are from one of three experiments performed in duplicate.

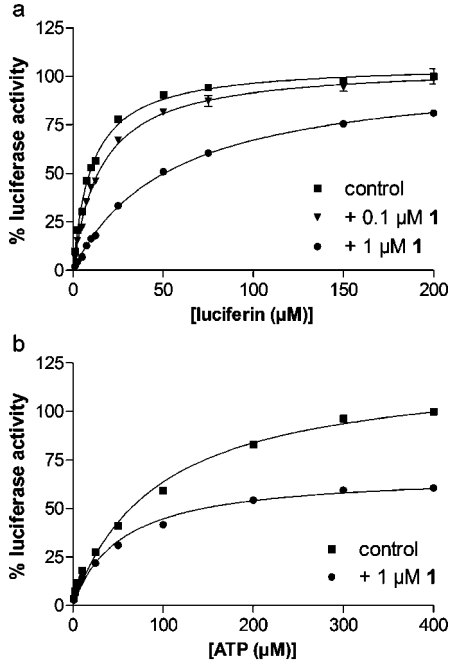


Figure 3. Saturation of luciferase activity by (a) luciferin or (b) ATP in the absence (control) or presence of 0.1 or 1 μM **1**. Representative graphs are from one experiment performed in duplicate (see Table 3 for K_M and V_{max} values).

of inhibitor (Table 3). In the presence of 0.1 or 1 μM **1**, the K_M value significantly increased while the V_{max} was unchanged. This indicated that compound **1** competitively inhibited the action of luciferin. As luciferase has a second catalytic site for ATP, the Michaelis−Menten kinetics of ATP in the absence and presence of compound **1** were examined as well (Figure 3b). The presence of 1 μM **1** resulted in a significantly decreased V_{max} value (Table 3), proof for a noncompetitive inhibition of ATP. Hence, **1** appears to solely compete for the luciferin binding site at luciferase. Notably, the ATP saturation curves were best analyzed by a two-site binding model ($p < 0.0001$). These results support earlier reports of two different ATP binding sites in the enzyme.[7,19] At one of these sites the Mg−ATP complex is bound and at the other ATP, where the latter is thought to promote the release of product.[20] The second site was therefore described as an allosteric site with positive cooperativity.

In our search for further evidence of direct luciferase inhibitors, we analyzed screening data deposited at the PubChem database (luciferase profiling assay AID 411).[13] Interestingly, one of the most active compounds (CID 649849) also contained an *N*-pyridin-2-ylbenzamide core and had an IC_{50} value of 0.25 μM in their assay (**3**, Figure 1). This compound was synthesized by us and tested in the luciferase assay described here, which yielded a similar IC_{50} value of 0.61 ± 0.09 μM (Figure 2). For compounds **1** and **3** the Hill coefficients of the inhibition curves were 1.07 ± 0.02 and 1.02 ± 0.02, respectively, which indicates that the binding of these ligands is independent of the presence of any other substrates.

Subsequently, analogues of *N*-pyridin-2-ylbenzamide (**4**−**12**) were synthesized and tested for luciferase inhibition to explore the structure−activity relationships of this compound class (Table 1). Replacement of the quinoline ring system of **1** for a pyridine ring (**4**) resulted in a dramatic loss of potency. Subsequent introduction of a 2-phenyl ring (**5**) did not improve the potency. Substitution with a 3-phenyl ring, however, resulted in a highly potent luciferase inhibitor (**6**) (Figure 2). This compound had an IC_{50} value of 0.069 ± 0.01 μM that was 10-fold lower than that of compound **3**. Interestingly, introduction of a *p*-methyl substituent as found in **3** decreased the potency by 100-fold (**7**, $IC_{50} = 6.4 \pm 0.4$ μM). Apparently, either the 2,4-dimethoxy substituted phenyl ring on the right-hand side or the phenyl-substituted pyridine ring was important for high potency. In addition, the effect of different linkers between the pyridine and the phenyl ring was studied. As follows from Table 1, neither the amine (**8**) nor the carbamic acid ester (**9**) nor the urea (**10**) linker resulted in an increase in potency. On the contrary, compounds **8**−**10** showed negligible inhibition, if any. With the amide as the preferred linker, two alkyl substituents were tested. A cyclohexyl group (**12**), but not an *n*-propyl group (**11**), also resulted in a potent luciferase inhibitor, although approximately 4-fold less potent than compound **3** (Table 1). Apparently, a larger substituent, either alkyl (**12**) or aryl (**6**), is preferred in the binding pocket of the enzyme.

The design of other analogues was based on a Topliss approach in which either one of the aromatic rings was modified with various substituents (Table 2).[17] In general, modifications both on the 5-phenyl ring (**22**−**30**) and on the 2-phenylamide group (**31**−**39**) of the pyridine ring resulted in a loss of potency in comparison to **6**. Apparently, the binding pocket does not tolerate any substituents on either one of the phenyl rings, indicating steric hindrance. Four compounds, however, showed inhibition of luciferase in the high nanomolar to low micromolar concentration range. On the 5-phenyl ring, introduction of a 4-chloro (**22**) or 4-methoxy (**23**) substituent resulted in IC_{50} values of 0.56 ± 0.02 or 0.31 ± 0.02 μM, respectively.

Table 3. Saturation of Luciferase Activity by Luciferin and ATP in the Absence and Presence of 0.1 or 1 μM Compound **1**, Represented by K_M and V_{max} Values[a]

	luciferin[b]		ATP[c]			
	K_M (μM)	V_{max} (%)	K_{M1} (μM)	V_{max1} (%)	K_{M2} (μM)	V_{max2} (%)
control	12 ± 1	100 ± 2	3.1 ± 0.3	100 ± 39	138 ± 16	100 ± 22
+0.1 μM **1**	16 ± 0.8*	96 ± 0.8	ND	ND	ND	ND
+1 μM **1**	53 ± 4***	95 ± 6	3.8 ± 0.5	87 ± 29	94 ± 4**	56 ± 13*

[a] Values are the mean (±SEM) of three separate assays performed in duplicate: (*) $p < 0.05$; (**) $p < 0.01$, (***) $p < 0.001$ versus control. [b] Saturation of luciferase activity by increasing concentrations of luciferin at 90 μM ATP. [c] Saturation of luciferase activity by increasing concentrations of ATP at 150 μM luciferin. ND: not determined.

Interestingly, the same substituents on the other side of the pyridine ring (almost) completely abolished the activity. This may indicate that these luciferase inhibitors bind in a certain pocket with specific sites of interaction. In addition, introduction of a 3-chloro (**34**) or 4-amino (**37**) substituent on the 2-phenylamide group resulted in IC_{50} values of 0.16 ± 0.01 or 1.2 ± 0.05 μM, respectively.

Finally, compound **6** was docked into a homology model of firefly luciferase based on its crystal structure including AMP and oxyluciferin.[21] From Figure 4, it becomes clear that AMP and oxyluciferin bind in two different pockets at the enzyme. Compound **6** was docked into the AMP and the oxyluciferin pocket. Interestingly, the best model was obtained when the binding pocket of compound **6** overlapped with that of oxyluciferin. There are two reasons for that. First, the binding pocket of AMP is curved while the pocket of oxyluciferin is flat. A largely planar compound as **6** is therefore accommodated best by the latter pocket. Second, when the phenyl ring attached to the amide group is superimposed on the thiazole ring of oxyluciferin, the "other" phenyl ring of **6** extends into an available pocket in the enzyme. The results obtained with this docking study therefore correspond with the competitive and noncompetitive inhibition of **1** that was found with respect to luciferin and ATP, respectively (Figure 3). In addition, from Figure 4 it follows that the further introduction of substituents on both phenyl rings would cause steric hindrance, which may explain the fact that the unsubstituted compound **6** is the most potent inhibitor.

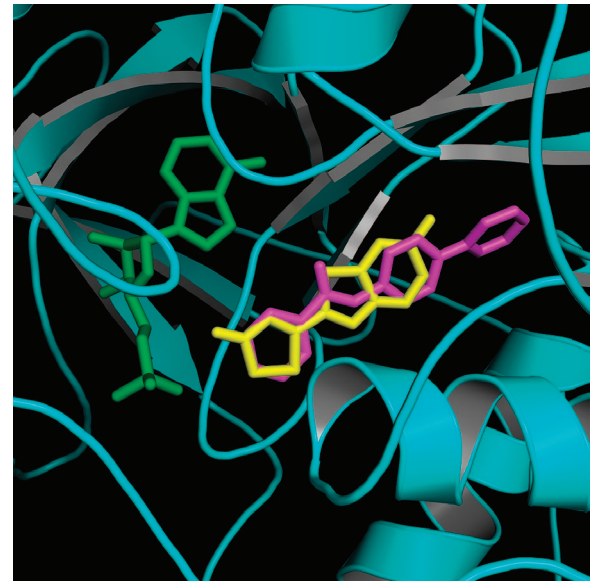


Figure 4. Docking results obtained using Autodock, showing firefly luciferase homology model (cyan) with AMP (green), oxyluciferin (yellow), and compound **6** (magenta). For details of the modeling procedure, see the Experimental Section.

Conclusion

In summary, we have shown that (druglike) compounds, such as **1** or **6**, are competitive inhibitors of luciferase with respect to luciferin. In addition, these compounds are noncompetitive inhibitors with respect to ATP. The inclusion of similar compounds would result in a high number of "false positives" in screening campaigns that rely on luciferase reporter-gene assays, an otherwise robust screening approach with good signal-to-noise ratio. Notably, we learned that the same structure of **1** has been patented for osteoporosis treatment.[22,23] However, also in these disclosures a luciferase reporter-gene assay was used to identify the compounds. The data presented in this paper are therefore meant to warn researchers of direct luciferase inhibitors that also have druglike properties. Such compounds may be "flagged" after their evaluation in an assay with, for example, purified luciferase.

Experimental Section

Chemistry: Material and Methods. All reagents used were obtained from commercial sources, and all solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (^{1}H NMR, 200 MHz; ^{13}C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm), and the following abbreviations are used: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, br = broad, Ar = aromatic protons. Elemental analyses were performed by the Leiden Institute of Chemistry and are within 0.4% of the theoretical values unless otherwise stated. Reactions were routinely monitored on TLC using Merck silica gel F_{254} plates. Microwave reactions were performed in an Emrys Optimizer (Biotage AB, formerly Personal Chemistry). Wattage was automatically adjusted to maintain the desired temperature. The yields of all products were not optimized. All the final products were purified by column chromatography.

General Procedure for the Preparation of 1, 3−7, 9, 11, 12, and 22−39.[24] The appropriate acid chloride (1.1 mmol) was added to a solution of 2-amino-5-phenylpyridine (1.0 mmol) in pyridine (4 mL) at room temperature under a nitrogen atmosphere. According to TLC the reaction went to completion after 2 h. The organic material was extracted with DCM, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by column chromatography, eluting with a mixture of 0.5−2% methanol and chloroform or 0.5−2% methanol and dichloromethane, resulting in a yield of 31−75% of the desired products.

***N*-Quinolin-2-ylbenzamide (1).** Starting from 2-aminoquinoline.[1] Yield 31%; white solid, recrystallized from ethanol. ^{1}H NMR δ ($CDCl_3$): 8.99 (br s, 1H, N*H*), 8.59 (d, 1H, J = 9.1 Hz, quinoline-*H*), 8.21 (d, 1H, J = 8.8 Hz, quinoline-*H*), 8.01−7.96 (m, 2H, phenyl-*H*), 7.84−7.77 (m, 2H, quinoline-*H*), 7.66 (dd, 1H, J^1 = 8.4 Hz, J^2 = 1.5 Hz, quinoline-*H*), 7.56−7.41 (m, 4H, phenyl-*H* + quinoline-*H*). ^{13}C NMR δ ($CDCl_3$): 151.1, 146.5, 141.0, 138.5, 134.1, 132.3, 129.9, 129.2, 128.7, 127.5, 127.2, 125.1, 114.3, 114.1. Anal. ($C_{16}H_{12}N_2O$·0.05EtOH) C, H, N.

2,4-Dimethoxy-*N*-(5-methylpyridin-2-yl)benzamide (3). Starting from 2-amino-5-methylpyridine. Yield 54%; white solid. ^{1}H NMR δ ($CDCl_3$): 10.17 (br s, 1H, N*H*), 8.31 (d, 1H, J = 8.4 Hz, pyridine-*H*), 8.24 (d, 1H, J = 9.1 Hz, Ar-*H*), 8.13 (s, 1H, pyridine-

H), 7.52 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 6.63 (dd, 1H, $J^1 = 8.8$ Hz, $J^2 = 2.2$ Hz, Ar-*H*), 6.53 (d, 1H, $J = 2.2$ Hz, Ar-*H*), 4.04 (s, 3H, OC*H*$_3$), 3.86 (s, 3H, OC*H*$_3$), 2.29 (s, 3H, C*H*$_3$). ^{13}C NMR δ ($CDCl_3$): 163.7, 162.9, 158.6, 149.7, 147.5, 138.4, 133.8, 128.3, 114.0, 113.8, 105.0, 98.2, 55.9, 55.2, 17.5. Anal. ($C_{15}H_{16}N_2O_3$) C, H, N.

***N*-(5-Phenylpyridin-2-yl)benzamide (6).** Yield 46%; white solid. ^{1}H NMR δ ($CDCl_3$): 8.83 (br s, 1H, N*H*), 8.50−8.46 (m, 2H, pyridine-*H*), 8.00−7.93 (m, 3H, pyridine-*H* + phenyl-*H*), 7.58−7.38 (m, 8H, phenyl-*H*). ^{13}C NMR δ ($CDCl_3$): 166.3, 150.9, 145.7, 137.0, 136.6, 134.5, 132.5, 131.9, 128.8, 128.5, 127.6, 127.4, 126.4, 114.1. Anal. ($C_{18}H_{14}N_2O \cdot 0.04CHCl_3$) C, H, N.

***N*-[5-(4-Chorophenyl)pyridin-2-yl]benzamide (22).** Yield 53%; white solid. ^{1}H NMR δ ($CDCl_3$): 8.86 (br s, 1H, N*H*), 8.48 (d, 1H, $J = 8.76$ Hz, pyridine-*H*), 8.43 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.97−7.91 (m, 3H, pyridine-*H* + phenyl-*H*), 7.59−7.40 (m, 7H, phenyl-*H* + Ar-*H*). ^{13}C NMR δ ($CDCl_3$): 165.8, 150.9, 145.6, 136.5, 135.5, 134.2, 133.8, 132.0, 131.4, 129.0, 128.6, 127.7, 127.2, 113.9. Anal. ($C_{18}H_{13}ClN_2O \cdot 0.04CHCl_3$) C, H, N.

***N*-[5-(4-Methoxyphenyl)pyridin-2-yl]benzamide (23).** Yield 68%; white solid. ^{1}H NMR δ ($CDCl_3$): 8.80 (br s, 1H, N*H*), 8.47−8.43 (m, 2H, pyridine-*H*), 7.97−7.90(m, 3H, pyridine-*H* + phenyl-*H*), 7.62−7.48 (m, 5H, phenyl-*H* + Ar-*H*), 7.00 (d, 2H, $J = 8.7$ Hz, Ar-*H*), 3.86 (s, 3H, OC*H*$_3$). ^{13}C NMR δ ($CDCl_3$): 165.8, 159.3, 150.2, 145.3, 136.1, 134.3, 132.4, 131.9, 129.5, 128.5, 127.5, 127.2, 114.3, 113.9, 55.1. Anal. ($C_{19}H_{16}N_2O_2 \cdot 0.06CHCl_3$) C, H, N.

3-Chloro-*N*-(5-phenylpyridin-2-yl)benzamide (34). Yield 59%; white solid. ^{1}H NMR δ ($CDCl_3$): 8.74 (br s, 1H, N*H*), 8.51 (dd, 1H, $J^1 = 2.2$ Hz, $J^2 = 0.7$ Hz, pyridine-*H*), 8.44 (dd, 1H, $J^1 = 8.4$ Hz, $J^2 = 0.7$ Hz, pyridine-*H*), 8.01 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.96 (dd, 1H, $J^1 = 3.5$ Hz, $J^2 = 1.8$ Hz, Ar-*H*), 7.83−7.78 (m, 1H, Ar-*H*), 7.60−7.35 (m, 7H, Ar-*H* + phenyl-*H*). ^{13}C NMR δ (DMSO): 164.7, 151.4, 145.8, 136.8, 133.3, 131.7, 130.3, 129.1, 128.0, 127.8, 126.8, 126.5, 114.7. Anal. ($C_{18}H_{13}ClN_2O$) C, H, N.

4-Amino-*N*-(5-phenylpyridin-2-yl)benzamide (37). Yield 33%; off-white solid. ^{1}H NMR δ ($CDCl_3$): 8.67 (br s, 1H, N*H*), 8.49−8.43 (m, 2H, pyridine-*H*), 7.95 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*), 7.78 (d, 2H, $J = 8.4$ Hz, Ar-*H*), 7.50−7.33 (m, 5H, phenyl-*H*), 6.70 (d, 2H, $J = 8.8$ Hz, Ar-*H*), 4.09 (br s, 2H, N*H*$_2$). ^{13}C NMR δ (DMSO): 165.7, 152.6, 152.1, 145.5, 137.0, 136.0, 130.9, 129.9, 129.2, 127.7, 126.4, 120.2, 114.3, 112.7. Anal. ($C_{18}H_{15}N_3O$) C, H, N.

Biology: Material and Methods. D-Luciferin was purchased from Duchefa (Haarlem, The Netherlands). Adenosine 5′-triphosphate (ATP), luciferase (*Luciola mingrelica*),[25] and bovine serum albumin (BSA, fraction V) were bought from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). All other chemicals were obtained from standard commercial sources.

All luciferase assays were performed according to a slightly adjusted protocol from Sigma-Aldrich (EC 1.13.12.7). In short, inhibition assays were performed using 32 pM firefly luciferase in buffer A (1 M glycine-Tris buffer containing 10 mM EDTA and 100 mM $MgSO_4$, pH 7.8) and 50 μM D-luciferin and 90 μM ATP in buffer B (50 mM glycine-Tris buffer supplemented with 5 mM $MgSO_4$, 0.5 mM EDTA, 0.1% (w/v) BSA, and 0.1% (w/v) sodium azide, pH 7.8), incubated with either a single concentration of inhibitor (10 μM) or 11 concentrations of inhibitor. Nonspecific luciferase activity was determined in the presence of 100 μM **1**. Saturation assays with respect to D-luciferin were performed using different luciferin concentrations (1−200 μM). Saturation assays with respect to ATP were performed under equal conditions, where the ATP concentration varied from 1 to 400 μM and the concentration luciferin was fixed at 150 μM. Typically, a well contained 80 μL of luciferin/ATP solution, 10 μL of buffer B or inhibitor, and 10 μL of luciferase solution. After 30 min of incubation in the dark at room temperature, the luminescence signal was quantified on a Microbeta Trilux 1450 luminescence counter (PerkinElmer, Groningen, The Netherlands).

Data Analysis. All enzymatic data were analyzed using the nonlinear regression curve-fitting program GraphPad Prism, version 5.00 (GraphPad Software Inc., San Diego, CA). Inhibitory binding constants (IC_{50}) were directly obtained from the concentration−effect curves. The K_M and V_{max} values of luciferin and ATP in the absence or presence of **1** were obtained by computer analysis of one- or two-site saturation curves, respectively. All values obtained are the mean values of three independent experiments performed in duplicate.

Docking Studies. The crystal structure of *Luciola cruciata* complexed with oxyluciferin and AMP was retrieved from the Brookhaven Protein Databank (PDB entry 2D1R).[21] Sequence alignment of *Luciola cruciata* and *Luciola mingrelica* was performed using CLUSTALW.[26] The structural homology models were created using InsightII 98 (San Diego, CA).

Docking simulations were performed with AutoDock 3.[27] Grid maps of 20 Å × 20 Å × 20 Å representing the protein were calculated with AutoGrid. Docking simulations were carried out using the Lamarckian genetic algorithm, with an initial population of 100 individuals, a maximum number of 10 000 000 energy evaluations, and a maximum number of 50 000 generations.[27] Resulting orientations lying within 1.5 Å in the rmsd were clustered together. Finally the configuration with the most favorable free energy of binding was further optimized by 1500 energy minimization steps with InsightII. PyMOL, version 1.0 (DeLano Scientific, Palo Alto, CA), was used to superimpose and visualize the model.

Supporting Information Available: Experimental details of the synthesis of the compounds described in this paper, their ^{1}H NMR and ^{13}C NMR spectroscopic data, and their elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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