Allosteric Modulation of 'Reproductive' GPCRs : a case for the GnRH and LH receptors

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CHAPTER 7

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

Luciferase reporter-gene assays are a commonly used technique in high-throughput screening campaigns. In this study, we report a luciferase inhibitor (**1**), which emerged from a 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-yl-benzamide core. Subsequently, several analogs 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 'drug-like' molecules due to direct luciferase inhibition.

This chapter is an adjusted version of a recent publication:

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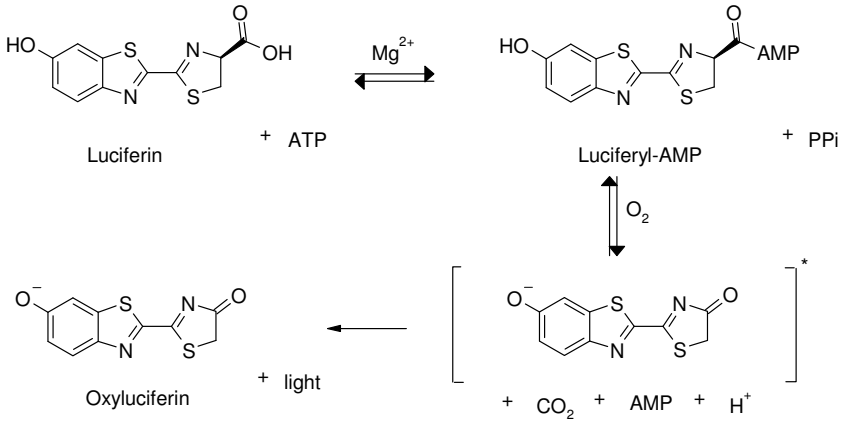
7.1 INTRODUCTION

Firefly luciferase has a long history of use in biology. This enzyme catalyses the formation of luciferyl-adenylate from the substrates luciferin and ATP (Scheme 7.1).[300] 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.[301,302] For example, luciferase has been proposed as a model for the μ-opioid receptor, as there are



Scheme 7.1 The luciferase-catalyzed reaction. 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.

structural similarities between the catalytic site of the enzyme and the opioid binding site of the receptor.[303] Nowadays, luciferase is applied widely as a reporter gene in high throughput screening processes, because of its high sensitivity and ease of use.[304] 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.[305,306] In addition, many substrate-like compounds such as and ATP-[306] and luciferin-analogues,[307] but also dissimilar compounds, such as pifithrin-α[308], lipoic acid[309] and N-tosylphenylalanine chloromethyl ketone (TPCK)[310] have been shown to inhibit luciferase activity.

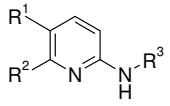
In an initial screen, several compounds were tested for activity at the human LH receptor using a radioligand dissociation assay (*Chapter 5* and *Chapter 6*) and the firefly luciferase reporter gene system (data not shown). In the latter case, this resulted in a high amount of false positive hits. In 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 coworkers.[198,311] That library contained several structural analogues of **1** of which one compound was a highly potent luciferase inhibitor (**3**). Therefore, different N-pyridin-2-yl-benzamide analogs (**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, in agreement with its competitive nature. Since these compounds are drug-like molecules, it should be taken into account that 'false positives' can easily emerge when luciferase activity is used as a read-out in high-throughput screens.

7.2 MATERIALS AND METHODS

7.2.1 Chemistry – Materials

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 so as to maintain the desired temperature. The yields of the products were not optimized. All the final products were purified by column chromatography.

7.2.2 General Procedure for the Preparation of 1, 3-7, 9, 11 and 12.[312]



The appropriate acid chloride (1.1 mmol) was added to a solution of 2-amino-(substituted)-pyridine (1.0 mmol) in pyridine (4 mL) at room temperature under a nitrogen atmosphere. According to TLC the reaction went to completion after 2 hours. 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.

N-Quinolin-2-yl-benzamide (1). Starting from 2-amino-quinoline.[313] 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-methyl-pyridin-2-yl)benzamide (3). Starting from 2-amino-5-methyl-pyridine. 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-Pyridin-2-yl-benzamide (4). Starting from 2-amino-pyridine. Yield 69%; white solid. ^{1}H-NMR δ (DMSO): 10.81 (br s, 1H, N*H*), 8.41 (d, 1H, J = 2.9 Hz, pyridine-*H*), 8.22 (d, 1H, J = 8.0, pyridine-*H*), 8.07-8.03 (m, 2H, phenyl-*H*), 7.87 (dd, 1H, J^1 = 7.3 Hz, J^2 = 1.5 Hz, pyridine-*H*), 7.66-7.49 (m, 3H, phenyl-*H*), 7.19 (dd, 1H, J^1 = 6.2 Hz, J^2 = 2.2 Hz, pyridine-*H*). ^{13}C-NMR δ ($CDCl_3$): 165.7, 151.4, 147.6, 138.2, 134.1, 132.0, 128.5, 127.1, 119.7, 114.0. Anal. ($C_{12}H_{10}N_2O$) C, H, N.

N-(6-Phenylpyridin-2-yl)benzamide (5). Starting from 2-amino-6-phenyl-pyridine. Yield 64%; white solid. ^{1}H-NMR δ (DMSO): 10.76 (br s, 1H, N*H*), 8.25-8.06 (m, 5H, phenyl-*H* + pyridine-*H*), 7.98-7.73 (m, 2H, pyridine-*H*), 7.60-7.43 (m, 6H, phenyl-*H*). ^{13}C-NMR δ ($CDCl_3$): 165.7, 155.9, 151.2, 139.1, 138.6, 134.2, 132.1, 129.0, 128.7, 128.6, 127.1, 126.7, 116.6, 112.3. Anal. ($C_{12}H_{10}N_2O$) C, H, N.

N-(5-Phenylpyridin-2-yl)benzamide (6). Starting from 2-amino-5-phenyl-pyridine. 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. ($C_{18}H_{14}N_2O$*0.04$CHCl_3$) C, H, N.

N-(5-Methylpyridin-2-yl)benzamide (7). Starting from 2-amino-5-methyl-pyridine.[314] Yield 49%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.93 (br s, 1H, N*H*), 8.29 (d, 1H, J = 8.4 Hz, pyridine-*H*), 7.98-7.89 (m, 3H, phenyl-*H* + pyridine-*H*), 7.58-7.43 (m, 4H, phenyl-*H* + pyridine-*H*), 2.28 (s, 3H, C*H*$_3$). ^{13}C-NMR δ ($CDCl_3$): 166.2, 149.7, 147.3, 138.6, 134.6, 131.4, 128.6, 128.2, 127.3, 113.8, 17.4. Anal. ($C_{13}H_{12}N_2O$) C, H, N.

Phenyl N-(5-phenylpyridin-2-yl)carbamate (9). Starting from phenyl chloroformate. Yield 47%; white solid. ^{1}H-NMR δ ($CDCl_3$): 9.60 (br s, 1H, N*H*), 8.67 (d, 1H, J = 2.6 Hz, pyridine-*H*), 8.13 (d, 1H, J = 8.4 Hz, pyridine-*H*), 7.96 (dd, 1H, J^1 = 8.7 Hz, J^2 = 2.2 Hz, pyridine-*H*), 7.56-7.23 (m, 10H, phenyl-*H*) . ^{13}C-NMR δ ($CDCl_3$): 151.5, 150.6, 150.2, 145.8, 136.9, 132.1, 129.3, 128.8, 127.5, 126.4, 125.7, 121.4, 112.3. Anal. ($C_{18}H_{14}N_2O_2$*0.05$CHCl_3$) C, H, N.

N-(5-Phenylpyridin-2-yl)butanamide (11). Yield 66%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.49 (br s, 1H, N*H*), 8.33-8.29 (m, 2H, pyridine-*H*), 7.92 (dd, 1H, J^1 = 8.8 Hz, J^2 = 2.2 Hz, pyridine-*H*), 7.57-7.37 (m, 5H, phenyl-*H*), 2.40 (t, 2H, J = 7.66 Hz, $COCH_2CH_2CH_3$), 1.84-1.73 (m, 2H, $COCH_2CH_2CH_3$), 1.02 (t, 3H, J = 7.3 Hz, $COCH_2CH_2CH_3$). ^{13}C-NMR δ (DMSO): 172.2, 151.5, 145.7, 136.9, 136.1, 131.0, 129.1, 127.6, 126.3, 113.4, 38.1, 18.5, 13.6. Anal. ($C_{15}H_{16}N_2O$*0.02$CHCl_3$) C, H, N.

N-(5-Phenylpyridin-2-yl)cyclohexanecarboxamide (12). Yield 69%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.49 (d, 1H, J = 2.2 Hz, pyridine-*H*), 8.31 (d, 1H, J = 8.4 Hz, pyridine-*H*), 8.16 (br s, 1H, N*H*), 7.91 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.5 Hz, pyridine-*H*), 7.58-7.37 (m, 5H, phenyl-*H*), 2.36-2.22 (m, 1H, J = 7.66 Hz, C*H*-cyclohexane), 2.02-1.25 (m, 10H, CH_2-cyclohexane). ^{13}C-NMR δ ($CDCl_3$): 174.9, 150.9, 145.4, 136.9, 137.2, 136.7, 132.4, 128.8, 127.5, 126.4, 114.0, 46.1, 29.3, 25.4. Anal. ($C_{18}H_{20}N_2O$ *0.02$CHCl_3$) C, H, N.

7.2.3 Synthesis of N-(5-Phenylpyridin-2-yl)benzylamine (8).

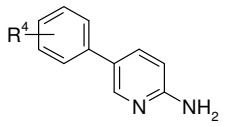
A solution of 2-amino-5-phenyl-pyridine (300 mg, 1.76 mmol) and benzyl chloride (405 µL, 3.52 mmol) in pyridine (3.5 mL) was heated in a closed vessel in the microwave at 180 °C for 2 hours. The reaction mixture was poured in water, adjusted to pH = 10 with 1 M NaOH and extracted with DCM. The combined organic layers were dried over $MgSO_4$, filtered and the solvents were evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using DCM/methanol (99/1), followed by recrystallization from methanol, yielding 78 mg (17% yield) of the crystalline product. Yield 17%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.36 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.65 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.5 Hz, pyridine-*H*), 7.52-7.27 (m, 10H, phenyl-*H*), 6.45 (d, 1H, J = 8.8 Hz, pyridine-*H*), 5.03 (br s, 1H, N*H*), 4.55 (d, 2H, J = 5.9 Hz, CH_2). ^{13}C-NMR δ ($CDCl_3$): 157.9, 146.3, 139.0, 138.3, 136.1, 128.7, 128.5, 127.3, 127.1, 126.5, 126.0, 106.4, 46.2. Anal. ($C_{18}H_{16}N_2$) C, H, N.

7.2.4 Synthesis of 1-Phenyl-3-(5-phenylpyridin-2-yl)urea (10).

The 2-amino-5-phenylpyridine (250 mg, 1.47 mmol) was dissolved in dioxane (2 mL) and phenyl isocyanate (160 µL, 1.47 mmol) was added at room temperature under a nitrogen atmosphere. After 4 hours the formed precipitate was collected by filtration and washed with

chloroform. Yield 84%; white solid. ^{1}H-NMR δ (DMSO): 10.41 (br s, 1H, N*H*), 9.55 (s, 1H, N*H*), 8.64 (d, 1H, J = 1.8 Hz, pyridine-*H*), 8.10 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.5 Hz, pyridine-*H*), 7.73-7.30 (m, 10H, phenyl-*H*), 7.08-7.01 (m, 1H, pyridine-*H*). ^{13}C-NMR δ (DMSO): 152.2, 144.8, 139.1, 136.9, 136.7, 129.5, 129.1, 127.6, 126.3, 122.6, 118.9, 111.9. Anal. ($C_{18}H_{15}N_3O$*0.12$CHCl_3$) C, H, N.

7.2.5 General Procedure for the Preparation of 2a and 13-21.[315]



Under a nitrogen atmosphere 2-amino-5-bromo-pyridine (1.0 equiv), the appropriate substituted boronic acid (1.3 equiv) and Na_2CO_3 (2.6 equiv) were dissolved in a mixture of water and DMF (1:4), followed by the addition of $Pd(OAc)_2$ (0.01 equiv). The reaction mixture was heated at 110 °C for 3 hours. After cooling to room temperature, it was diluted with water and the organic materials extracted with ethyl acetate. The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude products were purified by silica column chromatography, eluting with ethyl acetate or with a 0-3% methanol in dichloromethane mixture.

2-Amino-5-phenylpyridine (2a).[315] Yield 34%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.32 (d, 1H, J = 2.5 Hz, pyridine-*H*), 7.68 (dd, 1H, J^1 = 8.4 Hz, J^2 = 2.5 Hz, pyridine-*H*), 7.52-7.31 (m, 5H, phenyl-*H*), 6.58 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.51 (br s, 1H, NH_2).

5-(4-Chloro-phenyl)-pyridin-2ylamine (13). Yield 26%; off white solid. ^{1}H-NMR δ ($CDCl_3$): 8.28 (d, 1H, J = 2.2 Hz, pyridine-*H*), 7.63 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.2 Hz, pyridine-*H*), 7.45-7.35 (m, 4H, Ar-*H*), 6.57 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.55 (br s, 1H, NH_2).

5-(4-Methoxy-phenyl)-pyridin-2ylamine (14). Yield 64%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.28 (d, 1H, J = 2.5 Hz, pyridine-*H*), 7.62 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.42 (d, 2H, J = 8.8 Hz, Ar-*H*), 6.97 (d, 2H, J = 8.4 Hz, Ar-*H*), 6.56 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.50 (br s, 1H, NH_2), 3.84 (s, 3H, OCH_3).

5-(4-Methyl-phenyl)-pyridin-2ylamine (15). Yield 59%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.34 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.65 (dd, 1H, J^1 = 8.4 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.39 (d, 2H, J = 8.1 Hz, Ar-*H*), 7.23 (d, 2H, J = 8.4 Hz, Ar-*H*), 6.56 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.58 (br s, 1H, NH_2), 2.38 (s, 3H, CH_3).

5-(3-Chloro-phenyl)-pyridin-2ylamine (16). Yield 62%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.29 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.63 (dd, 1H, J^1 = 8.7 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.47 (s, 1H, Ar-*H*), 7.39-7.24 (m, 3H, Ar-*H*), 6.57 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.58 (br s, 1H, NH_2).

5-(4-Dimethylamino-phenyl)-pyridin-2ylamine (17). Yield 52%; yellowish solid. ^{1}H-NMR δ ($CDCl_3$): 8.28 (s, 1H, pyridine-*H*), 7.62 (d, 1H, J = 8.4 Hz, pyridine-*H*), 7.39 (d, 2H, J = 8.7 Hz, Ar-*H*), 7.79 (d, 2H, J = 9.1 Hz, Ar-*H*), 6.55 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.42 (br s, 1H, NH_2), 2.95 (s, 6H, N(CH_3)$_2$).

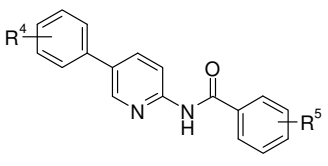
5-(4-Isopropoxy-phenyl)-pyridin-2ylamine (18). Yield 65%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.30 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.61 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.40 (d, 2H, J = 8.4 Hz, Ar-*H*), 6.93 (d, 2H, J = 8.4 Hz, Ar-*H*), 6.55 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.60-4.51 (m, 3H, C*H*(CH_3)$_2$ + NH_2), 1.37 (s, 3H, CH(CH_3)$_2$), 1.34 (s, 3H, CH(CH_3)$_2$).

5-(4-*tert*-Butyl-phenyl)-pyridin-2ylamine (19). Yield 46%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.35 (d, 1H, J = 2.2 Hz, pyridine-*H*), 7.67 (dd, 1H, J^1 = 8.5 Hz, J^2 = 2.4 Hz, pyridine-*H*), 7.45 (m, 4H, Ar-*H*), 6.55 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.55 (s br, 2H, NH_2), 1.35 (s, 6H, C(CH_3)$_3$).

5-(4-Trifluoromethyl-phenyl)-pyridin-2ylamine (20). Yield 45%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.36 (d, 1H, J = 2.2 Hz, pyridine-*H*), 7.71-7.57 (m, 5H, pyridine-*H* + Ar-*H*), 6.60 (d, 1H, J = 8.8 Hz, pyridine-*H*), 4.73 (br s, 1H, NH_2).

5-(3,4-Dichloro-phenyl)-pyridin-2ylamine (21). Yield 62%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.30 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.65-7.56 (m, 2H, Ar-*H* + pyridine-*H*), 7.47 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.30 (dd, 1H, J^1 = 8.3 Hz, J^2 = 2.2 Hz, Ar-*H*), 6.58 (d, 1H, J = 8.4 Hz, pyridine-*H*), 4.72 (br s, 2H, NH_2).

7.2.6 General Procedure for the Preparation of 22-39.[312]



The appropriate acid chloride (1.1 mmol) was added to a solution of 2-amino-5-phenyl-pyridine (1.0 mmol) in pyridine (4 mL) at room temperature under a nitrogen atmosphere. According to TLC the reaction went to completion after 2 hours. 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.

N-[5-(4-Chlorophenyl)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. ($C_{18}H_{13}ClN_2O$*0.04$CHCl_3$) 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. ($C_{19}H_{16}N_2O_2$*0.06$CHCl_3$) C, H, N.

N-[5-(4-Methylphenyl)pyridin-2-yl]benzamide (24). Yield 41%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.89 (br s, 1H, N*H*), 8.46-8.45 (m, 2H, pyridine-*H*), 7.97-7.94(m, 3H, pyridine-*H* + phenyl-*H*), 7.59-7.56 (m, 1H, phenyl-*H*), 7.52-7.49 (m, 2H, phenyl-*H*), 7.46 (d, 2H, J = 8.0 Hz, Ar-*H*), 7.28 (d, 2H, J = 8.0 Hz, Ar-*H*), 2.28 (s, 3H, C*H*$_3$). ^{13}C-NMR δ ($CDCl_3$): 165.8, 150.5, 145.8, 137.7, 136.6, 134.4, 134.3, 133.0, 132.2, 129.7, 128.7, 127.3, 126.6, 114.0, 21.1. Anal. ($C_{19}H_{16}N_2O$) C, H, N.

N-[5-(3-Chlorophenyl)pyridin-2-yl]benzamide (25). Yield 43%; white solid. ^{1}H-NMR δ ($CDCl_3$): 9.07 (br s, 1H, N*H*), 8.50 (d, 1H, J = 8.8 Hz, pyridine-*H*), 8.35 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.96-7.91 (m, 3H, pyridine-*H* + phenyl-*H*), 7.63-7.32 (m, 7H, Ar-*H* + phenyl-*H*). ^{13}C-NMR δ ($CDCl_3$): 166.2, 151.3, 145.7, 138.8, 136.6, 134.7, 134.4, 132.1, 131.1, 130.0, 128.6, 127.5, 127.3, 126.4, 124.5, 113.9. Anal. ($C_{18}H_{13}ClN_2O$*0.14CH_2Cl_2) C, H, N.

N-[5-(4-Dimethylamino-phenyl)pyridin-2-yl]benzamide (26). Yield 75%; off-white solid. ^{1}H-NMR δ ($CDCl_3$): 8.86 (br s, 1H, N*H*), 8.43 (s, 1H, pyridine-*H*), 8.41 (d, 1H, J = 4.7 Hz, pyridine-*H*), 7.97-7.89 (m, 3H, pyridine-*H* + phenyl-*H*), 7.57-7.43 (m, 5H, Ar-*H* + phenyl-*H*), 5.85 (s, 6H, N(C*H*$_3$)). ^{13}C-NMR δ ($CDCl_3$): 165.6, 150.0, 149.5, 144.7, 135.7, 134.3, 132.9, 131.8, 128.5, 127.1, 124.8, 113.9, 112.6, 40.2. Anal. ($C_{20}H_{19}N_3O$*0.02$CHCl_3$) C, H, N.

N-[5-(4-Isopropoxy-phenyl)pyridin-2-yl]benzamide (27). Yield 65%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.87 (br s, 1H, N*H*), 8.46-8.42 (m, 2H, pyridine-*H*), 7.97-8.89 (m, 3H,

phenyl-*H* + pyridine-*H*), 7.58-7.43 (m, 5H, Ar-*H* + phenyl-*H*), 6.98 (d, 2H, J = 9.1 Hz, Ar-*H*), 4.60 (q, 1H, J = 6.2 Hz, OC*H*(CH3)$_2$), 1.39 (s, 3H, CH_3), 1.36 (s, 3H, CH_3). ^{13}C-NMR δ ($CDCl_3$): 166.2, 156.6, 150.4, 145.2, 135.9, 134.6, 132.2, 131.8, 129.1, 128.4, 127.5, 116.0, 114.2, 69.7, 21.8. Anal. ($C_{21}H_{20}N_2O_2$) C, H, N.

N-[5-(4-tert-Butylphenyl)pyridin-2-yl]benzamide (28). Yield 34%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.98 (br s, 1H, N*H*), 8.49-8.43 (m, 2H, pyridine-*H*), 7.99-7.92 (m, 3H, pyridine-*H* + phenyl-*H*), 7.57-7.45 (m, 7H, Ar-*H* + phenyl-*H*), 1.37 (s, 9H, t-butyl). ^{13}C-NMR δ ($CDCl_3$): 166.1, 150.7, 145.6, 136.5, 134.4, 134.1, 132.4, 131.9, 128.5, 127.3, 126.1, 125.8, 114.1, 34.4, 31.1. Anal. ($C_{22}H_{22}N_2O$) C, H, N.

N-{5-[4-(Trifluoromethyl)phenyl]pyridin-2-yl}benzamide (29). Yield 52%; white solid. ^{1}H-NMR δ ($CDCl_3$): 9.11 (br s, 1H, N*H*), 8.52 (d, 1H, J = 8.4 Hz, pyridine-*H*), 8.40 (d, 1H, J = 2.2 Hz, pyridine-*H*), 8.00-7.93 (m, 3H, pyridine-*H* + phenyl-*H*), 7.73-7.47 (m, 7H, Ar-*H* + phenyl-*H*). ^{13}C-NMR δ ($CDCl_3$): 165.9, 151.4, 145.9, 140.6, 136.7, 134.1, 132.1, 131.1, 128.6, 127.2, 126.7, 125.7, 116.0, 113.9. Anal. ($C_{19}H_{13}F_3N_2O$) C, H, N.

N-[5-(3,4-Dichlorophenyl)pyridin-2-yl]benzamide (30). Yield 49%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.74 (br s, 1H, N*H*), 8.51-8.45 (m, 2H, pyridine-*H*), 7.96-7.90 (m, 3H, pyridine-*H* + phenyl-*H*), 7.65-7.37 (m, 6H, Ar-*H* + phenyl-*H*). ^{13}C-NMR δ (DMSO): 166.2, 152.2, 146.1, 137.5, 136.4, 134.0, 132.1, 132.0, 131.1, 130.5, 129.0, 128.4, 128.2, 128.1, 126.6, 114.4. Anal. ($C_{18}H_{12}Cl_2N_2O$ *0.15CH_2Cl_2) C, H, N.

4-Chloro-N-(5-phenylpyridin-2-yl)benzamide (31). Yield 41%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.85 (br s, 1H, N*H*), 8.47-8.43 (m, 2H, pyridine-*H*), 7.98 (d, 1H, J = 8.0 Hz, pyridine-*H*), 7.90 (dd, 2H, J^1 = 8.8 Hz, J^2 = 1.5 Hz, Ar-*H*), 7.58-7.35 (m, 7H, Ar-*H* + phenyl-*H*). ^{13}C-NMR δ (DMSO): 165.1, 151.4, 146.2, 136.9, 136.8, 136.3, 132.9, 131.7, 130.1, 129.2, 128.5, 127.9, 126.5, 114.6, 126.6. Anal. ($C_{18}H_{13}ClN_2O$*0.06$CHCl_3$) C, H, N.

4-Methoxy-N-(5-phenylpyridin-2-yl)benzamide (32). Yield 69%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.93 (br s, 1H, N*H*), 8.49-8.41 (m, 2H, pyridine-*H*), 7.99-7.90 (m, 3H, pyridine-*H* + Ar-*H*), 7.56-7.37 (m, 5H, phenyl-*H*), 6.98 (d, 2H, J = 8.8 Hz, Ar-*H*), 3.90 (s, 3H, OC*H*$_3$). ^{13}C-NMR δ ($CDCl_3$): 165.8, 162.5, 151.2, 145.7, 137.0, 136.5, 132.1, 129.4, 128.8, 127.5, 126.7, 126.4, 114.0, 113.7, 55.2. Anal. ($C_{19}H_{16}N_2O_2$*0.03 $CHCl_3$) C, H, N.

4-Methyl-N-(5-phenylpyridin-2-yl)benzamide (33). Yield 70%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.96 (br s, 1H, N*H*), 8.47 (dd, 1H, J^1 = 8.8 Hz, J^2 = 0.8 Hz, pyridine-*H*), 8.41 (d, 1H, J = 2.2 Hz, pyridine-*H*), 7.96 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.84 (d, 2H, J = 8.0 Hz, Ar-*H*), 7.57-7.27 (m, 7H, Ar-*H* + phenyl-*H*), 2.41 (s, 3H, C*H*$_3$). ^{13}C-NMR δ ($CDCl_3$):

166.2, 151.0, 145.8, 142.5, 137.1, 136.5, 132.3, 131.7, 129.1, 128.8, 127.4, 127.3, 126.4, 113.9, 21.2. Anal. ($C_{19}H_{16}N_2O$) 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.

3,4-Dichloro-N-(5-phenylpyridin-2-yl)benzamide (35). Yield 45%; white solid. ^{1}H-NMR δ (DMSO): 11.17 (br s, 1H, N*H*), 8.75 (s, 1H, pyridine-*H*), 8.32-8.17 (m, 3H, Ar-*H* + pyridine-*H*), 8.02 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.83-7.74 (m, 3H, Ar-*H* + phenyl-*H*), 7.55-7.41 (m, 3H, phenyl-*H*). ^{13}C-NMR δ (DMSO): 163.8, 151.2, 145.8, 136.7, 136.1, 134.8, 134.4, 131.8, 131.4, 130.6, 130.1, 129.1, 128.3, 127.8, 126.5, 114.6. Anal. ($C_{18}H_{12}Cl_2N_2O$) C, H, N.

2,4-Dimethoxy-N-(5-phenylpyridin-2-yl)benzamide (36). Yield 44%; white solid. ^{1}H-NMR δ ($CDCl_3$): 10.32 (br s, 1H, N*H*), 8.55 (d, 1H, J = 2.2 Hz, pyridine-*H*), 8.50 (d, 1H, J^1 = 8.8 Hz, pyridine-*H*), 8.26 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.93 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.6 Hz, pyridine-*H*), 7.60-7.37 (m, 5H, phenyl-*H*), 6.65 (dd, 1H, J^1 = 8.9 Hz, J^2 = 2.6 Hz, Ar-*H*), 6.53 (d, 1H, J = 2.2 Hz, Ar-*H*), 4.06 (s, 3H, OC*H*$_3$), 3.87 (s, 3H, OC*H*$_3$). ^{13}C-NMR δ ($CDCl_3$): 163.8, 163.0, 158.7, 151.2, 145.7, 137.3, 136.2, 133.8, 131.9, 128.8, 127.3, 126.4, 114.1, 113.9, 105.5, 98.2, 55.8, 55.2. Anal. ($C_{20}H_{18}N_2O_3$) 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.

4-Isopropoxy-N-(5-phenylpyridin-2-yl)benzamide (38). Starting from 4-isopropoxy-benzoyl chloride.[316] Yield 32%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.86 (br s, 1H, N*H*), 8.48-8.44 (m, 2H, pyridine-*H*), 7.98-7.87 (m, 3H, Ar-*H* + pyridine-*H*), 7.57-7.37 (m, 5H, phenyl-*H*), 6.95 (d, 2H, J = 8.8 Hz, Ar-*H*), 4.72-4.54 (m, 1H, OC*H*$(CH_3)_2$), 1.38 (s, 3H, OCH(C*H*$_3)_2$), 1.34 (s, 3H, OCH(C*H*$_3)_2$). ^{13}C-NMR δ ($CDCl_3$): 165.8, 161.0, 151.3, 145.7, 137.1, 136.5, 132.1, 129.4, 128.8, 127.4, 126.4, 126.2, 115.1, 114.0, 69.8, 21.6. Anal. ($C_{21}H_{20}N_2O_2$) C, H, N.

4-Dimethylamino-N-(5-phenylpyridin-2-yl)benzamide (39). Starting from the *tert*-butyloxycarbonyl protected 4-amino-benzoyl chloride.[317,318] Yield 52%; white solid. ^{1}H-NMR δ ($CDCl_3$): 8.64 (br s, 1H, N*H*), 8.49-8.46 (m, 2H, pyridine-*H*), 7.95 (dd, 1H, J^1 = 8.6 Hz, J^2 = 2.2 Hz, pyridine-*H*), 7.85 (d, 2H, J = 9.1 Hz, Ar-*H*), 7.60-7.37 (m, 5H, phenyl-*H*), 6.71 (d, 2H, J = 9.1 Hz, Ar-*H*), 3.05 (s, 6H, N(CH_3)$_2$). ^{13}C-NMR δ ($CDCl_3$): 165.9, 152.6, 151.5, 145.8, 137.3, 136.4, 131.8, 129.0, 128.8, 127.4, 126.4, 120.6, 113.7, 110.8, 39.7. Anal. ($C_{20}H_{19}N_3O$*0.10$CHCl_3$) C, H, N.

7.2.7 Biology – Materials

D-Luciferin was purchased from Duchefa (Haarlem, The Netherlands). Adenosine 5'-triphosphate (ATP), luciferase (*Luciola mingrelica*)[319] 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.

7.2.8 Luciferase Inhibition and Saturation Assays

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), either incubated with a single concentration of inhibitor (10 µM) or eleven concentrations of inhibitor. Non-specific 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 - 400 µM and the concentration luciferin was fixed at 150 µM. Typically, a well contained 80 µL luciferin/ATP solution, 10 µL buffer B or inhibitor and 10 µL 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).

7.2.9 Data Analysis

All enzymatic data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA, U.S.A.). 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 means of three independent experiments performed in duplicate.

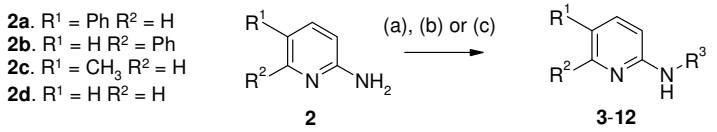
7.2.10 Docking Studies

The crystal structure of *Luciola cruciata* complexed with oxyluciferin and AMP was retrieved from the Brookhaven Protein Databank (PDB entry 2D1R).[320] Sequence alignment of *Luciola cruciata* and *Luciola mingrelica* was performed using CLUSTALW.[321] The structural homology models were created using InsightII 98 (San Diego, CA, USA). Docking simulations were performed with AutoDock 3.[322] Grid maps of 20x20x20 Ångstrom 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.[322] Resulting orientations lying within 1.5 Ångstrom 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 v. 1.0 (DeLano Scientific, Palo Alto, CA, USA) was used to superimpose and visualize the model.

7.3 RESULTS AND DISCUSSION

7.3.1 Chemistry

The initial active compound, N-quinolin-2-yl-benzamide (**1**), was obtained from the reaction of 2-aminoquinoline with benzoyl chloride in pyridine.[313] Based on the interesting behavior of quinoline **1** and the more active pyridine **3**[198] in the luciferase assay, N-pyridin-2-yl-benzamide analogs (**3**-**12**, **22**-**39**) were synthesized (Scheme 7.2 and 7.3). A Suzuki-coupling between phenylboronic acid and 2-amino-5-bromo-pyridine gave the 2-amino-5-phenyl-pyridine (**2a**).[315] The 2-amino-5-phenyl-pyridine (**2a**), the commercially available 2-amino-6-phenyl-pyridine (**2b**), 2-amino-5-methyl-pyridine (**2c**) and 2-amino-pyridine (**2d**) were benzoylated in pyridine at room temperature to the desired N-pyridin-2-yl-benzamides (**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 benzyl amine (**8**), phenyl carbamate (**9**), phenyl urea (**10**),[323] butanamide (**11**) and cyclohexanecarboxamide (**12**). The benzyl amine **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 phenyl urea 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[324] was done by Suzuki-coupling and acylation as described above, resulting in compounds **22**-**39**.

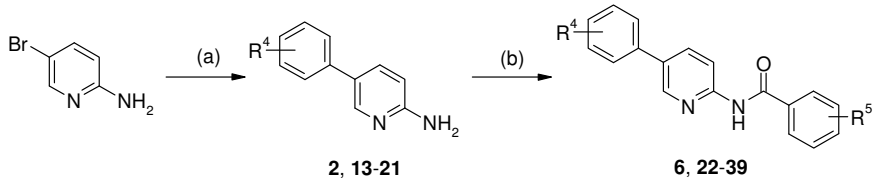


Scheme 7.2 Synthetic route to the compounds (a) **3-7**, **9**, **11** and **12**: acid chlorides, pyridine, (b) **8**: benzyl chloride, pyridine, MW, 180 °C, 2 h, (c) **10**: phenyl isocyanate, dioxane.

7.3.2 Structure-Activity Relationships

A small library of compounds was tested in an in-house luciferase reporter-gene assay screen for antagonistic activity at the human LH receptor (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.[325] 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 (Figure 7.1 and 7.2).



2, **13**-**21** **6**, **22**-**39**

Scheme 7.3 Synthetic route to the N-(5-phenylpyridin-2-yl)benzamides **22**-**39**. (a) (subst.) phenylboronic acid, Na_2CO_3, $Pd(OAc)_2$, DMF, H_2O, 110 °C; (b) (subst.) benzoyl chloride, pyridine.

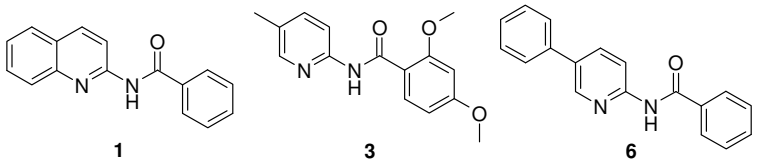


Figure 7.1 Chemical structures of compounds **1**, **3** and **6**.

Table 7.1 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.

	Luciferin[a]		**ATP**[b]			
Condition	**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] Saturation of luciferase activity by increasing concentrations of luciferin at 90 µM ATP.

[b] Saturation of luciferase activity by increasing concentrations of ATP at 150 µM luciferin.

Values are means (± S.E.M.) of three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control).
ND, not determined

As the luciferase assay is based upon a read-out of light production at 562 nm, quenching could cause false positive hits. Therefore, the absorbance spectra (240 – 600 nm) were determined of D-luciferin, **1** and 'Nile Red', which was identified as a luciferase inhibitor in the antibacterial screen mentioned above.[325] 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).

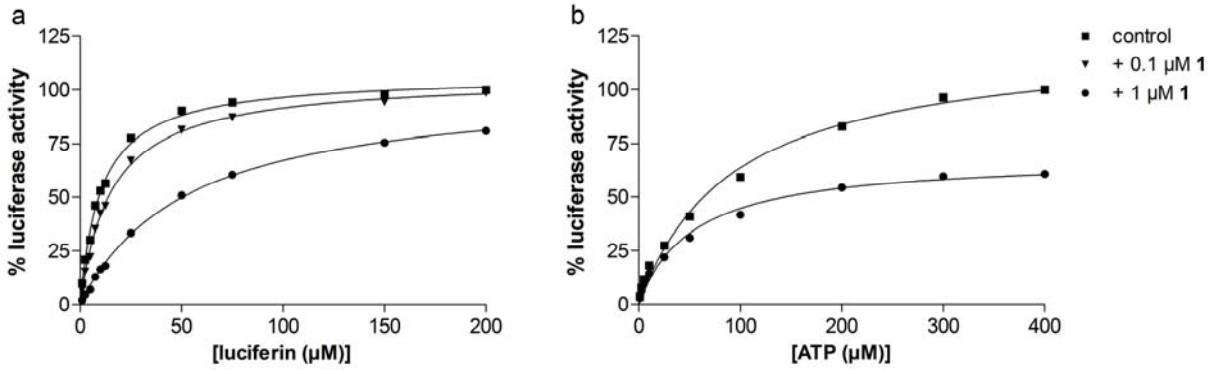
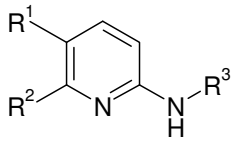
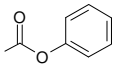
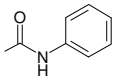


Figure 7.2 Saturation of luciferase activity by a) luciferin or b) ATP in the absence (control) or presence of 0.1 or 1 µM **1**. Representative graphs from one experiment performed in duplicate (see Table 7.1 for K_M- and V_{Max}-values).

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 7.2a). Saturation of luciferase activity by increasing concentrations of luciferin resulted in a K_M-value of 12 ± 2 µM in the absence of inhibitor (Table 7.2). In the presence of 0.1 or 1 µM of **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 7.2b). The presence of 1 µM of **1** resulted in a significantly decreased V_{max}-value (Table 7.1), proof for a non-competitive inhibition of ATP. Hence, **1** appears to solely compete for the luciferin binding site at luciferase. Notably,

Table 7.2 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} ± S.E.M. (µM), n = 3, duplicate) or % inhibition at 10 µM concentrations (n = 2, duplicate).

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.[306,326] 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.[327] 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).[198] Interestingly,

one of the most active compounds (CID: 649849) also contained an *N*-pyridin-2-yl-benzamide core and had an IC_{50}-value of 0.25 µM in their assay (**3**; Figure 7.1). 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 7.3). For compound **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.

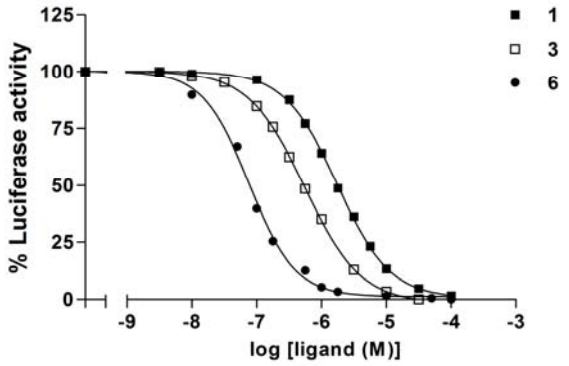
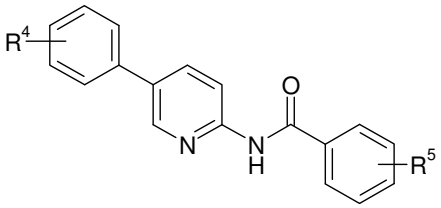


Figure 7.3 Inhibition of luciferase activity by compound **1**, **3** and **6**. The IC_{50}-values of **1**, **3** and **6** were 1.7 ± 0.1 µM, 0.61 ± 0.09 µM and 0.069 ± 0.01 µM, respectively. Representative graphs from one of three experiments performed in duplicate.

Subsequently, analogs of *N*-pyridin-2-yl-benzamide (**4**-**12**) were synthesized and tested for luciferase inhibition to explore the structure-activity relationships of this compound class (Table 7.2). 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 7.3). 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 *para*-methyl substituent as found in **3** decreased the potency by 100-fold (**7**; IC_{50} = 6.4 ± 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 7.2, 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 *cyclo*-hexyl (**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 7.2). Apparently, a larger substituent, either alkyl (**12**) or aryl (**6**), is preferred in the binding pocket of the enzyme.

Table 7.3 Inhibition of luciferase activity by compounds **6**, **22**-**39**, expressed as IC_{50}-values or as % inhibition at 10 µM.



Compound	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} ± S.E.M. (µM), n = 3, duplicate) or % inhibition at 10 µM concentrations (n = 2, duplicate).

The design of other analogs was based upon a Topliss approach in which either one of the aromatic rings was modified with various substituents (Table 7.3).[324] In general, modifications both on the 5-phenyl ring (**22**-**30**) and 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 μM or 0.31 ± 0.02 μM, respectively. 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 μM or 1.2 ± 0.05 μM, respectively.

Finally, compound **6** was docked into a homology model of firefly luciferase based upon its crystal structure including AMP and oxyluciferin.[320] From Figure 7.4, it becomes clear that AMP and oxyluciferin bind in two different pockets at the enzyme. Compound **6** was docked both 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. Firstly, 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. Secondly, 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 non-competitive inhibition of **1** that was found with respect to luciferin and ATP, respectively (Figure 7.2). In addition, from Figure 7.4 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.

In conclusion, we have shown that (drug-like) compounds, such as **1** or **6,** are competitive inhibitors of luciferase with respect to luciferin. In addition, these compounds are non-competitive 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,

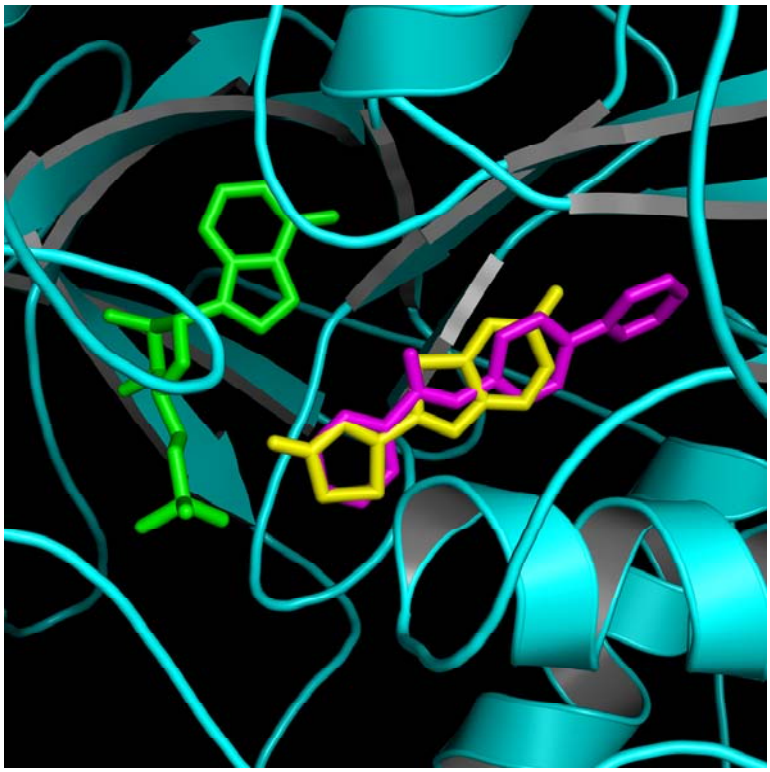


Figure 7.4 Docking results obtained using Autodock. Firefly luciferase homology model (cyan) with AMP (green), oxyluciferin (yellow) and compound **6** (magenta). For details of the modeling procedure see the Experimental Section.

we learned that compound **1** has been patented for osteoporosis treatment.[328,329] However, also in these disclosures a luciferase reporter gene assay was used to identify the compounds. The data presented in this paper is therefore meant to warn researchers for direct luciferase inhibitors that also have drug-like properties. Such compounds may be 'flagged' after their evaluation in an assay with e.g., purified luciferase.