2,6-Disubstituted benzothiazoles, analogues of the aromatic core of D-luciferin: synthesis and evaluation of the affinity for *Photinus pyralis* luciferase

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Abstract

A few 2,6-disubstituted benzothiazoles have been prepared as reference compounds or starting material for the preparation of derivatives containing positron emitting fluorine in the aromatic ring. Their affinity for *Photinus pyralis* luciferase has been evaluated and values of IC_{50} (8.8-45.2 μM) suggest that they are competitive inhibitors of the enzyme.

Keywords: Luciferase, D-luciferin, 2,6-disubstituted benzothiazoles

Introduction

The luciferase from the North American firefly *Photinus pyralis* (PpyLuc) catalyzes the conversion of D-luciferin [(*S*)-2-(6′-hydroxy-2′-benzothiazolyl)thiazoline-4-carboxylic acid] to oxyluciferin in the presence of ATP, Mg^{2+}, and oxygen with production of a yellow-green light characterized by a broad emission spectrum and a peak at 560 nm (Figure 1).[1]

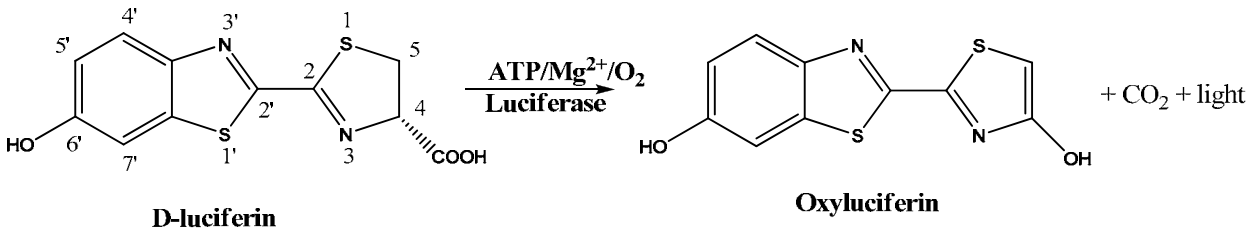
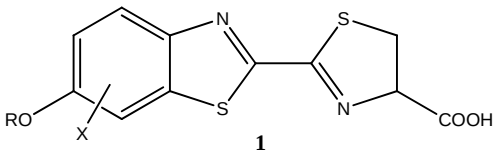


Figure 1. Luciferase-catalyzed production of bioluminescence.

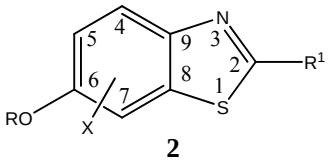
PpyLuc is a well characterized enzyme that finds a large number of biotechnological applications[2] and has been used, for example, as an indicator of cell proliferation, gene delivery or gene expression in cell culture and in living animals as a transgenic marker.[3] PpyLuc is at present the preferred enzyme for *in vivo* optical imaging of small animals,[4] a useful modality of molecular imaging that presents the limit of light absorption and scattering by organs or the high dose of the reporter probe required for a sufficient emission.[5] Furthermore, it is not certain to what degree the high contrast images obtained from the luciferin/luciferase bioluminescence are the result of selective substrate conversion or might be mainly dependent on substrate distribution. This could be better approached by other imaging modalities, such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) that use radionuclides for imaging of reporter genes.[6] In this respect, only two examples of radioactive isotopomers of D-luciferin (**1a**) are available, i. e. 6′-[^{11}C-methyl]-D-luciferin **1b**[7] and 7′-[^{123}I]iodo-D-luciferin **1c**.[8] However, **1b** and **1c** were unable to locate the tumor and showed poor cell uptake.[7,9]

We have recently undertaken a project aimed to prepare compounds related to D-luciferin labeled with positron emitting fluorine (^{18}F) for the *in vivo* imaging of a transgenic mouse that expresses a luciferase reporter gene under the control of activated estrogen receptors.[10] Initially, we evaluated the possibility of introducing ^{18}F into the benzothiazole moiety of D-luciferin (**1a**) or 6′-*O*-methyl luciferin **1d** that is a well known inhibitor of PpyLuc (IC_{50} 1μM).[11,12] However, due to the instability of the thiazoline moiety of **1a** or **1d**, chemical manipulations of these compounds were excluded.[13] We next considered to introduce ^{18}F into benzothiazoles corresponding to the aromatic core of compounds **1a** or **1d** and 2,6-disubstituted benzothiazoles **2a** were selected as starting material for the preparation of compounds **1e**.



1

a. R=X=H b. R=$^{11}CH_3$, X=H
c. R=H, X=7'-^{123}I d. R=CH_3, X=H
e. R=H, CH_3; X=^{18}F, $CH_2{}^{18}$F



2

a. R=H, CH_3; X=^{18}F, $CH_2{}^{18}$F; R^1=CN, COOH, $CONH_2$
b. R=H, CH_3; X=H; R^1=H, CN c. R and R^1 various; X=H
d. R=H, CH_3; X=H; R^1=CO-NH-$(CH_2)_n{}^{18}$F

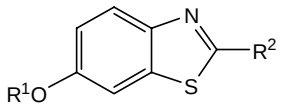
Figure 2. Structure of D-luciferin derivatives **1a-e** and 2-substituted-6-hydroxybenzothiazole analogues **2a-d**.

Furthermore, compounds such as **2a** could be used themselves as tracers, provided that they could show affinity for Ppyluc. We relied on early kinetic studies showing that 2,6-disubstituted benzothiazoles **2b** are competitive inhibitors of luciferase at pH 7.7, K_i ranging from 25 to 58 μM.[11] Moreover, in a recent paper other benzothiazoles **2c** have been examined as possible inhibitors of luciferase within a high-throughput screening (HTS) of a library of 70,000 small

molecules. Among these compounds, a series of benzothiazole analogues showed an activity approaching an IC_{50} 0.1 μM.[14] Based on this encouraging premise, we have prepared a few model compounds structurally related to compounds **2a** (X=H) and have evaluated their in vitro affinity for PpyLuc.

Results and Discussion

For our initial studies, we have selected 2-substituted-6-hydroxy and 6-methoxybenzothiazoles (compounds **3a-h,** Figure 3) that could be prepared from nitriles **3a** and **3e**. The synthesis of 2-cyano-6-methoxybenzothiazole **3e** has been fully described,[15-18] but we started from the commercially available compound. The nitrile **3e** can be transformed into the 6-hydroxy analogue **3a** carrying out the demethylation by fusion with pyridinium hydrochloride (Py.HCl) at 200 °C.[15]



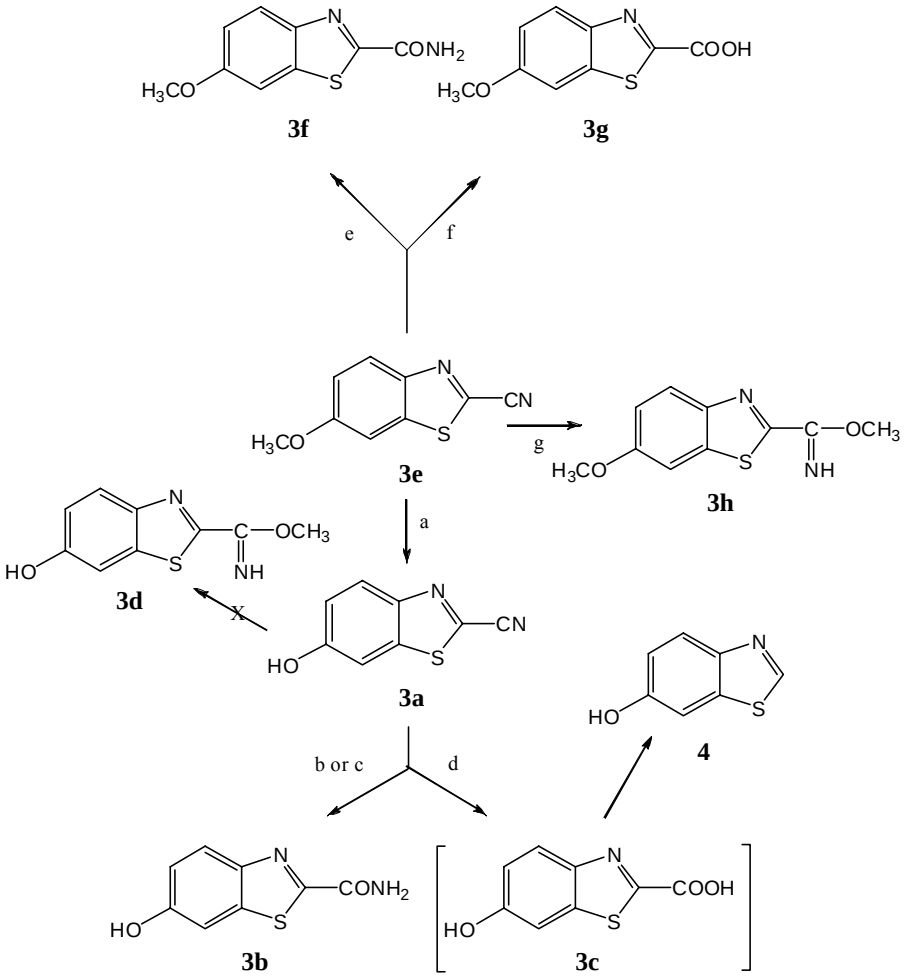
3

a. R^1 = H, R^2 = CN
b. R^1 = H, R^2 = $CONH_2$
c. R^1 = H, R^2 = COOH
d. R^1 = H, R^2 = $C(=NH)OCH_3$
e. R^1 = CH_3, R^2 = CN
f. R^1 = CH_3, R^2 = $CONH_2$
g. R^1 = CH_3, R^2 = COOH
h. R^1 = CH_3, R^2 = $C(=NH)OCH_3$

Figure 3. Structure of 6-hydroxybenzothiazole derivatives **3a-h**.

This method is still the most efficient for the preparation of 2-cyano-6-hydroxybenzothiazole **3a**, because the nitrile function remains intact under these conditions. A few attempts to prepare **3a** from **3e** with other reagents were much less efficient than Py.HCl.[19] The overall preparation of compounds **3a-h** is described in Scheme 1. Basic hydrolysis of the nitrile **3a** to the amide **3b** could not be controlled and this product was obtained in mixture with variable amounts of the acid **3c.** However, the 6-hydroxy acid **3c** is stable only for a few days as powder and in solution decarboxylates to 6-hydroxybenzothiazole **4** with a reported half-life of 31 hours.[20] We have verified the instability of the acid **3c** in aqueous solutions at room temperature and consequently the values of IC_{50} were not easily reproducible. The amide **3b** could also be prepared by a biocatalytic approach, using a specific enzyme that could catalyze the hydrolysis of a nitrile. The superfamily of nitrilases includes nitrilases and nitrile hydrolases and offers this opportunity.[21] The properly named nitrilase (EC 3.5.5.1) catalyzes the hydrolysis of a nitrile to the corresponding carboxylic acid and ammonia, whereas nitrile hydrolase (EC 4.2.1.84) transforms a nitrile into an amide.[22] Only nitrilases are commercially available, but occasional

formation of amide in nitrilase-catalyzed hydolysis of nitriles has been observed.[23] This partial hydrolysis may depend on the structure of the nitrile, as in the case reported for a nitrilase-catalyzed hydrolysis of a β-hydroxynitrile.[24] We have used the commercially available nitrilase from *Arabidopsis thaliana* and carried out the reaction in a 10% DMSO aqueous solution of the nitrile **3a**. In our case, a quantitative hydrolysis to the required amide **3b** was achieved with no formation of the corresponding acid **3c** or product of its decarboxylation (compound **4**). The same enzymatic reaction could not be repeated on the nitrile **3e**, only for the high insolubility of the substrate in the reaction media.



Scheme 1. Synthesis of compounds **3a-d** and **3f-h** from nitrile **3e.** Reagents and conditions: (a) PyHCl, 200°C, 1.5 h, 70%; (b) NaOH/THF, r.t., 1 h, 65%; (c) nitrilase from *Arabidopsis thaliana*, H_2O/DMSO (9:1), r.t., 2 h, 98%; (d) NaOH/THF, r.t., 3 h; (e) NaOH/THF, r.t., 1 h, 70%; (f) NaOH/THF, r. t., 3h, 98%; (g) MeOH/ NaOH, r.t., 3 h, 98%.

For the preparation of the 6-methoxy derivatives, the basic hydrolysis of the nitrile **3e** proceeded as described for nitrile **3a**. In fact, the amide **3f** was prepared in mixture with variable

amounts of the acid **3g**, that was, in turn, quantitatively obtained by a complete basic hydrolysis. We have also prepared the 6-methoxy imidate **3h** from the nitrile **3e**, by a modification of a published procedure.[25] Under the same conditions, the imidate **3d** could not be prepared, confirming the result obtained by Amess et al.[25] The bioluminescent assay was carried out using a recombinant PpyLuc and results are expressed as IC_{50} values (Table 1).

Table 1. IC_{50} values of 2-substituted-6-hydroxy and 6-methoxybenzothiazoles (compounds **3a-h**)[a]

Substrate	IC_{50}[b]
3a	8.8
3b	45.2
3e	14.7
3f	13.0
3g	308.0
3h	16.4

[a]Compound **3c** is unstable in solution and did not afford reproducible results. Compound **3d** could not be prepared from **3a** under the same reaction condition that afforded the imidate **3h** from **3e**

[b]IC_{50} refers to concentration (μM) of the compound in a competition assay that causes 50% of inhibition of PpyLuc-catalyzed bioluminescence at pH 7.8

Comparing the IC_{50} values of 6-hydroxybenzothiazole derivatives **3a** and **3b,** it can be observed that substitution of the 2-cyano with an amide group leads to a slight decrease of affinity (IC_{50} from 8.8 to 45.2 μM). In the 6-methoxybenzothiazole series, the presence of the methoxy group at the position 6 does not significantly affect the affinity of the 2-cyano derivatives **3a** and **3e** (IC_{50} 8.8 and 14.7 μM for nitrile **3a** and **3e**, respectively). Furthermore, the 6-methoxy amide **3f** shows an IC_{50} value nearly identical to that of the corresponding 6-methoxynitrile **3e**, in contrast with the difference observed between **3a** and **3b**. The 6-methoxy imidate **3h** is characterized by an IC_{50} value similar to **3e** and **3f**. The acid **3g** was prepared with a view to improving the limited solubility that all the benzothiazole derivatives prepared by us exhibited in the in vitro assay. However, this acid has revealed to be a poor substrate (IC_{50} 308.0 μM) for PpyLuc. A possible explanation of this result involves the recognition of the substrate by the enzyme that can be disfavored by the presence of a negatively charged group at position 2. This can interfere with some amino acid residue present in the part of the active site where the compound **3g** should be located.

Conclusions

Results from our study show that benzothiazoles **3a, 3e, 3f**, and **3h** present an interesting affinity for PpyLuc (IC50 values 8.8-16.4 μM), comparable to that of other benzothiazoles previously reported.[11,14] It is reasonable to assume that these small-sized compounds interact in a non-specific manner with different binding sites of the large enzymatic pocket of PpyLuc. This view of enzyme-substrate interaction has previously been proposed also to explain the magnitude of the competitive inhibition shown by other benzothiazoles[11,14] and several structurally diverse small molecules.[26-29] In any event, we have described preliminary observations about the affinity of PpyLuc for 2,6-disubstituted benzothiazole derivatives that can be considered as reference compounds or starting material for the preparation of derivatives containing positron emitting fluorine in the aromatic ring. We are currently investigating the introduction of fluorine or fluorine-containing groups in the benzothiazole nucleus of compounds **3a-h** in order to obtain benzothiazoles **2a** or **2d**, as potential tracers of luciferase reporter gene.

Experimental Section

General Procedures. Melting points were recorded on a Stuart Scientific SMP3 instrument and are uncorrected. IR spectra were recorded as KBr pellets on a Jasco FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan). ^{1}H NMR spectra were recorded in DMSO-d_6 solutions at 303 K on a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer, a process control and an array processor. The ^{1}H- chemical shifts are reported in parts per million, using as reference the signal for residual solvent protons. Mass spectra were recorded on a Finnigan LCQ-Deca (Termoquest) in ESI positive-ion mode (KV 5.00, 225 °C, 15 V). The progress of all reactions and column chromatography were monitored by TLC using Silica Gel 60 F_{254} precoated plates with a fluorescent indicator (Merck). Purification of products by chromatography was performed using silica gel 60 (230-400 mesh, Merck). All reagents were obtained from commercial sources and used without further purification. Nitrilase from *Arabidopsis thaliana* (0.26 U/mg) was purchased from Fluka. The nitrile **3a** was prepared from nitrile **3e** in 70% yield according to the literature method.[15] All products were crystallized from methanol/water.

Enzymatic assay. The luciferase assay was carried out in 96 multiwell plates using 100 pg recombinant luciferase (Quantilum, Promega, Madison W.I., U.S.A.). The enzyme was dissolved in a luciferine buffer [$(MgCO_3)_4Mg(OH)_2$*$5H_2O$, 1mM, Tricine (20 mM), EDTA (0,1 mM), $MgSO_4$*$7H_2O$ (2,5 mM), DTT (33 mM), ATP (530 mM), pH 7.8] D-luciferin potassium salt (Promega, Madison, WI U.S.A.) was added at the final concentration of 30μM in the presence of increasing concentrations of the synthetic compounds to be tested (10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, 1mM, 10 mM). The compounds were

solubilized in 0,1 M phosphate buffer pH 7.2 with 1% v/v DMSO. The reaction occurred at 37°C for 5 min, then the quantitative analysis of the luminescence was carried out for 10 sec using the Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA U.S.A.). The curve of luciferin activity was generated using 1:3 dilutions of luciferin (at concentrations from 10nM to 1mM). All reactions were carried out in quadruplicate and each experiment was repeated in triplicate. All reagents were from Sigma-Aldrich (Steinheim, Germany). Half maximal inhibitory concentration (IC_{50}) was calculated by means of the Program PRISM5 (GraphPad Software Inc., CA U.S.A.) using Sigmoidal dose-response (variable slope) equation.

General procedure of the hydrolysis of nitriles 3a and 3e.

To a stirred solution of **3a** or **3e** (500 mg) in 15 mL of tetrahydrofurane, 3 mL of NaOH 1N were added. The reaction was stirred at room temperature for 1 h to afford a mixture of the corresponding amide (**3b** or **3f**) and acid (**3c** or **3g**). The complete hydrolysis of nitriles to the acids was achieved stirring for 3 h at room temperature. At the end of the reaction, a solution of 1N HCl was added and the mixture extracted two times with ethyl acetate. The isolation of amides **3b** and **3f** required an additional step, consisting in the treatment of the ethyl acetate solution of the amide/acid mixture with a 10% $NaHCO_3$ solution to remove the sodium salt of the acid **3c** or **3g**. The organic phase was dried with sodium sulfate and evaporated.

6-Hydroxybenzothiazole-2-carboxyamide (3b). Yellow-brown solid (358 mg, 65%); mp 168-170 °C dec; IR (KBr): ν_{max} 3168, 1656, 1602, 1556, 1456, 1230 cm^{-1}; ^{1}H-NMR: δ 7.05 (dd, 1H, 4-H, J 14 Hz, 3.5Hz), 7.41 (d, 1H, 7-H, J 3.5Hz), 7.90 (m, 2H, 5-H and CONH), 8.32 (bs, 1H, CONH), 10.18 (bs, 1H, 6-OH); MS: (*m/z* , relative intensity) 194 (M^+, 100%), 151 (M^+- 44, 86%)]. *Anal*. Calcd. for $C_8H_6N_2O_2S$: C, 49.48; H, 3.09; N, 14.43. Found: C, 49.55; H, 3.14; N, 14.37.

6-Methoxybenzothiazole-2-carboxyamide (3f). Brown solid (383 mg, 70%); mp 248-250 °C dec; IR (KBr): ν_{max} 3256, 1661, 1604, 1500, 1226 cm^{-1}; ^{1}H-NMR: δ 3.85 (s, 3H, 6-OCH_3), 7.20 (d, 1H, 4-H, J 8.7 Hz), 7.75 (s, 1H, 7-H), 7.98 (d, 1H, 5-H, J 8.7 Hz), [7.95 (bs, 1H) and 8.35 (bs, 1H), $CONH_2$]; MS: (*m/z* , relative intensity) 207 (M^+-1, 10%), 165 (30), 151 (100). *Anal*. Calcd. for $C_9H_8N_2O_2S$: C, 51.92; H, 3.85; N, 13.46. Found:C,52.09;H,3.93;N,13.40.

6-Methoxybenzothiazole-2-carboxylic acid (3g). Yellow-brown solid (538 mg, 98%); mp >300 °C dec; IR (KBr): ν_{max} 3359-2971, 1602, 1440, 1388, 1267 cm^{-1};. ^{1}H-NMR: δ 3.80 (s, 3H, 6-OCH_3), 7.12 (dd, 1H, 4-H, J 8.7Hz, 2.0 Hz), 7.72 (d, 1H, 7-H, J 2.0 Hz), 7.95 (dd, 1H, 5-H, J 8.7 Hz, 2 Hz), 9.18 (s, 1H, COOH); MS: (*m/z* , relative intensity) 208 (M^+-1, 100%), 165 (45), 150 (67). *Anal*. Calcd. for $C_9H_7NO_3S$: C, 51.67; H, 3.35; N, 6.70. Found: C, 51.75; H, 3.40; N, 6.75.

Nitrilase-catalyzed hydrolysis of 2-cyano-6-hydroxybenzothizole (3a): enzymatic preparation of 6-hydroxybenzothiazole-2-carboxyamide (3b)

To a stirred solution of nitrile **3a** (120 mg, 0.68 mmol) in 5 mL of a water/dimethyl sulfoxyde solution (9/1) at room temperature, nitrilase from *Arabidopsis Thaliana* (10 mg) was added and

the reaction was stirred for 2 h and only the amide **3b** was revealed by TLC (dichloromethane/methanol 95:5). The mixture was filtered, diluted with water (3 mL) and extracted with ethyl acetate. The organic phase was dried over sodium sulfate and the product was recovered in 98% yield as a yellow-brown solid. All chemico-physical characteristics were in agreement with the structure of the amide **3b**.

Methyl 6-methoxybenzothiazole-2-carboxyimidate (3h)

To a stirred solution of the nitrile **3e** (500 mg, 2.62 mmol) in methanol (15 mL), 1N NaOH (3 mL) was added and the reaction was stirred at room temperature (3 h). The reaction was diluted with water (10 mL) and extracted with ethyl acetate. The organic phase was dried (sodium sulfate) and the product recovered as a white solid (570 mg, 98%). All chemico-physical characteristics were as described for **3h**.[25]

Acknowledgements

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