Tetrahedron Letters 56 (2015) 2437–2440

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Mechanism study of the thiol-addition reaction to benzothiazole derivative for sensing endogenous thiols

Olimpo García-Beltrán [a,d,*], José G. Santos [b], Sandra Fuentealba [b], Pedro De la Torre [c], Paulina Pavez [b], Natalia Mena [d], Marco T. Nuñez [d], Margarita E. Aliaga [b,*]

[a] Facultad de Ciencias Naturales y Matemáticas, Universidad de Ibagué, Carrera 22 Calle 67, Ibagué 730001, Colombia
[b] Facultad de Química, Pontificia Universidad Católica de Chile, Casilla 306, Santiago 6094411, Chile
[c] Laboratorio Síntesis Orgánica, Instituto de Química de Recursos Naturales, Universidad de Talca, Casilla 747, Talca, Chile
[d] Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 3 February 2015
Revised 10 March 2015
Accepted 18 March 2015
Available online 31 March 2015

Keywords:
Thiol-addition mechanism
Endogenous thiols
Fluorescent probe
Benzothiazole derivative

ABSTRACT

We kinetically studied the reaction between endogenous thiols such as -γ-glutamylcysteine (γ-Glu-Cys), cysteine (Cys), glutathione (GSH), homocysteine (Hcy), cysteinylglycine (Cys-Gly), and dihydrolipoic acid (DHLA)- with (*E*)-2-(benzo[*d*]thiazol-2-yl)-3-(4-morpholinophenyl)acrylonitrile (**JGB**). Studies conducted by NMR and ESI-MS/MS have demonstrated that this reaction occurs via thiol-addition toward the double bond present in **JGB**. Considering the product analysis and the pH-dependence of the second order rate constant (k_N), we proposed a mechanism that involves the rapid protonation of **JGB** giving place to an intermediate following by the thiolate attack yielding a final product. Moreover, this probe could successfully sense thiols in the human neuroblastoma SH-SY5Y cells.

Introduction

Endogenous thiols (RSH) are a set of small organic molecules (see Scheme S1; Supplementary material), featuring a –SH group, that plays a particularly crucial role in important biological functions.[1] Among these, the tripeptide glutathione (GSH), is the most abundant non-protein thiol compound.[2] Other thiols which contribute to the intracellular thiol pool are the precursors for the synthesis of GSH, the aminoacids homocysteine (Hcy) and cysteine (Cys), and the dipeptide γ-glutamylcysteine (γ-Glu-Cys), and as first product of GSH degradation, the dipeptide cysteinylglycine (Cys-Gly).[2] The lipoic acid is another biologically relevant compound with an eight-carbon centered disulfur, which is rapidly reduced in cells to dihydrolipoic acid (DHLA).[3] It is known that an increase or decrease of the levels of these molecules in the cell can generate a series of abnormality in the human, such as edema, cardiovascular, liver damage, and neurodegenerative diseases.[1a,c,4] Considering the biological relevance of the endogenous thiols, currently, there are a variety of methods for their quantitative measurement, such as electrochemical methods, capillary electrophoresis, mass spectrometry, and high performance liquid chromatography (HPLC).[5] However, due to the high sensitivity and easy operation of colorimetric and fluorescent methods, the development of novel chemical sensors for detecting biothiols has become very important.[6] Among the chemical strategies involved organic reactions include cyclization reaction with an aldehyde,[7] cleavage of sulfonamide, sulfonate esters,[8] disulfide bonds,[5a] and Michael addition by thiols.[9,10]

In connection with our interest, whether it be in the development of chemosensors for thiols, via Michael addition,[9,10,11a] or in establishing the mechanism of different reactions,[11b] in this work we focus on the study of a thiol-Michael addition reaction of a benzothiazole derivative. The choice of the benzothiazole-based fluorophore is based on its excellent photophysical properties; this fluorophore can also minimize cell damage and is more suitable for bioimaging studies.[12]

In addition, considering that detailed experimental studies for the sensing mechanism of Michael acceptors sensors are insufficient we will shed some light on these mechanisms. Finally, we will assess the applicability of our probe in living SH-SY5Y cells.

Firstly, the synthesis of compound (**JGB**) was realized in one step using commercial precursors according to the literature[13] (see Scheme S2; Supplementary material). A mixture of 2-(benzo[*d*]thiazol-2-yl) acetonitrile (**1**) and 4-morpholinobenzaldehyde, was stirred for 40 min at room temperature using ethanol as solvent and a catalytic amount of triethylamine. The

* Corresponding authors. Tel.: +57 8 270 9444; fax: +57 8 2709443.
E-mail addresses: jose.garcia@unibague.edu.co (O. García-Beltrán), mealiaga@uc.cl (M.E. Aliaga).

http://dx.doi.org/10.1016/j.tetlet.2015.03.083
0040-4039/

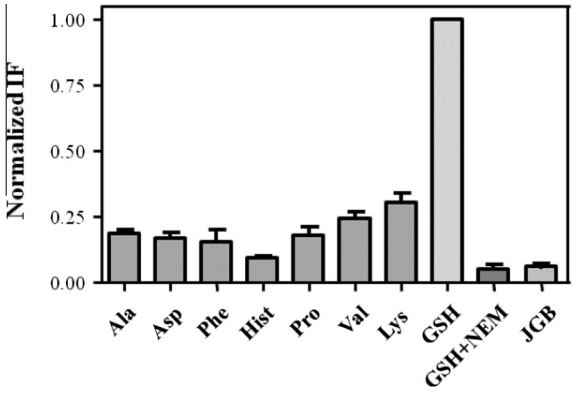


Figure 1. Fluorescence turn-ON response of probe **JGB** (10 μM) to different relevant analytes (200 μM), in buffer HEPES (pH 7.4).

compound was characterized using ^{1}H NMR and ^{13}C NMR spectroscopy (Figs. S1A and B; Supplementary material) and high-resolution mass spectrometry (Fig. S2; Supplementary material). ^{1}H NMR analysis of the benzothiazole derivative (**JGB**) revealed a single olefinic proton associated with the formation of a single isomer, which was assigned to have the thermodynamically more stable *E* configuration.

The absorption spectrum of **JGB** in DMSO–HEPES (50 mM pH = 7.4, 1/9 v/v) exhibited a band with a maximum at 484 nm (Fig. S3). In the fluorescence spectrum, compound **JGB** exhibited a weak fluorescence emission at 590 nm (Fig. S4; Supplementary material). However, upon addition of RSH, the fluorescence increased considerably, the increase depending on the tested thiol. This behavior is in line with another study on the fluorescent response toward biothiols of compounds containing a benzothiazole scaffold.[11a]

In particular, in the case of the glutathione (GSH) the fluorescence titration spectra of **JGB** with increasing concentrations of GSH (0–500 μM) is presented in Figure S5. When 200 μM of GSH was added to the solution of **JGB** a 10-fold enhancement in fluorescence was observed at 30 °C in DMSO–HEPES (50 mM, 1/9 v/v), as shown in Figure 1.

On the other hand, when the thiol alkylation reagent *N*-ethylmaleimide (NEM) was co-incubated with GSH and immediately added to the solution containing **JGB**, the level of fluorescent response obtained was similar to that observed for the probe in the absence of GSH. The latter effect suggests the participation of the sulfhydryl group in the interaction between RSH and **JGB**.

With the aim to explore the mechanism involved in the reaction between the series of endogenous thiols and **JGB**, experiments using ESI-MS/MS were carried out. As shown in Figure 2, the ESI-MS spectrum of a solution containing the probe in the presence of Cys, other relevant endogenous thiol, depicted a peak at *m*/*z* 468.0, which was associated with the adduct [**JGB-Cys**]$^{+}$.

The formation of the addition product was also confirmed using ^{1}H NMR spectroscopy. Figure 3 shows the NMR spectra for the probe **JGB** in the absence or presence of 2-mercaptoethanol (2-ME), a model compound for thiols.

As shown in the same figure, upon addition of 2-ME, the vinyl proton H_a (δ 8.16 ppm) of **JGB** disappeared and concurrently two new signals assigned to the thioether methylene protons H_b and H_a emerged at δ 5.57 and 4.71 ppm, respectively. This result is consistent with the formation of the **JGB**-ME adduct and support the thio-Michael addition of RSH on the electrophilic site present in **JGB**.

Once the nature of the product of the studied reaction was established, we proceeded to determine the pseudo-first-order rate

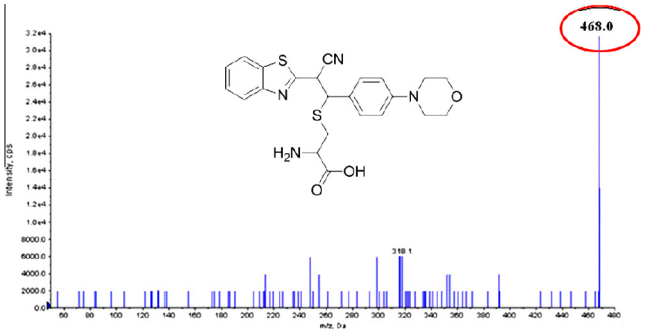


Figure 2. ESI-MS spectrum of a solution containing the probe **JGB** (20 μM) co-incubated with Cys (40 μM; buffer HEPES, pH 7.4) during 20 min.

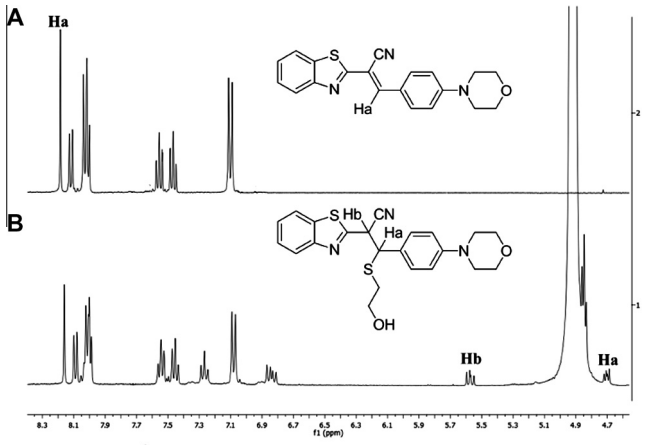


Figure 3. Partial ^{1}H NMR spectral changes (400 MHz, DMSO-d_6) of **JGB** (20 mM) upon addition of 2-ME (1.8 equiv) in DMSO-d_6 at 25 °C. (A) **JGB** only; (B) **JGB** + 2-ME (30 min).

Table 1
Values of pK_a for the sulfhydryl group of endogenous thiols (RSH; see structures Scheme S1) and k_N values for the reactions of them with **JGB**

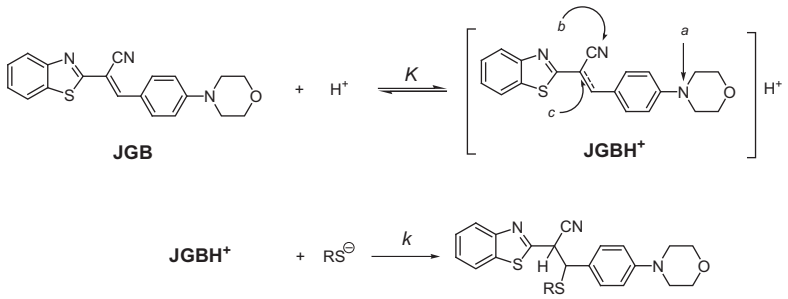
Biothiol (RSH)	pK_a	k_N (s^{-1} M^{-1})
Cys-Gly	7.00	0.50 ± 0.04 (pH = 6.8)
		0.39 ± 0.02 (pH = 7.1)
		0.31 ± 0.01 (pH = 7.4)
Cys	8.10	4.21 ± 0.60 (pH = 6.8)
		1.43 ± 0.14 (pH = 7.4)
Hcy	8.25	5.01 ± 0.38 (pH = 6.8)
		2.91 ± 0.22 (pH = 7.1)
		2.50 ± 0.05 (pH = 7.4)
GSH	8.72	14.46 ± 0.79 (pH = 6.8)
		4.19 ± 0.42 (pH = 7.4)
γ-Glu-Cys	9.98	110.3 ± 5.9 (pH = 6.8)
		42.2 ± 3.2 (pH = 7.4)
DHLA	10.7	331.2 ± 33.3 (pH = 7.1)
		183.0 ± 23.8 (pH = 7.4)

The pK_a values were taken from Refs. 10,14 and the k_N value for DHLA was corrected statistically considering equivalent both nucleophilic sites.[14]

constants (k_{obsd}) for each reaction. All these constants were obtained under thiol excess, at constant pH, and the reactions obey Eq. 1. Here k_0 and k_N are the rate coefficients for solvolysis and thiolysis of the probe, respectively and [RS^-] represents the concentration of thiolate.

$$k_{obs} = k_0 + k_N [RS^-] \quad (1)$$

The values of k_N obtained for the reaction of endogenous thiols with the benzothiazole derivative (**JGB**) and the pK_a values for the sulfhydryl group of RSH are summarized in Table 1.



Scheme 1. Proposed mechanism for the thiol-addition reaction to **JGB**. The letters a, b and c represent the probable sites of protonation of the intermediate (**JGBH⁺**) in our experimental conditions.

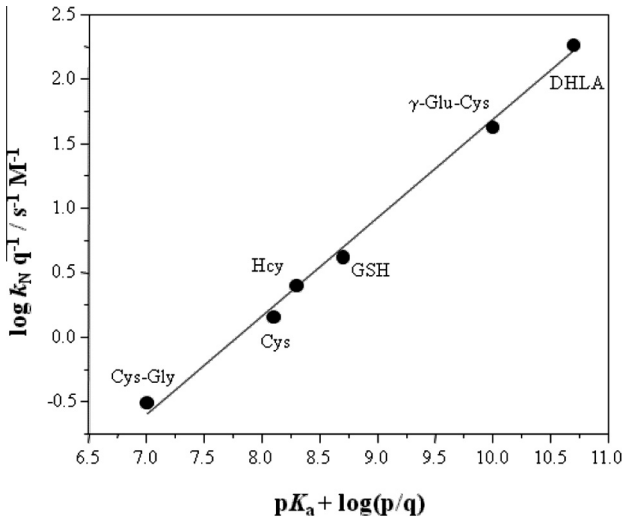


Figure 4. Brønsted-type plot (statistically corrected, see text) for the thiol-addition reaction to benzothiazole derivative (**JGB**), in aqueous solution, at 30.0 °C and ionic strength 0.2 M (KCl).

The following reactivity order for the series of the tested thiols toward **JGB** was obtained: Cys-Gly<Cys<Hcy<GSH<γ-Glu-Cys<DHLA. This order is in according to previous Letter conducted by us,[9,10] on the reactivity increases with the basicity of the sulfhydryl group of the corresponding thiol.

Interestingly, the studied reaction showed a dependence of their second rate constants (k_N) on the solution pH as shown in Table 1. Scheme 1 describes a reaction model consistent with the above mentioned pH effect and with the analysis of the reaction product where **JGB** and RS^- referring to the Michael acceptor, and thiolate, respectively. This mechanism would involve the rapid protonation of **JGB** giving place to an intermediate following by the thiolate attack yields the final product. As a consequence, the following expression (Eq. 2) can be obtained for the observed rate constant:

$$k_{obs} = \frac{kK[H^+][RS^-]}{1 + K[H^+]} \quad (2)$$

The linear pH-dependence observed in data from Table 1 is in according with in a situation where the equilibrium (K described in Scheme 1) will be shifted toward **JGB** (i.e., $K[H^+] << 1$).

For the studied reactions the k_N values, as well as those of the pK_a of the conjugate acids of the thiols were statistically corrected with $q = 2$ and $p = 2$ only for DHLA. The parameter q is the number of equivalent basic sites in the thiolate and p is the number of equivalent dissociable protons of the thiol. Figure 4 shows the statistically corrected Brønsted type plots for the biothiolysis studied, at pH = 7.4. The β value (0.7) obtained is in accordance with a charge development at the nucleophilic reaction center (S atom) in the transition state for a concerted mechanism. Nevertheless, it is not possible to accept this mechanism of simultaneous addition of the thiolate and proton toward the reaction site present in **JGB** because this is a less likely situation that would involve a trimolecular step. Thus, this β value could be related with the transition state of the addition of RS^- to **JGBH⁺** in Scheme 1.

Finally, the application of **JGB** was implemented with the aim to visualize endogenous thiols directly in living cells using changes in fluorescence intensity.

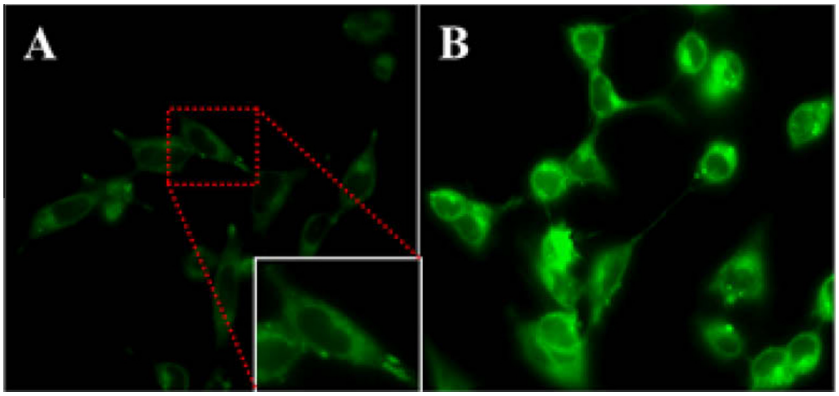


Figure 5. (A) SH-SY5Y cells were incubated for 20 min with **JGB** at a concentration of 5 μM. (B) The cells were treated for 12 h with NAC (1.5 mM), washed and then incubated for 20 min with **JGB** (5 μM) and then the fluorescence was measured using a microplate fluorescence reader and epifluorescence microscopy. Epifluorescence microscope, 63× objective.

As shown in Figure 5, the fluorescence due to **JGB** was concentrated in the cytoplasm and cytoplasmic structures. It is important to note that we discarded the presence of **JGB** in mitochondria through colocalization using the mitochondria-specific fluorescent probe Mito-Tracker (Supplementary material; Figs. S6A–C). Considering the latter and the fluorescence level induced only by **JGB** (Fig. 5A), we suggested that **JGB** could detect endogenous thiols (in cytoplasm) in living SH-SY5Y cells.

Moreover, other experiments were conducted in the presence of *N*-acetylcysteine (NAC), with the aim to provide an experimental condition where the cells stimulate of di novo GSH synthesis.[15] Results presented in Figure 5B confirm that the emission from the cells is caused by the reaction of the probe with intracellular thiols whose concentrations, mainly GSH, were incremented after the addition of NAC.

In summary, a benzothiazole-based fluorophore has been successfully synthetized to detect endogenous thiols. The probe can easily penetrate cell membranes and be utilized for fluorescence imaging in living cells. By comparing the reactions under investigation between each other, we propose a stepwise mechanism that involves the rapid protonation of **JGB** giving place to an intermediate following by the thiolate attack yields the final product.

Acknowledgments

This work was supported by PIA-ACT1114 from CONICYT, FONDECYT Grant #1130062 and FONDEQUIP EQM-130032.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2015.03.083.

References and notes

1. (a) Seshadri, S.; Beiser, A.; Selhub, J.; Jacques, P. F.; Rosenberg, I. H.; D'Agostino, R. B.; Wilson, P. W.; Wolf, P. A. N. *Engl. J. Med.* **2002**, *346*, 476–483; (b) Ball, R. O.; Courtney-Martin, G.; Pencharz, P. B. *J. Nutr.* **2006**, *136*, 1682S–1693S; (c) Schulz, J. B.; Lindenau, J.; Seyfried, J.; Dichgans, J. *Eur. J. Biochem.* **2000**, *267*, 4904–4911.
2. Meister, A. *Methods Enzymol.* **1995**, *251*, 3–7.
3. Handelman, G. J.; Han, D.; Tritschler, H.; Packer, L. *Biochem. Pharmacol.* **1994**, *47*, 1725–1730.
4. Dickinson, D. A.; Forman, H. J. *Biochem Pharmacol.* **2002**, *64*, 1019–1026.
5. For some reviews and recent examples, see: (a) Liu, Sh-R.; Chang, Ch-Y.; Wu, Sh-P. *Anal. Chim. Acta* **2014**, *449*, 64–69; (b) Shahrokhian, S. *Anal. Chem.* **2001**, *73*, 5972–5978; (c) Inoue, T.; Kirchhoff, J. *Anal. Chem.* **2002**, *74*, 1349–1354; (d) Li, J.; Ma, H.; Wang, X.; Xiong, S.; Dong, S.; Wang, S. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2608–2612; (e) Nolin, T. D.; Elizabeth McMenamin, M.; Himmelfrab, J. *J. Chromatogr. B* **2007**, *852*, 554–561.
6. For some reviews and recent examples, see: (a) Dasa, S.; Sarkar, Y.; Mukherjee, S.; Bandyopadhyay, J.; Samanta, S.; Prati Parui, P.; Ray, A. *Sensor Actuat. B-Chem.* **2015**, *209*, 545–554; (b) Chen, Z.; Zhou, Y.; Peng, X.; Yoon, J. *Chem. Soc. Rev.* **2010**, *39*, 2120–2135; (c) Yin, C.; Huo, F.; Zhang, J.; Martínez-Máñez, R.; Yang, Y.; Lv, H.; Li, S. *Chem. Soc. Rev.* **2013**, *42*, 6032–6059; (d) Zhu, L.; Yuan, Z.; Simmons, J. T.; Sreenath, K. *RSC Adv.* **2014**, *4*, 20398–20440.
7. Dai, X.; Zhang, T.; Liu, Y.-Z.; Yan, T.; Li, Y.; Miao, J.-Y.; Zhao, B.-X. *Sensor Actuat. B-Chem.* **2015**, *207*, 872–877.
8. Jiang, W.; Fu, Q.; Fan, H.; Ho, J.; Wang, W. *Angew. Chem. Int. Ed.* **2007**, *46*, 8445–8448.
9. García-Beltrán, O.; Mena, N.; Pérez, E. G.; Cassels, B. K.; Núñez, M. T.; Werlinger, V.; Zavala, D.; Aliaga, M. E.; Pavez, P. *Tetrahedron Lett.* **2011**, *52*, 6606–6609.
10. Aliaga, M. E.; Tiznado, W.; Cassels, B. K.; Nuñez, M. T.; Millan, D.; Pérez, E. G.; García-Beltrán, O.; Pavez, P. *RSC. Adv.* **2014**, *4*, 697–704.
11. (a) De la Torre, P.; García-Beltrán, O.; Tiznado, W.; Mena, N.; Astudillo Saavedra, L.; Gutierrez Cabrera, M.; Trilleras, J.; Pavez, P.; Aliaga, M. *Sensor Actuat B-Chem.* **2014**, *193*, 391–399; (b) Andrade-Acuña, D.; Santos, J. G.; Tiznado, W.; Cañete, A.; Aliaga, M. E. *J. Phys. Org. Chem.* **2014**, *27*, 670–675.
12. (a) Kim, T. I.; Kim, H.; Choi, V.; Kim, Y. *Chem. Commun.* **2011**, 9825–9827; (b) Zhang, H.; Xie, T.; Wang, P.; Chen, G.; Liu, R.; Lam, Y.-W.; Hu, Y.; Zhu, Q.; Sun, H. *Talanta* **2015**, *135*, 149–154.
13. De la Torre, P.; Astudillo-Saavedra, L.; Caballero, J.; Quiroga, J.; Alzate-Morales, J. H.; Cabrera, M. G.; Trilleras, J. *Molecules* **2012**, *17*, 12072–12085.
14. Trujillo, M.; Radi, R. *Arch. Biochem. Biophys.* **2002**, *397*, 91–98.
15. De Vries, N.; De Flora, S. *J. Cell. Biochem.* **1993**, *53*, 270–277.