



The development of a fluorescence turn-on sensor for cysteine, glutathione and other biothiols. A kinetic study

Olimpo García-Beltrán^{a,d}, Natalia Mena^b, Edwin G. Pérez^{c,e}, Bruce K. Cassels^{a,c}, Marco T. Nuñez^{b,c}, Francisca Werlinger^e, Daniel Zavala^e, Margarita E. Aliaga^{e,*}, Paulina Pavez^{e,*}

^a Department of Chemistry, Faculty of Sciences, University of Chile, Santiago, Chile

^b Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile

^c Institute for Cell Dynamics and Biotechnology, University of Chile, Santiago, Chile

^d Departamento de Ciencias Químicas, Facultad de Ciencias Exactas, Universidad Andrés Bello, Avenida República 275, Piso 3, Santiago, Chile

^e Facultad de Química, Pontificia Universidad Católica de Chile, Av. Vicuña Mackenna 4860, Santiago 6094411, Chile

ARTICLE INFO

Article history:

Received 22 August 2011

Revised 22 September 2011

Accepted 29 September 2011

Available online 6 October 2011

Keywords:

Biothiols

Coumarin-based derivatives

Fluorescence probes

Michael addition

ABSTRACT

Two fluorescence probes for the detection of cysteine (Cys), glutathione (GSH) and other biothiols, such as homocysteine (Hcy) and cysteinyl-glycine (Cys-Gly), were developed. These molecular probes are coumarin-based derivatives containing a chalcone-like moiety that reacts with biothiols through a Michael addition reaction, leading to strong fluorescence enhancements. The reactivity of the tested biothiols toward both probes (**ChC1** and **ChC2**) follows the order Cys > GSH > Hcy > Cys-Gly, **ChC1** being less reactive than **ChC2**. Possible interference with other amino acids was assessed. **ChC1** and **ChC2** display a highly selective fluorescence enhancement with thiols, allowing these probes to be used for fluorimetric thiol determination in SH-SY5Y cells.

© 2011 Elsevier Ltd. All rights reserved.

Biothiols (RSH) are compounds involved in many essential cellular functions.^{1–4} Among these, the tripeptide glutathione (GSH), γ -glutamyl-L-cysteinyl-glycine, is the most abundant low-molecular-mass non-protein thiol compound in cells.¹ Other low molecular mass thiols that contribute to the intracellular thiol pool are precursors for the synthesis of GSH: the aminoacids homocysteine (Hcy) and cysteine (Cys); and the first product of GSH degradation; the dipeptide cysteinyl-glycine (Cys-Gly).^{1,3} Diverse reports have demonstrated that abnormal levels of these biothiols can cause a number of health problems.^{5–7} Thus, development of optical probes for their detection has been an active research area in recent years.⁸ Among the available techniques to detect and quantify biothiols (Cys, Hcy and GSH) fluorescence methods have been extensively pursued due to their simplicity, sensitivity, and versatility.⁸

In this work, we focus on the interaction between these biothiols and coumarin-based compounds containing a chalcone-like moiety. Specifically, we describe here (*E*)-3-cinnamoyl-7-methoxy-2*H*-chromen-2-one (**ChC1**) and (*E*)-3-(3-(2-hydroxyphenyl)acryloyl)-7-methoxy-2*H*-chromen-2-one (**ChC2**) as fluorescence probes for the detection of Cys, Hcy, GSH and, for the first time, Cys-Gly, mediated by a Michael addition reaction (Scheme 1).

The probes **ChC1** and **ChC2** were readily synthesized in four steps from commercial precursors (Scheme 1; Supplementary data). We then proceeded to study the fluorescence response of these probes to 2-mercaptoethanol (ME), as a model compound for biothiols (Figs. S3(A) and S4; Supplementary data). Figure S3(B) shows the turn-on fluorescence of **ChC1** upon addition of GSH (1 mM/HEPES buffer). The formation of the **ChC1**-GSH adduct is apparently complete within 50 min.

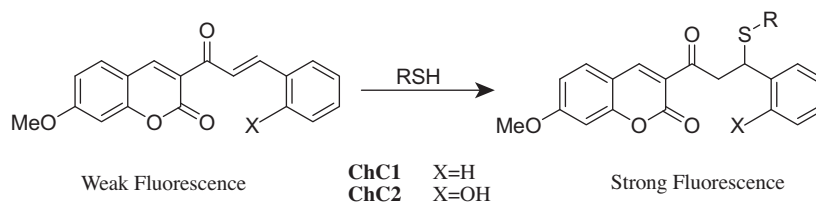
ChC1, excited at 340 nm, emitted with $\lambda_{\max} = 430$ nm and quantum yield $\Phi = 0.00083$. Upon addition of 10 equiv of Cys or GSH the quantum yield increased to 0.0013 and 0.0010, respectively, with no band shift. A similar experiment with **ChC2** ($\lambda_{\max} = 435$ nm, $\Phi = 0.0014$) led to the increase of the quantum yield to 0.0022 and 0.0016, respectively.

That these changes are due to a Michael addition was corroborated by ¹H NMR spectroscopy of the probes in the absence or presence of ME. As shown in Figure 1, upon addition of ME the vinyl proton resonances (H_b and H_a at δ 7.80 and 7.74 ppm, respectively) disappeared, and concurrently two new triplets, assigned to the thioether methylene protons H_b and H_a, emerged at δ 4.74 and 4.46 ppm, respectively, a result that is consistent with the formation of the **ChC1**-ME adduct.

The selectivity of **ChC1** and **ChC2** toward biothiols was investigated by incubating these probes with different species including non-sulfur amino acids (Asp, Lys, Ser, Hys, Pro, Phe, and Val). As shown in Figure 2 for the case of **ChC1**, none of these compounds

* Corresponding authors. Tel.: +56 2 3544742; fax: +56 2 3544744.

E-mail addresses: mealiaga@uc.cl (M.E. Aliaga), ppavezg@uc.cl (P. Pavez).



Scheme 1. Reaction of **ChC1** and **ChC2** with tested biothiols (RSH).

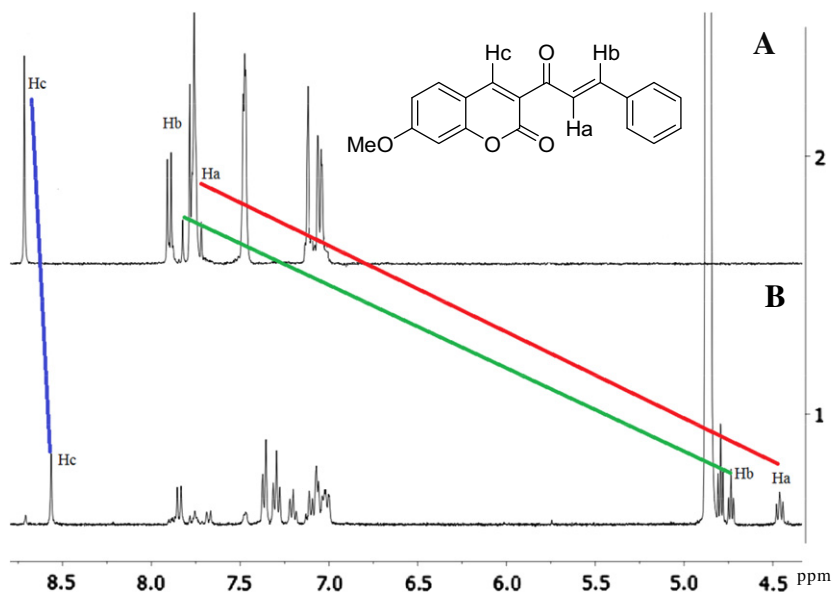


Figure 1. Change in the partial ^1H NMR spectrum of **ChC1** (20 mM) upon addition of ME (1.8 equiv) in $\text{DMSO-}d_6$ at $25\text{ }^\circ\text{C}$. (A) **ChC1** only; (B) **ChC1** + ME, (10 min).

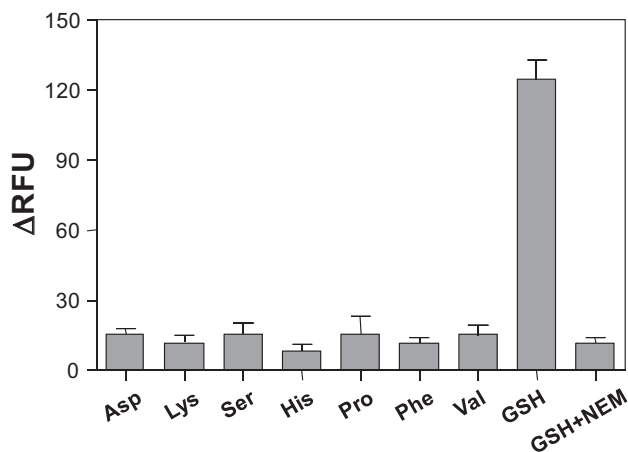


Figure 2. Fluorescence responses of **ChC1** (20 μM ; λ_{ex} 340 nm) in the presence of amino acids (2 mM), GSH (2 mM) or GSH plus NEM (500 μM).

interferes to any obvious extent with the detection of biothiols, even at a 100:1 molar ratio with respect to the probe. In addition, when GSH is co-incubated with *N*-ethylmaleimide (NEM), a sulfhydryl alkylating reagent, the fluorescence response of **ChC1** decreases considerably.

We then studied the kinetic profiles of the reactions of **ChC1** and **ChC2** with the tested biothiols under pseudo-first-order conditions (large excesses of the thiols). The observed rate constants (k_{obsd}) obtained at $30.0\text{ }^\circ\text{C}$ are shown in the [Supplementary data \(Table S1 and S2\)](#). For the purpose of comparison the relative second-order rate

Table 1

Values of $\text{p}K_{\text{a}}$ for the sulfhydryl group of biothiols and k_{N} values for the reactions of these thiols with **ChC1** and **ChC2**

Biothiols	$\text{p}K_{\text{a}}$	$k_{\text{N}}/\text{s}^{-1}\text{M}^{-1}$	
		ChC1	ChC2
Cysteinyglycine (Cys-Gly)	7.00	0.34 ± 0.02	20.14 ± 1.67
Cysteine (Cys)	8.10	5.17 ± 0.31	60.03 ± 2.34
Homocysteine (Hcy)	8.25	0.49 ± 0.04	20.92 ± 1.14
Glutathione (GSH)	8.72	1.56 ± 0.22	47.48 ± 1.91

constants were calculated. [Table 1](#) shows a comparison of the nucleophilic rate constants (k_{N}) for the reactions of **ChC1** and **ChC2** with the tested biothiols. It can be seen that in both cases the reactivity increases in the sequence $\text{Cys} > \text{GSH} > \text{Hcy} \geq \text{Cys-Gly}$. This order is in agreement with the basicity of the sulfhydryl group of each thiol ($\text{p}K_{\text{a}}$ values). The only exception is for Cys which, being one of the less basic thiols, is the most reactive compound. However, a similar result has been found very recently by other authors.^{8i,j}

Interestingly, we found that GSH is more reactive than Hcy in the Michael addition to **ChC1** and **ChC2**, in contrast to other probes which have proved to be less reactive toward GSH in comparison with Hcy.^{8i,j} However, a very recent paper describes a coumarin-substituted chalcone probe that reacts efficiently, via activation by an intramolecular hydrogen bond, with GSH and Cys but only slightly with non-thiol natural amino acids.⁹ In addition, a comparison of the nucleophilic rate constants (k_{N}) for the reactions with all four biothiols ([Table 1](#)) shows that under our experimental conditions **ChC2** is 12 to 60-fold more reactive toward thiolate ions than **ChC1**.

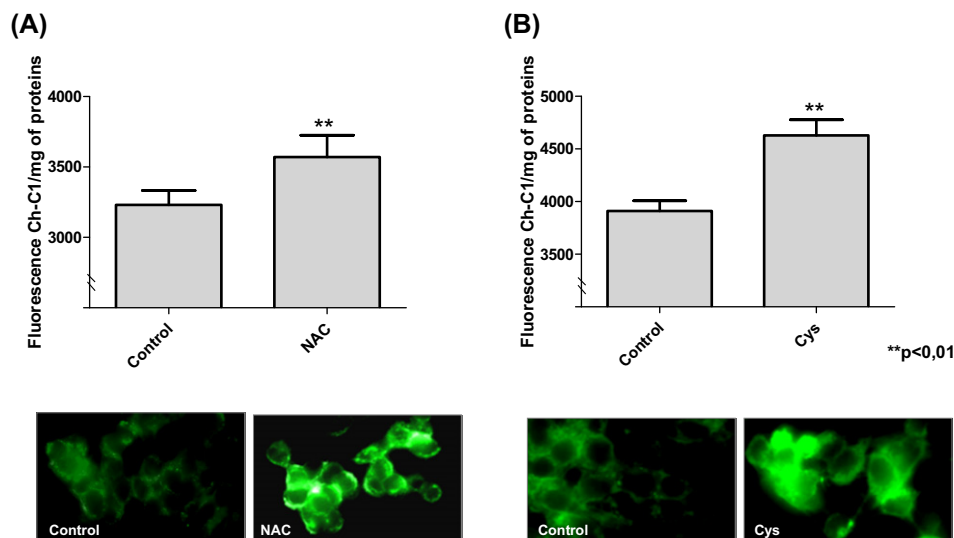


Figure 3. The **ChC1** fluorescence in SH-SY5Y cells increased by treatment with NAC or Cys. (A) The cells were treated for 12 h with NAC (30 mM), washed, and then incubated for 20 min with **ChC1** (5 μ M) and the fluorescence was measured using a microplate fluorescence reader and by epifluorescence microscopy. (B) The cells were incubated for 20 min with **ChC1** (5 μ M) and then Cys (5 mM) was added and the fluorescence was measured after 5 min using a microplate fluorescence reader and by epifluorescence microscopy. Data represent mean \pm SEM; $n = 6$, $P < 0.01$.

We propose that the higher reactivity of **ChC2** than **ChC1** toward thiolate ions might be a consequence of proton donation by the hydroxy group of **ChC2** to the vinyl carbon neighboring the carbonyl group, thus making the reaction site more prone to attack by the thiolate. While this manuscript was in preparation, it was reported that (*E*)-7-(diethylamino)-3-(3-(2-hydroxyphenyl)acryloyl)-2*H*-chromen-2-one, which bears an *ortho*-hydroxyl group in the same relative position as **ChC2**, showed an only threefold enhancement of its reaction rate with GSH as compared to (*E*)-3-cinnamoyl-7-diethylamino-2*H*-chromen-2-one, the corresponding analogue of **ChC1**.¹⁰ Those authors concluded that the participation of the hydroxyl group in this Michael addition was unlikely and therefore the presence of this group made little difference. In our hands, **ChC2** proved to be remarkably more reactive than **ChC1**, particularly toward Cys-Gly, Hcy, and GSH, warranting future studies of the detailed reaction mechanism.

A practical application of **ChC1** and **ChC2** was to evaluate the effect of *N*-acetylcysteine (NAC), an agent capable of stimulating the synthesis of glutathione,¹¹ on the fluorescence of both probes in human neuroblastoma SH-SY5Y cells. This cell line was treated for 12 h with NAC (30 mM) after which the cells were washed and incubated with the probes. The fluorescence was measured using a microplate fluorescence reader and by epifluorescence microscopy (Figs. 3A and 4A). The results showed that the fluorescence was significantly increased after NAC treatment. Similar results were observed when the cells were loaded with the probes and Cys (5 mM) was subsequently added, incubating for 5 min (Figs. 3B and 4B). The data represent mean \pm SEM; $n = 6$, $P < 0.01$.

In conclusion, we have prepared and characterized two new fluorescence turn-on sensors (**ChC1** and **ChC2**) that exhibit a highly selective response to biothiols both *in vitro* and *in vivo*. Unexpectedly, introduction of an *ortho*-hydroxyl group on the cinnamoyl

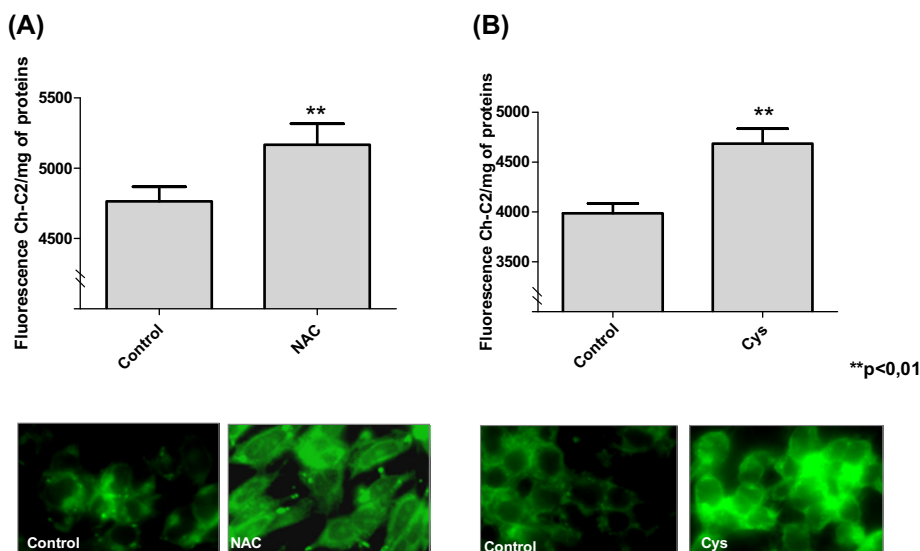


Figure 4. The **ChC2** fluorescence in SH-SY5Y cells increased by treatment with NAC or Cys. (A) The cells were treated for 12 h with NAC (30 mM) and then incubated for 20 min with **ChC2** (5 μ M) and the fluorescence was measured using a microplate fluorescence reader and by epifluorescence microscopy. (B) The cells were incubated for 20 min with **ChC2** (5 μ M) and then Cys (5 mM) was added and the fluorescence was measured after 5 min using a microplate fluorescence reader and by epifluorescence microscopy. Data represent mean \pm SEM; $n = 6$, $P < 0.01$.

moiety resulted in large rate enhancements of the Michael addition reactions that underlie the fluorescence response of our probes.

Acknowledgments

This work was supported by FONDECYT (Grants #11090115 and 1100640), and the Millennium Scientific Initiative (Grant P05-001-F).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.tetlet.2011.09.137](https://doi.org/10.1016/j.tetlet.2011.09.137).

References and notes

1. Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711.
2. Sies, H. *Free Rad. Biol. Med.* **1999**, *27*, 916.
3. Dickinson, D. A.; Forman, H. J. *Biochem. Pharmacol.* **2002**, *64*, 1019.
4. Ghezzi, P.; Bonetto, V.; Fratelli, M. *Antioxid. Redox Signal* **2005**, *7*, 964.
5. Heafield, M. T.; Fearn, S.; Steventon, G. B.; Waring, R. H.; Williams, A. C.; Sturman, S. G. *Neurosci. Lett.* **1990**, *110*, 216.
6. Nygard, O.; Nordrehaug, J. E.; Refsum, H.; Ueland, P. M.; Farstad, M.; Vollset, E. *N. N. Engl. J. Med.* **1997**, *337*, 230.
7. Jacobsen, D. W. *Clin. Chem.* **1998**, *44*, 1833.
8. For some reviews and recent examples, see: (a) Chen, X.; Zhou, Y.; Peng, X.; Yoon, J. *Chem. Soc. Rev.* **2010**, *39*, 2120; (b) Li, H.; Fan, J.; Wang, J.; Tian, M.; Du, J.; Sun, S.; Sun, P.; Peng, X. *Chem. Commun.* **2009**, 5904; (c) Yi, L.; Li, H.; Sun, L.; Liu, L.; Zhang, C.; Xi, Z. *Angew. Chem., Int. Ed.* **2009**, *48*, 4034; (d) Kwon, H.; Lee, K.; Kim, H.-J. *Chem. Commun.* **2011**, *47*, 1773; (e) Yang, Y.-K.; Shim, S.; Tae, J. *Chem. Commun.* **2010**, *46*, 7766; (f) Ruan, Y.-B.; Li, A.-F.; Zhao, J.-S.; Shen, J.-S.; Jiang, Y.-B. *Chem. Commun.* **2010**, *46*, 4938; (g) Yue, Y.; Guo, Y.; Xu, J.; Shao, S. *New J. Chem.* **2011**, *35*, 61; (h) Jung, H. S.; Ko, K. C.; Kim, G.-H.; Lee, A.-R.; Na, Y.-C.; Kang, C.; Lee, J. Y.; Kim, J. S. *Org. Lett.* **2011**, *13*, 1498; (i) Lin, W.; Yuan, L.; Cao, Z.; Feng, Y.; Long, L. *Chem. Eur. J.* **2009**, *15*, 5096; (j) Yuan, L.; Lin, W.; Yang, Y. *Chem. Commun.* **2011**, *47*, 6275.
9. Lim, S.-Y.; Lee, S.; Park, S. B.; Kim, H.-J. *Tetrahedron Lett.* **2011**, *52*, 3902.
10. Yoon, D.-H.; Park, S.; Ha, H.-J.; Kim, H.-J. *Tetrahedron* **2011**, *67*, 7759.
11. Kelly, G. S. *Altern. Med. Rev.* **1998**, *3*, 114.