Selective Mercuration of 2-Hydroxy Nile Red and Its Application towards Chemodosimetric Hg^{2+}-selective Signaling

Haekyung Lee, Myung Gil Choi, Hyo-Yeon Yu, Sangdoo Ahn, and Suk-Kyu Chang*

Department of Chemistry, Chung-Ang University, Seoul 156-756, Korea. *E-mail: skchang@cau.ac.kr

Received September 14, 2010, Accepted September 17, 2010

Selective mercuration at the 1,6-positions of 2-hydroxy derivative of Nile Red and its application towards Hg^{2+} ions over common coexisting physiologically important metal ions in aqueous environment. 1H NMR studies revealed that the mercuration was selectively effected at the 1,6-positions of 2-hydroxy Nile Red, which is quite different from that of the 6,8-positions for the parent Nile Red.

Key Words: Hg^{2+} signaling, Mercuration, Nile Red, Chemodosimeter, Fluorescence signaling

Introduction

Selective detection of transition and heavy metal ions is very important in various fields of chemical and biological sciences, as well as in the protection of our environment. Among many important transition metal ions, Hg^{2+} ions attract much research interest due to its toxic environmental impact. There are many sophisticated systems for the efficient and selective detection and visualization of Hg^{2+} ions. However, a great deal of effort has been continuously devoted toward the construction of devices that are able to signal and visualize the presence of Hg^{2+} in varying origins.

Recently, mercuration of important dyes has been used for the purpose of specific imaging and labeling of proteins. Mercuration of fluorescein and resorufin after subsequent transmetalation with AsCl3 afforded biarsenical ligands, FlAsH and ReAsH, respectively, which are employed for affinity chromatography, fluorescence measurements, and localization of tetracysteine-tagged proteins. Similarly, mercuration of Nile Red has provided dimercurated derivative for the preparation of biarsenical compound for the imaging of conformational changes of proteins containing tetracysteine motif.

Nile Red and related benzophenoxazine derivatives have been utilized as fluorescent dyes for the labeling of biomolecules. A series of water soluble Nile Red derivatives for the development of fluorescent probes for biotechnology have been prepared accordingly. Chemiluminescent energy-transfer cassettes based on fluorescein and Nile Red11 and near-IR fluorescence probes derived from Nile Red12 were also reported. Other Nile Red derivatives are currently used as probes for lipid- and drug-binding proteins, tools for hydrophobic characterization of intracellular lipids, and solvatochromic nucleoside for indicating micropolarity around DNA. During the course of search for a new probe system based on Nile Red derivatives, we found that Nile Red and its 2-hydroxy analogue exhibited quite different mercuration profile. In this paper, we report a selective mercuration of 2-hydroxy Nile Red and its potential for application toward chromogenic and fluorogenic Hg^{2+}-selective signaling. The process of the mercuration reaction mode of 2-hydroxy Nile Red could be confirmed by 1H NMR spectroscopy.

Results and Discussion

The 2-hydroxy derivative of Nile Red 1 was prepared by the reaction of 5-diethylamino-2-nitrosophenol, which was obtained by the reaction of 3-diethylaminophenol with NaNO2, with 1,6-dihydroxynaphthalene following the reported procedure (Scheme 1). First, the UV-vis signaling behavior of 1

Scheme 1. Preparation of the 2-hydroxy derivative of Nile Red 1

Figure 1. UV-vis spectra of 1 in the presence of various metal ions. [1] = 5.0 × 10^{-6} M, [M^{n+}] = 5.0 × 10^{-6} M. In acetate buffered (pH 4.7, 10 mM) H_2O-MeOH (50:50, v/v).
tion color changed from deep blue to light blue. Other surveyed metal ions was investigated. Compound 1 revealed a strong absorption at 580 nm in acetate buffered (pH 4.7, 10 mM) H₂O-MeOH (50:50, v/v).

The quenching was quite effective and the intensity ratio of fluorescence of the solution under illumination with a UV lamp. However, for 2-hydroxy Nile Red, mercuration was found to be affected at the 1,6-positions. The mercuration process was followed by measurements of the 1H-NMR spectrum of 1 in the presence of varying amounts of Hg(OAc)₂ in DMSO-d₆ solution.

With 1 equiv of Hg²⁺ ions, all the ¹H NMR resonances of compound 1 were somewhat broadened and downfield shifted without any significant changes in individual resonances (Figure 3). That might be due to the interaction of the Hg²⁺ ions with, most probably, the phenolic oxygen atom of Nile Red (Scheme 2). With 2 equiv of Hg²⁺ ions, the resonance of the 1-position proton at 8.0 ppm completely disappeared, while other resonances were not so significantly affected, consistent with the formation of monomercurated compound 1-HgOAc. Upon addition of 3 equiv of Hg²⁺ ions, subsequent disappearance of the resonance for the 6-position proton at 6.6 ppm was observed as expected for the formation of dimercurated product 1-(HgOAc)₂. With subsequent addition of Hg²⁺ ions (5 equiv), all the remaining resonances were significantly broadened.

The abovementioned chemodosimetric signaling behavior of 1 was further evidenced by treatment with EDTA (Figure 4). Diminished fluorescence of the 1-Hg²⁺ system, which was obtained by the treatment of 1 with 30 equiv of Hg²⁺ ions, was not affected by subsequent treatment with 50 equiv of the EDTA solution. This observation manifests the irreversible mercuration of representative alkali, alkaline earth, and transition metal ions was investigated. Compound 1 exhibited an intense fluorescence emission centered at 649 nm in acetate buffered aqueous 50% methanol solution at pH 4.7 (Figure 1). Upon treatment with various metal ions, the absorption spectrum was affected particularly with Hg²⁺ ions; the absorption intensity was significantly diminished with a slight blue-shift to 568 nm (Δλmax = 12 nm). The solution color changed from deep blue to light blue. Other surveyed metal ions revealed no significant changes.

Compound 1 exhibited an intense fluorescence emission centered at 649 nm in aqueous 50% methanol at pH 4.7. The Hg²⁺ ions quenched the fluorescence of 1 effectively and the fluorescence maximum was slightly red-shifted to 658 nm (Figure 2). The quenching was quite effective and the intensity ratio I/I₀ at 649 nm was 0.027 with 100 equiv of Hg²⁺ ions. The significant quenching of the fluorescence resulted in almost no fluorescence of the solution under illumination with a UV lamp. Other metal ions exhibited relatively minor effects on the fluorescence intensity and profile, and the I/I₀ values at 649 nm varied between 0.99 for Mg²⁺ and 1.04 for Ca²⁺.

The observed UV-vis and fluorescence signaling behaviors of 1 are due to the mercuration of 2-hydroxy Nile Red (Scheme 2). The dimercuration of parent Nile Red was known to be selectively affected at the 6,8-positions of the benzophenoxazine moiety. However, for 2-hydroxy Nile Red, mercuration was found to be affected at the 1,6-positions. The mercuration process was followed by measurements of the ¹H-NMR spectrum of 1 in the presence of varying amounts of Hg(OAc)₂ in DMSO-d₆ solution.

With 1 equiv of Hg²⁺ ions, all the ¹H NMR resonances of compound 1 were somewhat broadened and downfield shifted without any significant changes in individual resonances (Figure 3). That might be due to the interaction of the Hg²⁺ ions with, most probably, the phenolic oxygen atom of Nile Red (Scheme 2). With 2 equiv of Hg²⁺ ions, the resonance of the 1-position proton at 8.0 ppm completely disappeared, while other resonances were not so significantly affected, consistent with the formation of monomercurated compound 1-HgOAc. Upon addition of 3 equiv of Hg²⁺ ions, subsequent disappearance of the resonance for the 6-position proton at 6.6 ppm was observed as expected for the formation of dimercurated product 1-(HgOAc)₂. With subsequent addition of Hg²⁺ ions (5 equiv), all the remaining resonances were significantly broadened.

The abovementioned chemodosimetric signaling behavior of 1 was further evidenced by treatment with EDTA (Figure 4). Diminished fluorescence of the 1-Hg²⁺ system, which was obtained by the treatment of 1 with 30 equiv of Hg²⁺ ions, was not affected by subsequent treatment with 50 equiv of the EDTA solution. This observation manifests the irreversible mercuration.

Scheme 2. Selective 1,6-dimercuration of 2-hydroxy Nile Red
approximately 20 equiv of Hg$^{2+}$ ions. The emission maximum slightly increased, the fluorescence of [Ca$^{2+}$] = 3.0 mM, [Zn$^{2+}$] = 0.02 mM, and [Cu$^{2+}$] = 0.015 mM) possibility of mercuration of the Nile Red moiety.

6,8-dimercuration of the parent Nile Red. The results obtained 2-hydroxy Nile Red, which was quite different from that of Hg$^{2+}$ ions was estimated to be 2.8 × 10$^{-5}$ M. From this titration the detection limit of time course trace of the signaling of Hg$^{2+}$ ions of metal ions ([Na$^+$] = 138 mM, [K$^+$] = 4.0 mM, [Mg$^{2+}$] = 1.0 mM, [Ca$^{2+}$] = 3.0 mM, [Zn$^{2+}$] = 0.02 mM, and [Cu$^{2+}$] = 0.015 mM. In acetate buffered (pH 4.7, 10 mM) H$_2$O-MeOH (50:50, v/v).

Quantitative signaling behavior of I toward Hg$^{2+}$ ions was assessed by fluorescence titration. As the amount of Hg$^{2+}$ ions increased, the fluorescence of I decreased steadily until approximately 20 equiv of Hg$^{2+}$ ions. The emission maximum slightly shifted toward a longer wavelength. The signaling was not significantly interfered by the presence of other physiologically relevant metal ions. The fluorescence titration of I with Hg$^{2+}$ ions in the presence of representative physiologically important metal ions ([Na$^+$] = 138 mM, [K$^+$] = 4.0 mM, [Mg$^{2+}$] = 1.0 mM, [Ca$^{2+}$] = 3.0 mM, [Zn$^{2+}$] = 0.02 mM, and [Cu$^{2+}$] = 0.015 mM) as background resulted in similar titration curve (Figure 5). From this titration the detection limit of I for the signaling of Hg$^{2+}$ ions was estimated to be 2.8 × 10$^{-5}$ M.

To gain further insight into the signaling behavior of I, a time course trace of the signaling of Hg$^{2+}$ ions of I was measured. Changes in absorbance at 580 nm indicated that the signaling was somewhat slow and completed within 2 h after sample preparation, which is definitely undesirable behavior for an ideal system of fast chemodosimetric signaling. This sluggishness is due to the fact that signaling is based on the chemical transformation of the probe with analytes, as reported in other chemodosimeters. In fact, the mercuration of parent Nile Red required somewhat harsh conditions of overnight reaction using acetic acid at 50 °C. Attempts to signal Hg$^{2+}$ by Nile Red was also carried out, but the mercuration was much more sluggish and required several days to obtain a constant signal.

In summary, we have investigated selective mercuration of 2-hydroxy Nile Red and its chemodosimetric signaling behavior toward Hg$^{2+}$ ions. The 2-hydroxy Nile Red exhibited significant chromogenic and fluorescence responses toward Hg$^{2+}$ ions by selective mercuration in aqueous environments. The $^1$H NMR measurements evidenced the 1,6-dimercuration of 2-hydroxy Nile Red, which was quite different from that of 6,8-dimercuration of the parent Nile Red. The results obtained also suggest that one should pay extra attention to the design of Nile Red-based signaling and visualizing systems for the possibility of mercuration of the Nile Red moiety.

**Experimental**

**General.** Nile Red, N,N-diethyl-3-aminophenol, and 1,6-dihydroxy naphthalene were purchased from TCI Chemical Co. and used without further purification. The 2-hydroxy derivative of Nile Red was prepared following a reported procedure. All solvents were purchased from Aldrich Chemical Co. as ‘spectroscopic grade’. $^1$H NMR (600 MHz) and $^{13}$C NMR (150 MHz) spectra were obtained on a Varian VNS NMR spectrometer and referenced to the residual solvent signal. UV-vis spectra were recorded with a Jasco V-550 spectrophotometer. Fluorescence spectra were measured on an Amino-Bowman Series 2 Spectrophotometer.

**Preparation of compound 1.** 5-(Diethylamino)-2-nitrosophenol: Sodium nitrite (0.12 g, 1.8 mM) was dissolved in water (1 mL) and a solution of N,N-diethyl-3-aminophenol (0.20 g, 1.2 mM) in aqueous HCl (1.3 mL, 6 N) was added in several small portions at 0 °C. After stirring for 3 h at 0 °C, the resulting precipitate was filtered and dried to yield the product (85%). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 7.30 (d, J = 10.2 Hz, 1H), 6.88 (d, J = 10.2 Hz, 1H), 6.05 (s, 1H), 3.57 (m, 4H), 2.53-2.2 (m, 6H), 1.30 (s, 6H), 1.00 (s, 3H). $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 185.7, 138.8, 134.2, 130.4, 127.9, 124.3, 118.8, 110.4, 108.6, 104.5, 96.5, 44.9, 12.9.

**Mercuration of 1.** A mixture of compound I (10 mM) and Hg(OAc)$_2$ (10 - 50 mM) was dissolved in DMSO-d$_6$ solution and the $^1$H NMR spectrum was measured. Monomercurated product I-HgOAc; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 7.94 (d, J = 8.4 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.07 (d, J = 8.1 Hz, 1H), 6.82 (d, J = 7.8 Hz, 1H), 6.61 (s, 1H), 6.18 (s, 1H), 3.47 (br m, 4H), 1.15 (br m, 6H). $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 181.9, 173.6, 163.6, 152.7, 151.3, 147.4, 138.4, 137.0, 130.4, 128.4, 125.3, 122.7, 110.5, 105.0, 96.5, 44.9, 22.2, 12.9. Dimercurated product I-(HgOAc)$_2$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 7.98 (d, J = 8.7 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.4 Hz, 1H), 6.64 (s, 1H), 3.49 (br m, 4H), 1.17 (br m, 6H). $^{13}$C NMR spectral data could not be obtained due to the limited solubility of I-(HgOAc)$_2$ in common NMR solvents.

**UV-vis and fluorescence measurements.** Stock solutions of compound I and Nile Red were prepared as 5.0 × 10$^{-4}$ M in methanol. Stock solutions of metal perchlorates were prepared as 1.0 × 10$^{-2}$ M in water. The working solutions were prepared by adding different volumes of stock solutions of probe, metal perchlorates, and buffer, followed by dilution to 3.0 mL by

![Figure 5.](image-url)
adding calculated amounts of methanol and water. The pH of the solution was fixed at 4.7 with an acetate buffer solution. The final concentrations of the measuring solution were [1] = 5.0 × 10⁻⁴ M, [M⁺] = 5.0 × 10⁻⁴ M, and [acetate buffer] = 1.0 × 10⁻² M in a methanol-water solution (1:1, v/v) for UV-vis and fluorescence measurements (excitation wavelength = 580 nm). The measuring solution was thoroughly mixed and the absorption and fluorescence spectra were measured after 2 h of sample preparation. Fluorescence titration was carried out in a methanol-water (1:1, v/v) solution. Working solutions were prepared by adding 0 to 100 equiv of Hg²⁺ ions to the solution of 1 (5.0 × 10⁻⁴ M) and the fluorescence spectrum was measured with an excitation wavelength of 580 nm. Fluorescent titration of 1 with Hg²⁺ ions was also carried out similarly in the presence of physiologically relevant ions ([Na⁺] = 138 mM, [K⁺] = 4.0 mM, [Mg²⁺] = 1.0 mM, [Ca²⁺] = 3.0 mM, [Zn²⁺] = 0.02 mM, and [Cu²⁺] = 0.015 mM) as background.

Acknowledgments. This work was supported by a Fund from Chung-Ang University in 2010.

References