JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

A Mitochondria-Targetable Fluorescent Probe for Dual-Channel NO Imaging Assisted by Intracellular Cysteine and Glutathione

Yuan-Qiang Sun,[†,§] Jing Liu,[†,§] Hongxing Zhang,[†] Yingying Huo,[†] Xin Lv,[†] Yawei Shi,[‡] and Wei Guo*[,†]

[†]School of Chemistry and Chemical Engineering and [‡]Institute of Biotechnology, Shanxi University, Taiyuan 030006, China

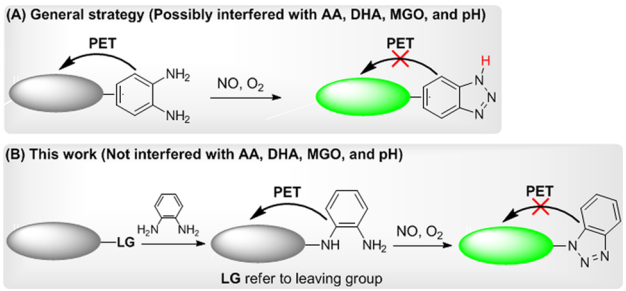
Supporting Information

ABSTRACT: A mitochondria-specific fluorescent probe for NO (**1**) was synthesized by the direct conjugation of a pyronin dye with one of the amino groups of *o*-phenylenediamino (OPD). The probe could selectively detect NO over dehydroascorbic acid (DHA), ascorbic acid (AA), and methylglyoxal (MGO) as well as the reactive oxygen/nitrogen species (ROS/RNS) with the significant *off*–*on* response due to the production of a red-emission triazole **2**. In the presence of cysteine/glutathione (Cys/GSH), **2** could be further transformed into a green-emission aminopyronin **4** and a red-emission thiopyronin **5**, respectively. Assisted by intracellular Cys and GSH, the probe demonstrated its potential to monitor mitochondrial NO in a dual-channel mode.

Nitric oxide (NO), produced by nitric oxide synthases, has been recognized as an ubiquitous signaling molecule. It plays key roles in various physiological systems, such as cardiovascular, immune, and the central and peripheral nervous systems.[1] Also, studies have shown that its down-regulation is closely linked with various pathophysiological conditions including the endothelial dysfunction, cancer, and neurodegenerative diseases.[2] However, the detailed mechanisms by which NO exerts its diverse biological functions still remain elusive. Thus, for better understanding of NO's origins, activities, and biological functions, it is very important to search for methods and tools that can sensitively and selectively probe NO in biological systems.

To this end, several techniques such as colorimetry, electron paramagnetic resonance, electrochemistry, and chemiluminescence have been developed.[3] Due to the simplicity and sensitivity as well as real-time and nondestructive imaging properties, fluorescence techniques have been considered ideal for sensing and imaging various cations, anions, and biomolecules in live cells or tissues. Accordingly, a number of fluorescent probes for NO have been exploited in the past decade by taking the advantage of some specific reactions of NO with *o*-phenylenediamino (OPD) moiety,[4,5] metal–ligand complexes,[6] and others.[7] Among them, the OPD-based fluorescent probes, pioneered by Nagano's group, have shown great potentials to image NO *in vitro* and *in vivo* under physiological conditions.[4,5] Moreover, some of such probes, such as DAF-2 DA and DAR-4 M AM, are commercially available[8] and have been widely applied in NO-related biological studies. The corresponding sensing mechanism is based on an irreversible reaction of OPD with NO^+ or N_2O_3 to form benzotriazole derivatives, whereby the fluorescence turn-on response can be achieved by inhibiting the quenching process via the photoinduced electron transfer (PET) from OPD to the excited fluorophore (Scheme 1A).

Scheme 1. Strategies for the OPD-Based Fluorescent NO Probes



However, although in specific biological systems such probes can provide useful information, some limitations still remain. For example, dehydroascorbic acid (DHA) and ascorbic acid (AA) were reported to be able to condense with OPD and turn on the fluorescence.[9] Therefore, the undesired interference may occur due to high physiological concentrations of AA and DHA (at micromolar to millimolar levels) colocalized with nanomolar to micromolar levels of NO in many cells.[10] Moreover, a OPD-based Bodipy fluorescent probe was recently reported to detect methylglyoxal (MGO),[11] a dicarbonyl metabolite produced by all living cells, under physiological conditions.[12] In addition, benzotriazole is pH sensitive, and its deprotonation at physiological pH can result in the formation of an electron-rich triazolate, which may induce the fluorescence quenching through a PET mechanism to lower the NO detection sensitivity.[4e,g] Therefore, just as pointed out by Anslyn and Shear: "the aforementioned limitations complicate NO detection using *o*-diamines".[13] On the other hand, mitochondria is the main organelle where NO is synthesized by the inducible NO synthase (iNOS) and plays important roles in regulating cell functions.[14] However, to the best of our knowledge, only one mitochondria-targetable fluorescent probe for NO has been reported to date.[5d]

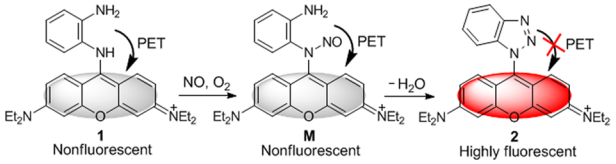
The aforementioned concerns encouraged us to develop a new strategy to sense this important signaling molecule. As shown in Scheme 1B, unlike the general design strategy that a fluorophore is linked with the phenyl ring of the OPD unit, our strategy features the direct conjugation of a fluorophore with one amino

Received: April 25, 2014
Published: August 14, 2014

   dx.doi.org/10.1021/ja504156a | *J. Am. Chem. Soc.* 2014, 136, 12520–12523

group of OPD. We speculated that the resulting probe may lose its capacity of condensing with DHA, AA, and MGO to form quinoxaline heterocycles, thereby increasing the specificity for NO. Also, the strategy allows the fluorescence *off*–*on* switching to be modulated by the widely used PET mechanism. Moreover, due to the absence of the NH proton in the resulting triazole product, the benzotriazolate-induced fluorescence quenching via PET under physiological conditions could be precluded, thereby improving the detection sensitivity. As a proof of concept, we constructed a fluorescent probe **1** by incorporating the OPD moiety to 9-position of pyronin dye (Scheme 2). Notably, like

Scheme 2. Proposed Sensing Mechanism of Probe 1 for NO



the mitochondria-selective probes rhodamine 123 and tetramethylrosamine,[8] probe **1** possesses a positive charge delocalized through resonance and was expected to be cell-permeant and readily sequestered by mitochondria to sense NO in this essential organelle.

To test the above-mentioned speculation, we synthesized **1** by following the procedure reported by Burgess.[15] The structure of **1** was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra (Supporting Information). Subsequently, we examined its reactivity toward NO through UV–vis and fluorescence spectra in PB buffers (50 mM, pH 7.4, containing 30% CH_3CN) at 25 °C. DEA·NONOate was used as the source of NO, which is commercially available with a half time of 16 min. As shown in Figure 1A, probe **1** itself showed a main absorption at 456 nm,

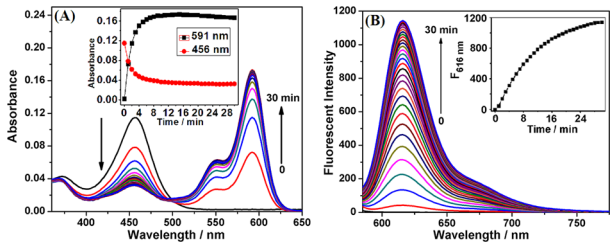


Figure 1. Time-dependent absorption (A) and fluorescence (B) spectra of **1** (2 μM) in the presence of 100 equiv DEA·NONOate in PB buffer (50 mM, pH 7.4, containing 30% CH_3CN) at 25 °C. Insets: the corresponding intensity changes as a function of time. λ_{ex} = 570 nm, λ_{em} = 616 nm. Slits: 5/10 nm, voltage: 600 V.

characteristic for aminopyronin dyes,[15] but no fluorescence when excited at 456 nm (Φ = 0.0003 with fluorescein in 0.1 N NaOH as the reference) due to an efficient PET from the electron-rich OPD moiety to the excited pyronin core. Upon the addition of 100 equiv of DEA·NONOate, the initial absorption band decreased along with a simultaneous emergence of the red-shifted absorption centered at 591 nm. Such a large bathchromic shift of 135 nm could eliminate any undesired background fluorescence from the unreacted probe (if any). Indeed, when excited at 570 nm (near the absorption maximum of the product), a significant fluorescence enhancement of 2743-fold at 616 nm (Φ = 0.25 with Cresyl Violet Perchlorate in EtOH as the reference) was observed from the dark background (Figure 1B). According to the well-established reaction mechanism, the new spectra could be assigned to triazole **2**, which was also supported by HRMS experiment (Figure S1). Notably, the fluorescence of **2** fell into the red region (>600 nm), which is especially preferable for *in vivo* bioimaging due to the deep penetration ability and low background autofluorescence. For explaining why the fluorescence response obviously lags behind the absorption changes (Figure 1, insets; details in Figure S2), an intermediate M was proposed (Scheme 2) and then observed in HRMS assay (Figure S3), which may have similar absorption wavelength to 2, but is nonfluorescent due to a PET quenching process (A detailed discussion was shown in Figure S3, caption).

Next, we performed fluorescence titration studies of **1** for NO at a time point of 30 min after the addition of DEA·NONOate. The spectra of the solution of **1** treated with a series of DEA·NONOate (0 to 200 μM) were recorded. As shown in Figure S4A, upon the treatment with the NO donor, a new emission emerged at 616 nm and gradually increased with the increasing concentrations of the NO donor. An excellent linear correlation (R^2 = 0.994) between the fluorescence intensities and the DEA·NONOate concentrations from 0 to 120 μM was observed (Figure S4B), and the detection limit was estimated to be 12 nM based on S/N = 3.

Next we evaluated the specificity of probe **1** for NO. We screened a wide array of the possible competitive species, including reactive oxygen/nitrogen species (ROS/RNS), DHA, AA, and MGO (Figure 2). Like most of the OPD-based

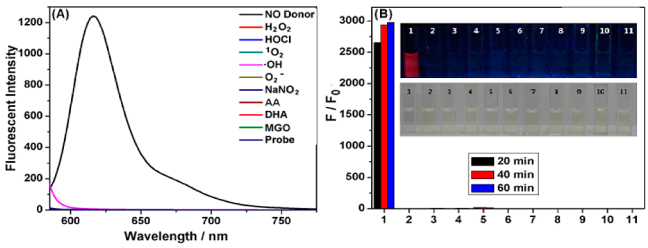


Figure 2. (A) Fluorescence spectra of **1** (2 μM) in the presence of 100 equiv various species after 60 min. (B) Fluorescence intensities of **1** (2 μM) upon addition of 100 equiv of various species after 20, 40, and 60 min, respectively. (1) DEA·NONOate, (2) H_2O_2, (3) HClO, (4) O_2^1, (5) •OH, (6) O_2^-, (7) NO_2^-, (8) AA, (9) DHA, (10) MGO, (11) **1** only. Condition: PB buffer (50 mM, pH 7.4, containing 30% CH_3CN); 25 °C; λ_{ex} = 570 nm, λ_{em} = 616 nm. Slits: 5/10 nm, voltage: 600 V.

fluorescent probes, probe **1** was inert to other ROS and RNS, such as H_2O_2, ClO^-, •OH, O_2^-, $^1O_2$, NO_2^-. Moreover, probe **1** did not show any response to DHA, AA, and MGO. By comparison, only NO elicited a dramatic increase in fluorescence intensity at 616 nm, suggesting the high selectivity of **1**. We attributed the high selectivity of **1** toward NO over DHA, AA, and MGO to the amino-protected OPD unit in **1**, which failed to condense with the 1,2-dicarbonyl group of DHA/AA/MGO to form the quinoxaline heterocycles. In this sense, probe **1** appeared to be superior to those commercial NO probes such as DAF-2 DA and DAR-4 M AM.[8]

Also, we studied the emission behavior of **1** treated with DEA·NONOate in different pH environments. As shown in Figure S5, probe **1** showed no emission in a pH region of 4–12 but displayed the best response for NO in the region of 7–8 due to the absence of the NH proton in triazole **2**, which precluded the benzotriazolate-induced fluorescence quenching via PET in physiological conditions. Thus, probe **1** is more advantageous for

dx.doi.org/10.1021/ja504156a | J. Am. Chem. Soc. 2014, 136, 12520–12523

practical applications in biological systems than most OPD-based fluorescent probes.

These *in vitro* assays confirmed the capability of **1** for the specific NO detection. Before testing its applications in biological systems we measured the fluorescence response of **1** toward NO in the presence of biothiols, such as cysteine (Cys) and glutathione (GSH), due to their high concentrations in cells.[16] Interestingly, the addition of DEA·NONOate to the mixtures of **1**/Cys and **1**/GSH, respectively, elicited a bright green fluorescence with λ_{max} = 536 nm for the former (Figure 3A)

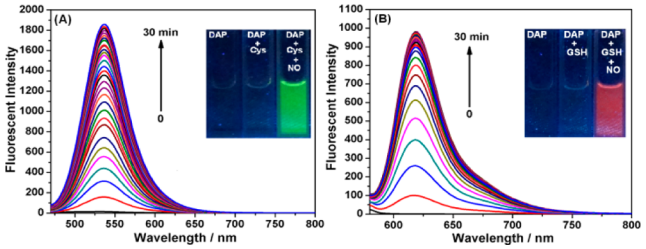
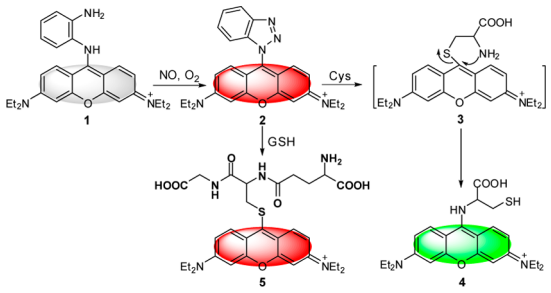


Figure 3. Time-dependent fluorescence spectra of **1** (2 μM) upon addition of DEA·NONOate (200 μM) in the presence of Cys (200 μM) (A) and GSH (200 μM) (B), respectively. For (A), λ_{ex} = 455 nm. For (B), λ_{ex} = 570 nm. Condition: PB buffer (50 mM, pH 7.4, containing 30% CH_3CN); 25 °C; Slits: 5/10 nm; voltage: 600 V.

and a bright red fluorescence with λ_{max} = 618 nm for the latter (Figure 3B). The same results were also observed when the solution of **1** was treated with DEA·NONOate for 30 min and then by adding Cys or GSH (Figure S6). Based on the observation that probe **1** did not react with Cys or GSH (Figure S7) as well as our recent studies on Cys (or GSH)-induced S_NAr substitution−rearrangement (or S_NAr substitution) reaction with a thiophenol−pyronin dye,[17] we proposed the following reaction mechanism. As shown in Scheme 3, the initial reaction

Scheme 3. Proposed Reaction Mechanisms of 1 with NO in the Presence of Cys and GSH, Respectively



of **1** with NO should produce triazole **2**. Because the benzotriazole unit was reported to be a leaving group in S-acylation reactions,[18] we speculated that a Cys-induced S_NAr substitution−rearrangement cascade reaction or a GSH-induced S_NAr substitution reaction could occur,[17,19] leading to the green-emission aminopyronin **4** via intermediate **3** or the red-emission thiopyronin **5**, respectively. In fact, the emission spectra of **4** and **5** matched well with those of the corresponding control compounds aminocouramin **6** and thiocoumarin **7** (Figure S8) and were also in line with our recent report, wherein the same products were obtained.[17] The HRMS results also confirmed our hypothesis, wherein **4** and **5** could be clearly observed (Figure S9). Overall, it is very possible that the intracellular NO could be detected from the green and red channels simultaneously assisted by intracellular Cys and GSH (More detailed information, see Figures S10 and S11).

Subsequently, we evaluated the capability of **1** to image NO in B16 cells. As shown in Figure 4A1 and A2, when the cells were

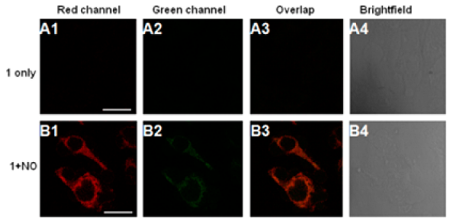


Figure 4. (A1−A4) Fluorescence images of B16 cells stained by **1** (2 μM, 30 min) only. (B1−B4) Fluorescence images of B16 cells stained by **1** (2 μM, 30 min) and then DEA·NONOate (200 μM, 30 min). Emission was collected at 480−550 nm for green channel (excited at 458 nm) and at 580−630 nm for red channel (excited at 543 nm). Scale bar: 20 μm.

incubated with **1** (2 μM), no fluorescence in both red and green channels was observed. However, when the cells were pretreated with **1** for 30 min, and then incubated with DEA·NONOate for 30 min, strong fluorescence in both red (Figure 4B1) and green channels (Figure 4B2) was observed, confirming that **1** could sense intracellular NO in multicolor imaging assisted by intracellular GSH and Cys. Moreover, the overlap image of Figure 4B1 and 4B2 indicated that **4** and **5** located in the same place in the cells (Figure 4B3). To confirm that **1** was a mitochondria-targetable probe for NO, B16 cells were treated in a sequence with **1** and MitoTracker Red FM (a known fluorescence marker for mitochondria). After washing with PBS, the cells were treated with DEA·NONOate in the culture media for 30 min. As shown in Figure 5A3, the fine merged image

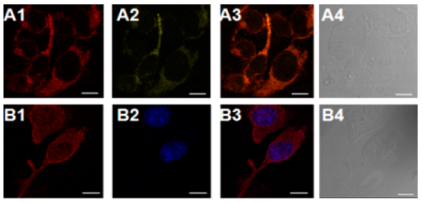


Figure 5. (A1−A4) Fluorescence images of B16 cells costained by **1** (2 μM, 30 min), MitoTracker Red FM (0.5 μM, 30 min) and then DEA·NONOate (200 μM, 30 min): (A1) image from band path of 580−630 nm upon excitation of **5** at 543 nm; (A2) image (reproduced in pseudocolor) from band path of 640−730 nm upon excitation of MitoTracker Red FM at 633 nm; (A3) the overlay imaging of A1 and A2; (A4) the bright-field image. (B1−B4) Fluorescence images of B16 cells costained by **1** (2 μM, 30 min), DIPA (1 μg/mL, 30 min), and then DEA·NONOate (200 μM, 30 min): (B1) image from band path of 580−630 nm upon excitation of **5** at 543 nm; (B2) image from band path of 410−490 nm upon excitation of DIPA at 405 nm; (B3) overlay of B1 and B2; (B4) the bright-field image. Scale bar: 10 μm.

clearly confirmed that the dye could specifically image mitochondrial NO. The bright-field measurements and nuclear staining with DIPA confirmed the viability of the cells over the course of the experiments (Figure 5B1−B4). The low cytotoxicity of **1** was also supported by MTT assay (Figure S12).

Finally, we performed fluorescence microscopic imaging of the endogenous NO in RAW264.7 murine macrophages.[6f] As shown in Figure S13, macrophages RAW264.7 incubated with **1** (2 μM) showed a weak fluorescence in both red and green channels,

dx.doi.org/10.1021/ja504156a | J. Am. Chem. Soc. 2014, 136, 12520−12523

indicating that **1** could detect the low level of endogenous NO in the cells. Stimulated with interferon-γ (IFN-γ), lipopolysaccharide (LPS), and L-arginine (L-Arg) for 12 h at 37 °C, the obvious fluorescence enhancements in both red and green channels were observed. These results were consistent with previous reports that IFN-γ/LPS/L-Arg could induce the production of NO in the cells,[5c,d] demonstrating the potential of **1** to image endogenously produced NO.

In summary, we constructed a novel fluorescence probe **1** by the direct conjugation of a pyronin dye with one amino group of OPD. The probe was proved to selectively sense NO over ROS/RNS as well as DHA, AA, and MGO. In the presence of Cys and GSH, triazole **2** produced by the reaction of **1** with NO could be further converted into green-emission aminopyronin **4** and red-emission thiopyronin **5**. Assisted by intracellular Cys and GSH, **1** could detect the exogenous and endogenous NO in mitochondria in cells from two distinct emission channels.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

guow@sxu.edu.cn.

Author Contributions

[§]These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the Natural Science Foundation of China (nos. 21172137 and 21302114) for support of this work. We thank professor Ming Xian for careful reviewing of the manuscript.

■ REFERENCES

(1) (a) Snyder, S. H. *Science* **1992**, *257*, 494. (b) Jasid, S.; Simontacchi, M.; Bartoli, C. G.; Puntarulo, S. *Plant Physiol.* **2006**, *142*, 1246. (c) Fukumura, D.; Kashiwagi, S.; Jain, R. K. *Nat. Rev. Cancer* **2006**, *6*, 521.

(2) (a) Taysi, S.; Uslu, C.; Akcay, F.; Sutbeyaz, M. Y. *Surg. Today* **2003**, *33*, 651. (b) Cobbs, C. S.; Brenman, J. E.; Aldape, K. D.; Bredt, D. S.; Israel, M. A. *Cancer Res.* **1995**, *55*, 727. (c) Biro, G. P. *Curr. Drug Discovery Technol.* **2012**, *9*, 194. (d) Giedt, R. J.; Yang, C. J.; Zweier, J. L.; Matzavinos, A.; Alevriadou, B. R. *Free Radicals Biol. Med.* **2012**, *52*, 348. (e) Choi, D. Y.; Lee, Y. J.; Hong, J. T.; Lee, H. J. *Brain Res. Bull.* **2012**, *87*, 144. (f) Gutowicz, M. *Postepy Hig. Med. Dosw.* **2011**, *65*, 104.

(3) (a) Lalezari, P.; Lekhraj, R.; Casper, D. *Anal. Biochem.* **2011**, *416*, 92. (b) Hogg, N. *Free Radicals Biol. Med.* **2010**, *49*, 122. (c) Rodriguez-Rodriguez, R.; Simonsen, U. *Curr. Anal. Chem.* **2012**, *8*, 485. (d) Mandon, J.; Hogman, M.; Merkus, J. F. M.; van Amsterdam, J.; Harren, F. J. M.; Cristescu, S. M. *J. Biomed. Opt.* **2012**, *17*, No. 017003.

(4) (a) Kojima, H.; Sakurai, K.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Akaike, T.; Maeda, H.; Nagano, T. *Biol. Pharm. Bull.* **1997**, *20*, 1229. (b) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446. (c) Nakatsubo, N.; Kojima, H.; Kikuchi, K.; Nagoshi, H.; Hirata, Y.; Maeda, D.; Imai, Y.; Irimura, T.; Nagano, T. *FEBS Lett.* **1998**, *427*, 263. (d) Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Hirata, Y.; Nagano, T. *Angew. Chem., Int. Ed.* **1999**, *38*, 21. (e) Kojima, H.; Hirotani, M.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. *Tetrahedron Lett.* **2000**, *41*, 69. (f) Kojima, H.; Hirotani, M.; Nakatsubo, N.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Hirata, Y.; Nagano, T. *Anal. Chem.* **2001**, *73*, 1967. (g) Gabe, Y.; Urano, Y.; Kikuchi, K.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 3357. (h) Sasaki, E.; Kojima, H.; Nishimatsu, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 3684. (i) Terai, T.; Urano, Y.; Izumi, S.; Hojima, H.; Nagano, T. *Chem. Commun.* **2012**, *48*, 2840.

(5) (a) Zheng, H.; Shang, G.-Q.; Yang, S.-Y.; Gao, X.; Xu, J.-G. *Org. Lett.* **2008**, *10*, 2357. (b) Yuan, L.; Lin, W.; Xie, Y.; Chen, B.; Zhu, S. *J. Am. Chem. Soc.* **2012**, *134*, 1305. (c) Yu, H.; Xiao, Y.; Jin, L. *J. Am. Chem. Soc.* **2012**, *134*, 17486. (d) Yu, H.; Zhang, X.; Xiao, Y.; Zou, W.; Wang, L.; Jin, L. *Anal. Chem.* **2013**, *85*, 7076. (e) Vegesna, G. K.; Sripathi, S. R.; Zhang, J.; Zhu, S.; He, W.; Luo, F.-T.; Jahng, W. J.; Frost, M.; Liu, H. *ACS Appl. Mater. Interfaces* **2013**, *5*, 4107. (f) Yu, C.; Wu, Y.; Zeng, F.; Wu, S. *J. Mater. Chem. B* **2013**, *1*, 4152.

(6) (a) Lim, M. H.; Lippard, S. J. *J. Am. Chem. Soc.* **2005**, *127*, 12170. (b) Smith, R. C.; Tennyson, A. G.; Lim, M. L.; Lippard, S. J. *Org. Lett.* **2005**, *7*, 3573. (c) Lim, M. H.; Xu, D.; Lippard, S. J. *Nat. Chem. Biol.* **2006**, *2*, 373. (d) Lim, M. H.; Wong, B. A.; Pitcock, W. H., Jr.; Mokshagundam, D.; Baik, M.-H.; Lippard, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 4363. (e) Pluth, M. D.; McQuade, L. E.; Lippard, S. J. *Org. Lett.* **2010**, *12*, 2318. (f) McQuade, L. E.; Ma, J.; Lowe, G.; Ghatpande, A.; Gelperin, A.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 8525. (g) Pluth, M. D.; Chan, M. R.; McQuade, L. E.; Lippard, S. J. *Inorg. Chem.* **2011**, *50*, 9385. (h) Hu, X.; Wang, J.; Zhu, X.; Dong, D.; Zhang, X.; Wu, S.; Duan, C. *Chem. Commun.* **2011**, *47*, 11507. (i) Mondal, B.; Kumar, P.; Ghosh, P.; Kalita, A. *Chem. Commun.* **2011**, *47*, 2964.

(7) (a) Sun, C.; Shi, W.; Song, Y.; Chen, W.; Ma, H. *Chem. Commun.* **2011**, *47*, 8638. (b) Shiue, T.-W.; Chen, Y.-H.; Wu, C.-M.; Singh, G.; Chen, H.-Y.; Hung, C.-H.; Liaw, W.-F.; Wang, Y.-M. *Inorg. Chem.* **2012**, *51*, 5400. (c) Lv, X.; Wang, Y.; Zhang, S.; Liu, Y.; Zhang, J.; Guo, W. *Chem. Commun.* **2014**, *50*, 7499.

(8) Johnson, I.; Spence, M. T. Z. *The Molecular Probes Handbook—A Guide to Fluorescent Probes and Labeling Technologies*, 11th ed.; Life Technologies: Carlsbad, CA, 2010.

(9) (a) Ye, X.; Rubakhin, S. S.; Sweedler, J. V. *J. Neurosci. Methods* **2008**, *168*, 373. (b) Zhang, X.; Kim, W.-S.; Hatcher, N.; Potgieter, K.; Moroz, L. L.; Gillette, R.; Sweedler, J. V. *J. Biol. Chem.* **2002**, *277*, 48472.

(10) (a) Grunewald, R. A. *Brain Res. Rev.* **1993**, *18*, 123. (b) Washko, P. W.; Wang, Y. H.; Levine, M. *J. Biol. Chem.* **1993**, *268*, 15531. (c) Ek, A.; Strom, K.; Cotgreave, I. A. *Biochem. Pharmacol.* **1995**, *50*, 1339. (d) Rice, M. E.; Russo-Menna, I. *Neuroscience* **1998**, *82*, 1213.

(11) Wang, T.; Douglass, E. F., Jr.; Fitzgerald, K. J.; Spiegel, D. A. *J. Am. Chem. Soc.* **2013**, *135*, 12429.

(12) (a) Kalapos, M. P. *Toxicol. Lett.* **1999**, *110*, 145. (b) Thornalley, P. I. *Drug Metab. Drug Interact.* **2008**, *23*, 125.

(13) Yang, Y.; Seidlits, S. K.; Adams, M. M.; Lynch, V. M.; Schmidt, C. E.; Anslyn, E. V.; Shear, J. B. *J. Am. Soc. Chem.* **2010**, *132*, 13114.

(14) (a) Marletta, M. A. *Cell* **1994**, *78*, 927. (b) Forstermann, U.; Gath, I.; Schwarz, P.; Closs, E. I.; Kleinert, H. *Biochem. Pharmacol.* **1995**, *50*, 1321. (c) Lopez-Figueroa, M. O.; Day, H. E. W.; Akil, H.; Watson, S. J. *Histol. Histopathol.* **1998**, *13*, 1243.

(15) Wu, L.; Burgess, K. *Org. Lett.* **2008**, *10*, 1779.

(16) (a) Chung, T. K.; Funk, M. A.; Baker, D. H. *J. Nutr.* **1990**, *120*, 158. (b) Park, S.; Imlay, J. A. *J. Bacteriol.* **2003**, *185*, 1942. (c) Hwang, C.; Sinskey, A. J.; Lodish, H. F. *Science* **1992**, *257*, 1496.

(17) Liu, J.; Sun, Y.-Q.; Zhang, H.; Huo, Y.; Shi, Y.; Guo, W. *Chem. Sci.* **2014**, *5*, 3183.

(18) (a) Katritzky, A. R.; Ibrahim, T. S.; Tala, S. R.; Abo-Dya, N. E.; Abdel-Samii, Z. K.; EI-Feky, S. A. *Synthesis* **2011**, *9*, 1494. (b) Katritzky, A. R.; Angrish, P.; Todadze, E. *Synlett* **2009**, *15*, 2392.

(19) (a) Niu, L.-Y.; Guan, Y.-S.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. *Chem. Commun.* **2013**, *49*, 1294. (b) Niu, L.-Y.; Guan, Y.-S.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. *J. Am. Chem. Soc.* **2012**, *134*, 18928.