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Designed To Dissolve: Suppression of Colloidal Aggregation of Cu(I)-Selective Fluorescent Probes in Aqueous Buffer and In-Gel Detection of a Metallochaperone

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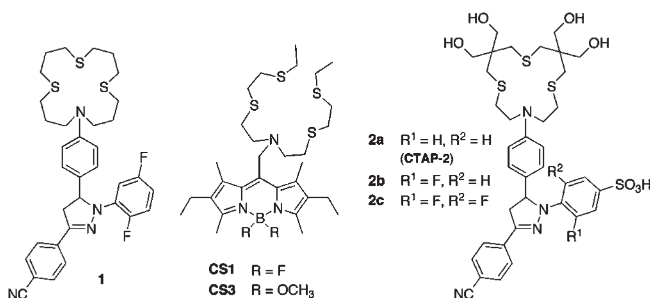
S Supporting Information

ABSTRACT: Due to the lipophilicity of the metal-ion receptor, previously reported Cu(I)-selective fluorescent probes form colloidal aggregates, as revealed by dynamic light scattering. To address this problem, we have developed a hydrophilic triarylpyrazoline-based fluorescent probe, **CTAP-2**, that dissolves directly in water and shows a rapid, reversible, and highly selective 65-fold fluorescence turn-on response to Cu(I) in aqueous solution. **CTAP-2** proved to be sufficiently sensitive for direct in-gel detection of Cu(I) bound to the metallochaperone Atox1, demonstrating the potential for cation-selective fluorescent probes to serve as tools in metalloproteomics for identifying proteins with readily accessible metal-binding sites.

Cation-selective fluorescent probes have become increasingly important analytical tools for the detection of metal ions in environmental samples, for visualizing metal ions in cells and tissues,[1] or as reagents for measuring metal affinities of biomolecules.[2] Such probes are typically comprised of a chelator for selective recognition of the target ion and a fluorophore to optically transduce binding of the analyte.[3] For passive diffusion across cellular membranes, the probes must be sufficiently lipophilic; however, in aqueous buffer the associated hydrophobic character might also lead to aggregate formation, which in turn may dramatically alter the photophysical properties, notably the brightness and emission wavelength of the probes. Because the majority of fluorescent probes used in biological research are lipophilic and often poorly water-soluble, incubation buffers are typically prepared starting from a stock solution in an organic solvent such as DMSO, which is then diluted into the buffer to a final probe concentration in the low micromolar range. Although this approach usually yields optically clear solutions, the absence of turbidity does not exclude the formation of a colloid composed of nanoparticles with sizes below the diffraction limit. In the course of our efforts in developing Cu(I)-selective fluorescent probes, we realized that the metal ion recognition site, typically composed of thioether donors,[4−8] further increases the lipophilicity and thus the propensity toward aggregation. Accordingly, we found that the Cu(I)-responsive probe **1** (Chart1), which we previously characterized in methanol,[7] forms a clear homogeneous solution when diluted from a 1 mM DMSO stock into aqueous buffer; however, dynamic light scattering measurements revealed the presence of colloidal aggregates with an average hydrodynamic radius of around 100 nm (Table 1, Figure 1). This

Chart 1



observation prompted us to also test other Cu(I)-responsive probes previously characterized in aqueous buffer for their ability to form colloidal aggregates. While the BODIPY-based copper sensors **CS1**[9] and **CS3**[10] both yielded optically clear solutions in aqueous buffer at a concentration of 5 μM, the autocorrelation curves obtained from dynamic light scattering measurements indicated the formation of nanoparticles with average sizes of 49 and 67 nm, respectively (Figure 1, Table 1). Surprisingly, our first-generation probe **CTAP-1**,[5] which is functionalized with a charged carboxylate group, also showed formation of colloidal aggregates under the same conditions (Table 1).

It has been recently recognized that many classes of bioactive organic molecules spontaneously form colloidal aggregates at micromolar concentrations, a problem noted to affect the reliability of high-throughput screening in early drug discovery.[11] Similar to drugs, fluorescent probes must reach their cellular targets by crossing lipid bilayers, and therefore they tend to be considerably lipophilic. The formation of colloidal aggregates might not necessarily jeopardize their utility in biological studies; however, the photophysical properties of the colloid may be dramatically different compared to those of the monomeric form. Therefore, great caution is advised when using fluorescent probes in a mixed-polarity environment as found in cells, which is likely to shift the equilibrium between the aggregated and monomeric forms.

To address the problem of colloid formation, we designed a series of new water-soluble, Cu(I)-selective probes **2a**−**c** in which the thiocrown receptor was modified with four hydroxymethyl groups and combined with triarylpyrazoline fluorophores (Chart 1). To balance the hydrophilicity between receptor and fluorescent reporter and to further increase the

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Table 1. Colloid Formation of Cu(I)-Selective Fluorescent Probes in Aqueous Buffer (10 mM MOPS/K^+, pH 7.2, 25 °C)[a]

probe	lit. ref	R_h (nm)[b]	SD (nm)[c]
1	7	100	12
CS1	9	49	6
CS3	10	67	9
CTAP-1[d]	5	63	6

[a] DMSO stock solution (1 mM) of the probe diluted into aqueous buffer to a final concentration of 5 μM. [b] Hydrodynamic radius. [c] Standard deviation. [d] Potassium salt.

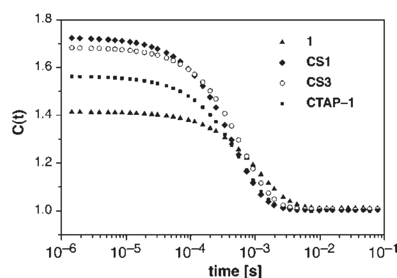


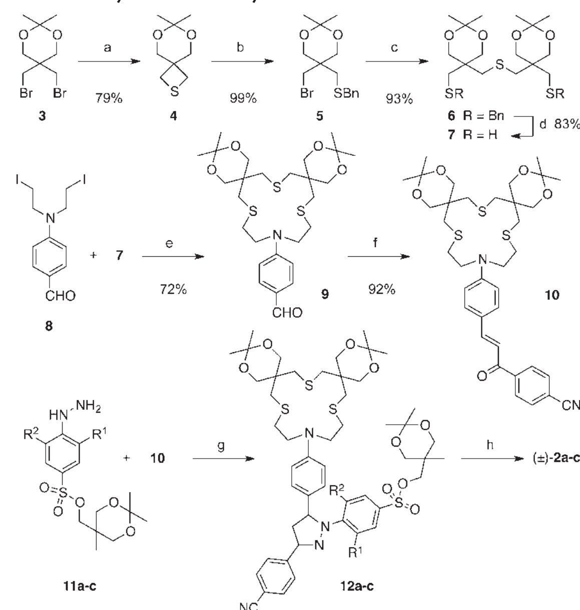
Figure 1. Autocorrelation curves from dynamic light scattering of fluorescent probe colloids in aqueous buffer at pH 7.2 (10 mM MOPS/K^+, 5 μM probe concentration).

overall water solubility, the pyrazoline moiety was functionalized with a sulfonate group, an established approach to solubilize organic fluorophores.[12] Binding of the analyte is translated into a fluorescence increase through a photoinduced electron transfer (PET) switching mechanism, as shown for a range of other pyrazoline-based fluorescent probes.[13,14] The variable number of fluoro substituents served to adjust the PET driving force and thus to optimize the fluorescence enhancement factor upon saturation with Cu(I), as previously demonstrated.[6,15]

For the synthesis of **2a**−**c**, we devised a modular approach in which the same cation receptor can be readily combined with various functionalized pyrazolines (Scheme 1). To this end, we sought a protective group strategy that would allow for masking of both the hydroxyl groups, which needed to be introduced early in the synthesis, and the sulfonate moiety, which could not be incorporated in the final step due to the presence of sensitive functional groups. Since the four hydroxyl groups could be efficiently protected pairwise as acetonides, we contrived an acetonide-based protective group for sulfonic acids. This was accomplished by combining a neopentyl sulfonate ester, which is sterically protected against external nucleophiles, with an acetonide moiety, which, upon hydrolysis, would provide hydroxyl groups that can intramolecularly displace the sulfonate under basic conditions. Compared to previously described neopentyl sulfonate ester protective groups,[17] this acetonide derivative can be synthesized in fewer steps and is sufficiently robust to allow preparation of arylhydrazines from the corresponding fluorinated sulfonate esters by nucleophilic aromatic substitution with hydrazine (see Supporting Information). Following this approach, we successfully prepared the protected pyrazolines **12a**−**c**, which were then converted to the desired products **2a**−**c**.

Probes **2a**−**c** readily dissolved in aqueous buffer and responded with strong fluorescence enhancements upon saturation with Cu(I), supplied either from a 2.5 mM stock solution of $[Cu(I)(CH_3CN)_4]PF_6$ in CH_3CN or by in situ reduction of $CuSO_4$ with ascorbate (Table 2). The absorption and emission bands shifted

Scheme 1. Synthesis of Pyrazoline Probes 2a−c[a]



[a] Reagents and conditions: (a) $Na_2S\cdot 9H_2O$, KI, MeOH−H_2O; (b) BnBr, K_2CO_3, CH_3CN; (c) $Na_2S\cdot 9H_2O$, KI, DMF−H_2O; (d) Na, NH_3(l), THF; (e) Cs_2CO_3, DMF; (f) 4-acetylbenzonitrile, pyrrolidine, C_6H_6−EtOH; (g) PPTS, pyridine; (h) TFA−H_2O (9:1), ^{t}BuOH−THF, KO^{t}Bu.

Table 2. Photophysical Properties of Pyrazoline Probes in Aqueous Solution (10 mM MOPS/K^+, pH 7.2, 22 °C)

	λ (nm)			Φ_F[c]			
compd[a]	abs	em	ΔE_{00}[b] (eV)	acidic[d]	neutral[e]	Cu(I)[f]	f_e[g]
2a[h]	396	508	2.79	0.25	0.0015	0.083	65
2b	376	498	2.89	0.31	0.0006	0.033	41
2c	358	467	3.06	0.62	0.0005	0.010	9
13	404	532	2.70	0.10	0.0026	0.077	32

[a] Ammonium salt. [b] Excited-state energy, estimated as the average of the peak absorption and emission energies. [c] Fluorescence quantum yield (norharmane in 0.1 N H_2SO_4 as reference[16]). [d] 5 mM HCl. [e] 10 mM MOPS/K^+, pH 7.20. [f] Saturated with Cu(I) at pH 7.2 (10 mM MOPS). [g] Fluorescence enhancement factor of Cu(I)-saturated probe relative to the analyte-free probe at neutral pH (λ_{ex} = 380 nm, integrated emission from λ_{em} − 10 to λ_{em} + 10 nm). [h] **CTAP-2**.

to shorter wavelengths with increasing electron-withdrawing ability of the 1-aryl ring (Figure S1), corresponding to a stepwise increase of the excited-state energies ΔE_{00} from 2.79 to 3.06 eV (Table 2). Consistent with a PET quenching mechanism, the quantum yields in the presence and absence of Cu(I) decreased with increasing ΔE_{00}, and the corresponding fluorescence enhancement f_e decreased from 65 to 9. Because the latter is expected to follow a bell-shaped distribution,[8] it was unclear whether **2a** already embodied the maximum obtainable contrast or whether a derivative with lower ΔE_{00} might yield an even better performance. To address this question, we synthesized probe **13** in which the electron-withdrawing sulfonate group is electronically separated from the fluorophore π-system through a methylene group (Supporting Information). As evident from Table 2, the lower excited-state energy of **13** (2.70 eV) produced an increased quantum yield in neutral buffer but not in the presence of Cu(I), thus resulting in a lower contrast than **2a**. Examination of the quantum yields under acidic

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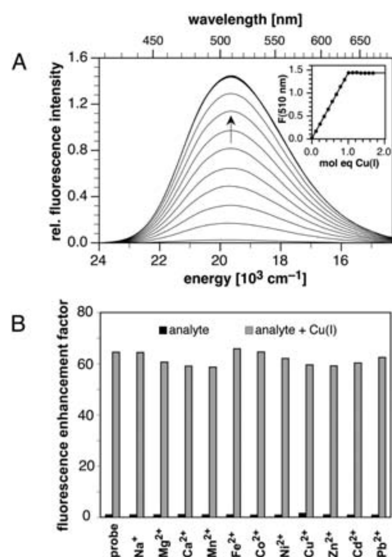

Figure 2. Fluorescence response of probe **CTAP-2** (4.5 μM) to Cu(I) in aqueous buffer at pH 7.2 (10 mM MOPS/K^+, 22 °C, λ_{ex} = 380 nm). (A) Titration of **CTAP-2** with Cu(I). (B) Fluorescence response of **CTAP-2** to various cations. Black bars, **CTAP-2** in the presence of an excess of the indicated analyte (10 mM for Na^+, Mg^{2+}, Ca^{2+}; 10 μM for other cations); gray bars, addition of 5 μM Cu(I) to the solution of **CTAP-2** and the indicated analyte. Cu(I) was supplied from 2.5 mM [Cu(CH_3CN)$_4$]PF_6 solution in CH_3CN.

conditions, where the arylamine is protonated and rendered inert toward oxidation, suggests that a quenching pathway other than acceptor-excited PET is responsible for the lower than expected quantum yield of **13**-Cu(I); anomalously low quantum yields in polar solvents have been previously reported for 1,3,5-triarylpyrazolines bearing electron-rich 1-aryl rings.[14,18]

Given the superior contrast of **2a** over the other probes, the remaining characterization focused exclusively on this compound, which we also named **CTAP-2** as an identifier for these and future studies. As expected for a high-affinity ligand, fluorescence titration of **CTAP-2** with Cu(I) showed a linear emission increase with sharp saturation at 1 molar equiv (Figure 2A).

The UV−vis absorbance of **CTAP-2** scaled linearly with concentrations from 0 to 5 μM, yielding a molar absorptivity of 2.9×10^4 M^{-1} cm^{-1} at 396 nm (Figure S3, inset). Similarly, the absorption and emission intensity of Cu(I)-bound **CTAP-2** increased linearly in the same concentration range (Figure S2). At values above 10 μM **CTAP-2**, the absorbance vs concentration plot deviated slightly from linearity (Figure S3), indicating the presence of weak self-association. Nonlinear least-squares fitting of the experimental data assuming a simple dimerization equilibrium yielded an equilibrium constant of $\log K = 3.98 \pm 0.06$ and a dimer molar absorptivity of 4.3×10^4 M^{-1} cm^{-1}. On the basis of these data, we estimated that at a concentration of 5 μM, approximately 4% of the probe is present as dimer. While not negligible, dimer formation was experimentally evident only at concentrations that substantially exceeded the working concentration typically used for fluorescence measurements. Similar dimerization constants in aqueous solution have been reported for xanthene dyes such as the cationic Rhodamine 6G.[19] Most importantly, dynamic light scattering experiments with a 5 μM solution of **CTAP-2** in MOPS buffer (10 mM, pH 7.2) gave count rates that were no higher than the background of the buffer alone, thus confirming the absence of colloidal aggregates.

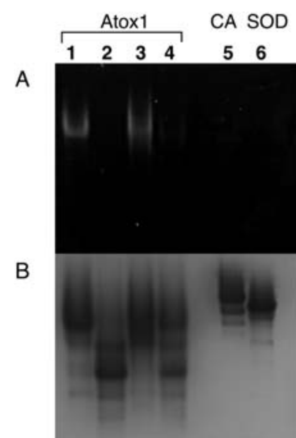

Figure 3. In-gel detection of a copper metallochaperone with **CTAP-2**. (A) Native PAGE incubated with a 5 μM aqueous solution of **CTAP-2** followed by visualization at 365 nm (emission 537/BP 35 nm; UV transillumination mode). (B) Same gel after staining with Coomassie blue. Lane 1, untreated hAtox1; lane 2, hAtox1, TCEP, KCN; lane 3, hAtox1, TCEP, [Cu(I)(CH_3CN)$_4$]PF_6; lane 4, hAtox1, TCEP, [Cu(I)(CH_3CN)$_4$]PF_6, then KCN; lane 5, carbonic anhydrase; and lane 6, superoxide dismutase (SOD1).

To determine the Cu(I) affinity, we used the formal potentials of the free and bound $Cu^{II/I}$ couples and the Cu(II) affinity of **CTAP-2** under mildly acidic conditions.[20] In the presence of Cu(II), **CTAP-2** showed a new quasi-reversible one-electron process with a half-wave potential of 0.226 V vs $Fc^{+/0}$, corresponding to 0.626 V vs SHE[21] (10 mM PIPBS, 0.1 M $KClO_4$, Figure S6). Under the same conditions, UV−vis titrations revealed a $\log K^{Cu(II)} = 2.97 \pm 0.07$ (Figure S5). On the basis of these data, we obtained $\log K^{Cu(I)} = 11.4 \pm 0.1$ or $K_d = 4 \pm 1$ pM for **CTAP-2** at pH 5.0 (I = 0.1 M), which compares well with the affinity of structurally related probes.[5,9] Given the low pK_a of 3.97 ± 0.03 for protonation of the thiazacrown receptor (Figure S4), the apparent Cu(I) affinity at pH 7.2 remains unchanged within experimental error. Furthermore, the fluorescence response of **CTAP-2** proved to be very selective toward Cu(I) and unaffected by other biologically relevant ions (Figure 2B).

Encouraged by the high fluorescence contrast and selectivity toward Cu(I), we decided to explore the utility of **CTAP-2** as a reagent for the in-gel detection of proteins containing a readily accessible Cu(I)-binding site, as present in copper metallochaperones such as Atox1.[22] For this purpose we purified recombinant hAtox1 and subjected the protein to native gel electrophoresis. As illustrated in Figure 3, incubation of the gel with **CTAP-2** revealed the presence of Atox1 in a copper-dependent manner. While untreated Atox1 gave rise to a fluorescence signal (lane 1), preincubation with KCN to remove Cu(I) from Atox1 abolished the response (lane 2). Furthermore, lanes 3 and 4 demonstrate reversible copper binding, as expected for a metallochaperone. In contrast, no staining was observed for carbonic anhydrase (CA), a Zn^{II}-containing enzyme, or superoxide dismutase (SOD1), an enzyme in which the copper site is sterically inaccessible. Post-staining with Coomassie blue revealed the presence of the proteins in each lane and confirmed the removal of Cu(I) from hAtox1, based on the different mobilities of the apo and holo forms.[23] Given the high Cu(I) affinity of Atox1 ($\log K = 17.4$),[24] it is probable that **CTAP-2** associates with the protein in a Cu(I)-dependent manner without actually removing the metal ion from the binding site. Such ternary complexes might also be formed in a biological environment,

as recently suggested in the case of the zinc-responsive fluorescent probes FluoZin-3 and TSQ.[25] Despite its net anionic charge at neutral pH, **CTAP-2** proved to be cell permeant and produced in live NIH 3T3 cells a perinuclear staining pattern (Figure S7), reminiscent of the subcellular copper distribution previously reported;[5,26] however, in view of the above findings, the interpretation of the observed cellular staining is nontrivial and will require further detailed studies.

In conclusion, we have developed a fluorescent probe **CTAP-2** that selectively responds to Cu(I) in aqueous buffer with a 65-fold fluorescence enhancement. The response of **CTAP-2** is rapid and reversible, making it suitable as an indicator for titrations with Cu(I) or for monitoring equilibrium concentrations of Cu(I). While previously described fluorescent probes for Cu(I) have not been reported to dissolve directly in water,[4,5,9,10,27] salts of **CTAP-2** quickly dissolve in pure water up to millimolar concentrations, circumventing the possibility of colloidal aggregate formation that exists when organic stock solutions of poorly soluble dyes are diluted into aqueous buffer. Because the majority of fluorescent probes utilized in biology are considerably lipophilic, the formation of colloids is likely not limited to the probes investigated here but a rather widespread phenomenon that deserves particular attention when interpreting fluorescence microscopy data. Adding to the previously described applications of Cu(I)-responsive fluorescent probes, **CTAP-2** was able to detect copper bound to a metallochaperone. As only proteins with accessible metal sites can give rise to a fluorescence response, the detection of metalloproteins with fluorescent indicators such as **CTAP-2** nicely complements the currently available techniques for in-gel metal profiling, namely laser ablation−inductively coupled plasma mass spectrometry (LA-ICP-MS)[28] and synchrotron-based X-ray fluorescence mapping,[29] both of which measure the total metal content regardless of its accessibility, thus further expanding the metalloproteomics toolbox.[30]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Synthesis, spectroscopic characterization, and additional information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Domaille, D. W.; Que, E. L.; Chang, C. J. *Nat. Chem. Biol.* **2008**, *4*, 168. McRae, R.; Bagchi, P.; Sumalekshmy, S.; Fahrni, C. J. *Chem. Rev.* **2009**, *109*, 4780.

(2) Demchenko, A. P. *Introduction to Fluorescence Sensing*; Springer: Berlin, 2009.

(3) De Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515. Fahrni, C. J. In *Chemosensors: Principles, Strategies, and Applicatons*; Wang, B., Anslyn, E. V., Eds.; John Wiley & Sons: New York, 2011; p 371.

(4) Cody, J.; Fahrni, C. J. *Tetrahedron* **2004**, *60*, 11099.

(5) Yang, L. C.; McRae, R.; Henary, M. M.; Patel, R.; Lai, B.; Vogt, S.; Fahrni, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11179.

(6) Verma, M.; Chaudhry, A. F.; Morgan, M. T.; Fahrni, C. J. *Org. Biomol. Chem.* **2010**, *8*, 363.

(7) Chaudhry, A. F.; Verma, M.; Morgan, M. T.; Henary, M. M.; Siegel, N.; Hales, J. M.; Perry, J. W.; Fahrni, C. J. *J. Am. Chem. Soc.* **2010**, *132*, 737.

(8) Chaudhry, A. F.; Mandal, S.; Hardcastle, K. I.; Fahrni, C. J. *Chem. Sci.* **2011**, *2*, 1016.

(9) Zeng, L.; Miller, E. W.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 10.

(10) Dodani, S. C.; Domaille, D. W.; Nam, C. I.; Miller, E. W.; Finney, L. A.; Vogt, S.; Chang, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 5980.

(11) Feng, B. Y.; Shelat, A.; Doman, T. N.; Guy, R. K.; Shoichet, B. K. *Nat. Chem. Biol.* **2005**, *1*, 146. Doak, A. K.; Wille, H.; Prusiner, S. B.; Shoichet, B. K. *J. Med. Chem.* **2010**, *53*, 4259.

(12) Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1993**, *4*, 105. Jose, J.; Burgess, K. *J. Org. Chem.* **2006**, *71*, 7835. Li, L. L.; Han, J. Y.; Nguyen, B.; Burgess, K. *J. Org. Chem.* **2008**, *73*, 1963.

(13) De Silva, A. P.; De Silva, S. A.; Dissanayake, A. S.; Sandanayake, K. R. A. S. *J. Chem. Soc., Chem. Commun.* **1989**, 1054. De Silva, A. P.; Nimal Gunaratne, H. Q. *J. Chem. Soc., Chem. Commun.* **1990**, 186. Rurack, K.; Bricks, J. L.; Schulz, B.; Maus, M.; Reck, G.; Resch-Genger, U. *J. Phys. Chem. A* **2000**, *104*, 6171. Rurack, K.; Resch-Genger, U.; Bricks, J. L.; Spieles, M. *Chem. Commun.* **2000**, 2103.

(14) Fahrni, C. J.; Yang, L. C.; VanDerveer, D. G. *J. Am. Chem. Soc.* **2003**, *125*, 3799.

(15) Cody, J.; Mandal, S.; Yang, L. C.; Fahrni, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 13023.

(16) Pardo, A.; Reyman, D.; Poyato, J. M. L.; Medina, F. *J. Luminesc.* **1992**, *51*, 269.

(17) Roberts, J. C.; Gao, H.; Gopalsamy, A.; Kongsjahju, A.; Patch, R. J. *Tetrahedron Lett.* **1997**, *38*, 355. Seeberger, S.; Griffin, R. J.; Hardcastle, I. R.; Golding, B. T. *Org. Biomol. Chem.* **2007**, *5*, 132. Rusha, L.; Miller, S. C. *Chem. Commun.* **2011**, *47*, 2038.

(18) Rivett, D. E.; Rosevear, J.; Wilshire, J. F. K. *Aust. J. Chem.* **1979**, *32*, 1601.

(19) Valdes-Aguilera, O.; Neckers, D. C. *Acc. Chem. Res.* **1989**, *22*, 171.

(20) Bernardo, M. M.; Schroeder, R. R.; Rorabacher, D. B. *Inorg. Chem.* **1991**, *30*, 1241.

(21) Koepp, H.-M.; Wendt, H.; Strehlow, H. *Z. Elektrochem.* **1960**, *64*, 483.

(22) Robinson, N. J.; Winge, D. R. *Annu. Rev. Biochem.* **2010**, *79*, 537.

(23) Narindrasorasak, S.; Zhang, X.; Roberts, E. A.; Sarkar, B. *Bioinorg. Chem. Appl.* **2004**, *2*, 105.

(24) Xiao, Z.; Wedd, A. G. *Nat. Prod. Rep.* **2010**, *27*, 768.

(25) Kay, A. R. *Trends Neurosci.* **2006**, *29*, 200. Meeusen, J. W.; Tomasiewicz, H.; Nowakowski, A.; Petering, D. H. *Inorg. Chem.* **2011**, *50*, 7563.

(26) McRae, R.; Lai, B.; Fahrni, C. J. *J. Biol. Inorg. Chem.* **2010**, *15*, 99.

(27) Taki, M.; Iyoshi, S.; Ojida, A.; Hamachi, I.; Yamamoto, Y. *J. Am. Chem. Soc.* **2010**, *132*, 5938. Domaille, D. W.; Zeng, L.; Chang, C. J. *J. Am. Chem. Soc.* **2010**, *132*, 1194. Dodani, S. C.; Leary, S. C.; Cobine, P. A.; Winge, D. R.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 8606. Lim, C. S.; Han, J. H.; Kim, C. W.; Kang, M. Y.; Kang, D. W.; Cho, B. R. *Chem. Commun.* **2011**, *47*, 7146.

(28) Becker, J. S.; Lobinski, R. *Metallomics* **2009**, *1*, 312.

(29) Ortega, R. *Metallomics* **2009**, *1*, 137. Finney, L.; Chishti, Y.; Khare, T.; Giometti, C.; Levina, A.; Lay, P. A.; Vogt, S. *ACS Chem. Biol.* **2010**, *5*, 577.

(30) Waldron, K. J.; Rutherford, J. C.; Ford, D.; Robinson, N. J. *Nature* **2009**, *460*, 823.