Bioorthogonal Probes

Deutsche Ausgabe: DOI: 10.1002/ange.201509396
Internationale Ausgabe: DOI: 10.1002/anie.201509396

Conferring Phosphorogenic Properties on Iridium(III)-Based Bioorthogonal Probes through Modification with a Nitrone Unit

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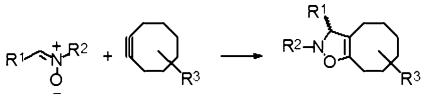
Abstract: *The use of bioorthogonal probes that display fluorogenic or phosphorogenic properties is advantageous to the labeling and imaging of biomolecules in live cells and organisms. Herein we present the design of three iridium(III) complexes containing a nitrone moiety as novel phosphorogenic bioorthogonal probes. These probes were non-emissive owing to isomerization of the C=N group but showed significant emission enhancement upon cycloaddition reaction with strained cyclooctynes. Interestingly, the connection of the nitrone ligand to the cationic iridium(III) center led to accelerated reaction kinetics. These nitrone complexes were also identified as phosphorogenic bioorthogonal labels and imaging reagents for cyclooctyne-modified proteins. These findings contribute to the development of phosphorogenic bioorthogonal probes and imaging reagents.*

Bioorthogonal probes have been developed as versatile tools for the specific and non-invasive labeling and imaging of biomolecules in their native environments.[1] In general, bioorthogonal labeling involves the incorporation of an abiotic functional group into the biomolecule of interest. After the biomolecule is introduced to live cells and organisms, the unique functional group serves as a chemical reporter and can be labeled with a bioorthogonal probe that carries the specific reaction partner. Recently, there has been a rapidly emerging interest in exploiting new bioorthogonal probes that display fluorogenic[2–5] or phosphorogenic[6] properties. These probes remain non-emissive as long as the appended bioorthogonal functional group is intact, but show emission turn-on upon the labeling reaction. Their use in detecting target biomolecules in live cells has attractive features, such as eliminating the need for stringent washout to remove unreacted probes, improving the signal-to-noise ratio, and offering the opportunity to monitor biological processes in real time. However, most of these probes developed thus far are limited to azide- and tetrazine-functionalized fluorophore scaffolds, with the quenching mechanisms being photo-induced electron transfer and fluorescence resonance energy transfer in most cases.[3,4]

In the search for bioorthogonal functional groups having the potential to be used in the construction of new probes, nitrone has captured our attention for two reasons. First, it can selectively undergo strain-promoted alkyne–nitrone cycloaddition (SPANC) reaction with a strained alkyne to give an *N*-alkylated isoxazoline as the product (Scheme 1).[7]



Scheme 1. Strain-promoted alkyne–nitrone cycloaddition reaction of a nitrone with a strained cyclooctyne.

Nitrone-modified biomolecules have been applied to site-specific modification of proteins[8] and labeling of mammalian cell surfaces[9] and bacterial cell walls.[10] Second, C=N isomerization is known to provide a facile non-radiative deactivation pathway for fluorescent[11] and phosphorescent[12] compounds. Thus, when the isomerization of the C=N group is inhibited, these compounds are expected to resume their emission behavior. To our knowledge, nitrone derivatives have never been explored for the construction of bioorthogonal probes.

We have been interested in the use of iridium(III) polypyridine complexes as a model luminophore owing to their structural variety and attractive photophysical properties.[13] The incorporation of a nitrone unit into these complexes should effectively quench their emission. Also, the modification of the nitrone ligand with the cationic complex is expected to increase the SPANC reaction kinetics since nitrones possessing an electron-withdrawing group at the α-C position react faster than their unsubstituted counterparts.[14] Herein, we designed three cyclometalated iridium(III) polypyridine complexes containing a nitrone moiety $[Ir(N^C)_2(bpy\text{-}nitrone)](PF_6)$ (HN^C = Hppy (**1**), Hpq (**2**), Hpqe (**3**); Scheme 2). The synthetic procedures and characterization data for the ligand bpy-nitrone and the complexes are in the Supporting Information. The ^{1}H NMR spectra revealed only one signal for the CH=N proton, indicating that bpy-nitrone and the complexes exist exclusively as the more stable *Z*-isomers.[15]

Upon photoexcitation, the complexes displayed extremely weak yellow to red emission (Table S1 in the Supporting Information). Complexes **1** and **3** showed a broad emission band with positive solvatochromism in fluid solu-

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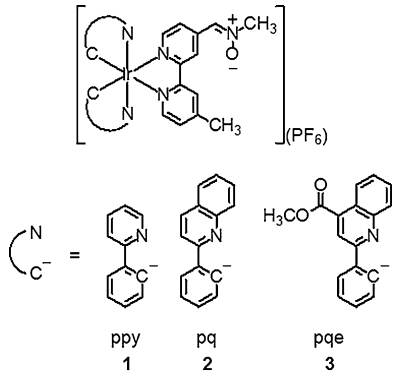
Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201509396.



Scheme 2. Structural formula of complexes **1**–**3**.

tions at 298 K. Also, their emission maxima displayed significant blue shifts upon cooling the samples to 77 K, typical of ^{3}MLCT (dπ(Ir)→π*(N^N/N^C)) emission. In contrast, complex **2** showed a structured band and the emission was longer-lived and less solvent-dependent, indicative of a ^{3}IL (π→π*) (pq) state. Importantly, the emission quantum yields of all three complexes ($\Phi_{em} < 0.021$; Table S1) were much lower than those of common cyclometalated iridium(III) polypyridine complexes,[16] which is a consequence of efficient quenching associated with isomerization of the C=N group.[12]

We selected the strained cyclooctyne BCN-OH as a model substrate to investigate the reactivity and possible phosphorogenic responses of the complexes (Figure 1 a). Upon the reaction in MeOH, the solutions showed substantial emission enhancement ($I/I_o = 24.2$ to 30.9; Table S2) as a result of the conversion of the quenching nitrone unit into a non-quenching isoxazoline derivative (Figure 1 a). The formation of products was confirmed by ESI-MS (Table S3). Interestingly, when the reactions were performed in aqueous buffer solutions, more significant emission enhancement ($I/I_o =$ 28.8 to 135.0; Table S2 and Figure 1 b–d) was observed. This is ascribed to the fact that the complexes displayed much lower emission intensity in aqueous solutions (Table S1). These results confirm that the incorporation of the nitrone unit into the iridium(III) complexes substantially quenches their emission, and allows them to exhibit significant emission enhancement upon reaction with cyclooctyne derivatives. To our knowledge, this is the very first example of nitrones being utilized in the design of bioorthogonal probes that show phosphorogenic properties.

As nitrones exhibit spin-trapping behavior[17] and reactivity toward thiols,[18] we examined whether the complexes would respond to reactive oxygen/nitrogen species (RONS) and biological thiols, leading to emission changes. Incubation of complexes **1**–**3** (10 μM) with RONS (100 μM) including H_2O_2, ^{t}BuOOH, ClO^-, O_2^-, $^1O_2$, HO·, NO_2^-, NO_3^-, and $ONOO^-$ and thiols (1 mM) including cysteine, homocysteine, and glutathione for 24 h did not result in substantial emission enhancement ($I/I_o < 1.83$; Figure S1). Mass spectrometric analyses of the reaction mixtures indicated that the complexes remained intact and no reaction products were isolated. Furthermore, the reaction kinetics of bpy-nitrone and the complexes with BCN-OH in MeOH at 298 K were studied. The second-order rate constant (k_2) of bpy-nitrone with BCN-OH was determined to be $0.040 \pm 0.002\ M^{-1}s^{-1}$ (Table S2), which is similar to those of other acyclic nitrones containing electron-withdrawing substituents ($k_2 = 0.011$ to $0.029\ M^{-1}s^{-1}$) and benzyl azide ($k_2 = 0.040\ M^{-1}s^{-1}$) with BCN-OH.[14b] Remarkably, the reactivity of complexes **1**–**3** was higher than that of bpy-nitrone by 2.9 to 6.0 fold (Table S2), illustrating that the coordination of the nitrone ligand to the cationic iridium(III) unit enhances the reactivity. Thus, the large phosphorogenic responses and accelerated reaction kinetics render the complexes useful bioorthogonal probes for biomolecules modified with a strained cyclooctyne.

To examine the bioorthogonal labeling properties of the complexes, three proteins including bovine serum albumin (BSA), human serum albumin (HSA), and holo-transferrin (HTf) were modified with a BCN unit (Scheme S1). The BCN-modified proteins and their unmodified counterparts were incubated with complexes **1**–**3** individually for 18 h, and the mixtures were analyzed by SDS-PAGE. We found that the BCN-modified proteins were labeled with the complexes and gave intensely phosphorescent bands in the gels (Figure 2 and Figure S2). In contrast, no phosphorescent bands were noted for the unmodified proteins, confirming that the labeling originated from the specific reaction between the nitrone complexes and the BCN units of the modified proteins. Impressively, reaction of the complexes with the BCN-modified proteins gave rise to very significant emission enhancement ($I/I_o = 92.1$ to 795.1; Table 1 and Figure S3), which is much more pronounced than the cases with BCN-OH ($I/I_o = 28.8$ to 135.0; Table S2 and Figure 1 b–d). We

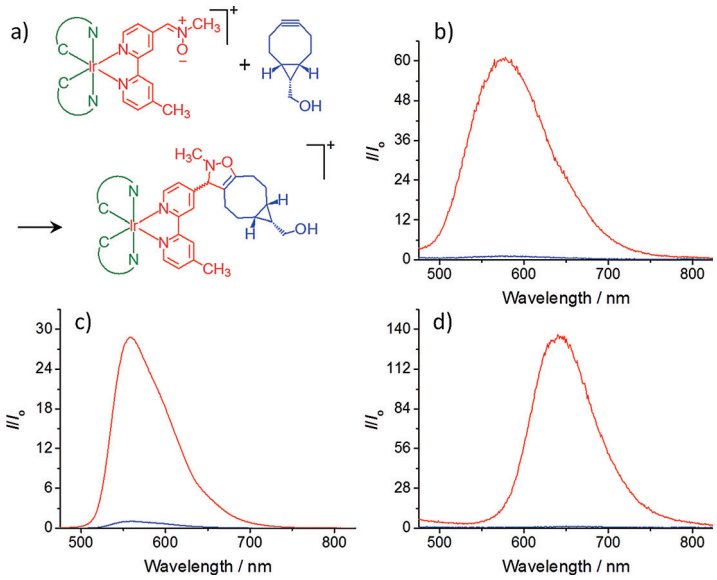


Figure 1. a) Reaction of the complexes with BCN-OH. b)–d) Emission spectra of complexes **1**–**3** (10 μM), respectively, in the absence (blue) and presence (red) of BCN-OH (250 μM) in aerated potassium phosphate buffer (50 mM, pH 7.4)/MeOH (9:1, *v*/*v*) at 298 K. Excitation wavelength = 350 nm.

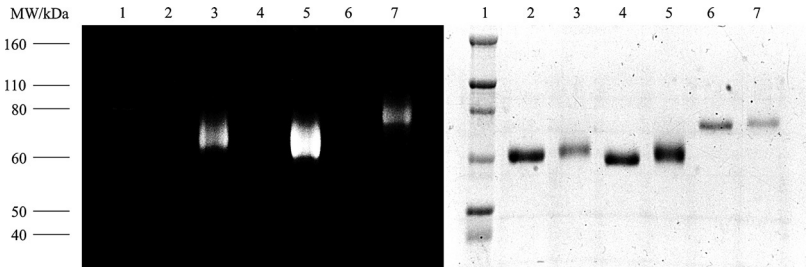


Figure 2. SDS-PAGE analysis. Left: UV transillumination; right: Coomassie Blue staining. Lane 1: protein ladder; lanes 2, 4, and 6: complex **2** with unmodified BSA, HSA, and HTf, respectively; lanes 3, 5, and 7: complex **2** with BCN-modified BSA, HSA, and HTf, respectively.

Table 1: Emission enhancement factors of complexes **1**–**3** upon incubation with BCN-modified BSA, HSA, and HTf, and their unmodified counterparts.[a]

Complex	I/I_o[b] BCN-BSA (BSA)	BCN-HSA (HSA)	BCN-HTf (HTf)
1	292.8 (1.9)	303.2 (1.8)	482.3 (2.0)
2	92.1 (1.1)	93.7 (1.0)	100.0 (0.9)
3	618.0 (5.9)	638.6 (2.1)	795.1 (3.2)

[a] Measurements in aerated potassium phosphate buffer (50 mM, pH 7.4)/MeOH (9:1, *v*/*v*) at 298 K. [b] I_o and I are the emission intensities of the complexes (10 μM) in the absence and presence of the proteins (2.5 μM), respectively.

argue that these are due to the increased hydrophobicity and enhanced rigidity of the local environment of the complexes after they react with the BCN-modified proteins,[19] which is in accordance with the observation that the complexes displayed higher emission intensity in less-polar solvents (Table S1). Control experiments involving the unmodified proteins did not give comparable emission enhancement (Table 1), illustrating the selective conjugation of the BCN-modified proteins with the complexes. These results show that the highly environment-sensitive emission of luminescent iridium(III) polypyridine complexes is an additional advantage in targeting BCN-modified biomolecules.

Since some nitrone compounds exhibit potent biological activity,[20] we studied the cellular properties of the complexes using live Chinese hamster ovary (CHO)-K1 cells as a model. ICP-MS measurements showed that an average cell contained 0.07 to 0.79 fmol of iridium after an incubation period of 1 h (Table S4). The cellular uptake efficiency of complex **3** was higher than those of complexes **1** and **2**. This finding should originate from intracellular esterases that convert the ester moieties of complex **3** into negatively charged carboxyl groups, enhancing its intracellular retention.[21] MTT assays showed that the complexes were essentially noncytotoxic toward the cells ($IC_{50} > 50$ μM) for an incubation period of 1 h (Table S4). Next, we investigated the phosphorogenic responses of the complexes toward a BCN-modified decane molecule BCN-C10 (Scheme S2) inside CHO-K1 cells. The cells were first incubated with BCN-C10 (125 μM) for 30 min, thoroughly washed with buffer, and further treated with complexes **1**–**3** (5 μM) for 1 h. The intracellular distribution of BCN-C10 was then imaged using laser-scanning confocal microscopy. The cytoplasmic region of the cells exhibited significant intracellular emission (Figure 3 and Figure S4). On the contrary, the cells treated with the complexes alone in the control experiments did not show similar emission, confirming that the observed emission is due to the specific labeling of BCN-C10 with the complexes inside the cells. These results are also supported by flow cytometry studies, which showed that complex **2** exhibited a 161.1-fold increase in emission intensity upon reaction with BCN-C10, and complexes **1** and **3** also displayed 2.0- and 6.4-fold increases, respectively (Table S5 and Figure S5).

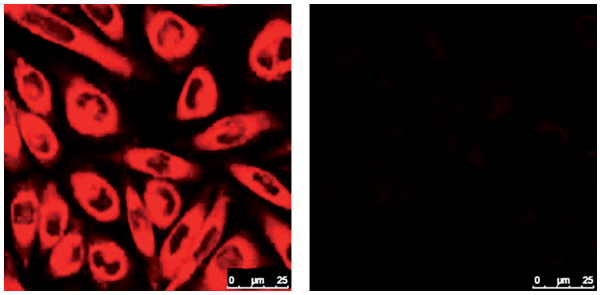


Figure 3. Laser-scanning confocal microscopy images of CHO-K1 cells incubated with complex **2** (5 μM) at 37 °C for 1 h with (left) or without (right) pretreatment with BCN-C10 (125 μM) at 37 °C for 30 min. Scale bar: 25 μm.

In conclusion, we have established a novel platform for the construction of phosphorogenic bioorthogonal probes containing the nitrone moiety, which serves as both the emission quencher and bioorthogonal functional group. Although the SPANC reaction has been applied in bioorthogonal labeling, this study is the very first demonstration of nitrone compounds as phosphorogenic bioorthogonal probes. We anticipate that this strategy can be readily extended to other fluorescent and phosphorescent compounds to develop probes for specific labeling and imaging of biomolecules in live cells and organisms.

Acknowledgements

We thank City University of Hong Kong (Project No. 7004210 and 7004396) for financial support. L.C.-C.L. acknowledges the receipt of a Postgraduate Studentship administered by City University of Hong Kong. K.K.-W.L. is grateful to the Croucher Foundation for the award of a Croucher Senior Research Fellowship.

Keywords: bioorthogonal · imaging agents · iridium · nitrone · phosphorogenic probes

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1046–1049
Angew. Chem. **2016**, *128*, 1058–1061

[1] See, for example: a) E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974; *Angew. Chem.* **2009**, *121*, 7108; b) C. P. Ramil, Q. Lin, *Chem. Commun.* **2013**, *49*, 11007; c) K. Lang, J. W. Chin, *Chem. Rev.* **2014**, *114*, 4764.
[2] P. Shieh, C. R. Bertozzi, *Org. Biomol. Chem.* **2014**, *12*, 9307.
[3] a) P. Shieh, M. J. Hangauer, C. R. Bertozzi, *J. Am. Chem. Soc.* **2012**, *134*, 17428; b) A. Herner, I. Nikić, M. Kállay, E. A. Lemke, P. Kele, *Org. Biomol. Chem.* **2013**, *11*, 3297; c) P. Shieh, M. S. Siegrist, A. J. Cullen, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5456; d) J.-J. Shie, Y.-C. Liu, Y.-M. Lee, C. Lim, J.-M. Fang, C.-H. Wong, *J. Am. Chem. Soc.* **2014**, *136*, 9953; e) P. Shieh, V. T. Dien, B. J. Beahm, J. M. Castellano, T. Wyss-Coray, C. R. Bertozzi, *J. Am. Chem. Soc.* **2015**, *137*, 7145.
[4] a) N. K. Devaraj, S. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, *Angew. Chem. Int. Ed.* **2010**, *49*, 2869; *Angew. Chem.* **2010**, *122*, 2931; b) J. C. T. Carlson, L. G. Meimetis, S. A. Hilderbrand, R. Weissleder, *Angew. Chem. Int. Ed.* **2013**, *52*, 6917; *Angew. Chem.* **2013**, *125*, 7055; c) A. Wieczorek, T. Buckup, R. Wombacher, *Org. Biomol. Chem.* **2014**, *12*, 4177; d) H. Wu, J. Yang, J. Šečkutė, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2014**, *53*, 5805; *Angew. Chem.* **2014**, *126*, 5915; e) L. G. Meimetis, J. C. T. Carlson, R. J. Giedt, R. H. Kohler, R. Weissleder, *Angew. Chem. Int. Ed.* **2014**, *53*, 7531; *Angew. Chem.* **2014**, *126*, 7661.
[5] a) M. Sawa, T.-L. Hsu, T. Itoh, M. Sugiyama, S. R. Hanson, P. K. Vogt, C.-H. Wong, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12371; b) L. Rong, L.-H. Liu, S. Chen, H. Cheng, C.-S. Chen, Z.-Y. Li, S.-Y. Qin, X.-Z. Zhang, *Chem. Commun.* **2014**, *50*, 667; c) F. Friscourt, C. J. Fahrni, G.-J. Boons, *J. Am. Chem. Soc.* **2012**, *134*, 18809.
[6] A. W.-T. Choi, K. K.-S. Tso, V. M.-W. Yim, H.-W. Liu, K. K.-W. Lo, *Chem. Commun.* **2015**, *51*, 3442.
[7] D. A. MacKenzie, A. R. Sherratt, M. Chigrinova, L. L. W. Cheung, J. P. Pezacki, *Curr. Opin. Chem. Biol.* **2014**, *21*, 81.
[8] a) X. Ning, R. P. Temming, J. Dommerholt, J. Guo, D. B. Ania, M. F. Debets, M. A. Wolfert, G.-J. Boons, F. L. van Delft, *Angew. Chem. Int. Ed.* **2010**, *49*, 3065; *Angew. Chem.* **2010**, *122*, 3129; b) R. P. Temming, L. Eggermont, M. B. van Eldijk, J. C. M. van Hest, F. L. van Delft, *Org. Biomol. Chem.* **2013**, *11*, 2772.
[9] C. S. McKay, J. A. Blake, J. Cheng, D. C. Danielson, J. P. Pezacki, *Chem. Commun.* **2011**, *47*, 10040.
[10] D. A. MacKenzie, A. R. Sherratt, M. Chigrinova, A. J. Kell, J. P. Pezacki, *Chem. Commun.* **2015**, *51*, 12501.
[11] a) J.-S. Wu, W.-M. Liu, X.-Q. Zhuang, F. Wang, P.-F. Wang, S.-L. Tao, X.-H. Zhang, S.-K. Wu, S.-T. Lee, *Org. Lett.* **2007**, *9*, 33; b) X. Cheng, H. Jia, T. Long, J. Feng, J. Qin, Z. Li, *Chem. Commun.* **2011**, *47*, 11978; c) M. Üçüncü, M. Emrullahoğlu, *Chem. Commun.* **2014**, *50*, 5884.
[12] a) N. Zhao, Y.-H. Wu, R.-M. Wang, L.-X. Shi, Z.-N. Chen, *Analyst* **2011**, *136*, 2277; b) G. Li, Q. Lin, L. Ji, H. Chao, *J. Mater. Chem. B* **2014**, *2*, 7918; c) M. Li, Y. Wang, Y. Yang, Y. Gao, M. Zhao, M. Zheng, S. Peng, *Dalton Trans.* **2015**, *44*, 14071.
[13] K. K.-W. Lo, *Acc. Chem. Res.* **2015**, *48*, DOI: 10.1021/acs.accounts.5b00211.
[14] a) C. S. Mckay, M. Chigrinova, J. A. Blake, J. P. Pezacki, *Org. Biomol. Chem.* **2012**, *10*, 3066; b) D. A. MacKenzie, J. P. Pezacki, *Can. J. Chem.* **2014**, *92*, 337.
[15] a) A. Hassan, M. I. M. Wazeer, S. A. Ali, *J. Chem. Soc., Perkin Trans. 2* **1998**, 443; b) P. Merino in *Science of Synthesis*, *Vol. 2010* (Ed.: E. Schaumann), George Thieme, Stuttgart, **2011**, pp. 325 – 403; c) A. Bădoiu, E. P. Kündig, *Org. Biomol. Chem.* **2012**, *10*, 114.
[16] a) S. P.-Y. Li, H.-W. Liu, K. Y. Zhang, K. K.-W. Lo, *Chem. Eur. J.* **2010**, *16*, 8329; b) S. P.-Y. Li, C. T.-S. Lau, M.-W. Louie, Y.-W. Lam, S. H. Cheng, K. K.-W. Lo, *Biomaterials* **2013**, *34*, 7519; c) K. K.-W. Lo, B. T.-N. Chan, H.-W. Liu, K. Y. Zhang, S. P.-Y. Li, T. S.-M. Tang, *Chem. Commun.* **2013**, *49*, 4271.
[17] See, for example: a) E. G. Janzen, *Acc. Chem. Res.* **1971**, *4*, 31; b) F. A. Villamena, J. L. Zweier, *Antioxid. Redox Signaling* **2004**, *6*, 619; c) C. L. Hawkins, M. J. Davies, *Biochim. Biophys. Acta, Gen. Subj.* **2014**, *1840*, 708.
[18] D. I. Potapenko, E. G. Bagryanskaya, Y. P. Tsentalovich, V. A. Reznikov, T. L. Clanton, V. V. Khramtsov, *J. Phys. Chem. B* **2004**, *108*, 9315.
[19] a) K. K.-W. Lo, K. Y. Zhang, C.-K. Chung, K.-Y. Kwok, *Chem. Eur. J.* **2007**, *13*, 7110; b) K. K.-W. Lo, J. S.-Y. Lau, *Inorg. Chem.* **2007**, *46*, 700; c) J. S.-Y. Lau, P.-K. Lee, K. H.-K. Tsang, C. H.-C. Ng, Y.-W. Lam, S.-H. Cheng, K. K.-W. Lo, *Inorg. Chem.* **2009**, *48*, 708.
[20] See, for example: a) R. A. Floyd, K. Hensley, M. J. Forster, J. A. Kelleher-Andersson, P. L. Wood, *Mech. Ageing Dev.* **2002**, *123*, 1021; b) R. A. Floyd, H. K. Chandru, T. He, R. Towner, *Anti-Cancer Agents Med. Chem.* **2011**, *11*, 373; c) R. A. Floyd, H. C. C. F. Neto, G. A. Zimmerman, K. Hensley, R. A. Towner, *Free Radical Biol. Med.* **2013**, *62*, 145.
[21] a) S. Izumi, Y. Urano, K. Hanaoka, T. Terai, T. Nagano, *J. Am. Chem. Soc.* **2009**, *131*, 10189; b) V. S. Lin, A. R. Lippert, C. J. Chang, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 7131; c) W. Chyan, D. Y. Zhang, S. J. Lippard, R. J. Radford, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 143.

Received: October 8, 2015
Published online: November 30, 2015

