Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron Letters 45 (2004) 9377–9381
Tetrahedron Letters

A new fluorescent metal sensor with two binding moieties

Asuka Ohshima,[a] Atsuya Momotake[b] and Tatsuo Arai[a,*]

[a]*Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1, Tennodai, Tsukuba-city, Ibaraki 305-8571, Japan*
[b]*Research Facility Centre for Science and Technology, University of Tsukuba, 1-1-1, Tennodai, Tsukuba-city, Ibaraki 305-8571, Japan*

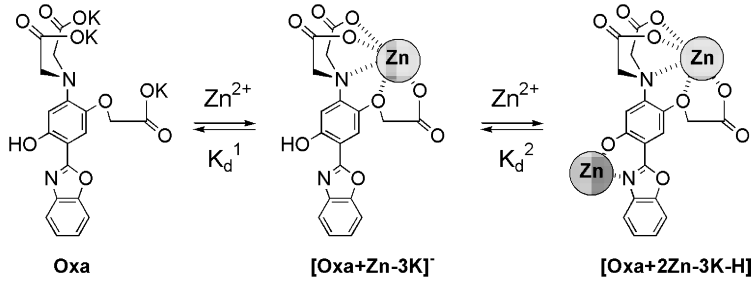
Received 28 July 2004; revised 8 October 2004; accepted 21 October 2004
Available online 5 November 2004

Abstract—A new fluorescent chelator **Oxa**, having two metal-binding sites, was designed and synthesized in six steps. **Oxa** exhibited two distinctive dissociation constants for Zn^{2+} ($K_d^1 = 1.50\,\mu M$ and $K_d^2 = 140\,\mu M$), with considerable fluorescence increase in aqueous buffer at pH 7.2. Affinities of **Oxa** for the other biologically important ions such as Ca^{2+} ($K_d^1 = 250\,\mu M$ and $K_d^2 = 275\,mM$) and Mg^{2+} ($K_d^1 = 975\,\mu M$ and K_d^2 could not be determined) in the same conditions were also obtained.

Our approach of the development of a new fluorescent sensor for biologically important ions is based on the idea that the fluorescent probes, which have two metal-binding sites with two distinctive dissociation constants (K_d^1 and K_d^2), may be applicable for measuring ions in wide concentration range depending upon the dissociation constants of each binding site. The first target for the new chelator is Zn^{2+} since Zn^{2+} is one of the most important transition metal ions in physiology. Fluorescent probes for Zn^{2+} have been developed[1–10,23] but they have only one metal-binding site.

2-(2′-Hydroxyphenyl)benzoxazole (HBO) is well-known as a typical molecule which undergoes excited state intramolecular proton transfer (ESIPT).[11–15] HBO also shows weak affinity for Zn^{2+} together with a wavelength shift in aqueous solution.[16] When other metal-chelating groups, which have much higher binding constants than HBO, are introduced on the HBO skeleton, the molecule would have two distinctive binding constants in different concentration ranges. In this respect, **Oxa** was designed and synthesized. **Oxa** has two chelating parts, aminophenol triacetic acid (APTRA) and HBO (Scheme 1).

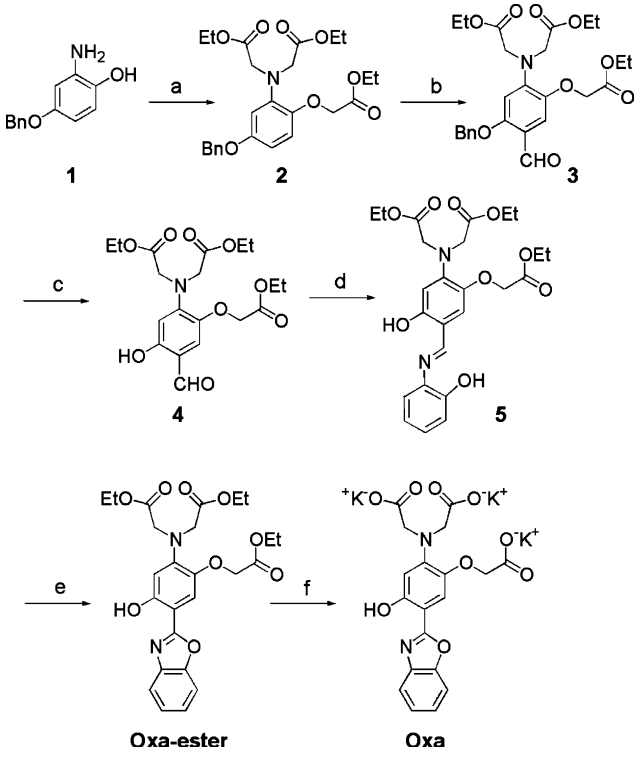
We employed the APTRA structure for a new fluorescent sensor. The APTRA chelator was developed by Levy et al.[17–19] The three carboxylate groups, the anilino nitrogen and the ether oxygen are available for complexation at physiological pH.[18] APTRA should have much higher affinity for Zn^{2+} than that of HBO due to the chelating effect and an electronic change in the aromatic ring, which directly reflects its fluorescence



Scheme 1. Tentative structures of Zn^{2+}- and $2Zn^{2+}$-bound forms of **Oxa**.

Keywords: Fluorescent sensors; Complexation.
* Corresponding author. Tel.: +81 298 53 4315; fax: +81 298 53 6503; e-mail: arai@chem.tsukuba.ac.jp

0040-4039/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2004.10.115



Scheme 2. Reagents and conditions: (a) *i*-Pr_2NEt, NaI, bromoacetic acid ethyl ester, acetonitrile, reflux, 15 h; (b) $POCl_3$, DMF, pyridine, 0 °C, 22 h; (c) H_2, Pd–C, CH_3CO_2H, rt, 48 h; (d) *o*-hydroxyaniline, ethanol, reflux, 2 h; (e) DDQ, chloroform, 70 °C, 40 h; (f) KOH, ethanol, rt, 15 h.

change, should occur when the amino nitrogen and ether oxygen contribute to form the complex with metal ions. Solubility to aqueous buffer at physiological pH is necessary for the chelating reagents if the targets are physiologically important metals. The APTRA derivatives would give enough solubility to an aqueous solution at medium pH.[20]

HBO derivatives have an extinction coefficient in the region of 300–400 nm, which is required because of the excitation wavelength. Chemical and thermal stability is also required for this study. In the course of our research,[21] we also tried to prepare fluorescent probes having a salicylideneaniline as a fluorophore. However, a salicylideneaniline analogue, such as **5** (Scheme 2), is unstable under alkali conditions. Thus, a benzoxazole structure is suitable from the viewpoint of not only a highly fluorescent but also hydrolysis-proof chromophore for this study.

The synthesis of **Oxa** is shown in Scheme 2. Compound **1**[22] was prepared from hydroquinone in four steps and was trialkylated with bromoacetic acid ethyl ester, followed by aromatic formylation to give aldehyde **3**, which was then reduced with catalytic Pd–C under H_2 atmosphere to form **4**. Schiff's base **5** was obtained by coupling reaction between **4** and 2-hydroxyaniline in refluxing ethanol. Oxidation of **5** with DDQ in chloroform gave **Oxa-ester** and following ester hydrolysis with KOH in water yielded a fluorescence chelator, **Oxa**.† This aqueous solution, including potassium salt of **Oxa** and little excess of KOH, was directly used as a stock solution for this research. The complexation studies of **Oxa** were performed in aqueous buffer solution at physiological pH (40 mM HEPES, 100 mM KCl, pH 7.20). It is noted that **Oxa** can be dissolved in buffer solution at pH 7.2 without using organic solvents such as DMSO.

When **Oxa** formed complexes with metal ions, the absorption and the fluorescence spectra changed. The change in the absorption spectra upon the addition of Zn^{2+} is shown in Figure 1a–c. As illustrated in Scheme 1, **Oxa** showed two distinctive binding constants with Zn^{2+} due to the formation of the corresponding two Zn-complexes **[Oxa+Zn–3K]**$^-$ and **[Oxa+2Zn–3K–H]**, revealed by the observation of the three-step absorption changes. First step (Fig. 1a and b): the absorbance maximum at 360 nm of **Oxa** decreased together with the blue-shift upon the addition of Zn^{2+} up to near the [Oxa]:[Zn^{2+}] = 1:1 ratio. The isosbestic point was observed at 315 nm (Fig. 1b), indicating a simple 1:1 complexation equilibrium between **Oxa** and **[Oxa+Zn–3K]**$^-$, where Zn^{2+} precedently binds to the APTRA group. The formation of **[Oxa+2Zn–3K–H]** is negligible as long as the isosbestic point remains at 315 nm. The apparent dissociation constant ($K_d^{1\ Zn} = 1.50\,\mu M$ at pH 7.2) for Zn^{2+} was determined by iterative least-squares fitting to a 1:1 model. Second step: when the concentration of Zn^{2+} reaches that of **Oxa**, the absorption band does not go through the isosbestic point at 313 nm due to the produced **[Oxa+2Zn-3K-H]**. Third Step: further addition of Zn^{2+} leads to a new isosbestic point at 358 nm (Fig. 1c), indicating that the concentration of free **Oxa** can be negligible in the high Zn^{2+} concentration range and the simple equilibrium between **[Oxa+Zn–3K]**$^-$ and **[Oxa+2Zn–3K–H]** (Scheme 1) should be operating. The plotting of the absorption bands at 400 nm upon the addition of Zn^{2+} gave the second dissociation constant ($K_d^{2\ Zn} = 140\,\mu M$).

The added Zn^{2+} also caused two-step fluorescence change. First step (Inset in Fig. 2): upon the addition of Zn^{2+}, slight decrease the fluorescence intensity with the isoemissive point at 459 nm was observed, indicating

† *Data for* **Oxa-ester**: ^1H NMR ($CDCl_3$, 500 MHz, Me_4Si) δ 11.20 (1H, s, ArOH), 7.66 (1H, m, ArH), 7.55 (1H, m, ArH), 7.43 (1H, s, ArH), 7.35–7.32 (2H, m, ArH), 6.47 (1H, s, ArH), 4.63 (2H, s, $ArOCH_2CO$), 4.30–4.20 (10H, m, CH_2), 1.33–1.28 (9H, m, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.7, 168.8, 162.8, 155.4, 149.0, 145.4, 142.4, 140.4, 124.8, 124.7, 118.8, 113.2, 110.4, 106.4, 102.4, 67.5, 61.2, 61.0, 53.9, 14.3, 14.2; Elemental Analysis. Anal. Calcd for $C_{25}H_{28}N_2O_9$: C, 59.99; H, 5.64; N, 5.60. Found: C, 59.84; H, 5.76; N, 5.54.

Oxa(*acid form*): Potassium salt of **Oxa** was reprecipitated by the addition of 0.1 N HCl in aqueous solution and the NMR of **Oxa** without salt (acid form) was measured in DMSO-d_6. ^1H NMR (DMSO-d_6, 500 MHz) δ 10.88 (1H, s, ArOH), 7.77–7.73 (2H, m, ArH), 7.40–7.36 (2H, m, ArH), 7.33 (1H, s, ArH), 6.15 (1H, s, ArH), 4.53 (2H, s, $ArOCH_2$), 4.15 (4H, s, $ArN(CH_2)_2$). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.7, 170.3, 162.8, 154.3, 148.6, 143.9, 141.3, 140.0, 125.1, 124.9, 118.4, 111.9, 110.8, 103.1, 99.0, 67.6, 59.3.

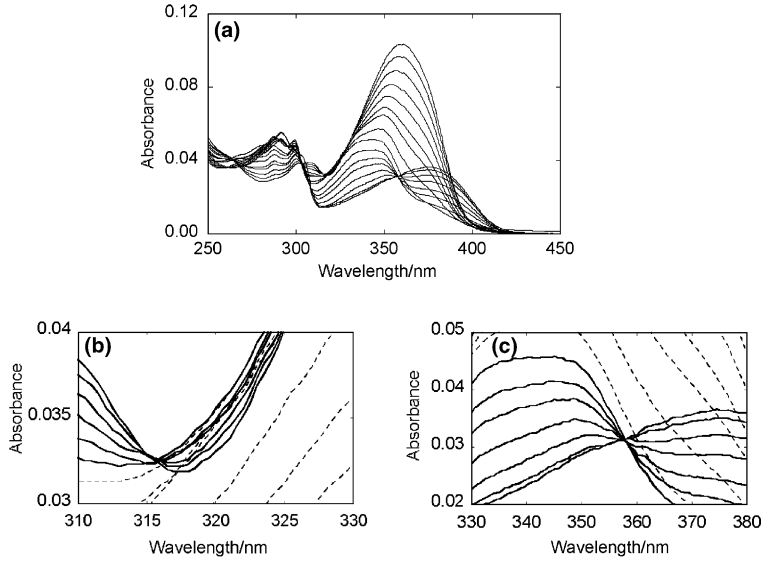


Figure 1. Change in the absorption spectra of **Oxa** (3.50 μM) upon the addition of $ZnCl_2$ in aqueous buffer (40 mM HEPES, 100 mM KCl, pH 7.20). $[Zn^{2+}]$ = 0–558 μM (a). Expanded spectra are shown in (b) and (c). Isosbestic points are observed at 315 nm when $[Zn^{2+}]$ = 0–3.23 μM (solid line in (b)) and at 358 nm when $[Zn^{2+}]$ = 42.1–558 μM (solid line in (c)).

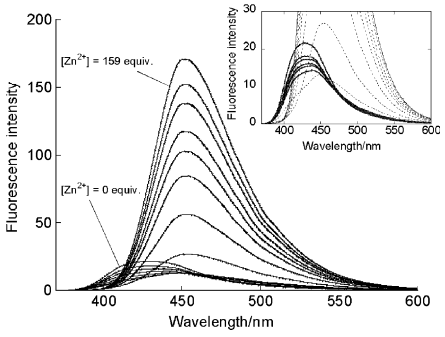


Figure 2. Change in the fluorescence spectra ($\lambda_{excitation}$ = 360 nm) of **Oxa** (3.50 μM) upon the addition of $ZnCl_2$ in buffer (40 mM HEPES, 100 mM KCl, pH 7.20) solution. $[Zn^{2+}]$ = 0–558 μM (0–159 equiv). Inset shows the expanded data of Figure 2. The isoemissive point at 459 nm was observed probably due to the equilibrium between **Oxa** and **[Oxa+Zn-3K]**$^{-}$.

1:1 complexation equilibrium between **Oxa** and **[Oxa+Zn–3K]**$^{-}$. Second step (Fig. 2): further addition of Zn^{2+} leads to the fluorescence red-shift with dramatic increase, which has also been observed in other chelators having a benzoxazole skeleton.[16,23] When Zn^{2+} binds to oxazole, the nonradiative decay pathway is inhibited due to the electronical change in the lowest excited singlet state.[16] Therefore, this fluorescence increase also strongly suggests the formation of **[Oxa+2Zn–3K–H]**. The emission maximum of **Oxa** and **[Oxa+2Zn–3K–H]** is observed at 430 nm and 451 nm, respectively.

The observed two distinctive dissociation constants for Zn^{2+} prompted us to further explore the affinity determination of **Oxa** to the other biologically important divalent cations. To Ca^{2+} and Mg^{2+}, the ATPRA unit in **Oxa** should have some affinities since the APTRA chelators have originally been developed as probes for Mg^{2+}.[17,18] On the other hand, Henary et al. reported

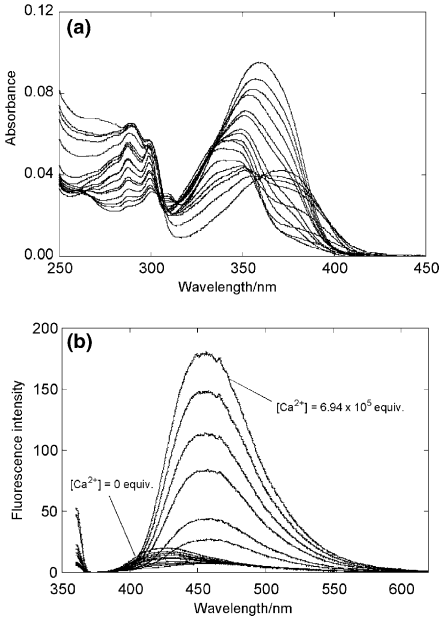


Figure 3. (a) Change in the absorption spectra of **Oxa** (3.50 μM) upon the addition of $CaCl_2$ in aqueous buffer (40 mM HEPES, 100 mM KCl, pH 7.20). $[Ca^{2+}]$ = 0–2.42 M. Isosbestic points are observed at 314 nm when $[Ca^{2+}]$ = 0–903 μM and at 360 nm when $[Ca^{2+}]$ = 1.53–2.42 M. (b) Change in the fluorescence spectra ($\lambda_{excitation}$ = 360 nm) of **Oxa** (3.50 μM) upon the addition of $CaCl_2$ in buffer (40 mM HEPES, 100 mM KCl, pH 7.20) solution. $[Ca^{2+}]$ = 0–2.42 M.

that HBO derivatives did not show any increase in fluorescence intensity by addition of divalent cations, such as Ca^{2+} and Mg^{2+}, even at high metal concentration,[16] indicating that HBO derivatives do not interact with Ca^{2+} and Mg^{2+}. In the case of **Oxa**, however, titration with Ca^{2+} gave similar spectral change to that with Zn^{2+} in both UV absorption and fluorescence measurements (Fig. 3), suggesting that the HBO moiety as

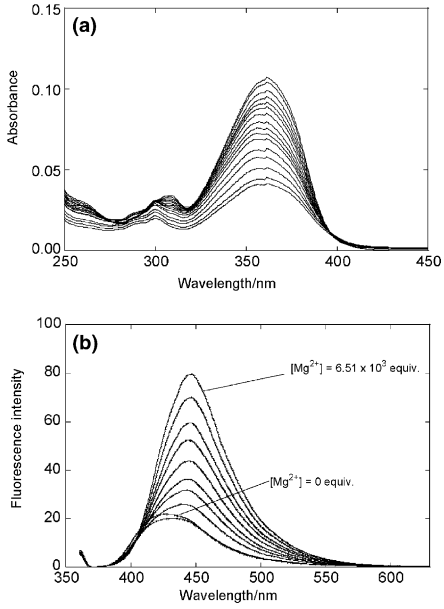


Figure 4. (a) Change in the absorption spectra of **Oxa** (3.50 μM) upon the addition of $MgCl_2$ in aqueous buffer (40 mM HEPES, 100 mM KCl, pH 7.20). $[Mg^{2+}] = 0$–22.8 mM. Isosbestic points are observed at 300 nm when $[Mg^{+}] = 0$ μM to 4 mM (b) Change in the fluorescence spectra ($\lambda_{excitation} = 360$ nm) of **Oxa** (3.50 μM) upon the addition of $MgCl_2$ in buffer (40 mM HEPES, 100 mM KCl, pH 7.20) solution. $[Mg^{2+}] = 0$–22.8 mM.

well as the APTRA unit bound to Ca^{2+}. The apparent dissociation constant for Ca^{2+} was much higher than that for Zn^{2+} ($K_d^{1\ Ca} = 250$ μM and $K_d^{2\ Ca} = 275$ mM). Titration with Mg^{2+} gave different spectral change from those with Zn^{2+} and Ca^{2+} (Fig. 4). In the absorption spectra, the absorbance intensity at 360 nm was decreased with increasing Mg^{2+}. Isosbestic point is observed at 300 nm when the concentration of Mg^{2+} is 0–4.0 mM and the dissociation constant for Mg^{2+} was calculated to be 943 μM, which is also much higher than that with Zn^{2+}. The second isosbestic point could not be observed during the titration with Mg^{2+}. Therefore, the second dissociation constant for Mg^{2+} could not be determined. In the fluorescence spectra, the intensity increased even at low Mg^{2+} concentration where the Mg^{2+} probably not binds to HBO unit in **Oxa** but the reason of fluorescence increase is still unclear. Isoemissive point was not observed during the titration. The calculated dissociation constants are shown in Table 1. From the results, one can say that **Oxa** preferably binds to Zn^{2+}, against Ca^{2+} and Mg^{2+} in this condition. **Oxa** can detect Zn^{2+} in micro molar range and increase its fluorescence in sub-milli molar range of Zn^{2+}. The exploration of the affinity of **Oxa** to the other cation species is ongoing.

Table 1. The apparent dissociation constants of **Oxa** for Zn^{2+}, Ca^{2+} and Mg^{2+} in aqueous buffer (40 mM HEPES, 100 mM KCl, pH 7.20)

	Zn^{2+}	Ca^{2+}	Mg^{2+}
K_d^1	1.50 μM	250 μM	943 μM
K_d^2	140 μM	275 mM	—

In conclusion, a new fluorescence chelating reagent **Oxa** was designed and synthesized and the complexation studies were performed to give the two distinctive dissociation constants for Zn^{2+} and Ca^{2+}. To the best of our knowledge, this is the first clear evidence of the spectroscopic properties of a chelating agent having two chelating parts in one molecule with considerably different binding constant capable of being dissolved in water.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (417), a Grant-in-Aid for Scientific Research (No. 16350005) and the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government, by University of Tsukuba Research Projects, by the Asahi Glass Foundation and by JSR Corporation.

References and notes

1. Fahrni, C. J.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1999**, *121*, 11448–11458.
2. Walkup, G. K. B.; Burdette, S. C.; Lippard, S. J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2000**, *122*, 5644–5645.
3. Burdette, S. C.; Walkup, G. K.; Springler, B.; Tsien, R. Y.; Rippard, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 1831–1841.
4. Pearce, D. A.; Jotterand, N.; Carrico, I. S.; Imperiali, B. *J. Am. Chem. Soc.* **2001**, *123*, 5160–5161.
5. Hirano, T.; Kikuchi, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *12*, 6555–6562.
6. Maruyama, S.; Kikuchi, K.; Hirano, T.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *12*, 10650–10651.
7. Gee, K. R.; Zhou, Z. L.; Qian, W. J.; Kennedy, R. *J. Am. Chem. Soc.* **2002**, *124*, 776–778.
8. Shults, M. D.; Pearce, D. A.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 10591–10597.
9. Woodroofe, C. C.; Lippard, S. J. *J. Am. Chem. Soc.* **2003**, *125*, 11458–11459.
10. Reany, O.; Gunnlaugsson, T.; Parker, D. *Chem. Commun.* **2000**, 473–474.
11. Arthen-Engeland, Th.; Bultmann, T.; Ernsting, M. P.; Rodriguez, M. A.; Thiel, W. *Chem. Phys.* **1992**, *163*, 43.
12. Wang, H.; Zhang, H.; Abou-Zied, O. K.; Yu, C.; Romesberg, F. E.; Glasbeek, M. *Chem. Phys. Lett.* **2003**, *367*, 599–608.
13. Abou-Zied, O. K.; Jimenez, R.; Thompson, E. H. Z.; Millar, D. P.; Romesberg, F. E. *J. Phys. Chem. A* **2002**, *106*, 3665.
14. Ogawa, A. K.; Abou-Zied, O. K.; Tsui, V.; Jimenez, R.; Case, D. A.; Romesberg, F. E. *J. Am. Chem. Soc.* **2000**, *122*, 9917–9920.
15. Yang, G.; Morelet-Savary, F.; Peng, Z.; Wu, S.; Fouassier, J.-P. *Chem. Phys. Lett.* **1996**, *256*, 536–542.
16. Henary, M. M.; Fahrni, C. J. *J. Phys. Chem. A* **2002**, *106*, 5210–5220.
17. Levy, L. A.; Murphy, E.; Raju, B.; London, R. E. *Biochemistry* **1988**, *27*, 4041–4048.
18. Otten, P. A.; London, R. E.; Levy, L. A. *Bioconjug. Chem.* **2001**, *12*, 76–83.
19. Raju, B.; Murphy, E.; Levy, L. A.; Hall, R. D.; London, R. E. *Am. J. Physiol.* **1989**, *256*, C540–C548.

20. Cielen, E.; Stobiecka, A.; Tahri, A.; Hoornaert, G. J.; De Schryver, F. C.; Gallay, J.; Vincent, M.; Boens, N. *J. Chem. Soc., Perkin Trans. 2* **2002**, 1197–1206.
21. Ohshima, A.; Momotake, A.; Arai, T. *J. Photochem. Photobiol. A, Chem.* **2004**, *162*, 473–479.
22. Haviv, F.; Ratajczyk, J. D.; DeNet, R. W.; Kerdesky, F. A.; Walters, R. L.; Schmidt, S. P.; Holms, J. H.; Young, P. R.; Carter, G. W. *J. Med. Chem.* **1988**, *31*, 1719–1728.
23. Taki, M.; Wolford, J. L.; O'Halloran, T. V. *J. Am. Chem. Soc.* **2003**, *126*, 712–713.