Organic LETTERS

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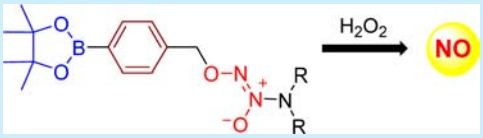
Arylboronate Ester Based Diazeniumdiolates (BORO/NO), a Class of Hydrogen Peroxide Inducible Nitric Oxide (NO) Donors

Allimuthu T. Dharmaraja,[†] Govindan Ravikumar,[†] and Harinath Chakrapani*

Indian Institute of Science Education and Research Pune, Dr. Homi Bhabha Road, Pashan, Pune 411 008, Maharashtra, India

Supporting Information

ABSTRACT: Here, we report the design, synthesis, and evaluation of arylboronate ester based diazeniumdiolates (BORO/NO), a class of nitric oxide (NO) donors activated by hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), to generate NO. We provide evidence for the NO donors' ability to permeate bacteria to produce NO when exposed to H_2O_2 supporting possible applications for BORO/NO to study molecular mechanisms of NO generation in response to elevated ROS.



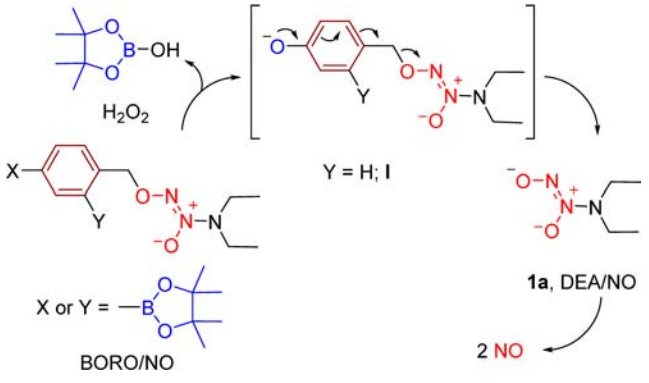
Although reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) mediate numerous physiological processes,[1] elevated levels of ROS can cause oxidative stress leading to cell death.[2−6] Similarly, nitric oxide (NO), a reactive nitrogen species (RNS), has multifarious functions and effects in cells, but increased NO has been associated with nitrosative stress and growth inhibition.[7−10] Both ROS and RNS are generated during immune response to counter infectious pathogens, presumably due to their ability to synergize[11,12] leading to increased damaging effects.[13−15] However, due to its antioxidant capability,[16,17] NO has also been implicated in protecting bacteria from oxidative stress and may hence contribute to bacterial drug resistance.[18−20] Thus, the precise effects of NO when generated in the presence of elevated ROS are yet to be completely understood.

Due to its diverse roles, NO for use in biological studies must be generated in a controlled manner and preferably in response to a stimulus.[21,22] A number of nitric oxide donors are available, but among these, sodium salts of diazeniumdiolates (such as DEA/NO **1a**, Scheme 1) are reliable sources of NO.[23] However, these NO donor salts lack specificity and would generate NO in the absence of H_2O_2 as well. Diazeniumdiolate anions can, however, be derivatized into "protected" forms, which can be activated in the presence of a specific metabolic trigger to generate NO.[24,25] Numerous methodologies[11,12,26−33] for cleavage of otherwise stable diazeniumdiolate derivatives are known, but to our knowledge a nitric oxide donor that is selectively activated by ROS is not available.

Boronate ester protecting groups are highly specific to cleavage by H_2O_2 and have been extensively used in imaging[34−37] as well as in drug delivery.[38−41] For mechanistic studies with bacteria, elevated H_2O_2 is frequently used to simulate conditions of oxidative stress.[2,42] Arylboronate ester based diazeniumdiolates (BORO/NO), a class of diazeniumdiolate derivatives that are attached to a pinacolboronate ester through a self-immolative aryl linker, were considered as H_2O_2 activated NO donors (Scheme 1). Reaction of BORO/NO with H_2O_2 should produce the phenolate intermediate **I**, which could rearrange to produce the diazeniumdiolate anion, which in pH 7.4 releases NO (Scheme 1.).

Following a reported procedure, compound **5** was synthesized by bromination of **4**[43,44] (Figure 1) and reacted with DEA/NO **1a** to obtain **2a** (Scheme 2). A similar procedure was used to synthesize **2b**−**2d** by the reaction of

Scheme 1. BORO/NO, H_2O_2 Activated NO Donors



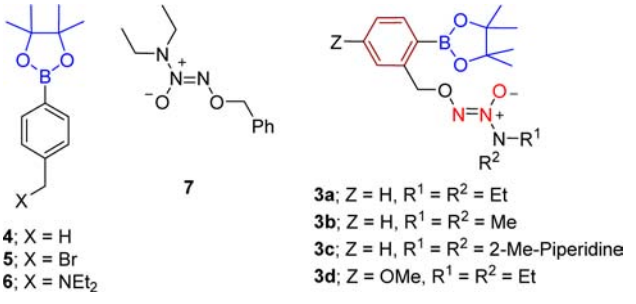
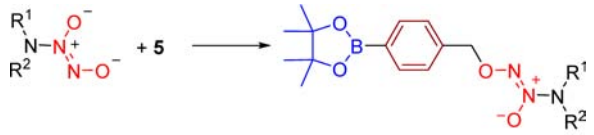


Figure 1. Structure of compounds **4**−**7** and **3a**−**3d**.

Received: March 11, 2014
Published: May 5, 2014

dx.doi.org/10.1021/ol5010643 | Org. Lett. 2014, 16, 2610−2613

Scheme 2. Synthesis of 4-BORO/NO Derivatives



1a; $R^1 = R^2$ = Et
1b; $R^1 = R^2$ = Me
1c; R^1R^2N = pyrrolidine
1d; R^1R^2N = 2-Me-piperidine

2a; R^1, R^2 = Et
2b; R^1, R^2 = Me
2c; R^1R^2N = pyrrolidine
2d; R^1R^2N = 2-Me-piperidine

the bromide **5** with the corresponding diazeniumdiolate salts, **1b**–**1d** (Scheme 2).[45] In order to study possible differences in 1,4- versus 1,2-elimination[38] of the phenolate intermediate generated during hydrogen peroxide activated NO release from this class of compounds, derivatives **1a**, **1b**, and **1d** were reacted with the corresponding 2-(pinacol boronate ester)benzyl bromides to produce **3a**–**3d** (Figure 1).

The 4-BORO/NO derivative **2a** was incubated in pH 7.4 buffer, and no evidence for NO production in the headspace was found as determined by a chemiluminescence-based assay that is selective for NO (Figure 2a). In the presence of H_2O_2,

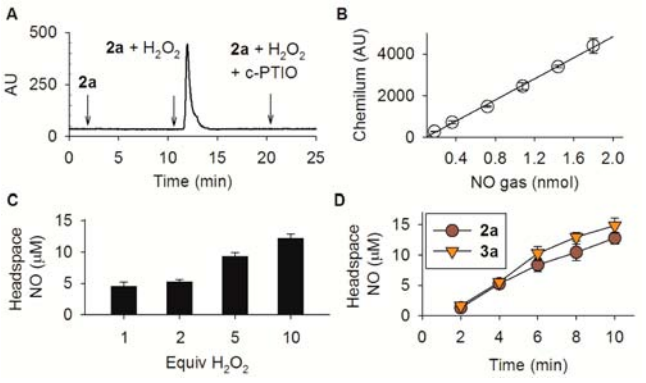


Figure 2. Nitric oxide analysis in the headspace[a] using a chemiluminescence detector: (a) Traces for **2a** alone, in the presence of H_2O_2 with and without a NO scavenger;[b] (b) Calibration curve generated using NO gas; (c) NO generated by **2a** with increasing equivalents of H2O2; (d) Time course of NO generated during incubation of **2a** and **3a** in the presence of 10 equiv H_2O_2. [a] Experiments were conducted in 10 mM pH 7.4 phosphate buffer containing 100 μM of diethylene triamine pentaacetic acid (DTPA), a metal ion chelating agent at 37 °C. Reaction mixtures from headspace of reaction vial were injected into a chemiluminescene-based nitric oxide analyzer using argon as the carrier gas. [b] Arrow indicates injection of sample.

we found significant levels of NO produced (Figure 2a). When **2a** was incubated in the presence of H_2O_2 and (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (c-PTIO), a scavenger for NO, we found nearly complete abrogation of signal attributable to NO in the headspace confirming NO generation by **2a** (Figure 2a). A calibration curve with nitric oxide gas was generated using authentic NO gas (linear regression analysis coefficient, R^2 = 0.998, Figure 2b).

Next, hydrogen peroxide induced NO generation in solution was analyzed. First, a calibration curve with sodium nitrite in a reducing mixture, which converts nitrite to NO, was generated (linear regression analysis coefficient, R^2 = 0.999, Figure 3a). We found evidence for nitric oxide production during

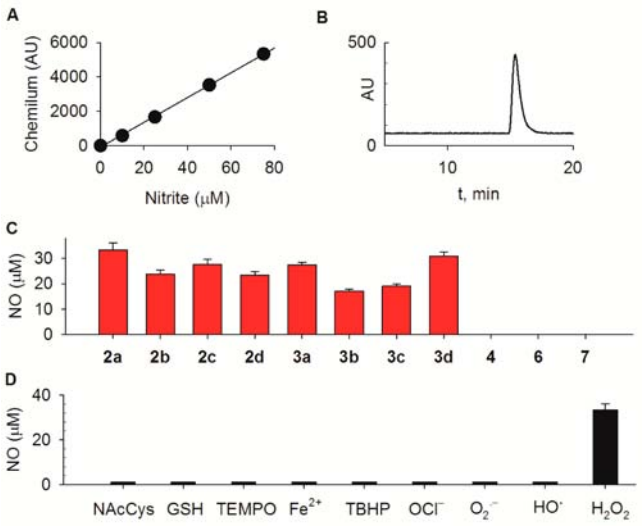


Figure 3. Solution phase nitric oxide analysis using a chemiluminescence detector: (a) Calibration curve generated using sodium nitrite; (b) Representative spectrum when a solution of **2a** in H_2O_2 was analyzed; (c) Nitric oxide analysis of BORO/NO derivatives and **4**, **6**, and **7**; (d) NO generated in solution when incubated with various biologically relevant species. Aliquots from the solution phase of the reaction vial were injected into a chemiluminescene-based nitric oxide analyzer using argon as the carrier gas containing a reducing mixture. Under these conditions, values of NO reported are NO + nitrite. NAcCys: *N*-acetyl cysteine; GSH: glutathione; TEMPO: 2,2,6,6-tetramethylpiperidinyloxy; Fe^{2+}; TBHP: *tert*-butyl hydroperoxide; OCl^-: sodium hypochlorite; $O_2^{-\bullet}$: generated using hypoxanthine/xanthine oxidase; $HO^\bullet$ was generated using H_2O_2 and Fe(II) mixed at a 1:10 ratio; here, DTPA was not added.

incubation of **2a** in the presence of H_2O_2 in buffer (Figure 3b; see Supporting Information Figure S3). When **2a** (25 μM) was incubated with varying equivalents of H_2O_2, we found a dose-dependent increase in nitric oxide produced after 10 min (Figure 2c; see Supporting Information, Table S6) and 10 equiv of H_2O_2 were chosen for further studies.

A time course of headspace NO produced during incubation of **2a** (25 μM) in the presence of H_2O_2 (10 equiv) showed a gradual increase of NO during 10 min, and the yield of NO was nearly 12 μM (Figure 2d). When the other analogues **2b**–**2d** and **3a**–**3d** were tested, we found nitric oxide production only when exposed to H_2O_2 and the yields of NO (see Supporting Information, Table S1 and Figure 3c) were comparable or lower than the yield of NO produced during incubation of **2a** under similar conditions. A time course of NO production during incubation of the 2-BORO/NO derivative **3a** in the presence of H_2O_2 showed no major difference in the rate of NO release possibly because 1,2- and 1,4-eliminations occurred at comparable rates (Figure 2d).

Compound **4** (Figure 1) which contained the boronate ester but no capability to generate NO did not produce significant levels of NO during incubation with H_2O_2 (Figure 3c). Next, the diethylamino derivative **6** and benzyl derivative of DEA/NO, **7** (Figure 1), were prepared using reported procedures (see Supporting Information). During incubation with H_2O_2 (10 equiv), as expected, we found no evidence for NO generation by these compounds suggesting the requirement for the boronate ester functionality as well as the diazeniumdiolate for NO production (Figure 3c).

dx.doi.org/10.1021/ol5010643 | *Org. Lett.* 2014, 16, 2610–2613

Having established that BORO/NO derivatives were capable of generating NO when triggered by H_2O_2, a number of biologically relevant nucleophiles, reductants, and oxidants were utilized to study the selectivity of activation of **2a** to produce NO (Figure 3d). The NO donor **2a** was found to be highly specific toward activation by H_2O_2 and did not generate significant levels of NO in the presence of biologically relevant thiols, metal ions, antioxidants, and other common ROS (Figure 3d). The observed specificity is consistent with previously published data on reactivity of boronate esters toward H_2O_2.[36,46]

In order to study if BORO/NO was capable of generating NO in bacteria, *Escherichia coli* (*E. coli*) cells were incubated with **2a** (25 μM). The ability of this compound to enhance NO was studied by measuring extracellular nitrite using a Griess assay. We found negligible NO during incubation of **2a** alone, but under similar conditions, when **2a** was cotreated with H_2O_2, we found a significant increase in extracellular nitrite, a marker for increased NO (Figure 4a). The amount of nitrite

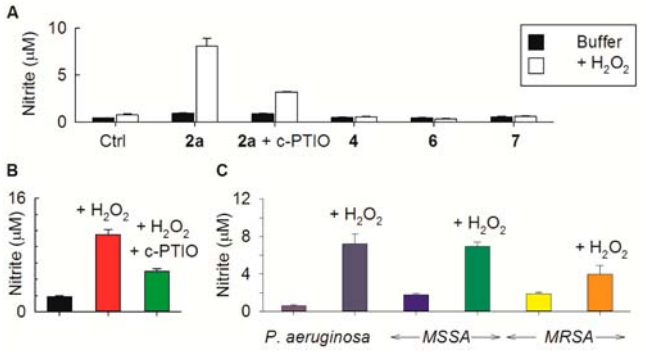


Figure 4. (a) Extracellular nitrite in *E. coli* after 1 h; (b) Intracellular NO from **2a** in *E. coli*; (c) Extracellular nitrite release upon incubation of **2a** in the presence of Gram-positive and Gram-negative bacteria.

generated was diminished when cotreated with c-PTIO, a scavenger for NO. Compounds **4**, **6**, and **7**, which did not generate NO upon reaction with H_2O_2 in buffer (Figure 3c), were incapable of enhancing nitrite when incubated with bacteria and H_2O_2 (Figure 4a).

We next studied the ability of **2a** to enhance intracellular NO levels in *E. coli* in the presence of elevated levels of H_2O_2.[47] Bacteria were incubated with **2a** (100 μM) for 30 min followed by centrifugation of the bacterial suspension to aspirate out any excess **2a** in the medium. The collected bacterial pellet was resuspended with buffer and plated in a 96-well microplate, and cells were incubated with and without added H_2O_2 at 37 °C. Any NO that is produced must be due to **2a** that has permeated bacteria. After 30 min, Griess reagent was added and we found significantly increased NO in bacteria treated with H_2O_2 in comparison with untreated cells (Figure 4b). Under similar conditions, when bacterial cells were treated with H_2O_2 and c-PTIO, a NO scavenger, we found diminished nitrite supporting the intermediacy of NO during decomposition of **2a** (Figure 4b). Taken together, our data support **2a** as a cell permeable hydrogen peroxide activated NO donor.

The capability of **2a** to enhance NO in other Gram-negative and Gram-positive bacteria, *Pseudomonas aeruginosa* (*P. aeruginosa*), methicillin-sensitive *Staphylococcus aureus* (MSSA), and methicillin-resistant *Staphylococcus aureus* (MRSA), was studied. We found that incubation of these bacteria with **2a** resulted in increased nitrite (see Supporting Information) in the presence of hydrogen peroxide (Figure 4c).

Taken together, we report BORO/NO, a novel class of cell permeable H_2O_2 activated NO donors. Antibiotic resistance is emerging as a major global health problem with millions affected each year, and the role of NO in bacteria developing drug resistance remains unclear. NO has been reported to synergize with silver(I) sulfadiazine, an oxidative stress inducing antimicrobial, against a host of bacteria including *S. aureus* suggesting the possible use of NO in combination therapy. In contrast, NO generated by bacterial nitric oxide synthase (bNOS) has been proposed as a possible cellular antioxidant that protects MRSA from oxidative stress,[18−20] and specific bNOS inhibitors are in development as possible drug candidates.[48] Thus, molecular mechanisms of NO-derived protective effects (or otherwise)[16,17] remain to be elucidated and it is anticipated that BORO/NO will be useful in deciphering such mechanisms.[42] The use of BORO/NO for localized delivery of NO to cancers,[24,25] which are known to have elevated ROS as a phenotype, and other applications are currently under investigation.[24,25,49,50]

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization data, assay protocols and associated data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: harinath@iiserpune.ac.in.

Author Contributions

†These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank IISER Pune and the Department of Biotechnology, Ministry of Science and Technology, India (DBT, BT/PR6798/MED/29/636/2012) for financial support. A.T.D. and G.R. acknowledge research fellowships from Council for Scientific and Industrial Research (CSIR). Diazeniumdiolate salts were a gift from Dr. Larry Keefer and Dr. Joseph Saavedra (Chemical Biology Laboratory, National Cancer Institute).

■ REFERENCES

(1) Imlay, J. A. *Nat. Rev. Microbiol.* **2013**, *11*, 443.
(2) Brynildsen, M. P.; Winkler, J. A.; Spina, C. S.; MacDonald, I. C.; Collins, J. J. *Nat. Biotechnol.* **2013**, *31*, 160.
(3) Foti, J. J.; Devadoss, B.; Winkler, J. A.; Collins, J. J.; Walker, G. C. *Science* **2012**, *336*, 315.
(4) Kohanski, M. A.; Dwyer, D. J.; Hayete, B.; Lawrence, C. A.; Collins, J. J. *Cell* **2007**, *130*, 797.
(5) Ruben Morones-Ramirez, J.; Winkler, J. A.; Spina, C. S.; Collins, J. J. *Sci. Transl. Med.* **2013**, *5*, 190ra81.
(6) Dharmaraja, A. T.; Alvala, M.; Sriram, D.; Yogeeswari, P.; Chakrapani, H. *Chem. Commun.* **2012**, *48*, 10325.
(7) Hirst, D.; Robson, T. *J. Pharm. Pharmacol.* **2007**, *59*, 3.
(8) Huerta, S.; Chilka, S.; Bonavida, B. *Int. J. Oncol.* **2008**, *33*, 909.
(9) Lu, Y.; Slomberg, D. L.; Shah, A.; Schoenfisch, M. H. *Biomacromolecules* **2013**, *14*, 3589.

(10) Sun, B.; Slomberg, D. L.; Chudasama, S. L.; Lu, Y.; Schoenfisch, M. H. *Biomacromolecules* **2012**, *13*, 3343.
(11) Kiziltepe, T.; Hideshima, T.; Ishitsuka, K.; Ocio, E. M.; Raje, N.; Catley, L.; Li, C.-Q.; Trudel, L. J.; Yasui, H.; Vallet, S.; Kutok, J. L.; Chauhan, D.; Mitsiades, C. S.; Saavedra, J. E.; Wogan, G. N.; Keefer, L. K.; Shami, P. J.; Anderson, K. C. *Blood* **2007**, *110*, 709.
(12) Maciag, A. E.; Chakrapani, H.; Saavedra, J. E.; Morris, N. L.; Holland, R. J.; Kosak, K. M.; Shami, P. J.; Anderson, L. M.; Keefer, L. K. *J. Pharmacol. Exp. Ther.* **2011**, *336*, 313.
(13) Fang, F. C. *Nat. Rev. Microbiol.* **2004**, *2*, 820.
(14) Nathan, C.; Cunningham-Bussel, A. *Nat. Rev. Immunol.* **2013**, *13*, 349.
(15) Privett, B. J.; Deupree, S. M.; Backlund, C. J.; Rao, K. S.; Johnson, C. B.; Coneski, P. N.; Schoenfisch, M. H. *Mol. Pharmaceutics* **2010**, *7*, 2289.
(16) Hummel, S. G.; Fischer, A. J.; Martin, S. M.; Schafer, F. Q.; Buettner, G. R. *Free Radical Biol. Med.* **2006**, *40*, 501.
(17) Joshi, M. S.; Ponthier, J. L.; Lancaster, J. R., Jr. *Free Radical Biol. Med.* **1999**, *27*, 1357.
(18) Van Sorge, N. M.; Beasley, F. C.; Gusarov, I.; Gonzalez, D. J.; Von Köckritz-Blickwede, M.; Anik, S.; Borkowski, A. W.; Dorrestein, P. C.; Nudler, E.; Nizet, V. *J. Biol. Chem.* **2013**, *288*, 6417.
(19) Gusarov, I.; Nudler, E. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13855.
(20) Gusarov, I.; Shatalin, K.; Starodubtseva, M.; Nudler, E. *Science* **2009**, *325*, 1380.
(21) Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091.
(22) Chakrapani, H.; Bartberger, M. D.; Toone, E. J. *J. Org. Chem.* **2009**, *74*, 1450.
(23) Hrabie, J. A.; Keefer, L. K. *Chem. Rev.* **2002**, *102*, 1135.
(24) Keefer, L. K. *Annu. Rev. Pharmacol. Toxicol.* **2003**, *43*, 585.
(25) Keefer, L. K. *ACS Chem. Biol.* **2011**, *6*, 1147.
(26) Chakrapani, H.; Kalathur, R. C.; Maciag, A. E.; Citro, M. L.; Ji, X.; Keefer, L. K.; Saavedra, J. E. *Bioorg. Med. Chem.* **2008**, *16*, 9764.
(27) Chakrapani, H.; Maciag, A. E.; Citro, M. L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2008**, *10*, 5155.
(28) Chakrapani, H.; Showalter, B. M.; Kong, L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2007**, *9*, 3409.
(29) Nandurdikar, R. S.; Maciag, A. E.; Hong, S. Y.; Chakrapani, H.; Citro, M. L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2010**, *12*, 56.
(30) Cai, T. B.; Lu, D. N.; Landerholm, M.; Wang, P. G. *Org. Lett.* **2004**, *6*, 4203.
(31) Saavedra, J. E.; Shami, P. J.; Wang, L. Y.; Davies, K. M.; Booth, M. N.; Citro, M. L.; Keefer, L. K. *J. Med. Chem.* **2000**, *43*, 261.
(32) Sharma, K.; Sengupta, K.; Chakrapani, H. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 5964.
(33) Sharma, K.; Iyer, A.; Sengupta, K.; Chakrapani, H. *Org. Lett.* **2013**, *15*, 2636.
(34) Chung, C.; Srikun, D.; Lim, C. S.; Chang, C. J.; Cho, B. R. *Chem. Commun.* **2011**, *47*, 9618.
(35) Au-Yeung, H. Y.; New, E. J.; Chang, C. J. *Chem. Commun.* **2012**, *48*, 5268.
(36) Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 15392.
(37) Miller, E. W.; Tulyanthan, O.; Isacoff, E. Y.; Chang, C. J. *Nat. Chem. Biol.* **2007**, *3*, 263.
(38) Cao, S.; Christiansen, R.; Peng, X. *Chem.—Eur. J.* **2013**, *19*, 9050.
(39) Stone, J. R. *Arch. Biochem. Biophys.* **2004**, *422*, 119.
(40) Hagen, H.; Marzenell, P.; Jentzsch, E.; Wenz, F.; Veldwijk, M. R.; Mokhir, A. *J. Med. Chem.* **2012**, *55*, 924.
(41) Marzenell, P.; Hagen, H.; Sellner, L.; Zenz, T.; Grinyte, R.; Pavlov, V.; Daum, S.; Mokhir, A. *J. Med. Chem.* **2013**, *56*, 6935.
(42) Yadav, R.; Samuni, Y.; Abramson, A.; Zeltser, R.; Casap, N.; Kabiraj, T. K.; L. Banach, M.; Samuni, U. *Free Radical Biol. Med.* **2014**, *67*, 248.
(43) Kuang, Y.; Balakrishnan, K.; Gandhi, V.; Peng, X. *J. Am. Chem. Soc.* **2011**, *133*, 19278.
(44) White, J. R.; Price, G. J.; Schiffers, S.; Raithby, P. R.; Plucinski, P. K.; Frost, C. G. *Tetrahedron Lett.* **2010**, *51*, 3913.
(45) Chakrapani, H.; Showalter, B. M.; Citro, M. L.; Keefer, L. K.; Saavedra, J. E. *Org. Lett.* **2007**, *9*, 4551.
(46) Chan, J.; Dodani, S. C.; Chang, C. J. *Nat. Chem.* **2012**, *4*, 973.
(47) Dharmaraja, A. T.; Chakrapani, H. *Org. Lett.* **2014**, *16*, 398.
(48) Holden, J. K.; Li, H.; Jing, Q.; Kang, S.; Richo, J.; Silverman, R. B.; Poulos, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 18127.
(49) Weinstain, R.; Savariar, E. N.; Felsen, C. N.; Tsien, R. Y. *J. Am. Chem. Soc.* **2013**, *136*, 874.
(50) Ballard, R. A.; Truog, W. E.; Cnaan, A.; Martin, R. J.; Ballard, P. L.; Merrill, J. D.; Walsh, M. C.; Durand, D. J.; Mayock, D. E.; Eichenwald, E. C.; Null, D. R.; Hudak, M. L.; Puri, A. R.; Golombek, S. G.; Courtney, S. E.; Stewart, D. L.; Welty, S. E.; Phibbs, R. H.; Hibbs, A. M.; Luan, X.; Wadlinger, S. R.; Asselin, J. M.; Coburn, C. E. *New Engl. J. Med.* **2006**, *355*, 343.