JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Co-opting a Bioorthogonal Reaction for Oncometabolite Detection

Thomas T. Zengeya,[†,§] Julie M. Garlick,[†,§] Rhushikesh A. Kulkarni,[†] Mikayla Miley,[†] Allison M. Roberts,[†] Youfeng Yang,[‡] Daniel R. Crooks,[‡] Carole Sourbier,[‡] W. Marston Linehan,[‡] and Jordan L. Meier*[,†]

[†]Chemical Biology Laboratory, National Cancer Institute, Frederick, Maryland 21702, United States

[‡]Urologic Oncology Branch, National Cancer Institute, Bethesda, Maryland 20817, United States

Supporting Information

ABSTRACT: Dysregulated metabolism is a hallmark of many diseases, including cancer. Methods to fluorescently detect metabolites have the potential to enable new approaches to cancer detection and imaging. However, fluorescent sensing methods for naturally occurring cellular metabolites are relatively unexplored. Here we report the development of a chemical approach to detect the oncometabolite fumarate. Our strategy exploits a known bioorthogonal reaction, the 1,3-dipolar cycloaddition of nitrileimines and electron-poor olefins, to detect fumarate via fluorescent pyrazoline cycloadduct formation. We demonstrate hydrazonyl chlorides serve as readily accessible nitrileimine precursors, whose reactivity and spectral properties can be tuned to enable detection of fumarate and other dipolarophile metabolites. Finally, we show this reaction can be used to detect enzyme activity changes caused by mutations in fumarate hydratase, which underlie the familial cancer predisposition syndrome hereditary leiomyomatosis and renal cell cancer. Our studies define a novel intersection of bioorthogonal chemistry and metabolite reactivity that may be harnessed to enable biological profiling, imaging, and diagnostic applications.

Dysregulated metabolism is a hallmark of many diseases, including cancer.[1] In addition to providing building blocks to support cell growth, metabolic changes can also directly stimulate cell signaling pathways.[2] For example, in hereditary leiomyomatosis and renal cell cancer (HLRCC), inactivating mutations in the fumarate hydratase (*FH*) gene cause the accumulation of fumarate.[3] High levels of this "oncometabolite" disrupt the activity of enzymes involved in epigenetic signaling, thereby facilitating malignant transformation.[4,5] Methods to sensitively detect fumarate have the potential to facilitate the rapid diagnosis of HLRCC, and also identify new disease settings in which fumarate may play a signaling role. However, the current gold standard method for fumarate detection, mass spectrometry, requires lengthy sample preparation, specialized equipment, and does not have the potential to form the basis for cellular imaging approaches.

In designing a chemical method to identify aberrant FH activity, we noticed that fumarate formally constitutes an electron-poor dipolarophile. Alkene dipolarophiles are rare in nature, but are well-known in the bioorthogonal reaction literature for their ability to undergo fluorogenic 1,3-cycloadditions with nitrileimine dipoles.[6,7] Although metabolites such as fumarate have not been explored as nitrileimine reaction partners, their similarity to previously reported substrates[8] suggested the novel possibility of exploiting this reactivity to monitor FH activity via fluorogenic detection of fumarate (Figure 1).

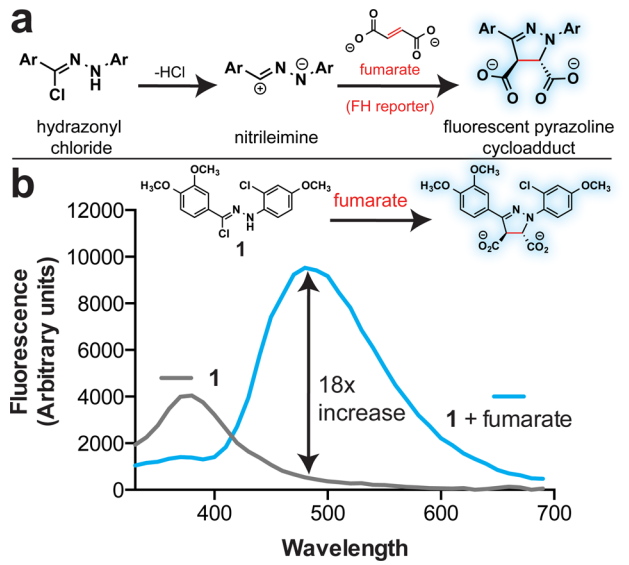


Figure 1. Fluorogenic detection of the oncometabolite fumarate via a [3+2] cycloaddition reaction. (a) Schematic for fumarate detection. (b) Fluorogenic reaction of fumarate with previously reported hydrazonyl chloride **1** (λ_{ex} = 320 nm) after 1 h. Note that only a single diasteromer of the pyrazoline product is shown.

To explore the feasibility of this approach, we first evaluated the reactivity of fumarate with hydrazonyl chloride **1**, a nitrileimine precursor known to form detectible fluorescent cycloadducts (Figure 1).[7] Fumarate is less polarizable and has a higher lying LUMO (12.2 eV) than the acrylyl dipolarophiles that have been commonly applied in bioorthogonal protein labeling applications ($LUMO_{ethyl\ acrylate}$ = 2.9 eV; Figure S1).[6,7] This has the potential to limit reaction-based detection strategies, as the rate of nitrileimine−alkene cycloadditions are inversely related to the free energy gap between the nitrileimine-HOMO and dipolarophile-LUMO.[9] However, we found that incubation of **1** with fumarate in buffer resulted in a

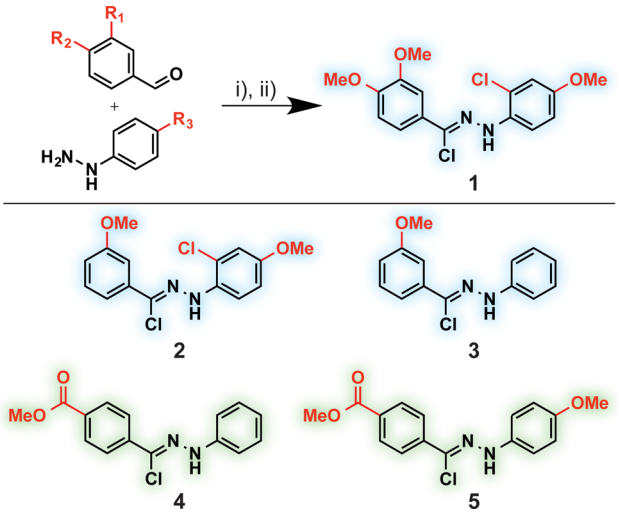
Received: September 15, 2016
Published: November 18, 2016

DOI: 10.1021/jacs.6b09706
J. Am. Chem. Soc. 2016, 138, 15813−15816

time-dependent increase in fluorescence, suggesting the HOMO−LUMO gap is not limiting. Reactions of **1** and fumarate were complete within 60 min, and formation of the cycloaddition product was verified by LC-MS (Figure S1). Fumarate reacted with **1** at a slower rate than ethyl acrylate, consistent with its higher LUMO energy (Figure S1). However, both dipolarophiles reacted to form products that display a 100 nm bathochromic shift in emission relative to the hydrazonyl chloride starting material. This results in high signal-to-noise, and for fumarate we observed an 18-fold increase in fluorescence at 490 nm upon reaction of **1** (Figure 1b). These studies establish the reactivity of fumarate with nitrileimines.

The sensitivity of fumarate detection by a nitrileimine dipole is expected to be highly dependent on the brightness of the resultant pyrazoline fluorophore.[10] However, the chemical optimization of hydrazonyl chlorides for use in fluorogenic 1,3-dipolar cycloadditions has not been described. To develop an optimized probe for FH profiling, we synthesized a small panel of diaryl hydrazonyl chlorides in which the electronic properties of the auxochrome subunits were systematically varied (Scheme 1). We characterized the optical properties of

Scheme 1. Synthesis of Nitrileimine Precursors 1−5[a]



[a]Reagents and conditions: (i) CH_2Cl_2, 100 °C (microwave), 15 min, (ii) CH_2Cl_2, *N*-chlorosuccinimide (1 equiv), dimethyl sulfide (1 equiv), −78 °C, 3 h.

these molecules by assessing each hydrazonyl chloride's fluorescence following reaction with ethyl fumarate, a fumarate analogue that reacts to completion with nitrileimines due to its low energy LUMO (Figure S2). Deletion of the 4-methoxy group from the C3 aryl unit of **1** alters the electronic character of this ring from electron-donating to inductively electron-withdrawing (**2**), but did not greatly affect excitation or emission properties of the ethyl fumarate-cycloadduct (Table 1). However, removal of an additional 4-methoxy group from the N3-aryl ring (**3**) results in a fluorophore that absorbs strongly from 330 to 370 nm and whose emission intensity is considerably increased. This is consistent with previous studies of diaryl pyrazoline dyes,[11,12] which found that reducing push−pull polarization decreases fluorophore Stokes shift (via a red-shift of excitation) and increases quantum yield. To build on this observation, we explored two additional hydrazonyl chlorides, **4** and **5**. These nitrileimine precursors each contain

Table 1. Optical Properties of Hydrazonyl Chlorides upon Reaction with Fumarate Dipolarophiles

comp.	λ_{ex} (nm)[a]	λ_{em} (nm)[a]	ε (M^{-1} cm^{-1})[a]	Φf_{app}[a]	fumarate fluorogenicity[b]
1	320	490	21,500	0.04	18×
2	320	490	15,000	0.05	7×
3	350	490	9,700	0.29	63×
4	390	530	13,800	0.12	31×
5	390	590	15,300	0.01	4×

[a]Measured values refer to the optical properties of the pyrazoline cycloadduct formed after reaction of ethyl fumarate (10 mM) with hydrazonyl chloride (0.1 mM) in a 1:1 mixture of CH_3CN/aqueous sodium phosphate (100 mM, pH 7). Quantum yields (Φf_{app}) were determined by comparison to a diphenylanthracene standard. [b]Relative fluorescence of fumarate (10 mM) versus vehicle control containing solutions upon addition of hydrazonyl chloride (0.1 mM). Comp. = compound.

electron withdrawing *p*-carboxymethyl esters in their N3-aryl ring, but form pyrazolines with vastly different electronics due to the presence or absence of an electron-donating methoxy group in the C3 aryl unit.[13] As above, the less polarized pyrazoline precursor **4** exhibited a reduced Stokes shift but increased fluorescence intensity relative to **5** upon reaction with ethyl acrylate (Table 1).[13] Examination of overall trends highlighted two optimal nitrileimine precursors: **3**, which forms the brightest pyrazolines of any hydrazonyl chloride tested, and **4**, which is strongly fluorogenic and excitable at higher wavelengths that may be less damaging to biomolecules.

Having identified brighter agents for dipolarophile detection, we next benchmarked their ability to detect oncometabolite fumarate. In model reactions, solutions of fumarate were treated individually with hydrazonyl chlorides **1**−**5**, and assessed for fluorescence. Fumarate reacted more slowly with **1**−**5** than ethyl fumarate, but formed fluorophores with identical excitation and emission maxima (Figure S3). This suggests the distinguishing carboxylates of fumarate and ethyl fumarate do not impact the pyrazoline fluorophore π system.[11,12] Consistent with trends observed for ethyl fumarate, we found that hydrazonyl chlorides forming bright pyrazolines also exhibit large fluorescence increases upon fumarate treatment, up to 63- and 31-fold in the case of **3** and **4**, respectively (Table 1, right column). Detailed analysis of fumarate reactions found **3** and **4** form approximately equivalent yields of pyrazoline, with the faster cycloaddition kinetics of **4** offset by a corresponding increased rate of hydrolysis (Figure S4). This emphasizes pyrazoline fluorophore brightness as a primary determinant of the sensitivity of nitrileimine-mediated fumarate detection. Optimized compound **4** was able to detect fumarate in the presence of proteinaceous cell extracts at concentrations as low as 100 μM (>2-fold fluorescence increase; Figure S5). Importantly, neither denatured proteomes, nor high concentrations of acetate or glutathione, caused the fluorescence of **3** or **4** to increase (Figures S5 and S6). This indicates that fluorogenic reactions of hydrazonyl chlorides require a dipolarophile partner, and that abundant biological nucleophiles should not affect background.[14] These studies define **3** and **4** as sensitive reagents for the detection of metabolite dipolarophiles.

Next, we evaluated the ability of our optimized fluorogenic reaction to profile FH activity. Because FH is a reversible enzyme, two potential approaches to monitor its activity can be envisioned: (1) incubating FH with fumarate, and monitoring

DOI: 10.1021/jacs.6b09706
J. Am. Chem. Soc. 2016, 138, 15813−15816

fumarate consumption (loss of signal), or (2) incubating FH with malate, and observing fumarate accumulation (gain of signal). The latter approach struck us as preferable, because it allows direct correlation of FH activity with fluorescence. We first explored this strategy using purified FH. Enzyme activity was monitored using a two-step protocol, first incubating FH with malate, and then "developing" the newly formed fumarate via reaction with **4** in a solution of 1:1 CH_3CN/sodium phosphate prior to fluorescence analysis. The use of CH_3CN in the developing buffer was found to be critical to fumarate detection, as it increases hydrazonyl chloride solubility and slows competing hydrolysis.[15] Using this approach, we found **4** was able to detect FH activity, exhibiting enzyme- and time-dependent increases in fluorescence (Figure 2). These studies establish the feasibility of using bioorthogonal ligation chemistry to monitor FH activity.

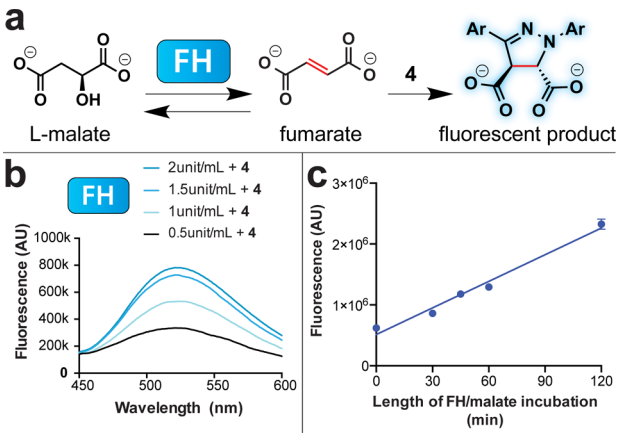


Figure 2. Fluorogenic profiling of FH activity. (a) Strategy for fluorogenic detection of FH activity. (b) Detecting recombinant FH-dependent conversion of malate to fumarate using **4** (λ_{ex} = 390 nm). (c) Detecting time-dependent conversion of malate to fumarate using **4**.

Finally, we sought to define the ability of our method to identify oncometabolic HLRCC cell lines on the basis of altered FH activity. Unfractionated extracts were prepared from a patient-derived HLRCC cell line harboring a homozygous *FH* mutation (UOK262EV), as well as an isogenic variant of this cell line engineered to stably express FH from a wild-type allele (UOK262WT).[16,17] Applying our optimized protocol, we found that adding malate/**4** to FH-deficient UOK262EV extracts resulted in negligible fluorescence, whereas applying an identical strategy to FH-wild-type UOK262WT proteomes caused a strong fluorescence increase (Figure 3a). Quantitative analysis found this signal reflects formation of ∼183 μM fumarate in the UOK262WT lysates (Figure S7). Analysis of additional kidney cell lines observed a diverse spectrum of FH activity (Figures 3a, S7). GC−MS analysis found that cells with low FH activity harbored high endogenous fumarate (Figures 3b, S7). This suggests the primary metabolic consequence of disrupted cellular FH is accumulation of fumarate in the TCA cycle, consistent with HLRCC's pathology. These studies define the nitrileimine-alkene cycloaddition as a novel approach to fluorescently profile endogenous cellular FH activity.

Bioorthogonal chemistry forms the critical underpinning for innumerable applications;[18−20] however, there are still few examples in which bioorthogonal reactions have been harnessed to detect endogenous biomolecules or enzyme

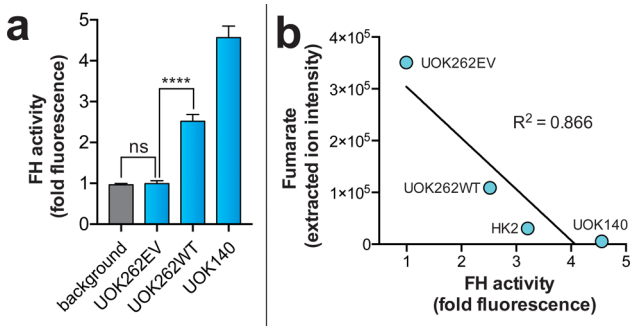


Figure 3. (a) Profiling FH activity in kidney cancer cell lines using **4** (λ_{ex} = 390 nm). Profiling data for additional kidney cancer cell lines is provided in the Supporting Information. (b) Correlation between FH activity measurements by **4** and GC−MS measurements of fumarate in kidney cell lines. Significance was analyzed by unpaired Student's *t* test (ns = $P > 0.05$, **** = $P < 0.0001$).

activities.[21−23] Here we co-opt a bioorthogonal reaction to detect the oncometabolite fumarate. This strategy unifies bioorthogonal chemistry with reaction-based sensing methods,[24] by identifying contexts in which biologically inert reactions can be harnessed to study endogenous biology. We define the tunable fluorescence and reactivity of hydrazonyl-chloride-derived nitrileimines, and show these agents can be used to report on *FH* mutation in cell models of the familial cancer syndrome HLRCC. A paradigm of hereditary cancer disorders is that they often highlight more widespread mechanisms of transformation.[25,26] From this perspective, we envision our fluorogenic probes may enable the rapid identification of alternative stimuli and cancer contexts that disrupt the tumor suppressive activity of FH. Importantly, this approach is not limited to FH and should, in theory, be applicable to study any enzyme that produces or consumes a dipolarophile detectable by our method. Examples of nitrileimine detectable metabolites include crotonate, itaconate, glutaconate, 4-hydroxynoneal, and prostaglandin A_1 (Figure S8).

Finally, we note some limitations of our method. First, our current probes are unable to directly detect differences in endogenous fumarate in FH mutant and wild-type cell lines, highlighting a need to improve sensitivity for future applications in imaging and diagnostics. Potential routes to address this include the use of diaryl tetrazoles, a nonlabile class of nitrileimine precursors,[27] as well as mitochondrial localization tags, which may facilitate reaction with concentrated subcellular dipolarophile pools.[28] Sensitivity will also likely benefit from analyzing FH-deficient tissue samples, which exhibit >10× increased accumulation of fumarate relative to the cell lines analyzed here.[3] Second, the broad scope of nitrileimine−metabolite reactivity (Figure S8) suggests future imaging methods based on this reaction may be limited to contexts such as HLRCC, where a single known metabolite contributes disproportionately to overall endogenous dipolarophile "load". Other disorders associated with metabolite dipolarophiles include hereditary tyrosinemia, glutaric aciduria, fluoroacetate poisoning, and enoyl-CoA hydratase deficiency.[25,29] The discovery of new dipolarophile-driven disease contexts will also benefit from integrating these probes with LC-MS metabolomic methods, where similar chemoselective reactions have been powerfully applied.[30,31] Overall, our studies define a novel intersection of bioorthogonal chemistry and metabolite

DOI: 10.1021/jacs.6b09706
J. Am. Chem. Soc. 2016, 138, 15813−15816

reactivity, and in so doing provide a chemical basis from which to pursue biological profiling, imaging, and diagnostic methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09706.

Figures, protocols, and synthetic characterization data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*jordan.meier@nih.gov

ORCID

Jordan L. Meier: 0000-0002-0537-7101

Author Contributions

§These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Martin Schnermann (NCI) and Dr. Rolf Swenson (Intramural Probe Development Center, NHLBI) for helpful discussions. This work was supported by the Intramural Research Program of the NIH, National Cancer Institute (ZIA BC011488-02).

■ REFERENCES

(1) Vander Heiden, M. G.; Cantley, L. C.; Thompson, C. B. *Science* **2009**, *324*, 1029−33.
(2) Meier, J. L. *ACS Chem. Biol.* **2013**, *8*, 2607−21.
(3) Pollard, P. J.; Briere, J. J.; Alam, N. A.; Barwell, J.; Barclay, E.; Wortham, N. C.; Hunt, T.; Mitchell, M.; Olpin, S.; Moat, S. J.; Hargreaves, I. P.; Heales, S. J.; Chung, Y. L.; Griffiths, J. R.; Dalgleish, A.; McGrath, J. A.; Gleeson, M. J.; Hodgson, S. V.; Poulsom, R.; Rustin, P.; Tomlinson, I. P. *Hum. Mol. Genet.* **2005**, *14*, 2231−2239.
(4) Isaacs, J. S.; Jung, Y. J.; Mole, D. R.; Lee, S.; Torres-Cabala, C.; Chung, Y. L.; Merino, M.; Trepel, J.; Zbar, B.; Toro, J.; Ratcliffe, P. J.; Linehan, W. M.; Neckers, L. *Cancer Cell* **2005**, *8*, 143−53.
(5) Xiao, M.; Yang, H.; Xu, W.; Ma, S.; Lin, H.; Zhu, H.; Liu, L.; Liu, Y.; Yang, C.; Xu, Y.; Zhao, S.; Ye, D.; Xiong, Y.; Guan, K. L. *Genes Dev.* **2012**, *26*, 1326−38.
(6) Song, W.; Wang, Y.; Qu, J.; Madden, M. M.; Lin, Q. *Angew. Chem., Int. Ed.* **2008**, *47*, 2832−5.
(7) Lee, Y. J.; Wu, B.; Raymond, J. E.; Zeng, Y.; Fang, X.; Wooley, K. L.; Liu, W. R. *ACS Chem. Biol.* **2013**, *8*, 1664−70.
(8) An, P.; Yu, Z.; Lin, Q. *Chem. Commun. (Cambridge, U. K.)* **2013**, *49*, 9920−2.
(9) Wang, Y.; Song, W.; Hu, W. J.; Lin, Q. *Angew. Chem., Int. Ed.* **2009**, *48*, 5330−3.
(10) Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2014**, *9*, 855−66.
(11) Lin, J.; Rivett, D. E.; Wilshire, J. F. K. *Aust. J. Chem.* **1977**, *30*, 629−637.
(12) Wagner, A.; Schellhammer, C.-W.; Petersen, S. *Angew. Chem., Int. Ed. Engl.* **1966**, *5*, 699−704.
(13) Song, W.; Wang, Y.; Qu, J.; Lin, Q. *J. Am. Chem. Soc.* **2008**, *130*, 9654−5.
(14) Li, Z.; Qian, L.; Li, L.; Bernhammer, J. C.; Huynh, H. V.; Lee, J. S.; Yao, S. Q. *Angew. Chem., Int. Ed.* **2016**, *55*, 2002−2006.
(15) Wang, X. S.; Lee, Y. J.; Liu, W. S. R. *Chem. Commun.* **2014**, *50*, 3176−3179.
(16) Yang, Y.; Valera, V. A.; Padilla-Nash, H. M.; Sourbier, C.; Vocke, C. D.; Vira, M. A.; Abu-Asab, M. S.; Bratslavsky, G.; Tsokos, M.; Merino, M. J.; Pinto, P. A.; Srinivasan, R.; Ried, T.; Neckers, L.; Linehan, W. M. *Cancer Genet. Cytogenet.* **2010**, *196*, 45−55.
(17) Sourbier, C.; Ricketts, C. J.; Matsumoto, S.; Crooks, D. R.; Liao, P. J.; Mannes, P. Z.; Yang, Y.; Wei, M. H.; Srivastava, G.; Ghosh, S.; Chen, V.; Vocke, C. D.; Merino, M.; Srinivasan, R.; Krishna, M. C.; Mitchell, J. B.; Pendergast, A. M.; Rouault, T. A.; Neckers, L.; Linehan, W. M. *Cancer Cell* **2014**, *26*, 840−50.
(18) Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. *ACS Chem. Biol.* **2014**, *9*, 592−605.
(19) Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13−21.
(20) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686−7.
(21) Wu, H.; Cisneros, B. T.; Cole, C. M.; Devaraj, N. K. *J. Am. Chem. Soc.* **2014**, *136*, 17942−5.
(22) Khidekel, N.; Arndt, S.; Lamarre-Vincent, N.; Lippert, A.; Poulin-Kerstien, K. G.; Ramakrishnan, B.; Qasba, P. K.; Hsieh-Wilson, L. C. *J. Am. Chem. Soc.* **2003**, *125*, 16162−3.
(23) Robinson, P. V.; Tsai, C. T.; de Groot, A. E.; McKechnie, J. L.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2016**, *138*, 10722−5.
(24) Chan, J.; Dodani, S. C.; Chang, C. J. *Nat. Chem.* **2012**, *4*, 973−84.
(25) Erez, A.; DeBerardinis, R. J. *Nat. Rev. Cancer* **2015**, *15*, 440−8.
(26) Herman, J. G.; Latif, F.; Weng, Y.; Lerman, M. I.; Zbar, B.; Liu, S.; Samid, D.; Duan, D. S.; Gnarra, J. R.; Linehan, W. M.; et al. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 9700−4.
(27) Lim, R. K.; Lin, Q. *Acc. Chem. Res.* **2011**, *44*, 828−39.
(28) Dickinson, B. C.; Chang, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 9638−9.
(29) Erez, A.; Shchelochkov, O. A.; Plon, S. E.; Scaglia, F.; Lee, B. *Am. J. Hum. Genet.* **2011**, *88*, 402−21.
(30) Carlson, E. E.; Cravatt, B. F. *Nat. Methods* **2007**, *4*, 429−35.
(31) Chang, J. W.; Lee, G.; Coukos, J. S.; Moellering, R. E. *Anal. Chem.* **2016**, *88*, 6658−61.

DOI: 10.1021/jacs.6b09706
J. Am. Chem. Soc. 2016, 138, 15813−15816