

## Installation of Minimal Tetrazines through Silver-Mediated Liebeskind–Srogl Coupling with Arylboronic Acids

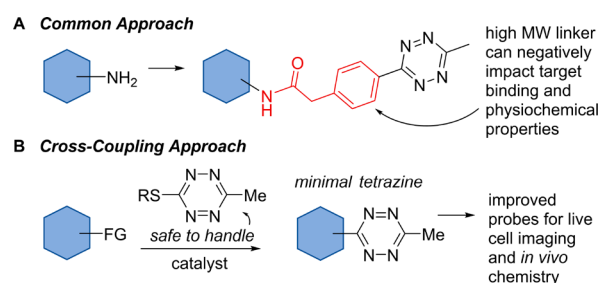
William D. Lambert,<sup>†</sup> Yinzhi Fang,<sup>†</sup> Subham Mahapatra,<sup>‡</sup> Zhen Huang,<sup>§</sup> Christopher W. am Ende,<sup>\*,‡,§</sup> and Joseph M. Fox<sup>\*,†,§</sup><sup>†</sup>Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States<sup>‡</sup>Pfizer Worldwide Research and Development, Eastern Point Road, Groton, Connecticut 06340, United States<sup>§</sup>Pfizer Worldwide Research and Development, 1 Portland Street, Cambridge, Massachusetts 02139, United States

## Supporting Information

**ABSTRACT:** Described is a general method for the installation of a minimal 6-methyltetrazin-3-yl group via the first example of a Ag-mediated Liebeskind–Srogl cross-coupling. The attachment of bioorthogonal tetrazines on complex molecules typically relies on linkers that can negatively impact the physiochemical properties of conjugates. Cross-coupling with arylboronic acids and a new reagent, 3-((*p*-biphenyl-4-ylmethyl)thio)-6-methyltetrazine (b-Tz), proceeds under mild, PdCl<sub>2</sub>(dppf)-catalyzed conditions to introduce minimal, linker-free tetrazine functionality. Safety considerations guided our design of b-Tz which can be prepared on decagram scale without handling hydrazine and without forming volatile, high-nitrogen tetrazine byproducts. Replacing conventional Cu(I) salts used in Liebeskind–Srogl cross-coupling with a Ag<sub>2</sub>O mediator resulted in higher yields across a broad library of aryl and heteroaryl boronic acids and provides improved access to a fluorogenic tetrazine-BODIPY conjugate. A covalent probe for MAGL incorporating 6-methyltetrazinyl functionality was synthesized in high yield and labeled endogenous MAGL in live cells. This new Ag-mediated cross-coupling method using b-Tz is anticipated to find additional applications for directly introducing the tetrazine subunit to complex substrates.

The bioorthogonal reactions of tetrazines have emerged as important tools for chemical biology over the past decade.<sup>1–6</sup> Cycloadditions involving a range of dienophiles including *trans*-cyclooctenes,<sup>1,7–10</sup> cyclopropenes<sup>11,12</sup> and norbornenes<sup>13</sup> have been developed as tools for a variety of applications including cellular labeling,<sup>14–17</sup> *in vivo* imaging,<sup>18–20</sup> unnatural amino acid mutagenesis,<sup>3,21,22</sup> targeted drug delivery,<sup>23–25</sup> proteomics,<sup>26</sup> as well as in the fabrication and patterning of biomaterials.<sup>27</sup> Tetrazines themselves have also found applications in explosives technology,<sup>28</sup> in metal–organic frameworks,<sup>29</sup> and in natural product synthesis.<sup>30</sup>

Conjugates of tetrazines are frequently prepared by amide bond forming reactions as represented in Figure 1A. A major limitation of this approach is that large and hydrophobic linkers can negatively impact the physiochemical properties of an attached ligand.<sup>6,17</sup> Complementary new methods for the



**Figure 1.** (A) The most common approach to tetrazine conjugation uses linkers to attach molecules of interest. (B) Cross-coupling approach described here.

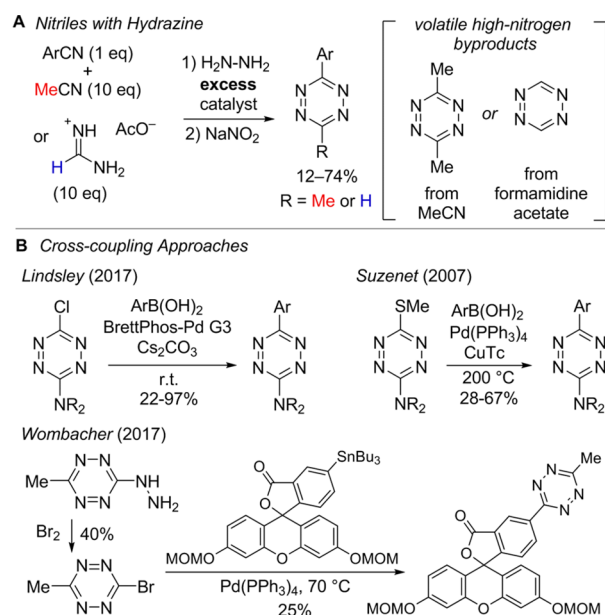
introduction of minimal tetrazines to small molecules may further advance their potential as bioorthogonal probes and chemical reporters. The replacement of bulky derivatives with smaller tetrazines has resulted in fluorophores with improved fluorogenic and cellular wash-out properties,<sup>31–33</sup> better substrates for enzyme-catalyzed protein modification,<sup>17,34</sup> and probes for <sup>18</sup>F-PET imaging.<sup>35</sup> However, there are currently few methods for the direct attachment of “minimal” tetrazine groups to target molecules.<sup>36</sup> Additionally, many approaches to tetrazine synthesis produce high-nitrogen byproducts and involve harsh reaction conditions that can limit scalability and scope. Herein, we describe the decagram synthesis and thermal stability of 3-((*p*-biphenyl-4-ylmethyl)thio)-6-methyltetrazine, (b-Tz, **1a**) and a method to directly introduce the 6-methyltetrazin-3-yl group to arylboronic acids through the first example of a Ag-mediated Liebeskind–Srogl reaction (Figure 1B).

Classical tetrazine synthesis involves the condensation of Pinner salts or nitriles with excess hydrazine followed by oxidation.<sup>37,38</sup> Catalytic nitrile condensation with neat anhydrous hydrazine, most notably with Zn(OTf)<sub>2</sub> and Ni(OTf)<sub>2</sub>, has expanded access to unsymmetrical tetrazines.<sup>39</sup> Further, thiol catalysis has been shown to promote tetrazine synthesis from nitriles using hydrazine-hydrate.<sup>40</sup> The most practiced procedures utilize excess acetonitrile or formamide acetate and produce volatile tetrazine byproducts with high-nitrogen content (Figure 2A). Recently, a sulfur-catalyzed

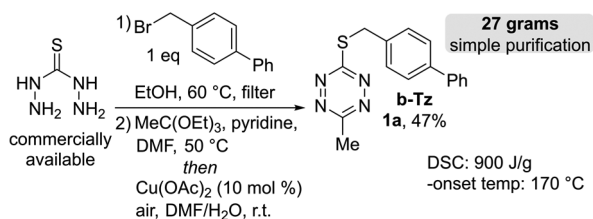
Received: August 11, 2019

Published: October 11, 2019





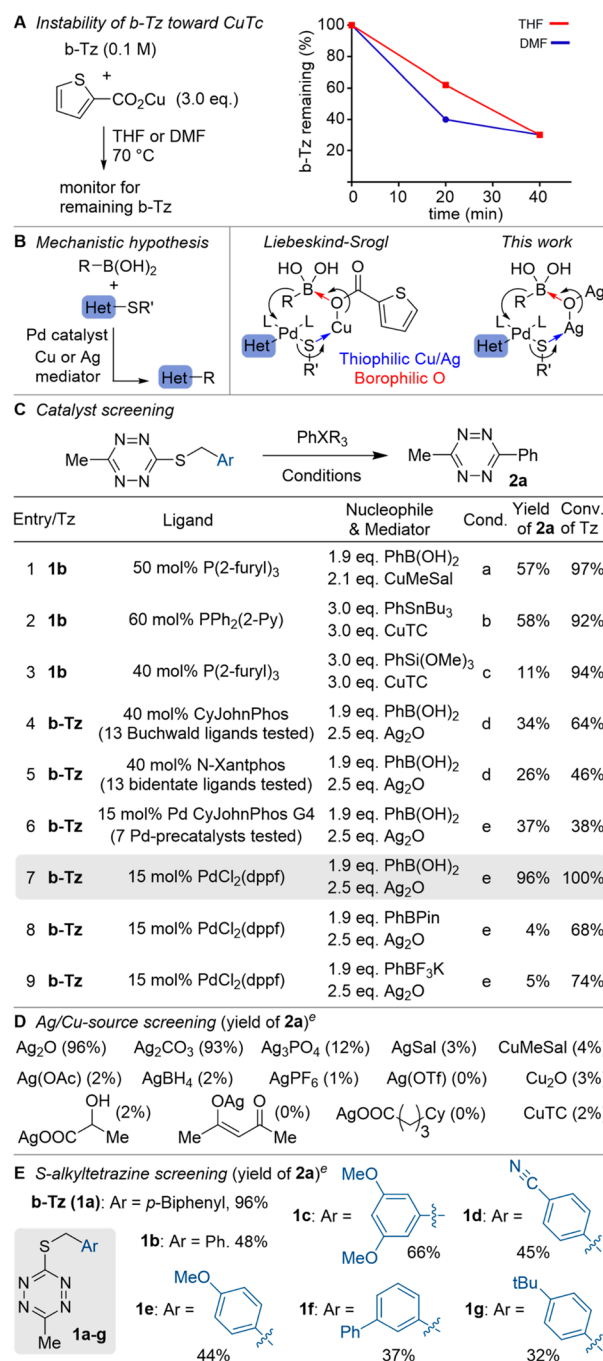
**Figure 2.** (A) Tetrazine synthesis based on condensation of nitriles or Pinner reagents with hydrazine. (B) Cross-couplings of tetrazine electrophiles with arylboronic acids have been limited to *N*-substituted tetrazines, which are deactivated for bioorthogonal chemistry applications. Stille coupling has been used to couple 3-bromo-6-methyltetrazine to fluorophores.



**Figure 3.** Decagram synthesis and thermal stability of b-Tz (1a).

reaction of nitriles with hydrazine hydrate and dichloromethane has been described for 3-aryltetrazine synthesis.<sup>41</sup> A safety consideration for all of these procedures is the direct addition of an oxidant to a reaction mixture containing hydrazine. While these methods for preparing tetrazines have been transformative to the field of bioorthogonal chemistry, there is a continuing need for safer alternatives with complementary functional group compatibility.

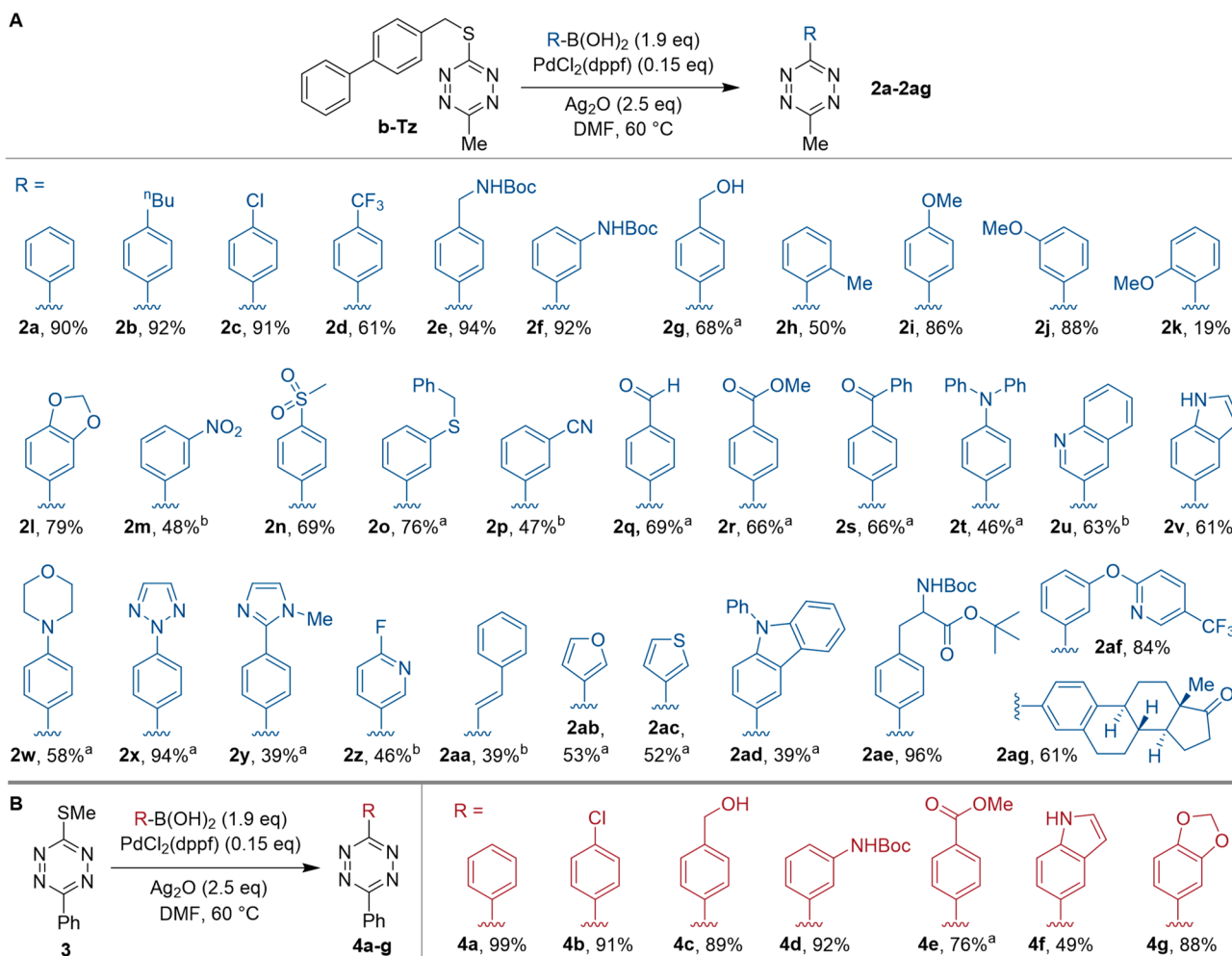
Tetrazines have been used in a limited number of metal-catalyzed CH activations<sup>42,43</sup> and cross-couplings<sup>32,33,44–51</sup> and in Heck reactions with *in situ*-generated 3-vinyl-6-methyltetrazine.<sup>32</sup> Recently, 3-amino-6-chlorotetrazines have been cross-coupled under Suzuki conditions (Figure 2B).<sup>45</sup> Liebeskind–Srogl cross-couplings have also been reported with 3-amino-6-thiomethyltetrazines at 200 °C (Figure 2B).<sup>46</sup> The 3-aminotetrazine products of these methods are valuable in medicinal chemistry, but their utility in bioorthogonal chemistry is attenuated by the deactivating amino substituent.<sup>21,32</sup> The tetrazines most useful to bioorthogonal chemistry are also sensitive to basic conditions, making them incompatible with many conditions commonly associated with cross-coupling chemistry. Currently, there is a single method of cross-coupling to introduce a 3-methyltetrazine group via Stille coupling with 3-bromo-6-methyltetrazine,



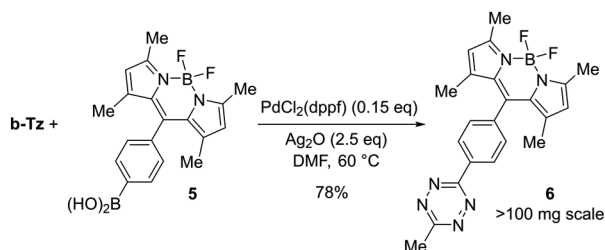
**Figure 4.** (A) Rapid decomposition of b-Tz in CuTC. (B) Proposed Liebeskind–Srogl transmetalation mechanism. (C) Optimized Pd-catalyzed cross-coupling of tetrazines b-Tz and 1b with various nucleophiles (yields determined by GC w/dodecane as a standard). Conditions: (a) Pd<sub>2</sub>dba<sub>3</sub> (12.5 mol %), Cs<sub>2</sub>CO<sub>3</sub> (3.0 equiv), dioxane, 70 °C, 90 min. (b) [Pd(allyl)Cl]<sub>2</sub> (10 mol %), THF, 70 °C, 2 h. (c) Pd(OAc)<sub>2</sub> (10 mol %), TBAF (1.0 equiv), dioxane, 70 °C, 2.5 h. (d) Pd<sub>2</sub>dba<sub>3</sub> (15 mol %), DMF, 60 °C, 20 h. (e) DMF, 60 °C, 20 h. (D) Screening of silver(I) and copper(I) additives for condition e. (E) Screening of tetrazines 1a–g under conditions e.

which is prepared from 3-hydrazino-6-methyltetrazine (Figure 2B).<sup>33</sup>

We considered that 3-thioalkyl-6-methyltetrazines might serve as useful reagents for the preparation of 3-aryl-6-methyltetrazines, which are attractive bioorthogonal reagents due to their balance of rapid kinetics toward dienophiles and



**Figure 5.** Reaction scope of **b-Tz** (A) and **3** (B). Typical conditions: thioalkyl tetrazine **b-Tz** or **3** (1.0 equiv),  $\text{RB(OH)}_2$  (1.9 equiv),  $\text{PdCl}_2(\text{dppf})$  (15 mol%),  $\text{Ag}_2\text{O}$  (2.5 equiv), DMF (0.1M), 60 °C, 19–21 h, average isolated yields of duplicate synthesis ( $\pm 5\%$ ). <sup>a</sup>3.0 equiv of  $\text{RB(OH)}_2$ . <sup>b</sup>3.0 equiv of  $\text{RB(OH)}_2$  did not significantly improve yield ( $< 5\%$ ), thus 1.9 equiv of  $\text{RB(OH)}_2$  was used.



**Figure 6.** Synthesis of 3-BODIPY-6-methyltetrazine **6**.

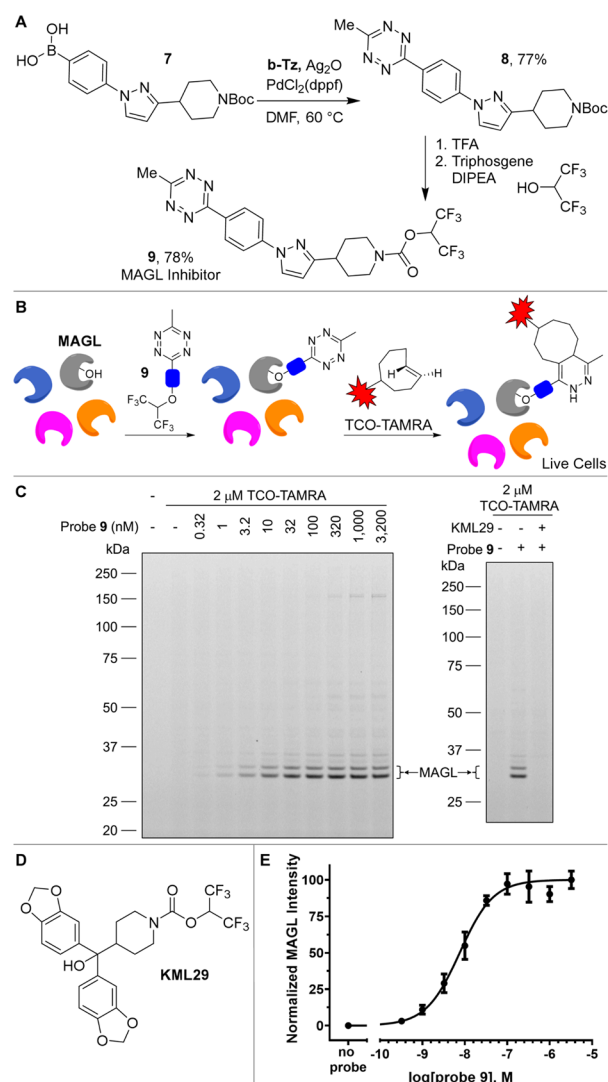
high stability in the cellular environment.<sup>17,21,52</sup> By modifying a method for the synthesis of 3-thiomethyl-6-methyltetrazine,<sup>53</sup> we prepared compounds **1a–g** with the rationale that a sacrificial *S*-benzylic substituent could serve to tune cross-coupling efficiency and improve the safety profile of the tetrazine. As shown in Figure 3, the 4-phenylbenzyl derivative **b-Tz** (**1a**) was prepared on large scale via alkylation of commercially available thiocarbohydrazide<sup>54</sup> with 4-bromomethylbiphenyl followed by one-pot condensation with triethylorthoacetate and a novel  $\text{Cu(OAc)}_2$ -catalyzed air-oxidation of the dihydrotetrazine intermediate. **b-Tz** was isolated on 27 g scale with a 47% overall yield after simple silica plug filtration and is a bench-stable crystalline solid (mp 141 °C). The differential scanning calorimetry (DSC) profile of **b-Tz** has an

onset temperature of 170 °C and a transition enthalpy of 900 J/g and is not flagged as potentially shock sensitive or explosive by a modified Yoshida correlation (Figure S-11).<sup>55</sup>

After extensive screening (Figures S-3–S-8), we found copper(I)-mediated Liebeskind–Srogl conditions<sup>56,57</sup> could promote cross-coupling of benzylic thioether tetrazines with  $\text{PhB(OH)}_2$ ,  $\text{PhSnBu}_3$ , and  $\text{PhSi(OMe)}_3$  (Figure 4C, entries 1–3). Under Cu-mediated conditions tetrazine **1b** was the best substrate; however, the generality under these conditions was modest. The rapid consumption of tetrazine starting materials during the reaction led us to test if Cu(I) was causing decomposition of the reagent. Indeed, heating **b-Tz** with Cu(I)-thiophene carboxylate ( $\text{CuTC}$ ) at 70 °C resulted in rapid decomposition and produced 4-phenylbenzaldehyde as the only identifiable side product (Figure 4A).

Copper has been proposed to promote the Liebeskind–Srogl reaction by facilitating transmetalation as shown in Figure 4B.<sup>57,58</sup> We hypothesized that silver(I) salts might be similarly capable as promoters, whereby transmetalation would be promoted in a dual role by the thiophilic capture of benzylic thiolate by silver and the borophilic capture by oxygen. Ag(I) additives have been shown to promote Rh-catalyzed coupling of arylboronic acids with arylmethylsulfides bearing *ortho*-directing groups,<sup>59,60</sup> and the Cu-catalyzed coupling of arylboronic acids with aromatic thioesters.<sup>61</sup> To our knowl-





**Figure 7.** (A) Synthesis of MAGL reactive probe 9. (B) Live cells were treated with probe 9 for 1 h, followed by 2  $\mu$ M TCO-TAMRA for 30 min, cell lysis, and analysis by in-gel fluorescence. (C) In-gel fluorescence signals for a dose response of probe 9. The labeling of probe 9 (32 nM, 1 h) was competed by pretreatment with MAGL inhibitor KML29 (300 nM, 1 h). (D) KML29 also incorporates a HFIP carbamate warhead. (E) Dose response fitting of the fluorescence signals of MAGL normalized by the total protein amount indicated by Coomassie staining. Data are reported as mean  $\pm$  SEM ( $n = 2$ ). See Figure S-21 for Coomassie staining.

edge, a Ag-mediated variation of the Liebeskind–Srogl reaction has not been reported. After extensive optimization (Figure 4C, entries 4–7, and Figures S-1 and S-2), PdCl<sub>2</sub>(dppf) (15 mol%) was found to be especially effective for cross-coupling of 3-thioalkyl-6-methyltetrazines with arylboronic acids in polar, aprotic solvents (DMF, DMSO) at 60 °C. A screening of silver(I) additives revealed Ag<sub>2</sub>O as the most general promotor, although Ag<sub>2</sub>CO<sub>3</sub> was also effective (Figure 4D). Substitution of Ag<sub>2</sub>O by Cu<sub>2</sub>O gave only trace product formation. Arylboronic acids are particularly effective nucleophiles, whereas PhBF<sub>3</sub>K and PhBPIn were both less effective under identical reaction conditions (Figure 4C, entries 8 and 9). Further, a series of 3-arylmethyl-6-methyltetrazines 1a–g were evaluated as coupling partners (Figure 4E). Of these, the 4-phenylbenzyl derivative b-Tz (1a)

was identified as the substrate with both the best cross-coupling yield as well as most favorable thermal stability. We also note that the cost of Ag<sub>2</sub>O (currently < \$3/g) is similar to the common promotor CuTC, and is minor in the context of bioorthogonal chemistry reagents which are typically required only in small amounts.

The scope of the Ag-mediated, Pd-catalyzed coupling of b-Tz with arylboronic acids is summarized in Figure 5A. Successful reactions were observed for arylboronic acids containing chloro, fluoro, secondary and tertiary amino, alcohol, ether, nitro, sulfonyl, thioether, nitrile, aldehyde, ester, ketone, carbamate, and styryl groups. Heterocyclic functionality tolerated on the boronic acid component included quinoline, indole, pyridine, triazole, *N*-methylimidazole, furan and thiophene groups. The protected amino acid 2ae coupled with b-Tz in 96% yield. Estrone-tetrazine 2ag was also synthesized in 61% yield. In general, couplings were carried out using 1.9 equiv of boronic acid, but 3.0 equiv was utilized in reactions where homocoupling of the boronic acid was pronounced. *Ortho*-substituted heteroatoms had a deleterious impact with a relatively low yield observed for *ortho*-methoxy tetrazine 2k and only trace product with *N*-Boc-2-aminophenylboronic acid and 2-hydroxyphenylboronic acid. While protected thiol and amine functionality was well tolerated (Figure 5), additives with free thiol or primary alkyl amine groups were not (Figure S-19). Also unsuccessful were 2-pyridyl- and 4-pyridylboronic acids which are regarded as problematic across other cross-coupling reactions.<sup>62</sup>

This cross-coupling method is not limited to *S*-benzyl thioethers or methyl-substituted tetrazines. 3-(Methylthio)-6-phenyl-tetrazine (3) was prepared from triethyl orthobenzoate and evaluated as a reagent in the synthesis of diaryltetrazines (Figure 5B). Successful reactions were observed for arylboronic acids bearing chloro-, alcohol, carbamate, ester, indole and ether groups with yields comparable to b-Tz. Included is an improved synthesis of 3-(4-hydroxymethylphenyl)-6-phenyl-tetrazine (4c), which is used to create cell-contact guiding microfibrillar materials for tissue-culture applications.<sup>27</sup>

We sought to demonstrate the application of b-Tz for the construction of fluorophore-tetrazine conjugates—compounds that have utility in live cell imaging.<sup>15</sup> BODIPY-dye 6 with a directly attached tetrazine has been developed as “superbright” bioorthogonal probe for fluorogenic labeling in live cells.<sup>31</sup> The condensation of nitriles with hydrazine produces 6 in 8% yield.<sup>31</sup> As shown in Figure 6, compound 6 can be accessed in 78% yield through the Ag-mediated cross-coupling of boronic acid 5 with b-Tz.

To demonstrate the utility of b-Tz in synthesizing chemical probes for studying endogenous levels of proteins in a native biological system, we constructed a tetrazine probe for monoacylglycerol lipase (MAGL). MAGL is a serine hydrolase in the endocannabinoid signaling pathway, and has attracted increasing interest as a target for neurological and metabolic disorders.<sup>63</sup> We designed a MAGL probe (9) by appending a 6-methyltetrazine moiety to a pyrazolypiperidine scaffold with an electrophilic hexafluoroisopropyl (HFIP) carbamate warhead for covalently labeling the active site serine (Figure 7A).<sup>64</sup> Synthesis was accomplished by cross-coupling of b-Tz with boronic acid 7 resulting in a 77% yield of 8. The reactive HFIP carbamate was installed by Boc deprotection followed by *in situ* addition to a triphosgene and hexafluoroisopropanol mixture, giving the MAGL reactive probe 9 in 78% yield. The reaction rate of 9 toward *trans*-cyclooctene is similar to that of 3-

methyl-6-(4-aminomethyl)tetrazine ( $k_{\text{rel}} = 1.1$ , Figure S-20).<sup>65</sup> Probe 9 inhibited MAGL activity with 31 nM  $\text{IC}_{50}$  in an *in vitro* assay.<sup>66</sup>

To test the labeling of endogenous MAGL in live cells, human brain vascular pericytes were treated with probe 9 for 1 h, followed by labeling with 2  $\mu\text{M}$  of TCO-TAMRA for 30 min in live cells (Figure 7B). After cell lysis, MAGL labeling was assessed with a gel-based activity-based protein profiling (ABPP) analysis (Figure 7C–E).<sup>67</sup> Strong fluorescence signals were observed for MAGL with minimal nonspecific labeling from TCO-TAMRA. The labeling by probe 9 was dose responsive with a cellular  $\text{IC}_{50}$  of 8 nM, and was competed by a MAGL inhibitor, KML29.<sup>64</sup> The HFIP warhead also labeled an additional protein at  $\sim 35$  kDa, which is consistent with reactivity with a known off-target  $\alpha/\beta$ -hydrolase domain 6 (ABHD6).<sup>64,67</sup>

In summary, a method has been described for installing a minimal 6-methyltetrazinyl-3-yl group through the first Ag-mediated Liebeskind–Srogl cross-coupling. A combination of  $\text{PdCl}_2(\text{dppf})$  catalyst and  $\text{Ag}_2\text{O}$  mediator was found to be uniquely effective for coupling 3-thioalkyl-6-methyltetrazines with arylboronic acids. Safety testing guided our design of the reactive substrate b-Tz (1a), which can be synthesized from commercially available materials on decagram scale in 47% overall yield. Cross-coupling of b-Tz with boronic acids proceeds under mild conditions with broad functional group tolerance. Alternatively, 3-(methylthio)-6-phenyl-tetrazine (3) undergoes cross-coupling with arylboronic acids to give 3,6-diaryltetrazines. Application to the synthesis of chemical biology tools was demonstrated. A BODIPY-tetrazine conjugate was synthesized in 78% yield—substantially higher than what is possible using traditional hydrazine-based synthesis. Finally, a tetrazine-functionalized probe for MAGL was synthesized in high yield and was shown to covalently label endogenous MAGL with good selectivity in live cells. We anticipate that this method for introducing minimal tetrazines to chemical probes will serve as an important tool for studying protein targets at endogenous levels in their native environment.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b08677.

Optimization tables, DSC data, experimental procedures, kinetics, inhibition assays, and  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

\*jmfox@udel.edu,

\*christopher.amende@pfizer.com

### ORCID

Christopher W. am Ende: 0000-0001-8832-9641

Joseph M. Fox: 0000-0002-8258-1640

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We would like to thank Jeffery Sperry for helpful discussions, Remzi Duzguner for DSC analysis, and Lucy Stevens for the *in vitro*

potency data on probe 9. This work was supported by NIH GM132460, NSF 1809612, and Pfizer. Instrumentation was supported by NIH awards P20GM104316, P30GM110758, S10RR026962, and S10OD016267 and NSF awards CHE-0840401, CHE-1229234, and CHE-1048367. We thank the State of Delaware CAT program for a grant that supported commercialization of b-Tz (catalog # AJ-0100, Affinity Research Chemicals, <http://www.affinitychem.com/>).

## ■ REFERENCES

- (1) Selvaraj, R.; Fox, J. M. *trans*-Cyclooctene—a stable, voracious dienophile for bioorthogonal labeling. *Curr. Opin. Chem. Biol.* **2013**, *17*, 753–760.
- (2) Patterson, D. M.; Nazarova, L. A.; Prescher, J. A. Finding the right (bioorthogonal) chemistry. *ACS Chem. Biol.* **2014**, *9*, 592–605.
- (3) Lang, K.; Chin, J. W. Bioorthogonal reactions for labeling proteins. *ACS Chem. Biol.* **2014**, *9*, 16–20.
- (4) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse electron demand Diels–Alder reactions in chemical biology. *Chem. Soc. Rev.* **2017**, *46*, 4895–4950.
- (5) Wu, H.; Devaraj, N. K. Advances in Tetrazine Bioorthogonal Chemistry Driven by the Synthesis of Novel Tetrazines and Dienophiles. *Acc. Chem. Res.* **2018**, *51*, 1249–1259.
- (6) Cañeque, T.; Müller, S.; Rodriguez, R. Visualizing biologically active small molecules in cells using click chemistry. *Nat. Rev. Chem.* **2018**, *2*, 202–215.
- (7) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine ligation: fast bioconjugation based on inverse-electron-demand Diels–Alder reactivity. *J. Am. Chem. Soc.* **2008**, *130*, 13518–13519.
- (8) Taylor, M. T.; Blackman, M. L.; Dmitrenko, O.; Fox, J. M. Design and synthesis of highly reactive dienophiles for the tetrazine–*trans*-cyclooctene ligation. *J. Am. Chem. Soc.* **2011**, *133*, 9646–9649.
- (9) Darko, A.; Wallace, S.; Dmitrenko, O.; Machovina, M. M.; Mehl, R. A.; Chin, J. W.; Fox, J. M. Conformationally Strained *trans*-Cyclooctene with Improved Stability and Excellent Reactivity in Tetrazine Ligation. *Chem. Sci.* **2014**, *5*, 3770–3776.
- (10) Lambert, W. D.; Scinto, S. L.; Dmitrenko, O.; Boyd, S. J.; Magboo, R.; Mehl, R. A.; Chin, J. W.; Fox, J. M.; Wallace, S. Computationally guided discovery of a reactive, hydrophilic *trans*-5-oxocene dienophile for bioorthogonal labeling. *Org. Biomol. Chem.* **2017**, *15*, 6640–6644.
- (11) Patterson, D. M.; Nazarova, L. A.; Xie, B.; Kamber, D. N.; Prescher, J. A. Functionalized cyclopropanes as bioorthogonal chemical reporters. *J. Am. Chem. Soc.* **2012**, *134*, 18638–18643.
- (12) Yang, J.; Seckute, J.; Cole, C. M.; Devaraj, N. K. Live-cell imaging of cyclopropane tags with fluorogenic tetrazine cycloadditions. *Angew. Chem., Int. Ed.* **2012**, *51*, 7476–7479.
- (13) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Tetrazine-based cycloadditions: application to pretargeted live cell imaging. *Bioconjugate Chem.* **2008**, *19*, 2297–2299.
- (14) Devaraj, N. K.; Upadhyay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. Fast and sensitive pretargeted labeling of cancer cells through a tetrazine/*trans*-cyclooctene cycloaddition. *Angew. Chem., Int. Ed.* **2009**, *48*, 7013–7016.
- (15) Devaraj, N. K.; Hilderbrand, S.; Upadhyay, R.; Mazitschek, R.; Weissleder, R. Bioorthogonal turn-on probes for imaging small molecules inside living cells. *Angew. Chem., Int. Ed.* **2010**, *49*, 2869–2872.
- (16) Murrey, H. E.; Judkins, J. C.; am Ende, C. W.; Ballard, T. E.; Fang, Y.; Riccardi, K.; Di, L.; Guilmette, E. R.; Schwartz, J. W.; Fox, J. M.; Johnson, D. S. Systematic Evaluation of Bioorthogonal Reactions in Live Cells with Clickable HaloTag Ligands: Implications for Intracellular Imaging. *J. Am. Chem. Soc.* **2015**, *137*, 11461–11475.
- (17) Baalman, M.; Ziegler, M. J.; Werther, P.; Wilhelm, J.; Wombacher, R. Enzymatic and Site-Specific Ligation of Minimal-Size Tetrazines and Triazines to Proteins for Bioconjugation and Live-Cell Imaging. *Bioconjugate Chem.* **2019**, *30*, 1405–1414.

- (18) Rossin, R.; Verkerk, P. R.; van den Bosch, S. M.; Vuldres, R. C.; Verel, I.; Lub, J.; Robillard, M. S. In vivo chemistry for pretargeted tumor imaging in live mice. *Angew. Chem., Int. Ed.* **2010**, *49*, 3375–3378.
- (19) Zeglis, B. M.; Sevak, K. K.; Reiner, T.; Mohindra, P.; Carlin, S. D.; Zanzonico, P.; Weissleder, R.; Lewis, J. S. A pretargeted PET imaging strategy based on bioorthogonal Diels-Alder click chemistry. *J. Nucl. Med.* **2013**, *54*, 1389–1396.
- (20) Wang, M.; Vannam, R.; Lambert, W. D.; Xie, Y.; Wang, H.; Giglio, B.; Ma, X.; Wu, Z.; Fox, J.; Li, Z. Hydrophilic (18)F-labeled *trans*-5-oxocane (oxoTCO) for efficient construction of PET agents with improved tumor-to-background ratios in neurotensin receptor (NTR) imaging. *Chem. Commun.* **2019**, *55*, 2485–2488.
- (21) Blizzard, R. J.; Backus, D. R.; Brown, W.; Bazewicz, C. G.; Li, Y.; Mehl, R. A. Ideal Bioorthogonal Reactions Using A Site-Specifically Encoded Tetrazine Amino Acid. *J. Am. Chem. Soc.* **2015**, *137*, 10044–10047.
- (22) Hoffmann, J. E.; Plass, T.; Nikic, I.; Aramburu, I. V.; Koehler, C.; Gillandt, H.; Lemke, E. A.; Schultz, C. Highly Stable *trans*-Cyclooctene Amino Acids for Live-Cell Labeling. *Chem. - Eur. J.* **2015**, *21*, 12266–12270.
- (23) Li, J.; Chen, P. R. Development and application of bond cleavage reactions in bioorthogonal chemistry. *Nat. Chem. Biol.* **2016**, *12*, 129–137.
- (24) Versteegen, R. M.; Rossin, R.; ten Hoeve, W.; Janssen, H. M.; Robillard, M. S. Click to release: instantaneous doxorubicin elimination upon tetrazine ligation. *Angew. Chem., Int. Ed.* **2013**, *52*, 14112–14116.
- (25) Mejia Oneto, J. M.; Khan, I.; Seebald, L.; Royzen, M. In Vivo Bioorthogonal Chemistry Enables Local Hydrogel and Systemic Pro-Drug To Treat Soft Tissue Sarcoma. *ACS Cent. Sci.* **2016**, *2*, 476–482.
- (26) Kang, K.; Park, J.; Kim, E. Tetrazine ligation for chemical proteomics. *Proteome Sci.* **2016**, *15*, 1–13.
- (27) Liu, S.; Moore, A. C.; Zerdoum, A. B.; Zhang, H.; Scinto, S. L.; Zhang, H.; Gong, L.; Burris, D. L.; Rajasekaran, A. K.; Fox, J. M.; Jia, X. Cellular interactions with hydrogel microfibers synthesized via interfacial tetrazine ligation. *Biomaterials* **2018**, *180*, 24–35.
- (28) DeLuca, L. T.; Shimada, T.; Sinditskii, V. P.; Calabro, M. *Chemical Rocket Propulsion: A Comprehensive Survey of Energetic Materials*; Springer International Publishing, 2017.
- (29) Xue, M.; Ma, S.; Jin, Z.; Schaffino, R. M.; Zhu, G. S.; Lobkovsky, E. B.; Qiu, S. L.; Chen, B. Robust metal-organic framework enforced by triple-framework interpenetration exhibiting high H<sub>2</sub> storage density. *Inorg. Chem.* **2008**, *47*, 6825–6828.
- (30) Hamasaki, A.; Zimpleman, J. M.; Hwang, I.; Boger, D. L. Total synthesis of ningalin D. *J. Am. Chem. Soc.* **2005**, *127*, 10767–10770.
- (31) Carlson, J. C.; Meimetis, L. G.; Hilderbrand, S. A.; Weissleder, R. BODIPY-tetrazine derivatives as superbright bioorthogonal turn-on probes. *Angew. Chem., Int. Ed.* **2013**, *52*, 6917–6920.
- (32) Wu, H.; Yang, J.; Seckute, J.; Devaraj, N. K. In situ synthesis of alkenyl tetrazines for highly fluorogenic bioorthogonal live-cell imaging probes. *Angew. Chem., Int. Ed.* **2014**, *53*, 5805–5809.
- (33) Wicczorek, A.; Werther, P.; Euchner, J.; Wombacher, R. Green-to far-red-emitting fluorogenic tetrazine probes - synthetic access and no-wash protein imaging inside living cells. *Chem. Sci.* **2017**, *8*, 1506–1510.
- (34) Best, M.; Degen, A.; Baalman, M.; Schmidt, T. T.; Wombacher, R. Two-step protein labeling by using lipoic acid ligase with norbornene substrates and subsequent inverse-electron demand Diels-Alder reaction. *ChemBioChem* **2015**, *16*, 1158–1162.
- (35) Denk, C.; Svatunek, D.; Filip, T.; Wanek, T.; Lumpi, D.; Frohlich, J.; Kuntner, C.; Mikula, H. Development of a (18) F-labeled tetrazine with favorable pharmacokinetics for bioorthogonal PET imaging. *Angew. Chem., Int. Ed.* **2014**, *53*, 9655–9659.
- (36) For the introduction of bioorthogonal triazines via Suzuki coupling, see: Kamber, D. N.; Liang, Y.; Blizzard, R. J.; Liu, F.; Mehl, R. A.; Houk, K. N.; Prescher, J. A. 1,2,4-Triazines Are Versatile Bioorthogonal Reagents. *J. Am. Chem. Soc.* **2015**, *137*, 8388–91.
- (37) Pinner, A. Ueber die Einwirkung von Hydrazin auf Imidoäther. *Ber. Dtsch. Chem. Ges.* **1893**, *26*, 2126.
- (38) Tolshchina, S. G.; Rusinov, G. L.; Charushin, V. N. 1,2,4,5-Tetrazines and Azolo[1,2,4,5]tetrazines: Synthesis and Reactions with Nucleophiles. *Chem. Heterocycl. Compd.* **2013**, *49*, 66–91.
- (39) Yang, J.; Karver, M. R.; Li, W.; Sahu, S.; Devaraj, N. K. Metal-catalyzed one-pot synthesis of tetrazines directly from aliphatic nitriles and hydrazine. *Angew. Chem., Int. Ed.* **2012**, *51*, 5222–5225.
- (40) Mao, W.; Shi, W.; Li, J.; Su, D.; Wang, X.; Zhang, L.; Pan, L.; Wu, X.; Wu, H. Organocatalytic and Scalable Syntheses of Unsymmetrical 1,2,4,5-Tetrazines by Thiol-Containing Promoters. *Angew. Chem., Int. Ed.* **2019**, *58*, 1106–1109.
- (41) Qu, Y.; Sauvage, F. X.; Clavier, G.; Miomandre, F.; Audebert, P. Metal-Free Synthetic Approach to 3-Monosubstituted Unsymmetrical 1,2,4,5-Tetrazines Useful for Bioorthogonal Reactions. *Angew. Chem., Int. Ed.* **2018**, *57*, 12057–12061.
- (42) Testa, C.; Gigot, E.; Genc, S.; Decreau, R.; Roger, J.; Hierso, J. C. Ortho-Functionalized Aryltetrazines by Direct Palladium-Catalyzed C-H Halogenation: Application to Fast Electrophilic Fluorination Reactions. *Angew. Chem., Int. Ed.* **2016**, *55*, 5555–5559.
- (43) Mboyi, C. D.; Testa, C.; Reeb, S.; Genc, S.; Cattey, H.; Fleurat-Lessard, P.; Roger, J.; Hierso, J.-C. Building Diversity in ortho-Substituted s-Aryltetrazines By Tuning N-Directed Palladium C–H Halogenation: Unsymmetrical Polyhalogenated and Biphenyl s-Aryltetrazines. *ACS Catal.* **2017**, *7*, 8493–8501.
- (44) Novak, Z.; Kotschy, A. First Cross-Coupling Reactions on Tetrazines. *Org. Lett.* **2003**, *5*, 3495–3497.
- (45) Bender, A. M.; Chopko, T. C.; Bridges, T. M.; Lindsley, C. W. Preparation of Unsymmetrical 1,2,4,5-Tetrazines via a Mild Suzuki Cross-Coupling Reaction. *Org. Lett.* **2017**, *19*, 5693–5696.
- (46) Suzenet, F.; Leconte, N.; Keromnes-Wuillaume, A.; Guillaumet, G. Efficient Palladium-Catalyzed Synthesis of Unsymmetrical (Het)-aryltetrazines. *Synlett* **2007**, 2004–210.
- (47) Pellegatti, L.; Vedrenne, E.; Leger, J.-M.; Jarry, C.; Routier, S. New heteroaromatic aminations on 5-aryl-1,2,4-triazines and 1,2,4,5-tetrazines by palladium catalysis. *Tetrahedron* **2010**, *66*, 4383–4389.
- (48) Quinton, C.; Alain-Rizzo, V.; Dumas-Verdes, C.; Clavier, G.; Vignau, L.; Audebert, P. Triphenylamine/tetrazine based  $\pi$ -conjugated systems as molecular donors for organic solar cells. *New J. Chem.* **2015**, *39*, 9700–9713.
- (49) Wicczorek, A.; Buckup, T.; Wombacher, R. Rigid tetrazine fluorophore conjugates with fluorogenic properties in the inverse electron demand Diels-Alder reaction. *Org. Biomol. Chem.* **2014**, *12*, 4177–4185.
- (50) Soloduchko, J.; Doskocz, J.; Cabaj, J.; Roszak, S. Practical synthesis of bis-substituted tetrazines with two pendant 2-pyrrolyl or 2-thienyl groups, precursors of new conjugated polymers. *Tetrahedron* **2003**, *59*, 4761–4766.
- (51) Li, Z.; Ding, J.; Song, N.; Lu, J.; Tao, Y. Development of a New s-Tetrazine-Based Copolymer for Efficient Solar Cells. *J. Am. Chem. Soc.* **2010**, *132*, 13160–13161.
- (52) Karver, M. R.; Weissleder, R.; Hilderbrand, S. A. Synthesis and evaluation of a series of 1,2,4,5-tetrazines for bioorthogonal conjugation. *Bioconjugate Chem.* **2011**, *22*, 2263–2270.
- (53) Fields, S. C.; Parker, M. H.; Erickson, W. R. A Simple Route to Unsymmetrically Substituted 1,2,4,5-Tetrazines. *J. Org. Chem.* **1994**, *59*, 8284–8287.
- (54) DSC data for commercially available thiocarbonylhydrazide: onset temperature of 172 °C, transition enthalpy of 1634 J/g. As a precaution, all experiments involving thiocarbonylhydrazide were carried out below 72 °C (100 °C below the DSC transition onset).
- (55) Sperry, J. B.; Minter, C. J.; Tao, J.; Johnson, R.; Duzguner, R.; Hawksworth, M.; Oke, S.; Richardson, P. F.; Barnhart, R.; Bill, D. R.; Giusto, R. A.; Weaver, J. D. Thermal Stability Assessment of Peptide Coupling Reagents Commonly Used in Pharmaceutical Manufacturing. *Org. Process Res. Dev.* **2018**, *22*, 1262–1275.
- (56) Cheng, H.-G.; Chen, H.; Liu, Y.; Zhou, Q. The Liebeskind–Srogl Cross-Coupling Reaction and its Synthetic Applications. *Asian J. Org. Chem.* **2018**, *7*, 490–508.



- (57) Liebeskind, L. S.; Srogl, J. Heteroaromatic Thioether-Boronic Acid Cross-Coupling under Neutral Reaction Conditions. *Org. Lett.* **2002**, *4*, 979–981.
- (58) Musaev, D. G.; Liebeskind, L. S. On the Mechanism of Pd(0)-Catalyzed, Cu(I) Carboxylate-Mediated Thioorganic-Boronic Acid Desulfative Coupling. A Non-innocent Role for Carboxylate Ligand. *Organometallics* **2009**, *28*, 4639–4642.
- (59) Hooper, J. F.; Young, R. D.; Pernik, I.; Weller, A. S.; Willis, M. C. Carbon–carbon bond construction using boronic acids and aryl methyl sulfides: orthogonal reactivity in Suzuki-type couplings. *Chem. Sci.* **2013**, *4*, 1568–1572.
- (60) Pan, F.; Wang, H.; Shen, P.-X.; Zhao, J.; Shi, Z.-J. Cross coupling of thioethers with aryl boroxines to construct biaryls via Rh catalyzed C–S activation. *Chem. Sci.* **2013**, *4*, 1573–1577.
- (61) Ghosh, P.; Ganguly, B.; Perl, E.; Das, S. A synthesis of biaryl ketones via the C–S bond cleavage of thiol ester by a Cu/Ag salt. *Tetrahedron Lett.* **2017**, *58*, 2751–2756.
- (62) Markovic, T.; Rocke, B. N.; Blakemore, D. C.; Mascitti, V.; Willis, M. C. Pyridine sulfinates as general nucleophilic coupling partners in palladium-catalyzed cross-coupling reactions with aryl halides. *Chem. Sci.* **2017**, *8*, 4437–4442.
- (63) Blankman, J. L.; Cravatt, B. F. Chemical probes of endocannabinoid metabolism. *Pharmacol. Rev.* **2013**, *65*, 849–871.
- (64) Chang, J. W.; Niphakis, M. J.; Lum, K. M.; Cognetta, A. B., 3rd; Wang, C.; Matthews, M. L.; Niessen, S.; Buczynski, M. W.; Parsons, L. H.; Cravatt, B. F. Highly selective inhibitors of monoacylglycerol lipase bearing a reactive group that is bioisosteric with endocannabinoid substrates. *Chem. Biol.* **2012**, *19*, 579–588.
- (65) Electron-withdrawing aromatic substituents have an accelerating effect on Diels–Alder rate constants with tetrazines; see refs 9 and 52.
- (66) Butler, C. R.; Beck, E. M.; Harris, A.; Huang, Z.; McAllister, L. A.; am Ende, C. W.; Fennell, K.; Foley, T. L.; Fonseca, K.; Hawrylik, S. J.; Johnson, D. S.; Knafels, J. D.; Mente, S.; Noell, G. S.; Pandit, J.; Phillips, T. B.; Piro, J. R.; Rogers, B. N.; Samad, T. A.; Wang, J.; Wan, S.; Brodney, M. A. Azetidine and Piperidine Carbamates as Efficient, Covalent Inhibitors of Monoacylglycerol Lipase. *J. Med. Chem.* **2017**, *60*, 9860–9873.
- (67) Chang, J. W.; Cognetta, A. B., 3rd; Niphakis, M. J.; Cravatt, B. F. Proteome-wide reactivity profiling identifies diverse carbamate chemotypes tuned for serine hydrolase inhibition. *ACS Chem. Biol.* **2013**, *8*, 1590–1599.