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Installation of Minimal Tetrazines Through Silver-mediated Liebeskind-Srogl Coupling with Arylboronic Acids

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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, $PdCl_2$(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_2O 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.

Graphical Abstract

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Supporting Information

Optimization tables, DSC data, experimental procedures, kinetics, inhibition assays, and ^{1}H and ^{13}C NMR data are displayed. The Supporting Information is available free of charge on the ACS Publications website at DOI: xxxxxxxxxxxxx

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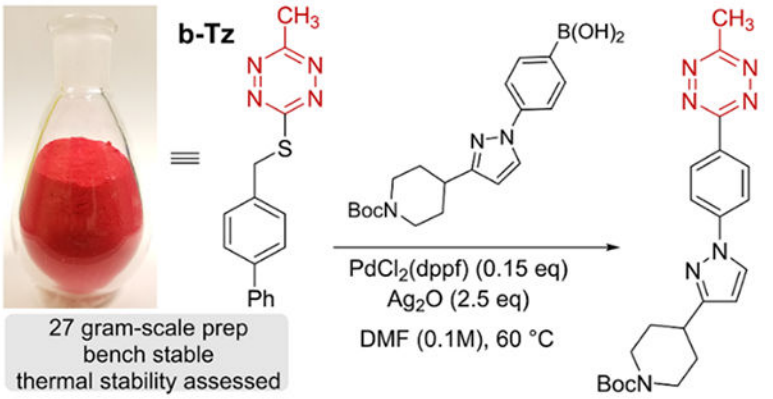

The bioorthogonal reactions of tetrazines have emerged as important tools for chemical biology over the last decade.[1–6] Cycloadditions involving a range of dienophiles including *trans*-cyclooctenes[1,7–10], cyclopropenes[11–12] and norbornenes[13] have been developed as tools for a variety of applications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

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.[6, 17] Complementary new methods for the 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[31–33], better substrates for enzyme-catalyzed protein modification,[17, 34] and probes for ^{18}F-PET imaging[35]. However, there are currently few methods for the direct attachment of ‘minimal’ tetrazine fragments to target molecules.[36] 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 (Fig 1B).

Classical tetrazine synthesis involves the condensation of Pinner salts or nitriles with excess hydrazine followed by oxidation.[37–38] Catalytic nitrile condensation with neat anhydrous hydrazine, most notably with $Zn(OTf)_2$ and $Ni(OTf)_2$, has expanded access to unsymmetrical tetrazines.[39] Further, thiol catalysis has been shown to promote tetrazine synthesis from nitriles using hydrazine-hydrate.[40] The most practiced procedures utilize excess acetonitrile or formamidine acetate and produce volatile tetrazine byproducts with high-nitrogen content (Fig 2A). Recently, a sulfur-catalyzed reaction of nitriles with hydrazine hydrate and dichloromethane has been described for 3-aryltetrazine synthesis.[41] A

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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,[42–43] cross-couplings,[32–33,44–51] and in Heck reactions with *in situ* generated 3-vinyl-6-methyltetrazine[32]. Recently, 3-amino-6-chlorotetrazines have been cross-coupled under Suzuki conditions (Fig 2B).[45] Liebeskind-Srogl cross-couplings have also been reported with 3-amino-6-thiomethyl-tetrazines at 200 °C (Fig 2B).[46] 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.[21, 32] 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-methylte-trazine fragment via Stille coupling with 3-bromo-6-methylte-trazine, which is prepared from 3-hydrazino-6-methylte-trazine (Fig 2B).[33]

We considered that 3-thioalkyl-6-methyltetrazines might serve as useful reagents for the preparation of 3-aryl-6-methylte-trazines, which are attractive bioorthogonal reagents due to their balance of rapid kinetics toward dienophiles and high stability in the cellular environment.[17, 21, 52] By modifying a method for the synthesis of 3-thiomethyl-6-methyltetrazine,[53] 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[54] with 4-bromomethylbiphenyl followed by one-pot condensation with triethylorthoacetate and a novel $Cu(OAc)_2$-catalyzed air-oxidation of the dihydrotetrazine intermediate. b-Tz was isolated on 27 gram scale with a 47% overall yield after simple silica plug filtration and is a bench-stable crystalline solid (m.p. 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 (Fig S-11).[55]

After extensive screening (Fig S-3 thru S-8), we found copper(I)-mediated Liebeskind-Srogl conditions[56,57] could promote cross-coupling of benzylic thioether tetrazines with $PhB(OH)_2$, $PhSnBu_3$, and $PhSi(OMe)_3$ (Fig 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 (CuTC) at 70 °C resulted in rapid decomposition and produced 4-phenylbenzaldehyde as the only identifiable side product (Fig 4A).

Copper has been proposed to promote the Liebeskind-Srogl reaction by facilitating transmetallation as shown in Figure 4B.[57–58] We hypothesized that silver(I) salts might be similarly capable as promotors, whereby transmetallation would be promoted in a dual role

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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[59–60], and the Cu-catalyzed coupling of arylboronic acids with aromatic thioesters[61]. To our knowledge, a Ag-mediated variation of the Liebeskind-Srogl reaction has not been reported. After extensive optimization (Fig 4C entries 4–7, S-1 and S-2), $PdCl_2$(dppf) (15 mol%) was found to be especially effective for cross-coupling of 3-thioalkyl-6-methylte-trazines with arylboronic acids in polar, aprotic solvents (DMF, DMSO) at 60 °C. A screening of silver(I) additives revealed Ag_2O as the most general promotor, although Ag_2CO_3 was also effective (Fig 4D). Substitution of Ag_2O by Cu_2O gave only trace product formation. Arylboronic acids are particularly effective nucleophiles, whereas $PhBF_3K$ and PhBPin were both less effective under identical reaction conditions (Fig 4C entries 8–9). Further, a series of 3-arylmethyl-6-methyltetrazines **1a-g** were evaluated as coupling partners (Fig 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 stabiliy. We also note that the cost of Ag_2O (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%. In general, couplings were carried out using 1.9 equiv. of boronic acid, but 3.0 equiv. were 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 (Fig 5), additives with free thiol or primary alkyl amine groups were not (Fig S-19). Also unsuccessful were 2-pyridyl- and 4-pyridylboronic acids which are regarded as problematic across other cross-coupling reactions.[62]

This cross-coupling method is not limited to *S*-benzylic 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 (Fig 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-phenyltetrazine (**4c**), which is used to create cell-contact guiding micro-fibrous materials for tissue-culture applications.[27]

We sought to demonstrate the application of b-Tz for the construction of fluorophore-tetrazine conjugates—compounds that have utility in live cell imaging.[15] BODIPY-dye **6** with a directly attached tetrazine has been developed as ‘superbright’ bioorthogonal probe for fluorogenic labeling in live cells.[31] The condensation of nitriles with hydrazine produces

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6 in 8% yield.[31] 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 a protein 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.[63] We designed a MAGL probe (**9**) by appending a 6-methyltetrazine moiety to a pyrazolylpiperidine scaffold with an electrophilic hexafluoroisopropyl (HFIP) carbamate warhead for covalently labeling the active site serine (Fig 7A).[64] 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_{rel} 1.1, Fig S-20).[65] Probe **9** inhibited MAGL activity with 31 nM IC_{50} in an *in vitro* assay.[66]

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 μM of TCO-TAMRA for 30 min in live cells (Fig 7B). After cell lysis, MAGL labeling was assessed with a gel-based activity-based protein profiling (ABPP) analysis (Fig 7C–E).[67] Strong fluorescence signals were observed for MAGL with minimal non-specific labeling from TCO-TAMRA. The labeling by probe **9** was dose responsive with a cellular IC50 of 8 nM, and was competed by a MAGL inhibitor, KML29.[64] The HFIP warhead also labeled an additional protein at ~35 kDa, which is consistent with its reactivity with α/β-hydrolase domain 6 (ABHD6), and other off-targets at higher concentrations.[64, 67]

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 $PdCl_2$(dppf) catalyst and Ag_2O 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.

Supplementary Material

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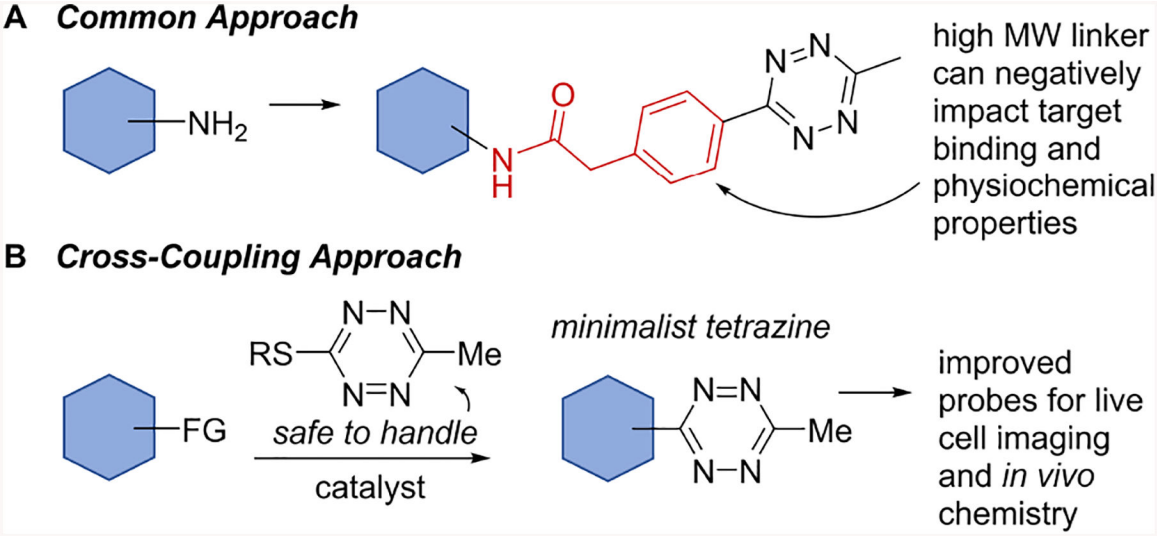

Figure 1:

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

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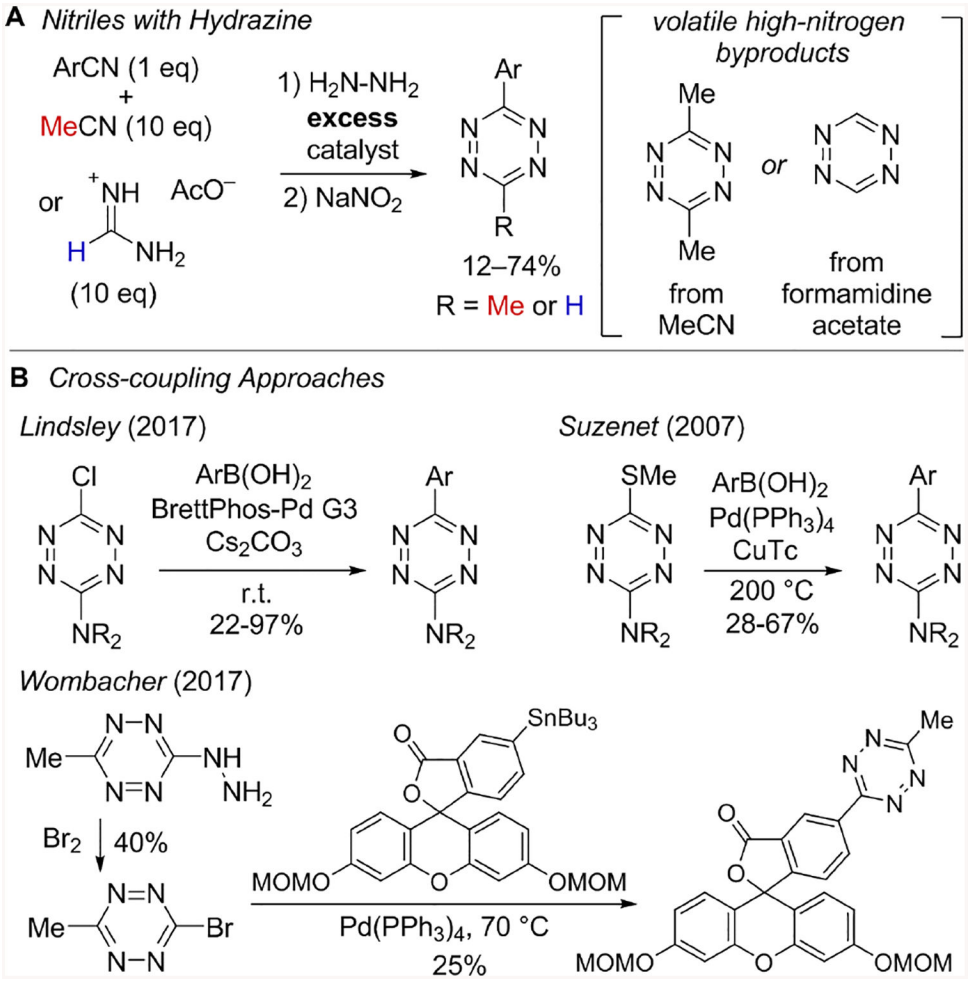

Figure 2:

(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-methyl-tetrazine to fluorophores.

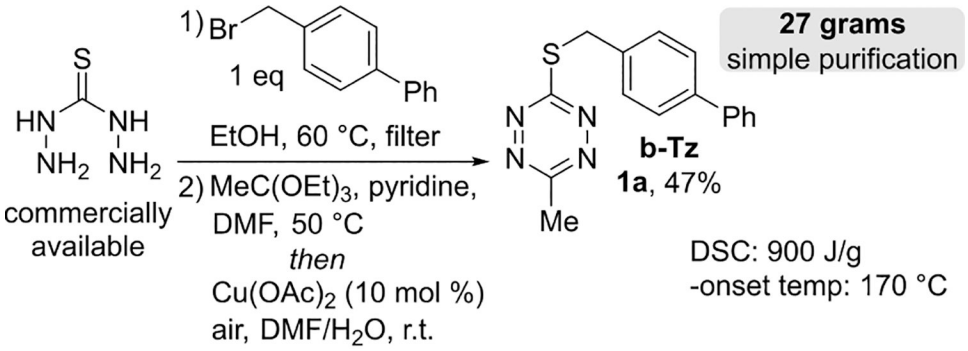

Figure 3:
Decagram synthesis and thermal stability of b-Tz (**1a**).

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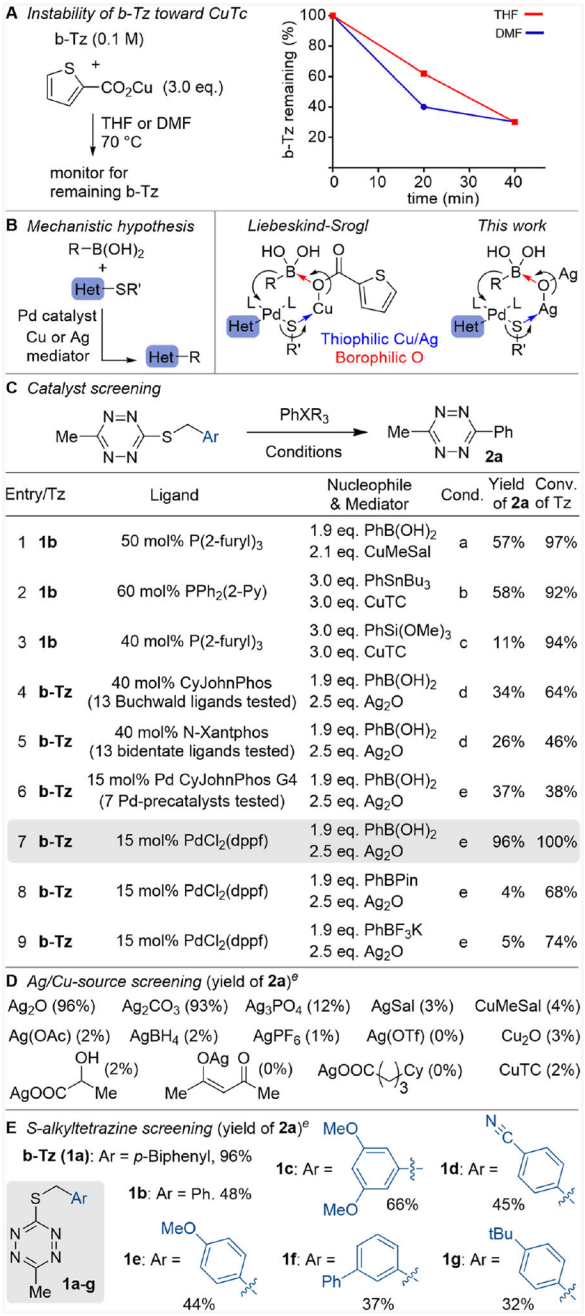

Entry/Tz		Ligand	Nucleophile & Mediator	Cond.	Yield of **2a**	Conv. of Tz
1	**1b**	50 mol% $P(2\text{-furyl})_3$	1.9 eq. $PhB(OH)_2$ 2.1 eq. CuMeSal	a	57%	97%
2	**1b**	60 mol% $PPh_2(2\text{-Py})$	3.0 eq. $PhSnBu_3$ 3.0 eq. CuTC	b	58%	92%
3	**1b**	40 mol% $P(2\text{-furyl})_3$	3.0 eq. $PhSi(OMe)_3$ 3.0 eq. CuTC	c	11%	94%
4	**b-Tz**	40 mol% CyJohnPhos (13 Buchwald ligands tested)	1.9 eq. $PhB(OH)_2$ 2.5 eq. Ag_2O	d	34%	64%
5	**b-Tz**	40 mol% N-Xantphos (13 bidentate ligands tested)	1.9 eq. $PhB(OH)_2$ 2.5 eq. Ag_2O	d	26%	46%
6	**b-Tz**	15 mol% Pd CyJohnPhos G4 (7 Pd-precatalysts tested)	1.9 eq. $PhB(OH)_2$ 2.5 eq. Ag_2O	e	37%	38%
7	**b-Tz**	15 mol% $PdCl_2(dppf)$	1.9 eq. $PhB(OH)_2$ 2.5 eq. Ag_2O	e	96%	100%
8	**b-Tz**	15 mol% $PdCl_2(dppf)$	1.9 eq. PhBPin 2.5 eq. Ag_2O	e	4%	68%
9	**b-Tz**	15 mol% $PdCl_2(dppf)$	1.9 eq. $PhBF_3K$ 2.5 eq. Ag_2O	e	5%	74%

Figure 4:

(A) Rapid decomposition of b-Tz in CuTC. (B) Proposed Liebeskind-Srogl transmetallation 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_2dba_3 (12.5 mol%), Cs_2CO_3 (3.0 eq.), dioxane, 70 °C, 90 min. (b) $[Pd(allyl)Cl]_2$ (10 mol %), THF, 70 °C, 2 h. (c) $Pd(OAc)_2$ (10 mol%), TBAF (1.0 eq.), dioxane, 70 °C, 2.5 h. (d) Pd_2dba_3 (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 condition e.

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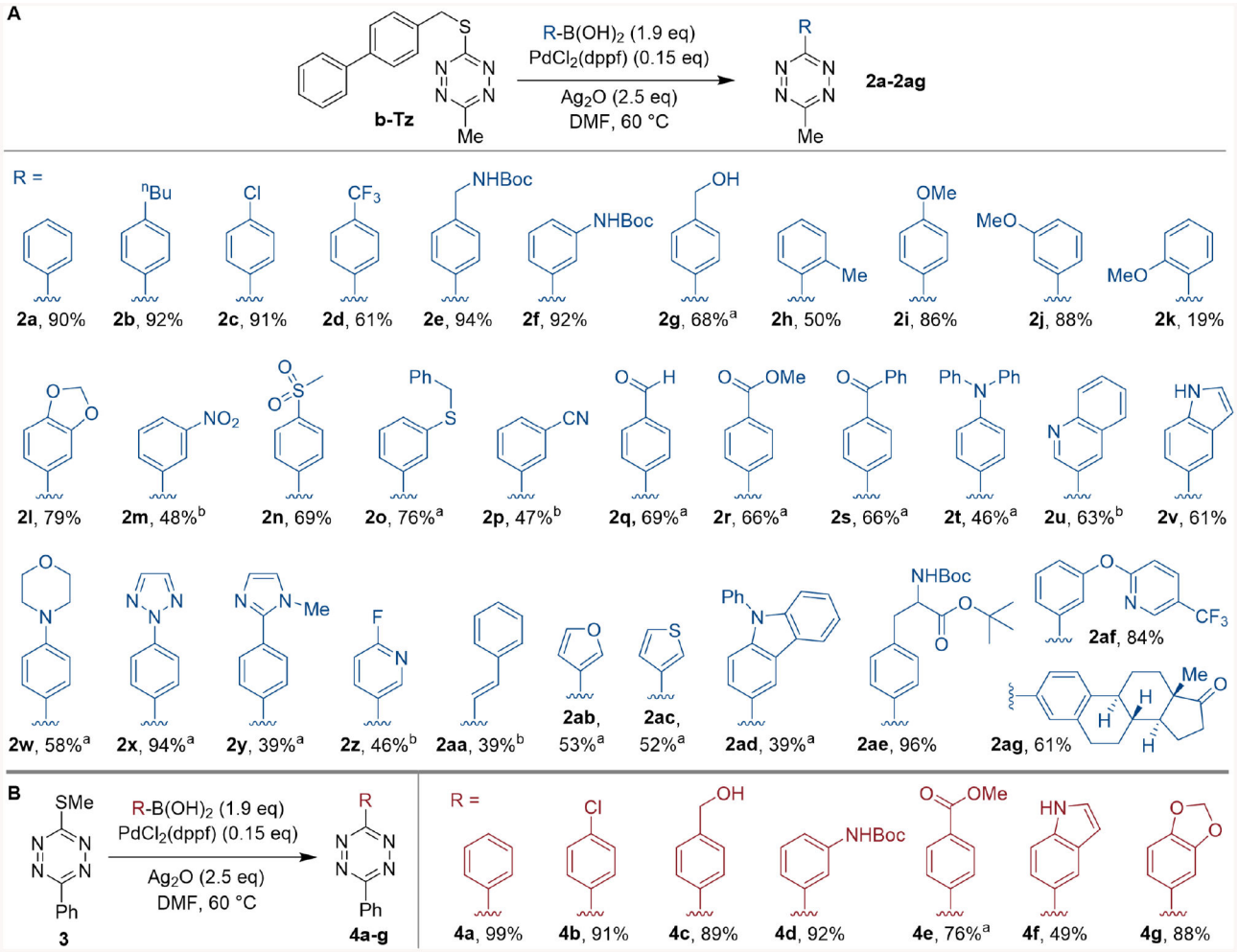

Figure 5:

Reaction scope of b-Tz (5A) and **3** (5B). Typical conditions: thioalkyl tetrazine b-Tz or **3** (1.0 equiv.), $RB(OH)_2$ (1.9 equiv.), $PdCl_2(dppf)$ (15 mol%), Ag_2O (2.5 equiv.), DMF (0.1M), 60°C, 19–21h, average isolated yields of duplicate synthesis (± 5%). [a] 3.0 equiv. of $RB(OH)_2$. [b] 3.0 equiv. of $RB(OH)_2$ did not significantly improve yield (< 5%), 1.9 equiv. of $RB(OH)_2$ was used.

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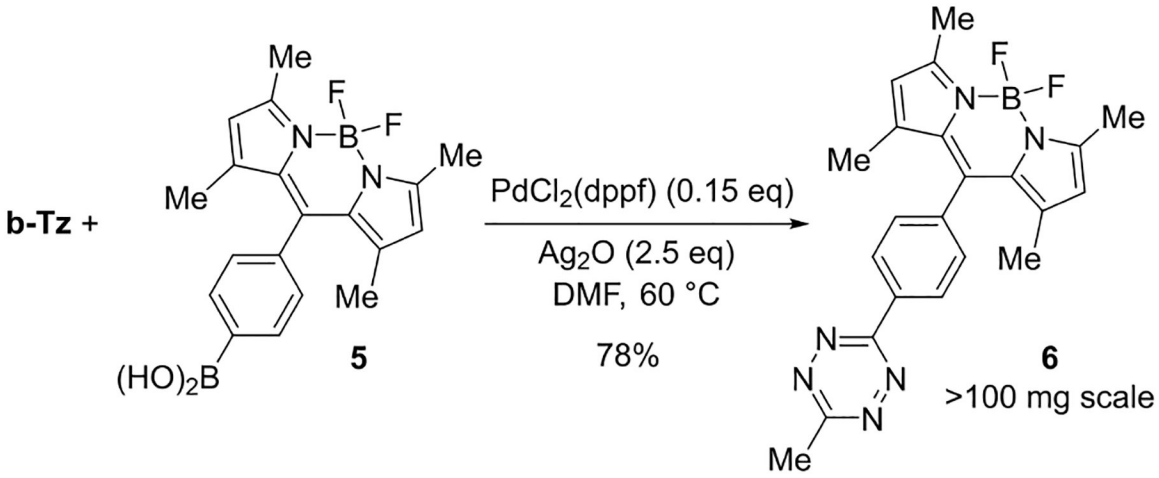

Figure 6:
Synthesis of 3-BODIPY-6-methyltetrazine **6**.

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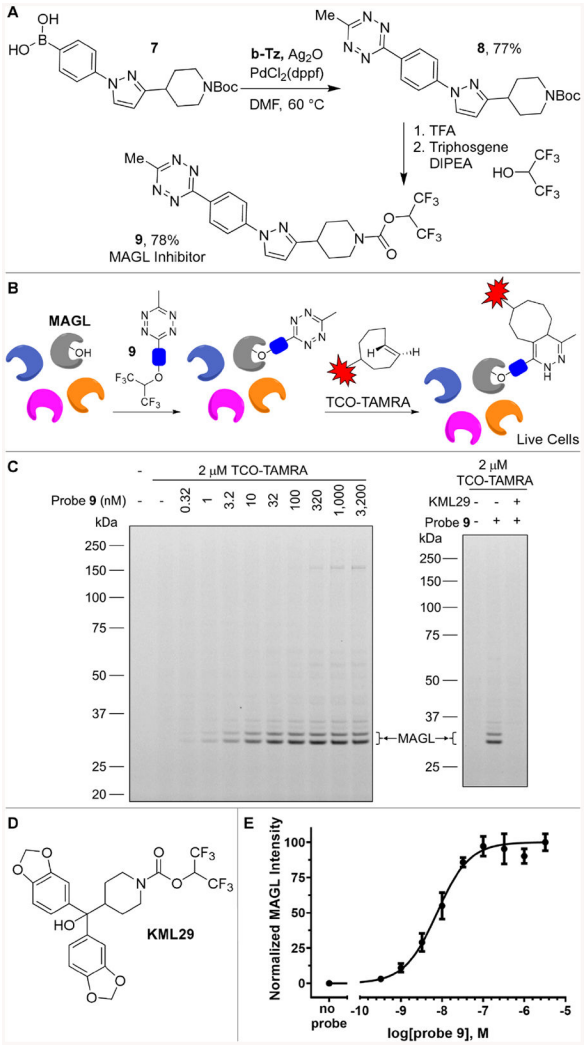

Figure 7:

(A) Synthesis of MAGL reactive probe **9**. (B) Live cells were treated with probe **9** for 1 h, followed by 2 μM 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**. Probe **9** (32 nM, 1 h) was competed by pre-treatment 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 ± SEM (n = 2). See Fig S-21 for Coomassie staining.