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Organocatalytic and Scalable Syntheses of Unsymmetrical 1,2,4,5-Tetrazines by Thiol-containing Promotors

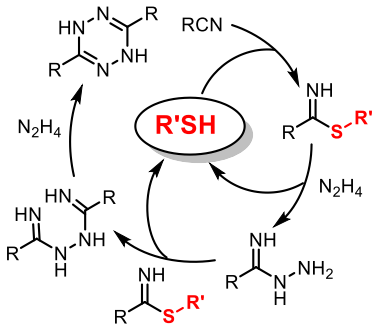
Wuyu Mao[+], Wei Shi[+], Jie Li, Dunyan Su, Xiaomeng Wang, Lyuye Zhang, Lili Pan, Xiaoai Wu, Haoxing Wu*

Dedicated to the 100th Anniversary of West China School of Pharmacy, Sichuan University

Abstract: Despite the growing application of tetrazine bioorthogonal chemistry, it is still challenging to access tetrazines conveniently from easily available materials. Here we describe *de novo* formation of tetrazine from nitriles and hydrazine hydrate using a broad array of thiol-containing catalysts, including peptides. Using this facile methodology, we describe 14 syntheses of unsymmetric tetrazines containing a range of reactive functional groups on the gram scale with satisfactory yield. Using tetrazine methylphosphonate as a building block, we develop a highly efficient Horner-Wadsworth-Emmons reaction for further derivatization under mild conditions. Tetrazine probes with diverse functions can be scalably produced in yields of 87–93%. This methodology may facilitate the widespread application of tetrazine bioorthogonal chemistry.

Tetrazine, a centuried molecule, has been used for many years for coordination chemistry, total synthesis and design of materials with high energy density.[1] With the emergence of bioorthogonal chemistry early this century,[2] tetrazine has become extremely attractive to chemical biologists and biomedical researchers.[3] In living systems, tetrazine can serve as a magic bullet for delivering imaging probes or drugs in targeted manner,[4] as an unnatural amino acid for site-specific protein labeling,[5] as fluorescence storage,[6] as a key for decaging proteins[7] or as a precursor of biomaterials.[8]

Although it is one of the most attractive bioorthogonal tools,[9] 1,2,4,5-tetrazines are not easy to synthesize. In the classical two-step tetrazine synthesis, the first step of condensation of hydrazine with nitriles tolerates a narrower range of substrates than the subsequent oxidation of 1,2-dihydrotetrazine.[1a, 10] In recent years, several groups have tried to broaden the range of functional tetrazines that can be produced.[11] In 2012, the Devaraj group pioneered a metal-catalyzed approach to unsymmetrical tetrazine, producing a series of alkyl tetrazines for the first time.[11a] Widespread use of this reaction is some hindered by the fact that it employs hazardous anhydrous hydrazine, which is not commercially available in several countries. In a recent approach based on an ethanol solution of hydrazine hydrate, Audebert and co-workers demonstrated that dichloromethane can be used instead of regular formamidine as a starting material in the synthesis of monosubstituted tetrazines.[11b] However, this reaction requires prolonged microwave irradiation, limiting its usefulness, especially for scale-up. Sequentially, several groups have bypassed these issues by using metal-catalyzed cross-coupling reactions to generate functionalized tetrazines.[6c, 6e, 12] Nevertheless, mild, scalable syntheses are still needed in order to endow tetrazine with new functions and develop downstream applications. Inspired by the interaction of nitriles with the biological media, here we report an organocatalytic approach to prepare unsymmetrical tetrazines. Our approach is mild and economical, and it is compatible with water. It can be carried out in most chemistry or biochemistry laboratories because it does not require dangerous reagents, harsh conditions or sophisticated equipment. In the presence of simple thiol as catalyst, commercially available nitriles bearing multiple functional groups, including free acid, azide, phosphonate, or hydroxy, can be activated smoothly. Tetrazines can be formed from nitriles and hydrazine hydrate at room temperature with satisfactory yields on a gram-scale. Furthermore, the resulting tetrazine methylphosphonate is a key building block for further derivatizations, and a mild Horner-Wadsworth-Emmons (HWE) reaction is developed to efficiently construct a linkage between tetrazine and other molecules of biomedical interest. This route should remove major obstacles to tetrazine synthesis, facilitating further development of bioorthogonal solutions to chemical, biological and medical challenges.



Scheme 1. Proposed organocatalytic role for tetrazine synthesis.

We began designing our strategy by searching for an appropriate catalyst to activate sluggish aliphatic nitriles. An ideal methodology to overcome the present limitations should be able to perform with a wide range of commercially available materials, it should be compatible with water and most functional groups, and it should be easy to scale up. Recently, the Rao, Chin and Lin

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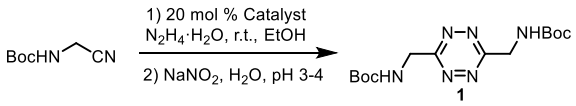
groups achieved the bioorthogonal ligation of electron-deficient cyano groups, typically cyanobenzothiazole, with an aminothiol or cysteine tag.[13] We also noted that nitrile moieties in some pharmaceuticals can interact reversibly with the thiol group in the active site of target proteins or enzymes.[14] Inspired by these discoveries in biological milieux, we hypothesized that thiols could reversibly activate weakly electrophilic aliphatic and aromatic nitriles at room temperature, generating a more reactive thioimidate ester intermediate *in situ*. Hydrazine nucleophilically attacks this intermediate, regenerating the thiol so that it can drive another catalytic cycle of activation in tetrazine synthesis (Scheme 1). And this catalytic process would be compatible with a wide range of functional groups in aqueous solution.

To determine whether our approach was feasible, we treated *N*-(*tert*-Butoxycarbonyl)-2-aminoacetonitrile (2 mmol) with an ethanol solution of hydrazine hydrate (16 mmol) in the presence of L-cysteine (0.2 mmol) as catalyst. To our delight, the reaction occurred efficiently at room temperature, affording symmetric 3,6-(di-*N*-Boc-aminomethyl)-tetrazine **1** in 69% yield, which is twice as high as the yield of a previous method (entry 1, Table 1).[11a]

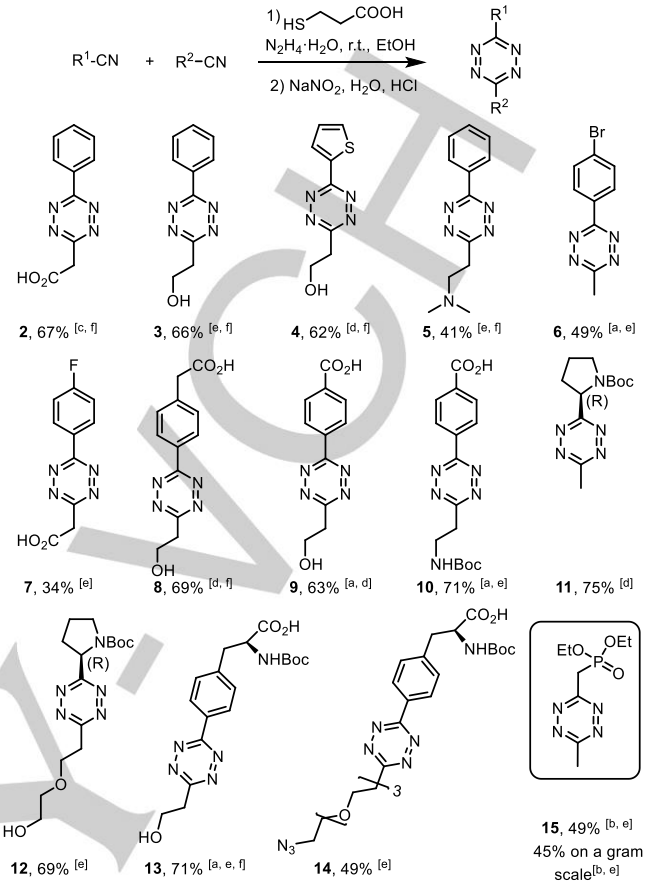
Table 1. Optimization of the reaction conditions.



Entry	Catalyst	Yield [%][a]
1	L-Cysteine	69
2	1,3-Propanedithiol	49
3	2-Aminoethanethiol	46
4	3-Mercaptopropionic Acid	77
5	Thioglycolic acid	70
6	*N*-Acetyl-L-Cysteine	71
7	Glutathione	66
8	3-Mercaptopropionic Acid	75[b]

All reactions in table 1 were conducted on the 1-mmol scale with 16 equivalents of $N_2H_4{\cdot}H_2O$ in an EtOH solution overnight, except as otherwise noted. [a] Isolated yields are reported after silica flash chromatography. [b] Gram scale.

We then surveyed a variety of commercial thiols to determine the best catalyst for tetrazine synthesis. All thiols were able to promote tetrazine synthesis, giving moderate to high yields (Table 1). Interestingly, thiols containing adjacent nucleophile substituents gave relatively modest yields (entries 2 and 3). We hypothesized that the thioimidate may undergo a side reaction of intramolecular nucleophilic attack instead of being attacked by hydrazine. In contrast, 3-mercaptopropionic acid (entry 4), thioglycolic acid (entry 5), and *N*-acetyl-L-cysteine (entry 6) exhibited satisfactory catalytic efficiency, generating tetrazine **1** in up to 77% isolated yield. Impressively, a catalytic amount of the thiol-containing bioactive peptide glutathione also promoted tetrazine formation in good yield (entry 7). After brief screening of solvents (see Supporting Information), we found conditions to work best with 3-mercaptopropionic acid, which is also the least expensive in China (<15 USD for 100 gram) and ethanol as solvent. Notably, we successfully demonstrated the scalability of our approach by preparing **1** on multigram-scale in consistent yield (entry 8).



Scheme 2. Substrate scope. Reaction isolated yields are given based on R^1CN. Reactions were conducted with 4 equiv R^2CN to R^1CN, except as otherwise noted. [a] 8 equiv R^2CN, [b] 20 equiv R^2CN, [c] 0.2 equiv catalyst, [d] 0.4 equiv catalyst, [e] 1 equiv catalyst, [f] 40 °C.

With optimized reaction conditions in hand, we next investigated the scope of our approach to prepare unsymmetric tetrazines. By tuning the molar ratio of two nitriles and slightly increasing catalyst loading, we smoothly synthesized 14 unsymmetric tetrazines in good yield (Scheme 2). All these tetrazines bear at least one functional group and show broad application prospects. For example, unsymmetric tetrazines with aryl and small alkyl substituents at positions 3 and 6, respectively, usually offer a balance of bioorthogonal reactivity and stability, and are quite efficient in "click to release" reactions.[15] Our mild organocatalytic approach afforded aromatic tetrazines **2**–**7** bearing small alkyl carboxylic acid, hydroxy or amino handles in yields of 34–67%. Even higher yields of 63–71% were obtained of tetrazines **8**–**10** bearing different functional groups at positions 3 and 6. This is likely due to the greater solubility of nitrile materials. Similarly high yields of 69–75% were obtained for tetrazines **11**-**13** containing proline or phenylalanine derivatives for site-specific protein labeling. Remarkably, we prepared an unnatural amino acid tetrazine probe **14** in 49% yield; the probe bears a PEG-azide handle for orthogonal Staudinger ligation or click reaction.[9a, 9b] To our knowledge, this tetrazine provides the first example of *de novo* synthesis with the potential for multiplexed bioorthogonal analysis.

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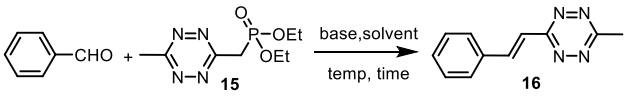
Table 2. Optimization of the tetrazine-HWE reaction

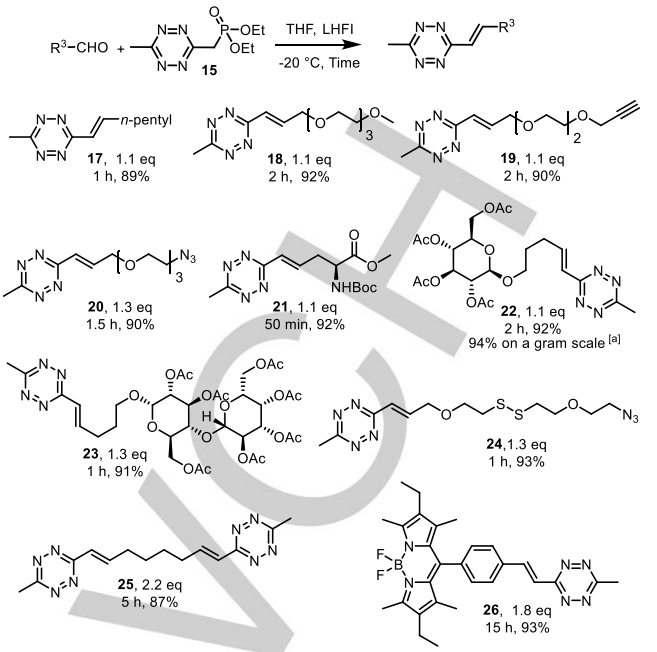


Entry	Base	Solvent	T [°C]	Time [h]	Yield [%][a]
1	NaH	THF	-20	1.5	58
2	*t*-BuOK	THF	-20	1.5	60
3	DBU, LiCl	MeCN	0	4	31
4	TEA, LiCl	THF	RT	4	<20
5	LiHFI	THF	0	0.5	81
6	LiHFI	THF	-20	3	98

All reactions in table 2 were conducted with 0.1 mmol of benzaldehyde and 1.1 equiv **15**. [a] Isolated yield.

We demonstrated that our approach tolerates a wide range of substrates, so we wanted to ask whether it could work beyond the relatively limited range of commercially available nitriles. We envisioned that we could use this mild organocatalytic methodology to develop a simple tetrazine as a robust building block for further derivatizations. First, we synthesized tetrazine methylphosphonate **15**, an HWE reaction precursor, with 45% isolated yield on the gram scale (Scheme 2). Rapid screening of reaction conditions (Table 2) identified lithium 1,1,1,3,3,3-hexafluoroisopropoxide (LiHFI) as the ideal weak base for the HWE reaction: it afforded product **16** in nearly quantitative yield.

Using the optimal reaction conditions, we examined substrate scope of the tetrazine-HWE reaction. We focused our investigation on alkyl derivatizations that were challenging by other cross-coupling strategies.[6c-e] As shown in Scheme 3, starting from **15** and the corresponding aldehydes**,** we prepared a panel of tetrazine derivatives **17**-**25** in excellent yields of 87-94%. These molecules included a series of novel probes in which tetrazine was decorated with azide, alkynyl, amino acid analogues, cleavable multiplexed probe or glucopyranose moieties via a double bond linkage. We were pleased to find that we could reproducibly conduct the tetrazine-HWE reaction on the gram scale (**22**, Scheme 3). The robustness of the mild HWE reaction meant that we could obtain di-tetrazine product **25** in 87% yield; this molecule can be used as a crosslink precursor for biomaterial formation. We also tested the ability of our HWE reaction to create coupling with complex aromatic aldehyde. As the test reaction, we synthesized a highly fluorogenic BODIPY-tetrazine probe **26**, previously prepared in modest yield.[6c] To our delight, our mild coupling approach significantly increased the yield of **26** to 95%. This tetrazine-HWE reaction may be useful for designing fluorogenic bioorthogonal probe in future.



Scheme 3. Substrate scope. Reactions were conducted on scales of 0.05-0.13 mmol (see Supporting Information). Equivalents of **15**, reaction time and isolated yield are shown under each product. [a] 1.2 equivalents of **15**, 3 h.

In conclusion, we have discovered an organocatalytic approach for unsymmetric tetrazine synthesis. Tetrazines can be facilely prepared on the gram scale from accessible materials in the presence of thiol-containing organic catalysts such as 3-mercaptopropionic acid or glutathione. We easily endowed tetrazine with a variety of functions in good yield, including tetrazine methylphosphonate as a novel building block for further HWE derivatization. Our approach eliminates the need for hydrazine anhydride and harsh reaction conditions, and it substantially improves scale-up possibilities. By greatly increasing the accessibility of tetrazines, our approach may lead to new bioorthogonal tools for biomedical, organometallic and materials research and applications.

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Keywords: bioorthogonal chemistry • tetrazines • scalable • organocatalysts

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Layout 1:

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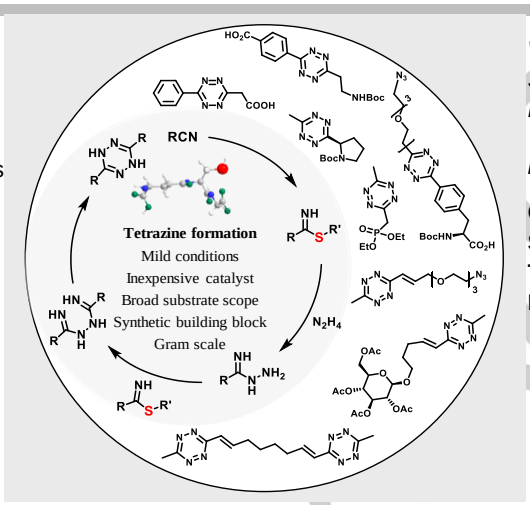
Thiols such as glutathione can be served as novel catalysts for the tetrazine formation. This simple, mild, scalable approach is inspired by the interaction of nitriles with proteins, has broad substrate tolerance and allows access to unsymmetric tetrazines including robust building block for further derivatizations. This methodology could enable most chemical laboratories to prepare tetrazine bioorthogonal probes and other reagents to address challenges in biomedicine, organometallic chemistry and materials research.



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