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**Title**
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**Permalink**
https://escholarship.org/uc/item/0372j20k

**Journal**
Angewandte Chemie International Edition, 58(27)

**ISSN**
1433-7851

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**Publication Date**
2019-07-01

**DOI**
10.1002/anie.201903877

Peer reviewed

# Stable, Reactive and Orthogonal Tetrazines: Dispersion Forces Promote the Cycloaddition with Isonitriles

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## Abstract

Bioorthogonal reactions are of great value in the life sciences. The isocyano group is a structurally compact bioorthogonal functional group that reacts with tetrazines under physiological conditions. Here we report that bulky tetrazine substituents accelerate this cycloaddition. Computational studies suggest that dispersion forces between the isocyano group and the tetrazine substituents in the transition state contribute to the atypical structure-activity relationship. Stable asymmetric tetrazines that react with isonitriles at rate constants as high as 57 $M^{-1}s^{-1}$ were accessible by combining bulky and electron-withdrawing substituents. Sterically encumbered tetrazines react selectively with isonitriles in the presence of strained alkenes/alkynes, which allows for the orthogonal labeling of three proteins. The established principles will open new opportunities for developing tetrazine reactants with improved characteristics for diverse labeling and release applications with isonitriles.

## Graphical Abstract

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Supporting information for this article is given via a link at the end of the document.
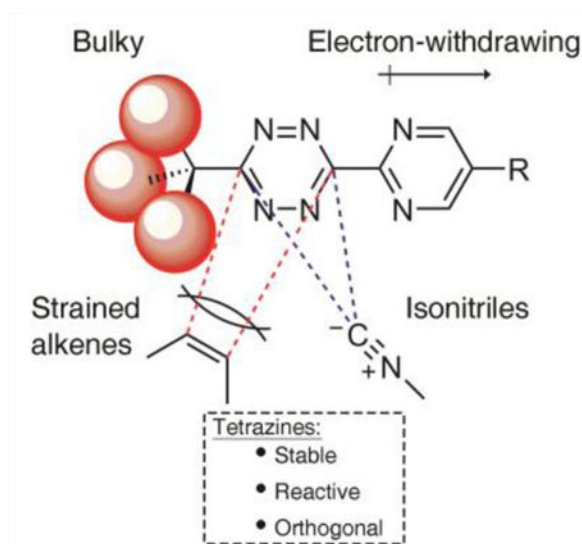
**Rise of bulky tetrazines**: Highly stable tetrazines that react rapidly with isonitriles are presented. Bulky tetrazine substituents sterically attract the isocyano group in the cycloaddition transition state and block the approach of strained alkenes. These characteristics enable rapid bioorthogonal labelling and release reactions and allow for triple-orthogonal protein labelling.

## Keywords

Bioorthogonal chemistry; Cycloadditions; Dispersion forces; Chemoselectivity; Bioconjugation

Bioorthogonal reactions proceed in biological systems without interference from cellular components.[1] Such reactions are finding widespread application in the life sciences, for example to study biomolecules in their native environment[2] and to control the activity of proteins in vivo.[3] Furthermore, drug-delivery approaches utilizing bioorthogonal reactions are under development.[4] Therefore, the pursuance of bioorthogonal ligation[5] and release[6] reactions with improved attributes is actively ongoing. The inverse-electron demand Diels-Alder (iEDDA) cycloaddition between dienophiles and tetrazines has emerged as an especially impactful reaction because of its fast rate and high selectivity.[7] As applications are becoming more complex, there is a need for bioorthogonal groups that can be orthogonally actuated.[8]

Isonitriles are unique among dienophiles that react with tetrazines to form adducts[9] or release leaving groups[10] (Fig. 1). The isocyano group is structurally compact, stable in biological fluids, synthetically facile to access and has a distinct reactivity profile. Rate constants of up to $k_2 = 4$ M$^{-1}$s$^{-1}$ have been reported for reactions of isonitriles with tetrazines;[10] accelerating the cycloaddition without destabilizing the reactants would expand its utility to study biomolecules with high temporal resolution and in drug-delivery. [4b, 11] However, making isonitriles more reactive is challenging because it is impractical to use ring strain, which allowed increasing the reactivity of cyclic alkenes/alkynes by orders of magnitude.[2] Enhancing the reactivity of tetrazines is also problematic because more reactive tetrazines are typically also less stable.[12] Electron-withdrawing groups make

tetrazines more reactive; however, they also render them susceptible to nucleophilic attack.[12] While bulky groups protect tetrazines, they generally obstruct the approach of dienophiles.[5l, 12–13] Here we report that it is possible to design tetrazines that react rapidly with isonitriles and are stable in thiol-containing buffer. Furthermore, sterically encumbered tetrazines react chemoselectively with isonitriles in the presence of strained alkenes and alkynes.

We hypothesized that the linear isocyano group would experience minimal steric repulsion by bulky tetrazine substituents in the (4+1) cycloaddition transition-state.[9a, 14] We synthesized symmetrical 3,6-dialkyl-1,2,4,5-tetrazines (**1a-d**) with substituents of different sizes and measured the rate of the reaction with 2-phenylethyl isonitrile (PhEtNC, Fig. 2a). Unexpectedly, the reaction of PhEtNC with tetrazines accelerated with increasing Taft's steric parameter $(E_s)$[15] of the alkyl group (Fig. 2a). PhEtNC reacted 10.8-fold faster with 3,6-bis-tert-butyl tetrazine (**1d**) than with 3,6-dimethyltetrazine (**1a**). This result is remarkable because **1d** is highly stable (See Supporting Information) and showed no reactivity with norbornene under these conditions (DMSO:$H_2O$, 4:1, v/v; T = 37°C; Fig. 2a). Furthermore, as LUMO levels on tetrazines decreased, reaction rates with both isonitrile and norbornene increased proportionally (Fig. S1).

To rationalize the unexpected relationship between reactivity and sterics, we conducted a computational study to calculate the activation energies required for model compound methylisonitrile to react with tetrazines **1a-1d**. The potential energy surfaces for these reactions were explored at the M06–2X-D3/def2-TZVP[16] level of theory in water (SMD)[17] and the first step was identified as rate determining (Fig. S2–3). In agreement with the experimental results, a 1.7–2.5 kcal/mol lower Gibbs free energy of activation was found for the reaction of methylisonitrile with **1d** relative to **1a-1c** (Fig. 2b). Furthermore, the ratio of the rate constants for the reaction between PhEtNC and **1a** and **1d** is independent of the solution's water content (Fig. S4), and it is therefore unlikely that a hydrophobic effect is responsible for the observed results. To investigate the effect of the tetrazine substituents in more detail, calculations using B3LYP/def2-TZVP with and without Grimme's D3 dispersion correction were conducted.[16c] Structures were reoptimized using both methods. Comparison of the reaction of **1a** and **1d** with methylisonitrile demonstrate the importance of dispersion forces (Fig 2c). Without dispersion correction $\Delta G^\ddagger$ is 1.0 kcal/mol higher for **1d** compared to **1a**. However, the bulkier t-Bu substituents lead to a 6.0 kcal/mol stabilization of the transition state through dispersion, in contrast to 3.1 kcal/mol for **1a**, and resulted in an overall 1.9 kcal/mol lowered free energy of activation. Therefore, not only are steric repulsions with tetrazine substituents less impactful for isonitriles than for other dienophiles, but dispersion forces significantly lower the activation energy. While other interactions may be involved, these computational studies clearly established that dispersion is a major causative factor underlying the observed structure-activity relationship.

These results are significant because they indicate that certain tetrazine modifications can simultaneously increase the reactivity towards isonitriles and the stability of the diene. We postulated that asymmetric tetrazines with both a bulky tert-butyl group and an electron-withdrawing substituent would react rapidly with isonitriles and be highly stable in aqueous solutions (Fig. 3a). We synthesized 3-tert-butyl-6-pyrimidine tetrazines (**3a-3c**; Fig. 3a) and

measured reaction rates with PhEtNC (DMSO:H$_2$O, 4:1, v/v at T = 37°C) and stabilities in buffer containing 10 mM glutathione (GSH) (DMSO:PBS (pH 7.4), 4:1, v/v at T = 37°C). Tetrazines were synthesized by a zinc-catalyzed reaction between nitriles and hydrazine followed by oxidation;[18] the preparation of **3c** required an alternative oxidation strategy (BAIB, cat. TEMPO, See Supporting Information). PhEtNC indeed reacted faster with these tetrazines than with 3,6-dipyridyltetrazine (**DPTz**; 0.297 ± 0.012 M$^{-1}$s$^{-1}$), which is a commonly-used reactant in iEDDA reaction studies. **3a** and **3b** reacted 3.9-fold (1.15 ± 0.20 M$^{-1}$s$^{-1}$) and 5.2-fold (1.53 ± 0.01 M$^{-1}$s$^{-1}$) faster than **DPTz**, respectively, and also exhibited high stability in the nucleophile-containing buffer/DMSO solution, whereas **DPTz** gradually decomposed under these conditions (Fig. 3b). The CF$_3$-substituent enhanced the reactivity further and **3c** reacted 8.1-fold faster (2.42 ± 0.10 M$^{-1}$s$^{-1}$) than **DPTz** and showed comparable stability to **DPTz** in the GSH-containing buffer/DMSO mixture. The reaction of PhEtNC with **3c** was even faster than the reaction with 3,6-dipyrimidyl tetrazine (**DPmTz**, 2.30 ± 0.04 M$^{-1}$s$^{-1}$), which is highly unstable and rapidly degraded in buffer (Fig. 3b, S5).

Water typically accelerates iEDDA reactions,[19] and we measured the rates of isonitriles reacting with tetrazine **3c** in DMSO:H$_2$O, 1:4, v/v at T = 37°C. PhEtNC reacted with **3c** at a second-order rate constant of 29.4 ± 0.8 M$^{-1}$s$^{-1}$ (Fig. 3c). Tertiary isonitriles are more reactive than primary ones[9a] (Table S6) and form stable conjugates with tetrazines suitable for bioorthogonal labeling applications.[9a] **3c** reacted with tert-octyl isocyanide (t-OcNC) at a rate of 57 ± 5 M$^{-1}$s$^{-1}$ (Fig. 3c), and the pyrazole adduct was stable over 24 h (HPLC analysis; Fig. S6). The reaction of these optimized tetrazines with isonitriles is therefore faster than popular bioorthogonal reactions such as the strain-promoted azide/alkyne cycloaddition,[5d, 5e] the Staudinger-Bertozzi ligation,[5a] and the iEEDA reactions between tetrazines and norbornene[5b, 5h] or methylcyclopropene.[5f, 5g, 5l] The principles underlying the design of **3a-c** should allow for designing tetrazines with further improved properties.

The observation that the dependence of iEDDA reaction rates on the size of tetrazine substituents is opposite for isonitriles and alkene-based dienophiles (e.g. norbornene; Fig. 2a), suggests that it should be possible to design reactant pairs that are orthogonal to each other. We investigated the chemoselectivity of three tetrazines (**1d**, **3c**, **DPTz**) towards several dienophiles (TCO, norbornene, methylcyclopropene, primary/tertiary isonitriles; Table 1, Fig. S7). The mono-tBu tetrazine **3c** reacted significantly faster with the isonitriles than the strained alkenes, TCO and norbornene. This result is interesting because sterically unconstrained tetrazines such as **DPTz** react orders of magnitude faster with TCO than with isonitriles (Table 1), and this outcome is in agreement with reports by Devaraj et al.[5l] The two t-Bu groups of **1d** further deterred the cycloaddition with strained alkenes, and even with methylcyclopropene reactants, which retain appreciable reactivity to tetrazines with one t-Bu substituent[5l] such as **3c.** In contrast, the reaction of **1d** with isonitriles proceeds reasonably; however, the kinetic results indicate a possible steric repulsion between tertiary isonitriles and the tert-butyl groups of **1d** (Fig. S8). Nevertheless, tetrazines preferentially react with isonitriles or strain-activated alkenes depending on the bulkiness of their substituents.

Tetrazines that react selectively with isonitriles over other dienophiles open the possibility for orthogonal labeling and release applications. It has been previously shown that the

reaction of both TCO and isonitriles with tetrazines is orthogonal to strain-promoted azide/alkyne cycloadditions.[8, 9b] Considering the increasing interest in multiplexed labeling[20] and release[6d, 21] applications employing orthogonal biocompatible reactions, we sought to determine whether the isonitrile/tetrazine iEDDA reaction could be used for the simultaneous triple-labeling of biomolecules. As a demonstration, we attempted to label three model proteins in a mixture with different fluorophores. Lysine residues on bovine serum albumin (BSA), ovalbumin (OVA), and lysozyme (LYSO) were modified with **1d'**, azide, and MeTzCOOH (Fig. S9) to generate **BSA-1d'**, **OVA-N₃**, and **LYSO-Tz** (Fig. 4a). The functionalized proteins were exposed to reactant-modified fluorophores with different emission wavelengths (Fig. S10)—a green-fluorescent bodipy-tertiary isonitrile conjugate (**BDPY-Fl-NC**), a red-fluorescent bodipy-TCO conjugate (**BDPY-TR-TCO**), and a blue-fluorescent AlexaFluor-DBCO conjugate (**AF405-DBCO**). Each reactive conjugate labeled one protein selectively as demonstrated by in-gel fluorescence analysis (Fig. 4b). **BDPY-Fl-NC** reacted with **BSA-1d'**, **BDPY-TR-TCO** with **LYSO-Tz**, and **AF405-DBCO** with **OVA-N₃**. In a one-pot labeling experiment combining the three proteins and the three dye-conjugates, each protein was labeled with the matching fluorophore and with minimal cross-reaction (Lane 9, Fig. 4b). These results establish that sterically encumbered tetrazines enable the orthogonal labeling of biomolecules. Importantly, while there are a handful of reports using two reactions simultaneously, only few reports of performing three bioorthogonal reactions concurrently are available.[22] It should be possible to combine isonitrile/tetrazine chemistry with additional transformations (e.g. photoinduced click chemistry[23]) to execute even more reactions in parallel.

Dissociative bioorthogonal reactions that release molecules also have many potential applications.[4b, 24] We previously developed tetrazine-responsive 3-isocyanopropyl (ICPr) groups,[10] and we tested whether the improved tetrazines reported here also effectively removed ICPr groups. Indeed, **3c** near-quantitatively removed ICPr groups from a ratiometric 4-hydroxy-1,8-naphthalimide fluorophore (Fig. S13). Given that **3c** reacts preferentially with isonitriles relative to other dienophiles (Table 1), it could be used to control the release of two different molecules by separate chemical stimuli.[25] We further tested whether the optimized tetrazines are compatible with in vivo use by implanting a bead modified with **3a** (Tz-PS) into the yolk sac of zebrafish embryos and monitoring the release of an ICPr-caged resorufin fluorophore (Fig. 5a, for in vitro release, see Fig. S15). Fish implanted with Tz-PS exhibited a significantly higher fluorescence signal compared to control fish implanted with unmodified beads (5.5-fold, t = 4 h; Fig. 5b, 5c). Furthermore, compared to previously disclosed Tz-beads modified with MeTzCOOH,[10] a in vivo resorufin release was faster with **3a**-modified beads (Fig. S16).

In conclusion, we discovered an atypical structure-activity relationship for the reaction of tetrazines and isonitriles. Computational studies revealed that dispersion forces between the isocyano group and the tetrazine substituents in the cycloaddition transition state primarily caused this trend. This effect made it possible to design 3-tert-butyl-6-pyrimidine-tetrazines that react rapidly with isonitriles and are stable under simulated physiological conditions. Sterically encumbered tetrazines further react preferentially with isonitriles over strained alkenes/alkynes, which enabled the triple-orthogonal labeling of proteins. Considering the

structural compactness and ease of synthesis of isonitriles, the developed tetrazines will be valuable for bioorthogonal conjugation and release applications. Importantly, the discovered basic principles lay a foundation towards the development of tetrazines with further enhanced reactivity and orthogonality. This versatile chemistry may open doors to diverse applications in chemical biology and therapeutics.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

## References

[1]. a)McKay CS, Finn MG, Chem. Biol 2014, 21, 1075–1101 [PubMed: 25237856] b)Carell T, Vrabel M, Top. Curr. Chem 2016, 374, 9.

[2]. Chen X, Wu YW, Org. Biomol. Chem 2016, 14, 5417–5439. [PubMed: 26940577]

[3]. Zhang G, Li J, Xie R, Fan X, Liu Y, Zheng S, Ge Y, Chen PR, ACS Cent. Sci 2016, 2, 325–331. [PubMed: 27280167]

[4]. a)Neumann K, Gambardella A, Bradley M, ChemBioChem 2019, 20, 872–876; [PubMed: 30394615] b)Tu J, Xu M, Franzini R, ChemBioChem 2019, In Press, doi: 10.1002/cbic. 201800810;c)Ji X, Pan Z, Yu B, De La Cruz LK, Zheng Y, Ke B, Wang B, Chem. Soc. Rev 2019, 48, 1077–1094. [PubMed: 30724944]

[5]. a)Lin FL, Hoyt HM, Van Halbeek H, Bergman RG, Bertozzi CR, J. Am. Chem. Soc 2005, 127, 2686–2695 [PubMed: 15725026] b)Devaraj NK, Weissleder R, Hilderbrand SA, Bioconjugate Chem 2008, 19, 2297–2299c)Blackman ML, Royzen M, Fox JM, J. Am. Chem. Soc 2008, 130, 13518–13519 [PubMed: 18798613] d)Jewett JC, Bertozzi CR, Chem. Soc. Rev 2010, 39, 1272–1279 [PubMed: 20349533] e)Debets MF, van Berkel SS, Dommerholt J, Dirks AT, Rutjes FP, van Delft FL, Acc. Chem. Res 2011, 44, 805–815 [PubMed: 21766804] f)Yang J, Seckute J, Cole CM, Devaraj NK, Angew. Chem., Int. Ed. Engl 2012, 51, 7476–7479 [PubMed: 22696426] g)Patterson DM, Nazarova LA, Xie B, Kamber DN, Prescher JA, J. Am. Chem. Soc 2012, 134, 18638–18643 [PubMed: 23072583] h)Vrabel M, Kolle P, Brunner KM, Gattner MJ, Lopez-Carrillo V, de Vivie-Riedle R, Carell T, Chem. Eur. J 2013, 19, 13309–13312 [PubMed: 24027163] i)Peewasan K, Wagenknecht HA, ChemBioChem 2017, 18, 1473–1476 [PubMed: 28485853] j)Gu H, Chio TI, Lei Z, Staples RJ, Hirschi JS, Bane S, Org. Biomol. Chem 2017, 15, 7543–7548 [PubMed: 28853481] k)Siegl SJ, Vrabel M, Eur. J. Org. Chem 2018, 2018, 5081–5085l)Yang J, Liang Y, Seckute J, Houk KN, Devaraj NK, Chem. Eur. J 2014, 20, 3365–3375. [PubMed: 24615990]

[6]. a)Versteegen RM, Rossin R, ten Hoeve W, Janssen HM, Robillard MS, Angew. Chem., Int. Ed. Engl 2013, 52, 14112–14116 [PubMed: 24281986] b)Matikonda SS, Orsi DL, Staudacher V, Jenkins IA, Fiedler F, Chen J, Gamble AB, Chem. Sci 2015, 6, 1212–1218 [PubMed: 29560207] c)Xu M, Tu J, Franzini RM, Chem. Commun 2017, 53, 6271–6274d)Zheng Y, Ji X, Yu B, Ji K, Gallo D, Csizmadia E, Zhu M, Choudhury MR, De La Cruz LKC, Chittavong V, Pan Z, Yuan Z, Otterbein LE, Wang B, Nat Chem 2018, 10, 787–794. [PubMed: 29760413]

[7]. a)Wu H, Devaraj NK, Top. Curr. Chem 2016, 374, 3b)Oliveira BL, Guo Z, Bernardes GJL, Chem. Soc. Rev 2017, 46, 4895–4950. [PubMed: 28660957]

[8]. a)Patterson DM, Prescher JA, Curr. Opin. Chem. Biol 2015, 28, 141–149; [PubMed: 26276062] b)Row RD, Prescher JA, Acc. Chem. Res 2018, 51, 1073–1081. [PubMed: 29727171]

[9]. a)Stockmann H, Neves AA, Stairs S, Brindle KM, Leeper FJ, Org. Biomol. Chem 2011, 9, 7303–7305; [PubMed: 21915395] b)Wainman YA, Neves AA, Stairs S, Stockmann H, Ireland-Zecchini H, Brindle KM, Leeper FJ, Org. Biomol. Chem 2013, 11, 7297–7300. [PubMed: 24065211]

[10]. Tu J, Xu M, Parvez S, Peterson RT, Franzini RM, J. Am. Chem. Soc 2018, 140, 8410–8414. [PubMed: 29927585]

[11]. Patterson DM, Nazarova LA, Prescher JA, ACS Chem. Biol 2014, 9, 592–605. [PubMed: 24437719]

[12]. Karver MR, Weissleder R, Hilderbrand SA, Bioconjugate Chem 2011, 22, 2263–2270.

[13]. Fan X, Ge Y, Lin F, Yang Y, Zhang G, Ngai WS, Lin Z, Zheng S, Wang J, Zhao J, Li J, Chen PR, Angew. Chem., Int. Ed. Engl 2016, 55, 14046–14050. [PubMed: 27735133]

[14]. Imming P, Mohr R, Muller E, Overheu W, Seitz G, Angew. Chem., Int. Ed. Engl 1982, 21, 284–284.

[15]. Hansch C, Leo A, Taft RW, Chem. Rev 1991, 91, 165–195.

[16]. a)Weigend F, Ahlrichs R, Phys. Chem. Chem. Phys 2005, 7, 3297–3305 [PubMed: 16240044] b)Zhao Y, Truhlar DG, Theor. Chem. Acc 2007, 120, 215–241c)Grimme S, Antony J, Ehrlich S, Krieg H, J. Chem. Phys 2010, 132, 154104. [PubMed: 20423165]

[17]. Marenich AV, Cramer CJ, Truhlar DG, J. Phys. Chem. B 2009, 113, 6378–6396. [PubMed: 19366259]

[18]. Yang J, Karver MR, Li W, Sahu S, Devaraj NK, Angew. Chem., Int. Ed. Engl 2012, 51, 5222–5225. [PubMed: 22511586]

[19]. a)Selvaraj R, Fox JM, Curr. Opin. Chem. Biol 2013, 17, 753–760 [PubMed: 23978373] b)Xu M, Galindo-Murillo R, Cheatham TE, Franzini RM, Org. Biomol. Chem 2017, 15, 9855–9865. [PubMed: 29139516]

[20]. a)Willems LI, van der Linden WA, Li N, Li KY, Liu N, Hoogendoorn S, van der Marel GA, Florea BI, Overkleeft HS, Acc. Chem. Res 2011, 44, 718–729 [PubMed: 21797256] b)Kovalova A, Pohl R, Vrabel M, Org. Biomol. Chem 2018, 16, 5960–5964; [PubMed: 30091427] c)Reisacher U, Ploschik D, Rönicke F, Cserép GB, Kele P, Wagenknecht H-A, Chem. Sci 2019, 10, 4032–4037 [PubMed: 31015943] d)Svatunek D, Houszka N, Hamlin TA, Bickelhaupt FM, Mikula H, Chem. Eur. J 2019, 25, 754–758. [PubMed: 30347481]

[21]. a)Devaraj NK, ACS Cent. Sci 2018, 4, 952–959 [PubMed: 30159392] b)De La Cruz LKC, Benoit SL, Pan Z, Yu B, Maier RJ, Ji X, Wang B, Org. Lett 2018, 20, 897–900. [PubMed: 29380605]

[22]. a)Willems LI, Li N, Florea BI, Ruben M, van der Marel GA, Overkleeft HS, Angew. Chem., Int. Ed. Engl 2012, 51, 4431–4434 [PubMed: 22438272] b)Simon C, Lion C, Spriet C, Baldacci-Cresp F, Hawkins S, Biot C, Angew. Chem., Int. Ed. Engl 2018, 57, 16665–16671 [PubMed: 30370981] c)Schart VF, Hassenruck J, Spate AK, Dold J, Fahrner R, Wittmann V, ChemBioChem 2019, 20, 166–171. [PubMed: 30499611]

[23]. a)Ramil CP, Lin Q, Chem. Commun 2013, 49, 11007–11022b)Li J, Kong H, Huang L, Cheng B, Qin K, Zheng M, Yan Z, Zhang Y, J. Am. Chem. Soc 2018, 140, 14542–14546. [PubMed: 30351919]

[24]. Li J, Chen PR, Nat. Chem. Biol 2016, 12, 129–137. [PubMed: 26881764]

[25]. Shao Z, Liu W, Tao H, Liu F, Zeng R, Champagne PA, Cao Y, Houk KN, Liang Y, Chem. Commun 2018, 54, 14089–14092.
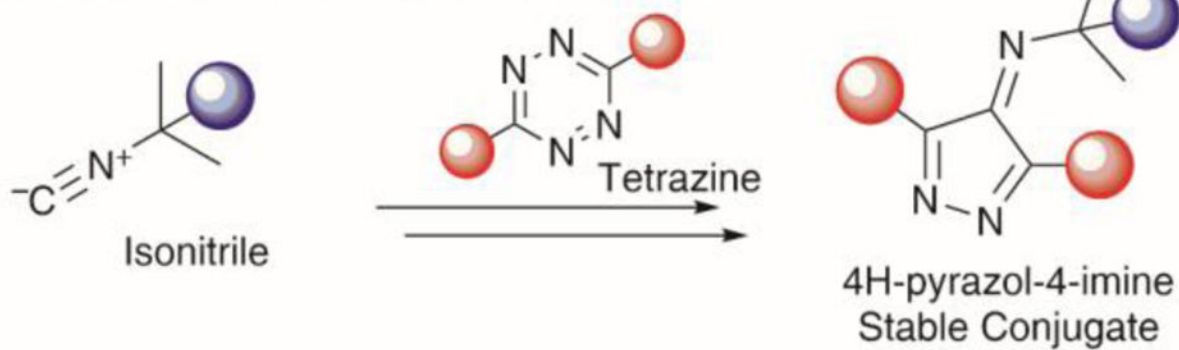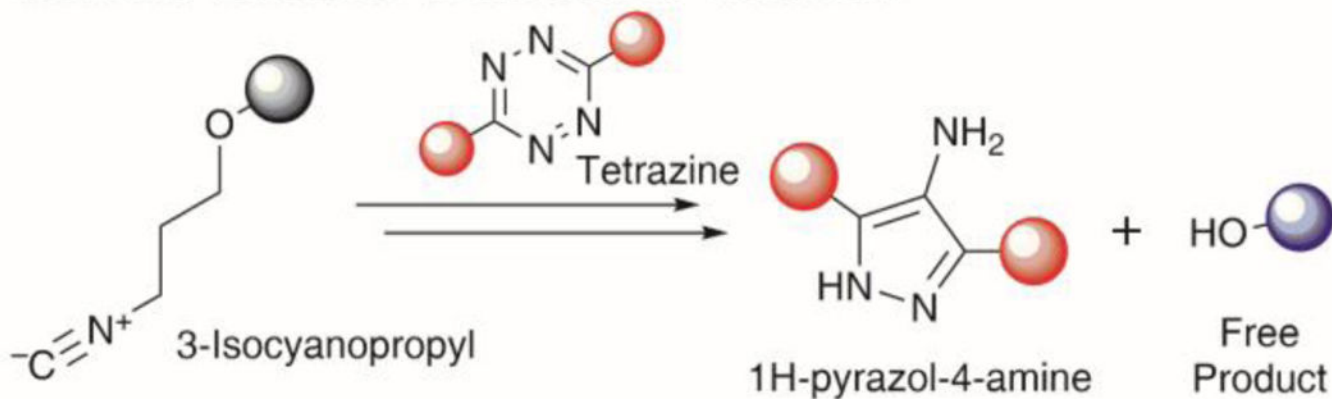
**Figure 1.**
The cycloaddition of isonitriles and tetrazines is a bioorthogonal reaction that can form stable conjugates or elicit the release of molecules.
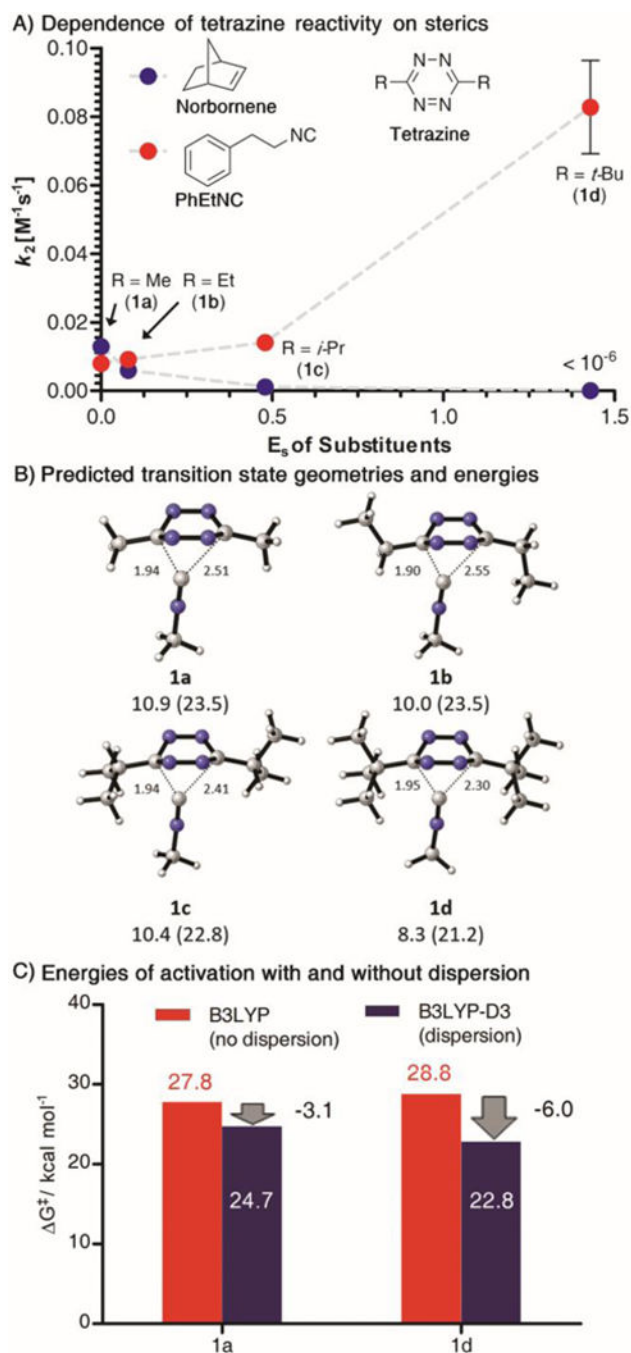
**Figure 2.**
Dispersion forces increase the reactivity of isonitriles to tetrazines with bulky substituents.
A) Bimolecular reaction rates recorded for PhEtNC and norbornene as a function of the
Taft's steric parameter $E_s$ of tetrazine alkyl substituents. B) M06–2X-D3(SMD) calculated
transition state geometries and energies of activation for the rate determining step of the
reaction of tetrazines **1a-d** with methylisonitrile. Energies in kcal/mol, distances in Å,
electronic energy and Gibbs free energy (in brackets) is shown. C) Gibbs free energies of
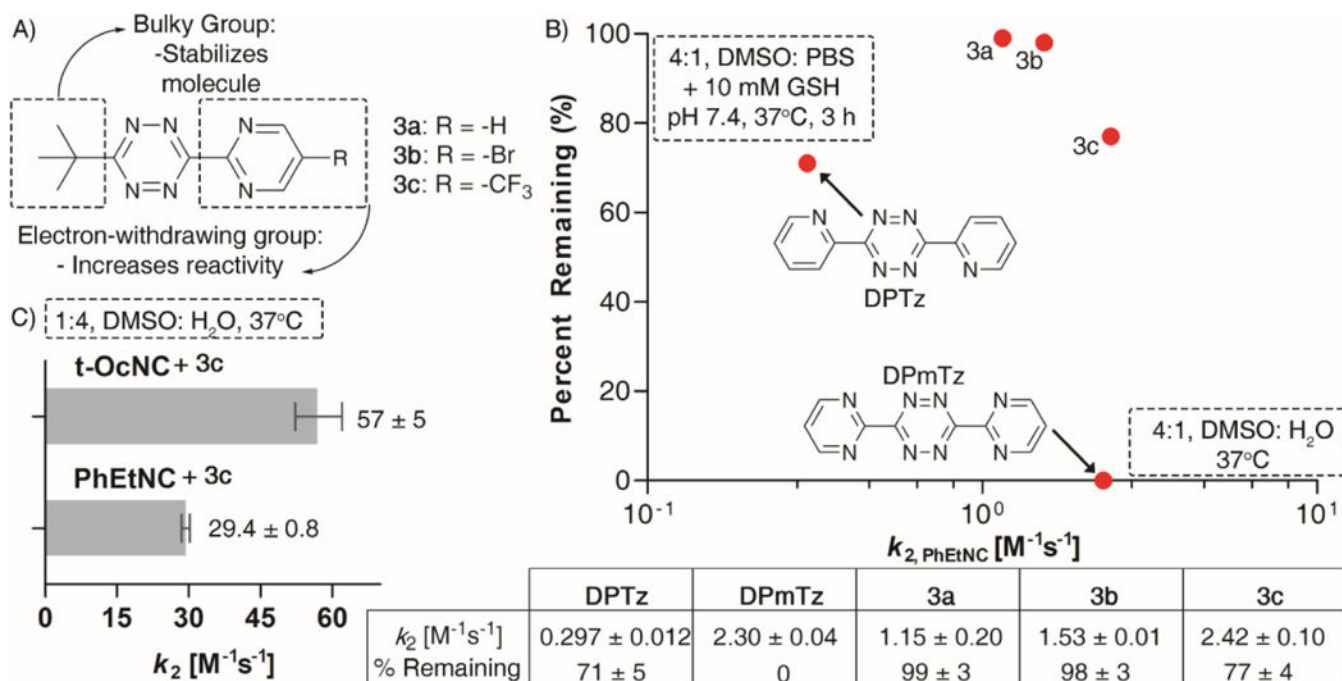
activation (kcal/mol) for the reaction of methylisonitrile and tetrazines **1a** and **1d** were calculated using the B3LYP functional with and without correction for dispersion forces.
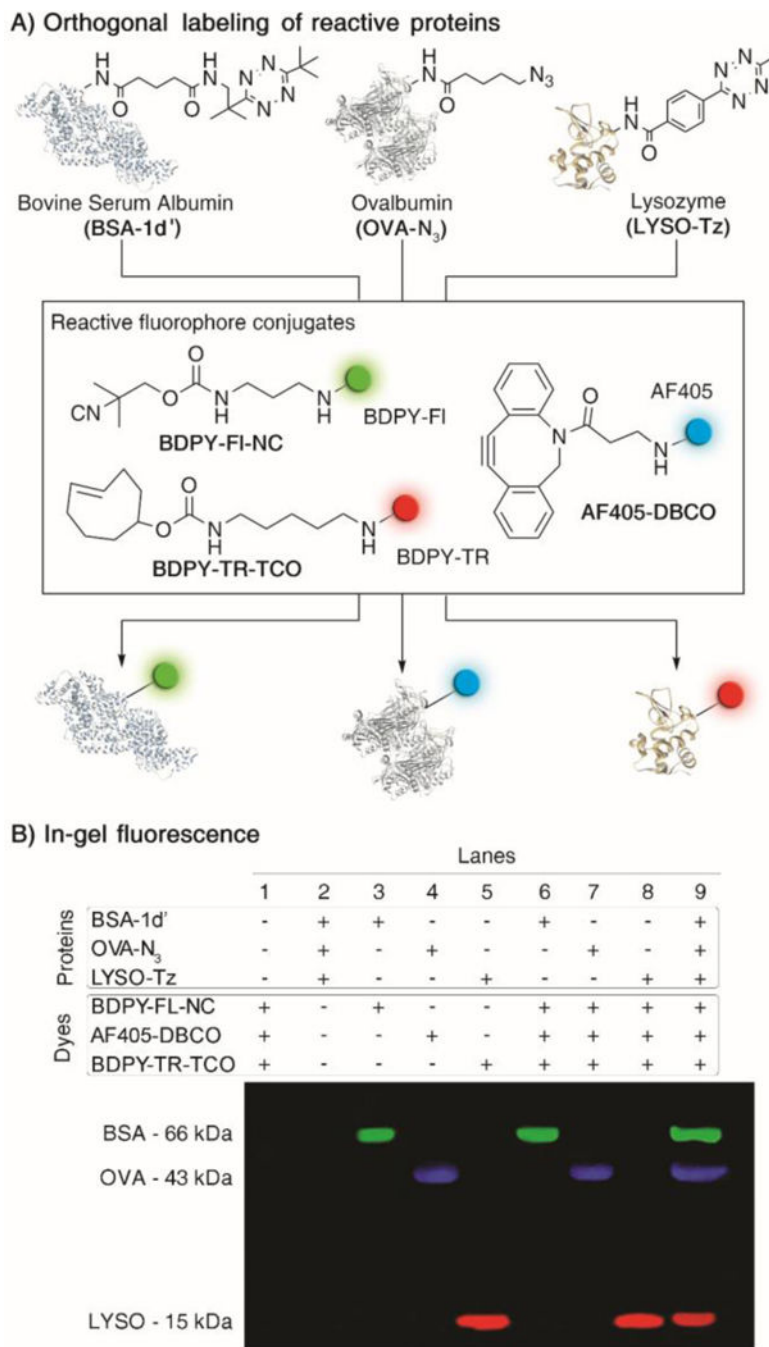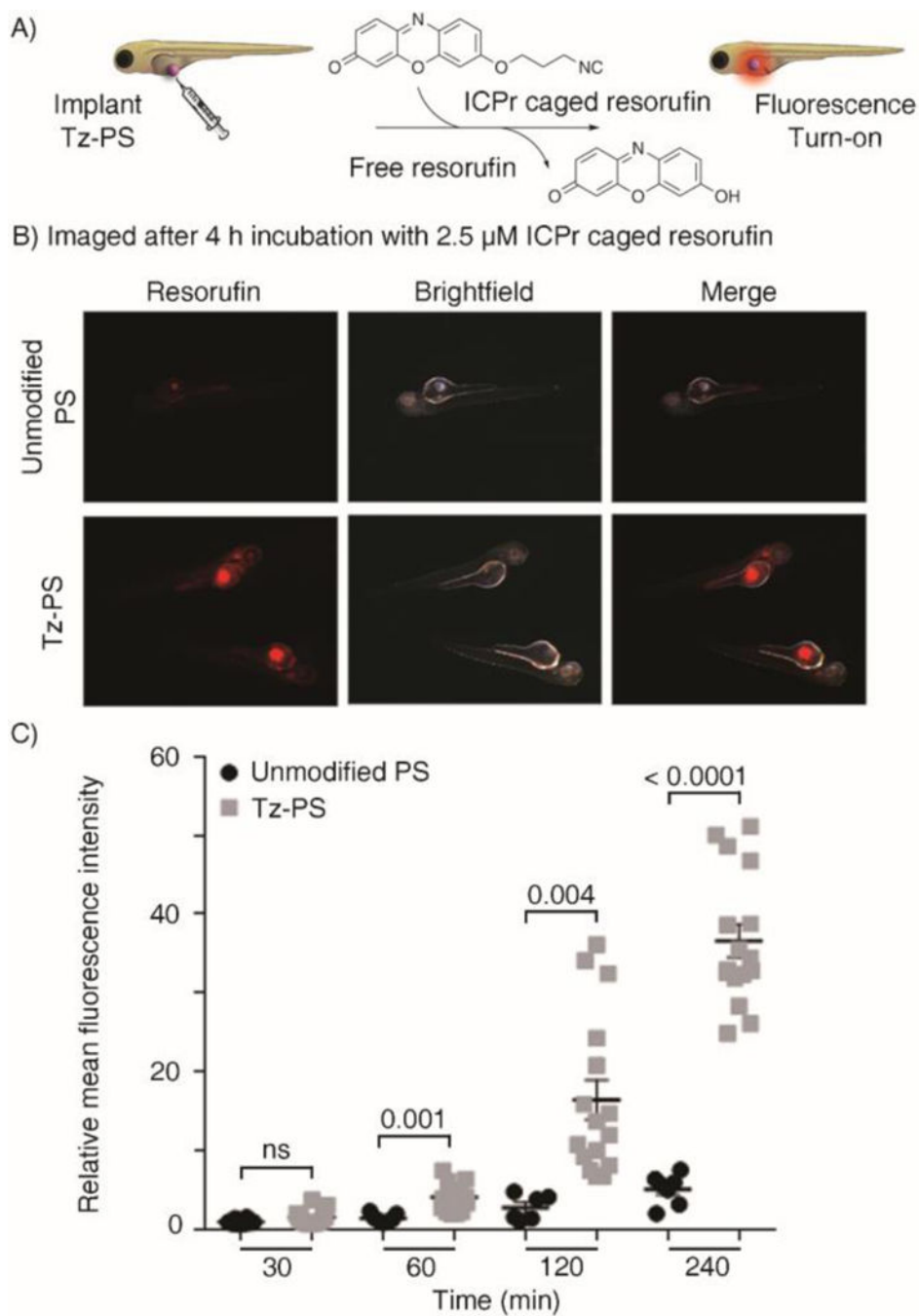
**Figure 3.**
Asymmetric tetrazines containing t-butyl and pyrimidyl substituents (**3a-c**) exhibit high stability and robust reactivity with isonitriles. A) Structures of **3a-c** and principles underlying their design. B) Rates of the reaction of tetrazines with PhEtNC (DMSO:$H_2O$ = 4:1, T = 37°C) and stability of tetrazines in thiol-containing buffer (t = 3 h, DMSO:PBS (pH 7.4) = 4:1 plus 10 mM GSH, T = 37°C). Experimental values are provided in table. C) Rate of the reaction of **3**c and a primary (PhEtNC) and tertiary (t-OcNC) isonitrile under high-water conditions (DMSO:$H_2O$ = 1:4, T = 37°C).

**Figure 4.**
Triple-orthogonal labelling of BSA, ovalbumin (OVA), and lysozyme (LYSO) proteins. A) Schematic of the triple-orthogonal protein labelling experiment. B) In-gel analysis of the fluorescent labelling of BSA-1d', OVA-N$_3$, and LYSO-Tz through chemoselective reactions with BDPY-FL-NC (200 μM), AF405-DBCO (200 μM), and BDPY-TR-TCO (100 μM), respectively. The stained protein gel is shown in Fig. S10.

**Figure 5.**
Demonstration of in-vivo use of isonitrile/tetrazine chemistry. A) Schematic for the release of fluorescent resorufin in zebrafish implanted with a tetrazine-modified resin. B) Efficient turn-on of fluorescence signal. C) Quantification of fluorescence turn-on in the presence of Tz-PS versus unmodified beads over time.

**Table 1.**

Second-order rate constants of reactions between tetrazines **DPTz**, **3c**, **1d** and several dienophiles.



| Tetrazine[a] | k$_{2,TCO}$ [M$^{-1}$s$^{-1}$] | k$_{2,Nb}$ [M$^{-1}$s$^{-1}$] | k$_{2,Cp}$ [M$^{-1}$s$^{-1}$] | k$_{2,PhEtNC}$ [M$^{-1}$s$^{-1}$] | k$_{2,t\text{-}OcNC}$ [M$^{-1}$s$^{-1}$] |
|---|---|---|---|---|---|
| **DPTz** | > 100 | 0.827 ±0.042 | 0.563 ±0.025 | 0.297 ±0.012 | 1.48 ±0.03 |
| **3c** | 0.234 ±0.002 | 0.013 ±0.005 | 0.588 ±0.055 | 1.53 ±0.01 | 6.27 ±0.05 |
| **1d** | n.d.[b] | n.r.[c] | n.d.[b] | 0.070 ±0.007 | 2.25e-03 ±3.54e-05 |

[a] Conditions: 0.2 mM tetrazine, 2 mM – 20 mM dienophile, DMSO:H$_2$O (4:1), T = 37°C, triplicate.

[b] not determinable; slow kinetics prevented appropriately fitting curves; $k_2 < 10^{-3}$ M$^{-1}$s$^{-1}$

[c] no reaction; $k_2 < 10^{-6}$ M$^{-1}$s$^{-1}$