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Published on: 23 Oct 2019 - Angewandte Chemie (John Wiley & Sons, Ltd)

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Submitted on 6 Feb 2020

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Bridge-Clamp Bis(tetazine)s with [N]_8 \pi-Stacking Interactions and Azido-s-Aryl Tetrazines: Two Classes of Doubly Clickable Tetrazines

A double-double! Two complementary new classes of s-aryl tetrazines are presented with two independent and selectively clickable functions. Ortho-constraint in their building generates unprecedented structural features such as [N]_8 London dispersion forces.


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Bridge-Clamp Bis(tetrazine)s with [N]8 π-Stacking Interactions and Azido-s-Aryl Tetrazines: Two Classes of Doubly Clickable Tetrazines

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Abstract: Tetrazine core “click-chemistry” is a blooming method for bioorthogonal labeling and crosslinking. We introduce two new classes of doubly clickable s-aryl tetrazines synthesized by Cu-catalyzed cross-coupling. Homocoupling arylation applied to o-brominated s-aryl tetrazines leads to bis-tetrazines structurally-characterized by tetrazine cores arranged “face-to-face”. Their previously unseen bridge clamp structure was investigated by DFT. London dispersion forces, which are experimentally evidenced for the first time as [N]8-π-stacking interactions, are essential to the conformation. Upon inverse Electron Demand Diels-Alder (iEDDA) cycloaddition the o-biphenyl motif of bis-tetrazines produces a unique structuring stapling tool. The α-azidation of s-aryltetrazines introduces a second proximal intermolecular clickable function that opens the way to double clicking chemistry opportunities. The stepwise facile introduction of fluorophores (coumarin, pyrene) then iEDDA cycloaddition, including bioconjugation to antibodies, was achieved on this class of tetrazines. The Cu-based synthetic toolbox extends to (thio)-etherification, phosphination, trifluoromethylation and the introduction of various bioactive nitrogen-based heterocycles. s-Tetrazines (Tz) are the object of high interest in biochemistry and photophysics.\[1-8] Notably, the preparation of tailored s-tetrazine allows their implementation via pyridazine reductive condensation or halide tetrazines SNX reactions.[9]

In our investigation dedicated to generalize copper-catalyzed s-aryltetrazines functionalization (Tables S1-S4) we devised C–C homocoupling from o-bromo-s-aryltetrazines 1a-e to produce in a single step biphenyl-bis-tetrazines 2a-e (Figure 1). These compounds were analyzed by X-ray diffraction (XRD), showing a previously unseen “bridge clamp” structure together with the existence of various π-stacking, possibly including puzzling [C2N2]...[C2N2] core interactions. Noncovalent π-interactions are of fundamental interest for structuring and molecular recognition within chemical and biological systems.[23,24] Aromatic interactions with very electron-poor heterocycles such as tetrazines are ill-known, despite the booming of supramolecular and biochemistry applications of tetrazines.[25] Thus, the bridge clamp bis-tetrazines 2a-e are ideal models for studying such π-stacking.

structures present previously unseen [N]8-π-stacking interactions, which operate between the electron-poor heterocycles Tz core. A second family of doubly clickable tetrazine was designed from azidation of s-aryltetrazines. The selective click-chemistry properties of both families were also illustrated in inverse Electron Demand Diels-Alder (iEDDA) cycloaddition. Finally, we generalized nucleophilic Cu-catalyzed cross-coupling in o-bromo-s-tetrazines in order to obtain s-aryltetrazines incorporating O, S, N and P heteroatoms, which are not easily reachable by the current synthetic methodologies like Pinner condensation or halide tetrazines SNX reactions.[1]

Figure 1. Bis-tetrazines obtained from oxidative homocoupling of o-bromo-s-aryltetrazines. Molecular views of “bridge clamp” structure of 2a (bottom, for XRD structures of 2b-d see crystal data SI). Isolated yields are given.

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We envisioned that DFT studies may help highlighting the role of London dispersion forces in the bridge clamp structures.\[26\] We thus performed DFT calculations using two complementary approaches: a standard functional that is recognized to neglect dispersion effect and another functional which properly describes dispersion effects from robust empirical corrections (details in SI, Tables STh1-8 and Figures STh1-7). We first computed the energy for the rotation of tetrazine cores around the O–C–C bond formed (Figure 2, \(\theta\) angle). Two minima were identified, the most stable one (Fig. 2, \(\theta = 54^\circ\)) corresponds to the clamp conformers experimentally obtained for all bis-Tz [XRD data (in \(\circ\)): 2a, \(\theta = 51.1(3)\); 2b, \(\theta = -57.7(3)\); 2c, \(\theta = 52.5(3)\), 2d, \(\theta = 55.7(2)\)]. Interestingly, a second conformer is stabilized, which corresponds to a gauche mutual conformation of the tetrazine cores (Fig. 2, \(\theta = 123^\circ\)). These conformations are isoenergetic when dispersion effects are not included, giving \(\theta = 64^\circ\) and 125°, respectively. Conversely, dispersion clearly stabilizes the clamp geometry by about 4.8 kcal mol\(^{-1}\). In the absence of London dispersion forces between Tz cores the interconversion barrier between the stable rotamers is low at 2 kcal mol\(^{-1}\) (conformer with pseudo-orthogonal Tz core, \(\theta = 79^\circ\)) while their inclusion significantly enhances TS barrier up to 6.5 kcal mol\(^{-1}\).

Figure 2. i) Relative electronic energy for rotational isomerization of the bis-Tz 2a taking into account dispersion effect (values in black) or not (values in blue); ii) Visualization of the \(\pi\)-stacking interactions in 2a.

These calculations were also conducted with bis-Tz halide derivatives 2b-e, which confirmed that the clamp conformation is stabilized by c.a. 3.3 to 4.8 kcal mol\(^{-1}\). This stabilization is exclusively due to London dispersion (visualization in ii) Fig. 2),\[26\] and comes mainly from \(\pi\)-stacking between the tetrazine cores (estimated for 2a at 1.9 kcal mol\(^{-1}\), see SI), completed by the dispersion attraction between the terminal phenyl groups (1.4 kcal mol\(^{-1}\)).\[27\] In addition, we calculated that \(\pi\)-stacking in the bis-Tz clamp conformers 2a-e is greater than in intermolecular benzene dimer and in the tetrazine dimer that accounts respectively for 3.2 and 2.7 kcal mol\(^{-1}\), respectively. Our results supported by DFT provide the first experimental proof that very significant noncovalent \(\pi\)-interactions involving s-tetrazine cores are possible.\[28\] These are pertinent in relation to binding and molecular recognition behavior of electron-poor heteroaromatics, especially with the general use of tetrazine derivatives in biological milieu.\[29\]

Owing to its high reactivity and bioorthogonal nature, iEDDA cycloaddition is used with tetrazines for bioconjugation and has also been applied successfully in materials synthesis.\[30\] We illustrated the potential of our new class of bridge clamp doubly clickable bis-tetrazine by reaction of 2a with dienophiles to form an adduct in iEDDA reaction using the bicyclononynes (BCNs) 3a and 3b (Figure 3). Cycloaddition of aryltetrazines with BCNs was monitored by UV-Vis spectroscopy from decay of the typical large absorption band around 550 nm (Figures S1-S2).\[164\] The double cycloaddition to 2aa was achieved in 30% in 90 min showing a fairly slow reaction rate \(k_{ap} = 0.004\) min\(^{-1}\).\[164\] The strong steric influence of the bridge clamp was even more pronounced when the hindered dienophile 3b was used at room temperature since then only a single cycloaddition to 2ab was selectively achieved (\(k_{ap} = 0.0013\) min\(^{-1}\), c. a. 12% in 120 min, 100% in 24 h). The second cycloaddition is accessible above 40 °C. Therefore, the constraint dimeric bridge clamp structure of 2a provides a way for temperature-controlled selective monocycloaddition in these compounds. The XRD of 2aa (see crystal data SI) shows the rigid staple structuring effect insured by the constraint o-biphenyl motif, which distinguishes bis-tetrazines 2a-e from any other clickable tetrazine reported to date.

![Figure 3. iEDDA di- and monocycloaddition of bis-tetrazine 2a with BCNs at rt.](image)

We further envisioned that the introduction of a second clickable functionality on s-tetrazines, different and complementary to the tetrazine core used in iEDDA cycloaddition, would be highly valuable for the development of synthetic routes to more diverse tetrazine structures.\[15,16,29-31\] In particular, the introduction of the versatile azide function would be advantageous for stepwise introduction of various functionalities by click-chemistry. The synthesis of the azide tetrazine derivative 5a was achieved in dioxane by using Cu-catalyzed nucleophilic coupling of 1a with azido-trimethylsilane (Figure 4). As expected the reactive azido-aryl-s-tetrazine 5a easily achieved click coupling with alkyne 6a at 25 °C, yielding after 30 min the new triazole-tetrazine 7a (61%, a XRD structure of 7a was resolved, Fig. 4). Huisgen azide-alkyne cycloaddition occurs selectively and the tetrazine core remains untouched. One-pot azidation/cycloaddition was optimized in good yield,
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We investigated the potential of our new classes of clickable molecules in iEDDA cyclization by reaction of the tetrazines 5a, 7b and 7e with bicyclononyne dienophile 3a (Figure 5 and Figures S3-S5). Pleasingly, the formation of 7ba and 7ea from fluorophore-containing 7b and 7e respectively, went to completion in less than 1 h, with fast rate of $k_{app} = 0.070 \text{ min}^{-1}$ and 0.085 min$^{-1}$, respectively.

Figure 4. i) Cu-catalyzed azidation of o-bromophenyl-α-tetrazine 1a and alkyne cycloaddition; ii) One-pot azidation/cycloaddition. Isolated yields are given.

Interestingly, the iEDDA cyclization of azide-functionalized 5a was also achieved, showing that at room temperature Tz core cyclization function ($k_{app} = 0.026 \text{ min}^{-1}$) mostly preserved the azide, for which a much slower decay was estimated with $k_{app2} = 0.0023 \text{ min}^{-1}$. Therefore, a conversion up to 90% in azide-functionalized 5aa is obtained in less than 3 h that opens the possibility of further click-chemistry via the mostly preserved azide function.

Figure 5. Doubly clickable tetrazine 5a stepwise used in iEDDA cycloaddition with fluorophore incorporation or azide function preserved.

As a definitive proof-of-concept illustrating a bioconjugation scenario, 5a was conjugated with the antigen-binding fragment (Fab) of Pertuzumab (M=50 kDa) and Trastuzumab (full-length antibody, M=150 kDa, Figures S6a-d). Those were beforehand randomly attached to activated esters of BCN (an average two), BCN-p-NPE (bicyclononyne p-nitrophenol ester, Figures 6a, and 6b–blue). The MALDI/ToF analyses show that the expected iEDDA reaction is smoothly proceeding, giving an average mass increase of 509 Da, which is attributable to the coupling of two tetrazines 5a to BCN-modified Pertuzumab (Figure 6b–green).

Figure 6. (a) Bioconjugation reaction of 5a and Fab fragment of Pertuzumab via BCN iEDDA. (b) MALDI/ToF comparative analysis. (c) ESI-MS spectra superposition before (orange) and after (blue) BCN introduction, thus orange
The Orbitrap mass analysis (Figures 6c-d) details the distribution of the conjugation mixtures, which includes zero to four BCN attached per Fab (blue peaks in Figures 6c), which can then randomly undergo coupling to one to three tetrazine 5a (green peaks in Figure 6d). This clearly confirmed the easy and versatile course of iEDDA from Tz core mostly preserving the azide function. The minor presence of probes attachment to the protein via azide–alkyne cycloaddition (SPAAC) between the BCN and the azide function is however illustrated in Figure 6d by the minor peaks 1-3, for which a mass increase of 275 Da is observed instead of the 247 Da expected from iEDDA.[33] Naturally, bioconjugation by iEDDA was selectively accessible by using antibodies modified with trans-cyclooctene (TCO).[31,34] Instead of BCN (Figures S6e-g), thus fully preserving the azide function for further conjugation.

Encouraged by the synthetic compatibility of tetrazine core with copper in homocoupling and azidation, we extended our catalysis toolbox to a range of valuable nucleophilic C–C bond formation. Cu(OAc)$_2$·H$_2$O combined with TMEDA was efficient for Cu–C coupling of 3-(2-bromophenyl)-6-phenyl-1,2,4,5-tetrazine with a large range of functionalized phenols (13a-n, Figure 7, XRD resolved for 13d, see SI). Nitrogen-rich tetrazines provide relevant coordination compounds and energy materials.[4] Recently, air-stable Fe(II) coordination complexes with triazolotetrazine ligands are controllable as low-energy laser initiation explosives.[44] We thus extended the C–O bond protocol to C–N bond formation for the straightforward production of novel tetrazine-azole derivatives (14a-i, Fig. 7).

Pyrazole-tetrazines 14a-d formed from C–N coupling using CuI as catalyst. CH$_3$ and CF$_3$ groups in C3 position of the pyrazole was tolerated to give 14b and 14c (XRD structure resolved for 14c, Fig. 7 and SI). A methyl group in C4 was tolerated and 14d formed in high yield. Indazole coupling gave 14e, while imidazole coupling limited the formation of 14f. We achieved pyrrole coupling to form pyrrole-tetrazine 14g and indole-tetrazine 14h in satisfactory yield. Coupling of the nonaromatic amine pyrrolidinone successfully gave 14i. Cu-catalysis allowed also thioetherification of s-aryltetrazine via C–S bond formation using thiophenols. In contrast to C–O and C–N bond formation, we observed the reduction of the tetrazine core that was re-oxidize in situ during workup. We thus isolated sulfur tetrazine 15a in good yield. Cu-catalyzed C–P bond formation that is more less developed,[35] was also achieved from secondary aryl and alkyl phosphines 11a,b. Re-oxidation to tetrazine was necessary to yield phosphine oxide 16a and 16b (XRD structure solved for 16b, Fig. 6 and SI).[36] Trifluoromethylated compounds are also topical in medicinal chemistry,[37] and we successfully achieved nucleophilic Cu-catalyzed alkylation to s-tetrazine using CF$_3$-silane.[38] The halogenated trifluoromethylated-Tzs 17a-d were synthesized (XRD resolved for 17a, see SI) using dioxane as solvent that limits Tz dehalogenation side-reaction. All these o-substituted s-aryltetrazines are highly colored in solution and in the solid state (red to purple, Figures S7-S8) but are not fluorescent.[1,39] Notably, in C–X bond formation we showed that Cu redox-processes (expected from catalysis) do not systematically interfere with the reducible tetrazine core. Our ongoing studies are related to materials and energy chemistry applications for 14a-i and ligand chemistry for 16a,b (as relevant Buchwald’s biphényl phosphines analogues).

In summary, we generalized copper-catalyzed s-aryltetrazines functionalization to efficient Csp$_2$–Csp$_2$ homocoupling, and to obtain s-aryltetrazines selectively incorporating ortho-positioned O, S, N and P heteroatoms that were not reachable by the current Tz synthetic methodologies. Accordingly, several hitherto not reported ortho-ethers, -thioethers, -N-heterocyclic and phosphine-oxide s-aryl tetrazines were synthesized. From an applied perspective, we then described two new classes of doubly clickable s-aryltetrazines readily accessible for bioorthogonal applications and materials “clicking”. Bis-tetrazines displaying a unique bridge clamp structure are ideal models for studying weak π-stacking interactions in heteroaromatics. They also allow for unprecedented rigid Tz-stapling by double iEDDA cyclization, which can be optionally conducted stepwise by temperature-control. Azide s-aryltetrazines open opportunities in fast rate bioconjugation from a pre- or post-clickable practical tool.

**Acknowledgements**

This work was jointly supported by the CNRS, the Université de Bourgogne (CoMUE BFC and ISITE-BFC via UB180013.MUB. IS_SmartTZ), the Conseil Régional de Bourgogne through the plan d’actions régional pour l’innovation (PARI) and the
Keywords: Tetrazines • Click Chemistry • London Dispersion Forces • Heteroareomatons • IEDDA Cycloadition