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Bridge Clamp Bis-Tetrazines Stacked by $[N]_8$-π-Interactions and Azido-*s*-Aryl Tetrazines: New Classes of Doubly Clickable Tetrazines

Clève D. Mboyi, Delphine Vivier, Ahmad Daher, Paul Fleurat-Lessard,* Hélène Cattey, Charles H. Devillers, Claire Bernhard, Franck Denat, Julien Roger* and Jean-Cyrille Hierso*

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 o-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 formation for bioconjugate chemistry (Tz click-chemistry).[9-15] This field experienced blooming applications in supramolecular chemistry and bioorthogonal chemistry.[16] Accordingly, the development of synthetic routes to more diverse tetrazine structures has become very useful since it diversifies current applications, and facilitates the emergence of new ones.[17,18] In this context, we envisioned that a facile synthetic access to bis-tetrazines,[6,19,20] would be pertinent regarding general tetrazine click-chemistry, usable for bridging (and stapling) in cycloaddition reactions for biological systems,[21] and materials applications.[22] Herein, we devised a Cu-catalysed protocol which allows the efficient synthesis of constraint bis-tetrazines in good yield. The influence of London forces in these new biphenyl-bis-tetrazines were studied by DFT. Such polyaromatic 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 SN_{Ar} reactions.[1]

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 $[C_2N_4]$…$[C_2N_4]$ 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.

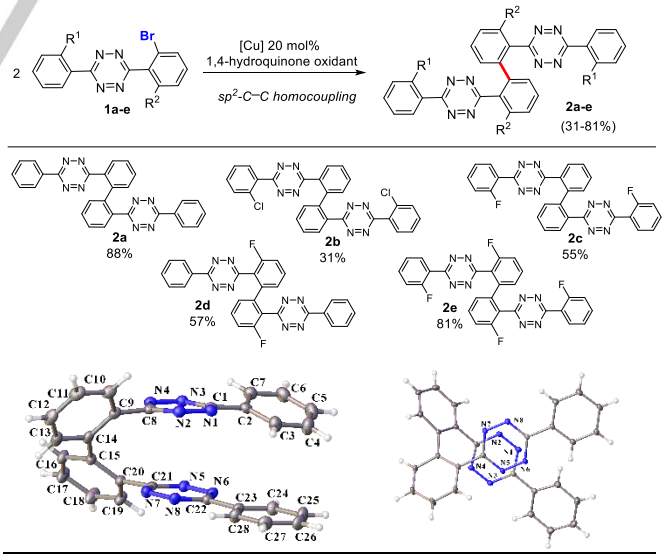

Figure 1. Bis-tetrazines obtained from oxidative homocoupling of *o*-bromo-*s*-aryltetrazines. Molecular views of "bridge clamp" structure of **2a** (bottom, for XRD structures of **2b-d** see crystal data SI). Isolated yields are given.

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

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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, θ angle). Two minima were identified, the most stable one (Fig. 2, θ = 54 °) corresponds to the clamp conformers experimentally obtained for all bis-Tz [XRD data (in °): **2a**, θ = 51.1(3); **2b**, θ = –57.7(3); **2c**, θ = 52.5(3), **2d**, θ = 55.7(2)]. Interestingly, a second conformer is stabilized, which corresponds to a *gauche* mutual conformation of the tetrazine cores (Fig. 2, θ = 123 °). These conformations are isoenergetic when dispersion effects are not included, giving θ = 64 ° 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, θ = 79 °) while their inclusion significantly enhances TS barrier up to 6.5 kcal mol^{-1}.

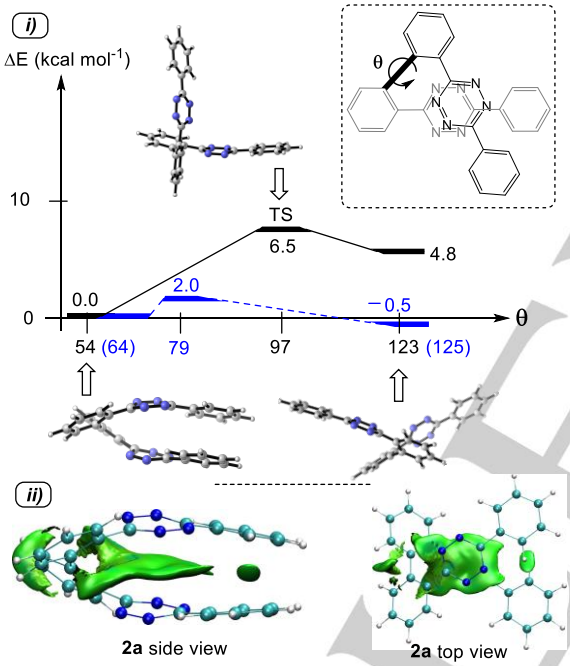

Figure 2. *i)* Relative electronic energy for rotational isomerization of the bis-Tz **2a** taking into account dispersion effect (values in black) or not (values in blue); ii) Visualization of the π-stacking interactions in **2a.**

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 π-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 π-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 π-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).[18a] The double cycloaddition to **2aa** was achieved in 30% in 90 min showing a fairly slow reaction rate k_{app} = 0.004 min^{-1}.[18a] 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_{app} = 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.

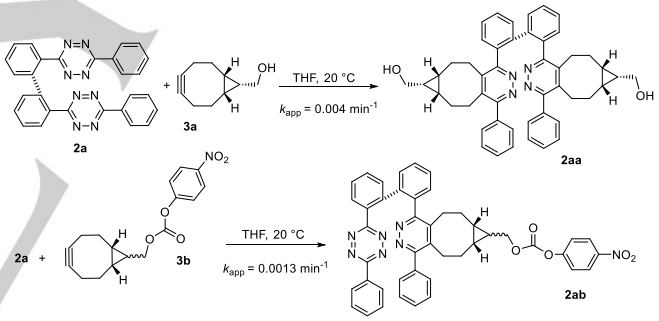

Figure 3. iEDDA di- and monocycloaddition of bis-tetrazine **2a** with BCNs at rt.

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.[5,9-16,31] 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, leading to a series of new tetrazines incorporating fluorophores such as coumarin and pyrene (Fig. 4).

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

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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 min^{-1} and 0.085 min^{-1}, respectively.

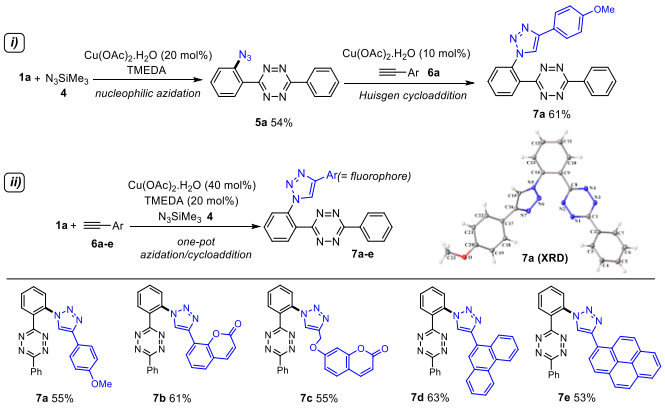

Figure 4. *i*) Cu-catalyzed azidation of *o*-bromophenyl-*s*-tetrazine **1a** and alkyne cycloaddition; *ii*) One-pot azidation/cycloaddition. Isolated yields are given.

Interestingly, the iEDDA cyclization of azide-functionalized **5a** was also achieved, showing that at room temperature Tz core cyclization function (k_{app1} = 0.026 min^{-1}) mostly preserved the azide, for which a much slower decay was estimated with k_{app2} = 0.0023 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.

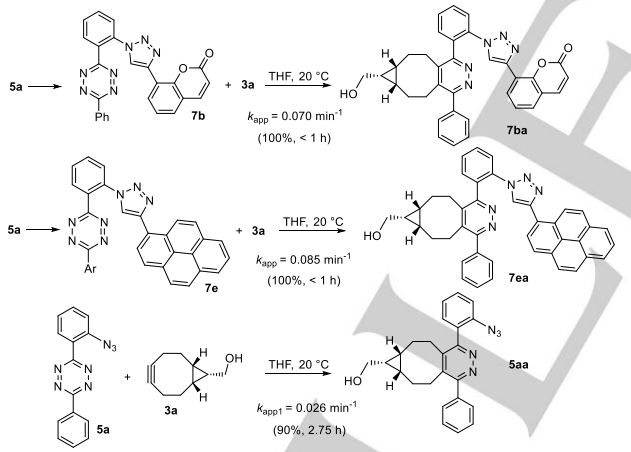

Figure 5. Doubly clickable tetrazine **5a** stepwise used in iEDDA cycloaddition with fluorophore incorporation or azide function preserved.

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).[32] Those were beforehand randomly attached to activated esters of BCN (*in 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).

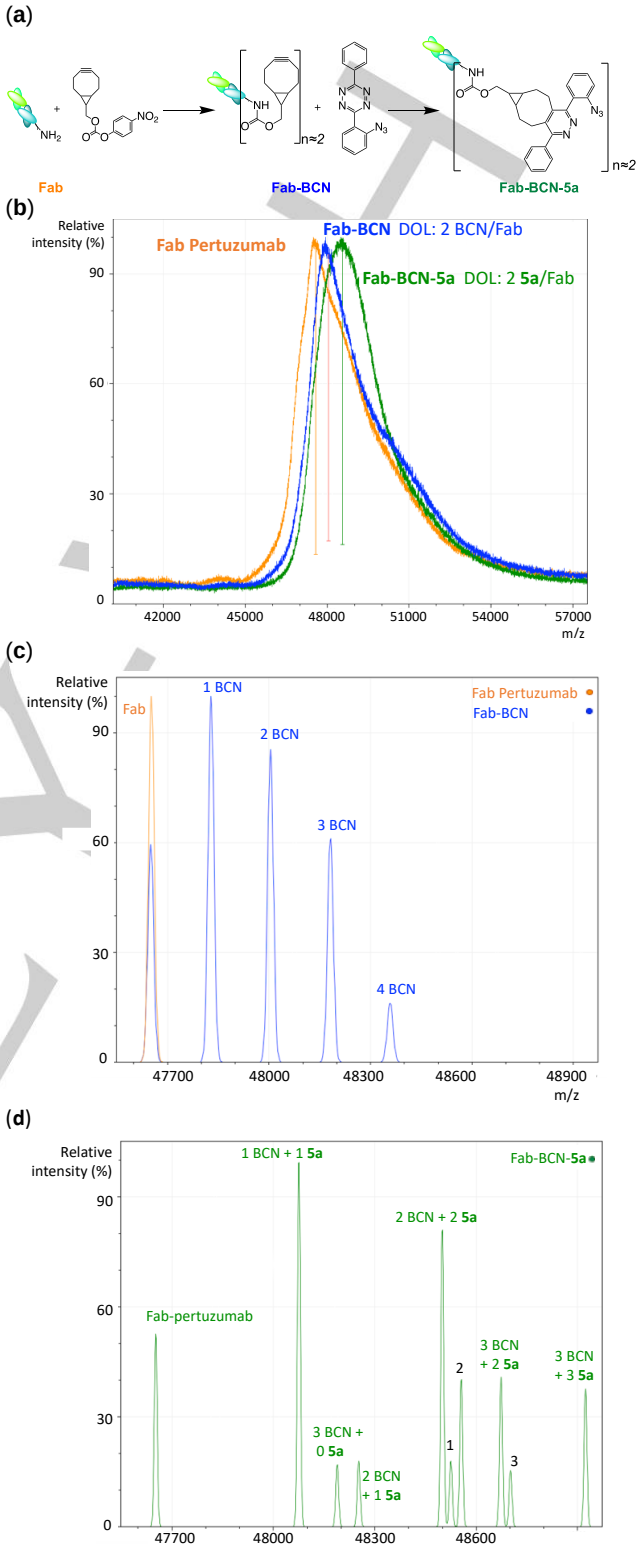

Figure 6. (**a**) Bioconjugation reaction of **5a** and Fab fragment of Pertuzumab *via* BCN iEDDA. (**b**) MALDI/ToF comparative analysis. (**c**) ESI-MS spectra superposition before (orange) and after (blue) BCN introduction, thus orange peaks account for the pristine Fab, and blue peaks account for the Fab randomly conjugated to BCN moieties. (**d**) ESI-MS spectra after bioconjugation, green peaks account for the Fab–BCN clicked to **5a**. DOL = degree of labeling. Minor peaks 1, 2 and 3 in (**d**) illustrate the presence of probe attachment via SPAAC.

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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),[31a] 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–X bond formation. $Cu(OAc)_2.H_2O$ combined with TMEDA was efficient for C–O 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.[34] 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).

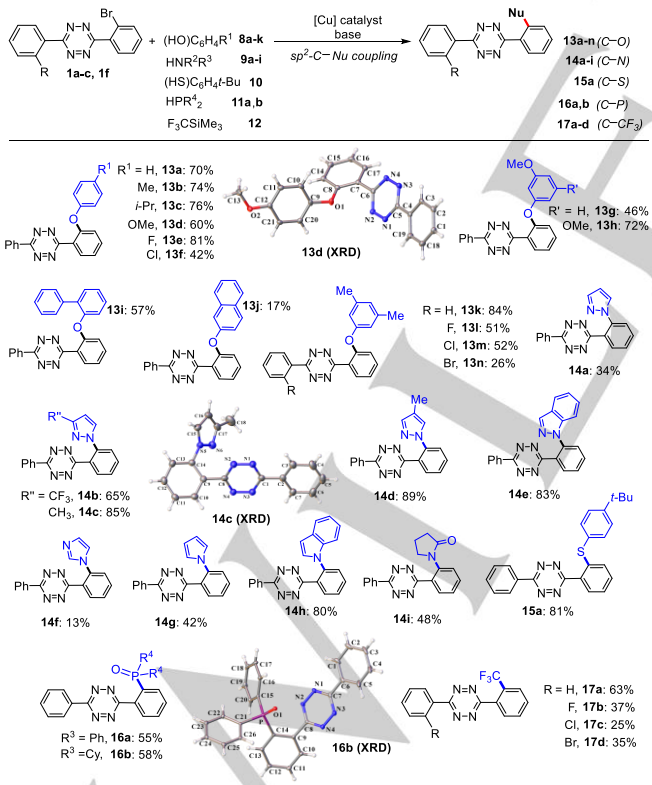

Figure 7. Generalization of Cu-catalyzed nucleophilic coupling of *o*-bromoaryltetrazines: (thio)-etherification, amination and phosphination.

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-catalysed C–P bond formation that is much 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 biphenyl 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.

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Crystallographic data (CIF). CCDC 1908606(**2a**), 1908607(**2b**), 1908608(**2c**), 1908609(**2d**), 1908612(**2aa**), 1908611(**7a**), 1828690(**13d**), 1828689(**13k**), 1828691(**14c**), 1828692(**16b**) and 1908610(**17a**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

Keywords: Tetrazines • Click Chemistry • London Dispersion Forces • Heteroaromatics • iEDDA Cycloaddition

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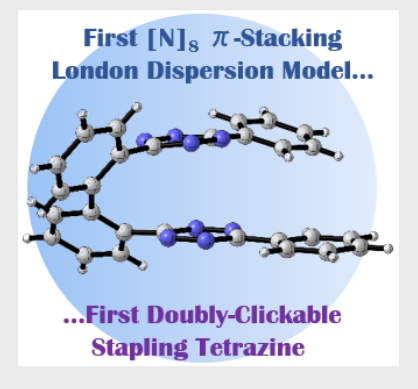
A double-double! Two complementary new classes of *s*-aryl tetrazines are presented with two independent and selectively clickable functions. *Ortho*-constraint in their building generates unprecedented structural features such as $[N]_8$ London dispersion forces.



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