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Acylation-Mediated 'Kinetic Turn-On' of 3-Amino-1,2,4,5-tetrazines

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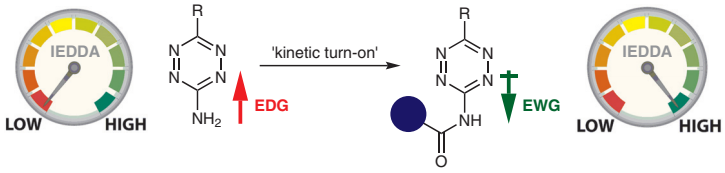

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Abstract The fast and biocompatible ligation of 1,2,4,5-tetrazines with strained alkenes has found numerous applications in biomedical sciences. The reactivity of a 1,2,4,5-tetrazine can generally be tuned by changing its electronic properties by varying the substituents in the 3- and/or 6-position. An increased reactivity of such bioorthogonal probes upon conjugation or attachment to a target molecule has not previously been described. Such an approach would be beneficial, as it would minimize the impact of residual tetrazine reagents and/or impurities. Herein, we describe such a 'kinetic turn-on' of 1,2,4,5-tetrazines upon conjugation. On the basis of the significant increase in reactivity following N-acylation predicted by quantum chemical calculations, we prepared 3-aminotetrazines and their corresponding acetylated derivatives. An investigation of the reaction kinetics indeed revealed a remarkable increase in reactivity upon acylation.

Key words click chemistry, tetrazines, kinetics, bioorthogonal chemistry, Diels–Alder reaction, acylation

The challenge of engineering chemical transformations that can proceed within the complex environment of living systems has led to the research field of bioorthogonal chemistry.[1] To enable a bioorthogonal reaction, the chemical probes that are involved need to exhibit high reactivity, high selectivity, biocompatibility, and metabolic stability. The Staudinger ligation[2] and the strain-promoted azide–alkyne cycloaddition (SPAAC),[3] both developed by Bertozzi and co-workers, were the first bioorthogonal reactions to be described. The SPAAC ligation is based on Sharpless's click chemistry,[4] but can proceed without toxic copper(I), and is therefore suitable for in vivo applications.[1]

The tetrazine ligation between 1,2,4,5-tetrazines and strained alkenes such as norbornene or *trans*-cyclooctene (TCO; **1**) was first described in 2008 by the groups of Fox and Weissleder.[5,6] These inverse electron-demand Diels–Alder (IEDDA)-initiated ligations (Figure 1) have attracted interest because of their in vivo compatibility, selectivity, and exceptionally high reaction rates. In recent years, tetrazine ligations have been applied in the development of numerous applications in biomedical research, including, but not limited to, (i) bioconjugation;[7] (ii) molecular imaging of proteins,[8–10] surface antigens,[11] small molecules/modified drugs,[12,13] lipids,[14] or glycans;[15] (iii) cell modification with nanomaterials for clinical diagnostics;[16] (iv) the development of smart fluorogenic probes;[17–20] (v) bioorthogonal approaches to the identification of drug targets in living cells;[21] and (vi) healthcare materials.[22] Additionally, the outstanding reaction kinetics of tetrazine ligations have led to an emerging application of bioorthogonal chemistry in the fields of radiolabeling (in vitro click) and pretargeted single-proton emission computed tomography or positron emission tomography (in vivo click), in which high reaction rates are essential due to the very low concentrations of the radiolabeled compounds in vivo.[23–26]

Second-order rate constants of up to 3.3×10^6 M^{-1} s^{-1} (25 °C, H_2O) have been reported for the ligation of tetrazines with highly strained *trans*-cyclooctenes (TCOs) as dienophiles;[27] this makes the tetrazine/TCO ligation the fastest bioorthogonal reaction to be discovered so far.

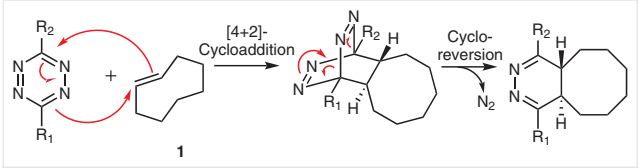
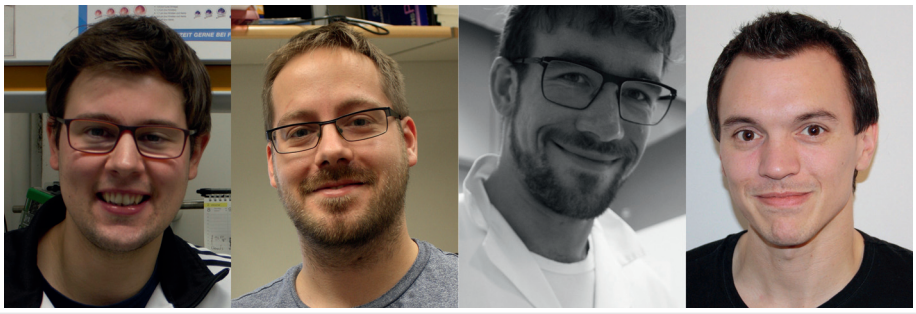


Figure 1 Mechanism of the bioorthogonal ligation between *trans*-cyclooctene (**1**) and 1,2,4,5-tetrazines

The first 1,2,4,5-tetrazines had already been reported by the end of the nineteenth century by Adolf Pinner, and were prepared from imino ester hydrochlorides (Pinner



(from left to right) **Stefan Kronister** graduated from TU Wien in March 2016 with an M.Sc. in Technical Chemistry. He is currently a second–year Ph.D. student at the Institute of Applied Synthetic Chemistry at TU Wien. His Ph.D. studies focus on the development of bioorthogonal tools and targeted prodrug activation inside tumor cells through in vivo cleavage reactions.
Dennis Svatunek received his Ph.D. (2016) from TU Wien, working on the kinetics of bioorthogonal ligations and their application in pretargeted imaging under the supervision of Professor Günter Allmaier. During his Ph.D. studies, Dennis joined the group of Professor Joseph M. Fox at the University of Delaware (2014), working on improved bioorthogonal reactions for rapid radiolabeling. He is currently a postdoctoral researcher at TU Wien, focusing on investigations on the kinetics of cycloadditions by computational and experimental methods.
Christoph Denk obtained his Ph.D. from TU Wien in 2016, and is currently a postdoctoral researcher at the Institute of Applied Synthetic Chemistry. His research focuses on radiolabeled bioorthogonal agents for pretargeted nuclear imaging and therapy. Christoph gains ideas and inspiration for his scientific work while running long distances or while observing life in his saltwater reef tank.
Hannes Mikula obtained his Ph.D. from TU Wien in 2014, working under the supervision of Professor Johannes Fröhlich. He then joined the laboratory of Professor Ralph Weissleder at the Massachusetts General Hospital/Harvard Medical School as a postdoctoral fellow funded by the Austrian Science Fund (FWF) within the Erwin-Schrödinger-Program. His postdoctoral studies focused on molecular imaging and the development of bioorthogonal tools for in vivo chemistry. In 2016, Hannes returned to TU Wien to continue research in the fields of chemical biology, bioorthogonal reactions, in vivo medical imaging, and the development of diagnostic tools.

salts) and hydrazine, which form an amidrazone intermediate. This reacts with excess hydrazine to give a dihydrotetrazine that is finally oxidized to give a 1,2,4,5-tetrazine (Figure 2a).[28]

In recent years, tetrazines have most commonly been synthesized by condensation of two nitrile molecules with hydrazine, followed by oxidation (Figure 2b).[29,30] However, the preparation of the alkyl-substituted tetrazines by this method often results in low yields. A major improvement was achieved by Devaraj and co-workers, who used Lewis acids for the activation of the nitriles, leading to significantly increased reaction yields.[31] A wide variety of aryl- and alkyl-substituted tetrazines are accessible by using this approach. A remaining drawback of the Lewis-acid-mediated tetrazine synthesis is that a statistical mixture is often obtained when different nitriles are used to prepare asymmetrically substituted tetrazines.[32] Alternatively, tetrazines can be synthesized by nucleophilic aromatic substitution of precursors mono- or difunctionalized with 3,5-dimethylpyrazolyl or chloro groups (Figure 2c).[33,34] In general, these methods afford tetrazines with decreased or even very low reactivities. By the 1960s, Takimoto and co-workers had presented a robust and straightforward method to produce unsymmetrical 3-aminotetrazines,[35] in which 3-azido-1,2,4-triazole-4-amines are thermally decomposed to give 3-aminotetrazines in good yields (Figure 2d).

The reaction rate constants of tetrazine ligations can be tuned within a range of several orders of magnitude by changing the electronic properties of the tetrazine moiety by varying the substituents in the 3- and 6-positions. In general, electron-withdrawing substituents increase the reactivity, whereas electron-donating groups decrease it.[36] Tetrazines bearing an amino group are useful because of their straightforward conjugation to target molecules or the ease with which they undergo further modification. An overview of selected amino-functionalized tetrazines and their respective second-order rate constants for the reaction with TCO (**1**) is presented in Figure 3.

Aminotetrazine **2** exhibits a low reactivity because of the electron-donating effect of the NH_2 group directly attached to the tetrazine moiety. Dialkyltetrazine **3** shows only moderate reactivity due to the donating effect of the alkyl substituents,[37] whereas aryl/alkyl-substituted tetrazines such as **4** are slightly more reactive.[10,37] Monosubstituted tetrazines such as **5** show high reaction rates because of a lower steric hindrance;[38] the reaction rates are similar to those of tetrazines bearing electron-withdrawing heteroaryl substituents such as pyridyl or pyrimidyl moieties (e.g., **6**).[10] However, the applicability of highly reactive tetrazines is often limited because of their low stability in biological media.[39]

When a target molecule is modified with a tetrazine tag, excess reagent needs to be completely removed before further application of the conjugate, as unbound tetrazines compete with the tetrazine-labeled molecule in the reaction with TCO (**1**). We surmised that a tetrazine showing a significant increase in IEDDA reactivity upon attachment to a target molecule ('kinetic turn-on') might be highly benefi-

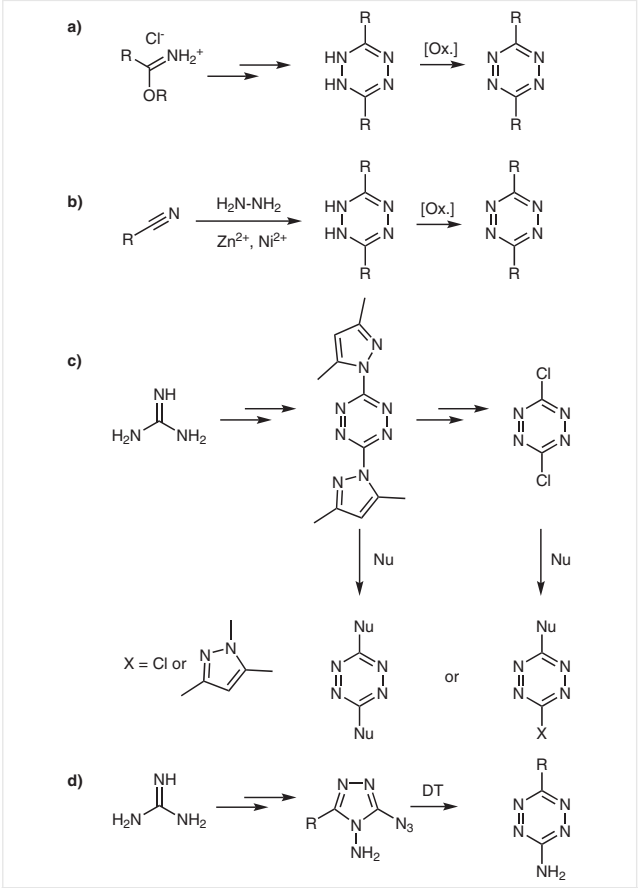


Figure 2 Methods for the preparation of 1,2,4,5-tetrazines. (a) First tetrazine synthesis by Pinner;[28] (b) Lewis-acid-mediated tetrazine synthesis;[29–31] (c) 3,5-dimethylpyrazol-1-yl- or chloro-substituted tetrazines, and subsequent nucleophilic aromatic substitution;[33,34] (d) Synthesis of unsymmetrical aminotetrazines by thermolytic decomposition of 3-azido-4*H*-1,2,4-triazol-4-amines.[35] Ox = oxidation; Nu = nucleophile.[32]

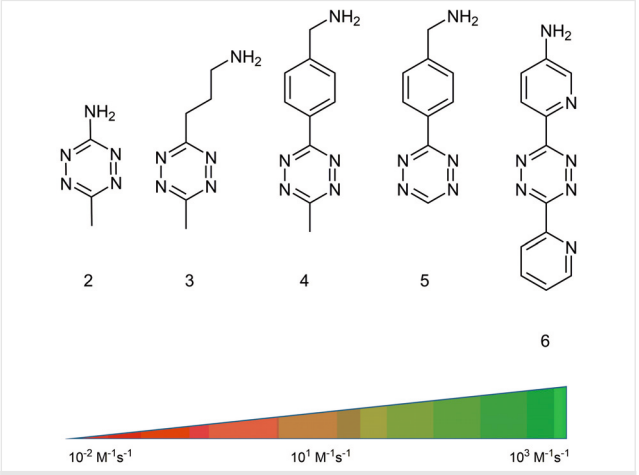


Figure 3 Overview of various aminotetrazines used in IEDDA ligations and their respective second-order rate constants for reaction with TCO (**1**) at room temperature under aqueous conditions.[32,37]

cial because of the minimalized impact of residual tetrazine reagent and/or tetrazine impurities. Lengthy purification procedures could be shortened or even omitted, which would be of particular importance in cases where short-lived nuclides (e.g., carbon-11) are involved.

N-Derivatization of aminotetrazines **3–6** is likely to have only a low, or even no, impact on cycloaddition reactivity because of the limited influence of the electronic properties of the 1,2,4,5-tetrazine moiety. In contrast, N-acylation of **2** appeared to be likely to have a pronounced influence on the reaction kinetics. We therefore investigated the kinetic turn-on of compound **2** and the 3-aminotetrazines **7** and **8** upon N-acylation. Acetylation affording the corresponding 3-acetamidotetrazines **9–11** (Figure 4) was chosen as a simple model for conjugation reactions yielding N-acylated 3-aminotetrazines. Substrates **7** and **8** were chosen because of their expected higher reactivity compared with the methyltetrazine **2**.

Gibbs free energies of activation ($\Delta G^{\ddagger}$) for the reaction of aminotetrazines **2**, **7**, and **8** and their respective acetylated derivatives **9–11** with TCO (**1**) were calculated by means of density-functional theory [M06-2X/6-311+G(d,p), gas phase, *Gaussian 09*]. The $\Delta G^{\ddagger}$ values for the reaction of acetylated compounds were around 4 kcal/mol lower than those of the corresponding aminotetrazines, resulting in a predicted increase in reactivity of around 600-fold (Table 1).

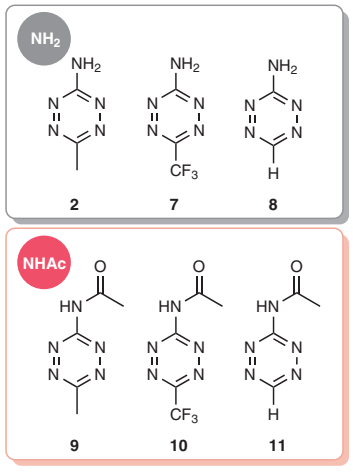
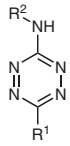


Figure 4 : 3-Aminotetrazines **2**, **7**, and **8**, and corresponding 3-acetamidotetrazines **9–11**

Table 1 Predicted Gibbs Free Energies of Activation (ΔG‡) for the Reactions of 3-Amino- and 3-Acetamidotetrazines with TCO (**1**)

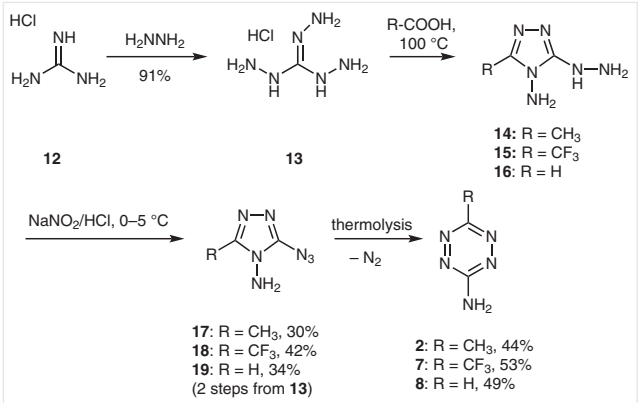
		ΔG‡ [kcal/mol]		
	R^1	R^2 = H	R^2 = Ac	Predicted increase in reactivity ('kinetic turn-on')
	Me	23.1	18.8	660-fold
	CF_3	18.9	14.9	430-fold
	H	21.7	17.4	640-fold

This increase can be attributed to the electron-withdrawing effect of the acetamido group in comparison with the amino group, as reflected in the calculated molecular-orbital energies [level of theory: HF/6-311+G(d,p)//M06-2X/6-311+G(d,p)] for the low-lying unoccupied orbitals involved in the reaction. The acetylated compounds show orbital energies that are 0.6–0.8 eV lower than those of the corresponding aminotetrazines (Supporting Information, Figure S8).

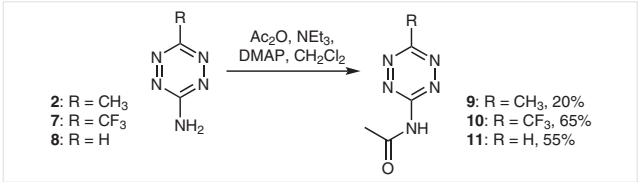
In addition, a distortion/interaction analysis[40] was performed for the reaction between TCO (**1**) and the monosubstituted tetrazines **8** and **11**. As expected, the acetylated derivative **11** shows a lower free energy of activation and an earlier transition state. Distortion energies are slightly elevated compared with **8**; however, interaction energies are much more favorable over the whole intrinsic reaction coordinate, thus lowering the energy of activation considerably and leading to an earlier transition state (Supporting Information, Figure S9).

3-Aminotetrazines **2**, **7**, and **8** were each prepared in four steps (Scheme 1). Triaminoguanidine hydrochloride (**13**) was prepared from guanidine hydrochloride (**12**) and hydrazine hydrate.[41] The 3-hydrazino-4*H*-1,2,4-triazol-4-amine intermediates **14**–**16** were synthesized by cyclocondensation of **13** with the appropriate carboxylic acid.[42] The crude products were directly converted into the corresponding 3-azido-1,2,4-triazol-4-amines **17**–**19**[43] by diazotization of the hydrazino group.[44] Aminotetrazoles **2**, **7**, and **8**[45] were obtained by thermolytic decomposition of the corresponding 3-azido-1,2,4-triazoles in overall yields of 12% (**2**), 20% (**7**), and 15% (**8**).[35] Notably, anhydrous hydrazine (not commercially available in Europe) was not required for these syntheses. Although we did not encounter any problems during this study, all compounds with a high nitrogen content are potentially energetic materials and should be handled and stored accordingly.

Acetylation was carried out by applying commonly used esterification protocols, including (i) acetic anhydride, triethylamine, and 4-(dimethylamino)pyridine,[46] (Scheme 2), or (ii) acetyl chloride and triethylamine (Supporting Information),[47] to give the N-acetylated tetrazines **9**–**11**. The 20% yield for compound **9** was due to the formation of a diacetylated byproduct (as indicated by LC/MS). The stability of **11** in phosphate-buffered saline was examined by monitoring its absorbance at 525 nm over a period of 24 hours, revealing a recovery of >90% (Supporting Information, Figure S7).



Scheme 1 Synthesis of 3-aminotetrazines **2**, **7**, and **8**



Scheme 2 Synthesis of 3-acetamidotetrazines **9**–**11**

The reactions kinetics of aminotetrazines **2**, **7**, and **8** and those of their corresponding N-acetyl derivatives **9**–**11** with TCO (**1**) in 1,4-dioxane at 25 °C were investigated by stopped-flow measurements. Pseudo-first-order conditions were used (an excess of **1**), and the decrease in the concentration of the tetrazole was monitored by absorbance measurement (Supporting Information). The results revealed a significant 'kinetic turn-on' upon N-acylation (Figure 5).

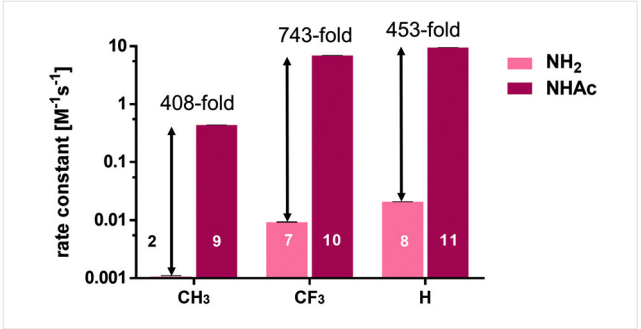


Figure 5 Stopped-flow measurements showing a significantly increased IEDDA reactivity of N-acetylated aminotetrazoles **9**–**11** in comparison with **2**, **7**, and **8**

The greatest increase in reactivity of 743-fold was observed for compound **10**. The second-order rate constant for the reaction of the most reactive 3-amidotetrazine **11** and TCO (**1**) was determined to be 9.5 $M^{-1}s^{-1}$, which is approximately an order of magnitude greater than that of the dialkyltetrazines that have been used in many applications in bioorthogonal chemistry.[48–51]

3-Aminotetrazines were prepared by a straightforward method without the need for anhydrous hydrazine, a reagent that is not commercially available in Europe. Acetylation of these compounds by acetic anhydride or acetyl chloride gave the corresponding 3-acetamidotetrazines. Kinetic investigations revealed a remarkable 'kinetic turn-on' in agreement with quantum chemical calculations. The most reactive amidotetrazine **11** was shown to be sufficiently stable and to react with TCO (**1**) approximately ten times faster than dialkyltetrazines. Overall, we are convinced that the presented concept can be applied in the development of new bioorthogonal tools, labeling strategies, and improved protocols.

Acknowledgment

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1591764.

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(43) **3-Azido-4*H*-1,2,4-triazole-4-amine (19)**
A suspension of guanidine hydrochloride (**13**; 2.2 g, 0.014 mol, 1 equiv) in HCO_2H (40 mL) was heated to 100 °C for 16 h. The acid was evaporated and the residue was dissolved in 6 N HCl (30 mL) to give a solution that was refluxed for 2 h. Upon removal of volatiles, the hydrazinotriazolylamine intermediate **16** was obtained as a white crystalline solid and used in the next step (diazotization) without further purification. A solu-

tion of $NaNO_2$ (0.97 g, 0.014 mol, 1 equiv) in H_2O (4 mL) was added dropwise to a solution of crude **16** in 1 N HCl (20 mL) at 0 °C. The solution was stirred at 0 °C for 30 min, then allowed to warm to r.t. The mixture was neutralized with to pH 9–10 with Na_2CO_3 and extracted with Et_2O in a continuous extractor for 72 h. The solvent was evaporated, and the crude product was purified by column chromatography (silica gel, CH_2Cl_2–MeOH) to give a beige solid; yield: 610 mg (34%); mp 53–55 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.34 (s, 1 H, CH), 5.97 (br s, 2 H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 148.3 (s, 1 C), 145.1 (d, 1 C). MS-ESI: *m/z* [M + H]$^+$ calcd for $C_2H_4N_7^+$: 126.0; found: 125.4.

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(45) **1,2,4,5-Tetrazine-3-amine (8)**
Amine **19** (600 mg, 4.80 mmol, 1 equiv) was suspended in PhCl (15 mL) and the mixture was refluxed for 18 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, hexanes–EtOAc) to give a red solid; yield: 228 mg (49%); mp 170–172 °C.
^{1}H NMR (400 MHz, CD_2Cl_2): δ = 9.71 (s, 1 H, CH), 5.74 (br s, 2 H, NH_2). ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 164.8 (s, 1 C), 154.7 (d, 1 C). MS-ESI: *m/z* [M + H]$^+$ calcd for $C_2H_4N_5^+$: 98.0; found: 97.6.
For the synthesis and characterization of 1,2,4,5-tetrazine-3-amines **2** and **7**, see the Supporting Information.

(46) ***N*-1,2,4,5-Tetrazin-3-ylacetamide (11)**
DMAP (7.5 mg, 0.06 mmol, 0.1 equiv), Ac_2O (292 µL, 315 mg, 3.09 mmol, 5 equiv), and Et_3N (103 µL, 75 mg, 0.74 mmol, 1.2 equiv) were added to a solution of amine **8** (60 mg, 0.62 mmol, 1 equiv) in anhyd CH_2Cl_2 (4 mL), and the mixture was stirred at r.t. overnight. Purification by column chromatography (silica gel, hexane–EtOAc) gave a red solid; yield: 47 mg (55%); mp 203–205 °C.
^{1}H NMR (400 MHz, acetone-d_6): δ = 10.18 (s, 1 H, CH), 2.82 (s, 3 H, CH_3). ^{13}C NMR (100 MHz, acetone-d_6): δ = 168.3 (s, 1 C), 162.1 (s, 1 C), 156.2 (d, 1 C), 24.0 (q, 1 C). MS-ESI: *m/z* [M + H]$^+$ calcd for $C_4H_6N_5O^+$: 140.0; found: 139.3.
For the syntheses and characterization of the *N*-(1,2,4,5-tetrazin-3-yl)acetamides **9** and **10**, see the Supporting Information.

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