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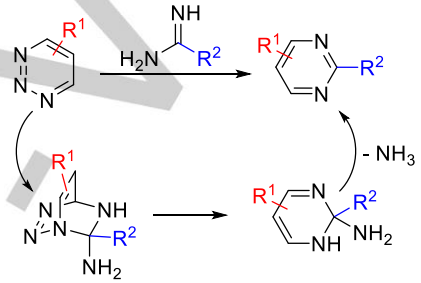
Probing the scope of the amidine–1,2,3-triazine cycloaddition as a prospective click ligation method

Sebastian J. Siegl, and Milan Vrabel*[a]

Abstract: Despite recent achievements in the development of chemical reactions enabling selective modification of complex biomolecules, the demand for fast and efficient methodologies allowing attachment of various functional groups to these systems is the subject of intense research. Here, we report on the study of the amidine–1,2,3-triazine cycloaddition reaction, which has the potential to address many of the challenges associated with the development of such chemistry. We describe an optimized protocol leading to the *in situ* formation of free amidine bases, which directly react in the cycloaddition reaction with 1,2,3-triazines. Our kinetic studies reveal the structural features determining the reaction rates. Finally, we show that the amidine–1,2,3-triazine cycloaddition is extraordinarily selective and orthogonal to other popular ligation reactions. The pros and cons of the methodology are presented.

Introduction

Given the vast number of functional groups present within the structure of biomolecules, selective chemical modification of such complex systems represents an immense challenge for organic chemists.[1] Although a number of reactions targeting e.g. the functional groups of natural amino acids exist, these methodologies often suffer from the lack of control over the modification site.[2] This is simply because the naturally occurring functional groups are present in multiple copies within the structure. On the other hand, the desired high degree of selectivity can be achieved by embedding an orthogonal reacting group into structure of biomolecules. This reactive group is then used in the next step for attachment of the desired tag/modification via selective chemical reaction using the appropriate complementary reagent. These, so-called bioorthogonal reactions, already proved to be an extremely powerful way not only for modifying biomolecules, but also to study their delicate structure and understand their numerous functions.[3] In our continuous effort to identify chemical reactions having such attributes we became particularly interested in the inverse electron-demand Diels-Alder reaction of 1,2,3-triazines with amidines (Scheme 1). Our inspiration came from the pioneering studies of Boger who demonstrated the exquisite reactivity of 1,2,3-triazines with various dienophiles.[4] Especially intriguing is the regioselectivity of these chemical transformations, which proceed exclusively across C4/N1. This allows for excellent control over product formation giving well defined pyrimidines as sole reaction products. Another important feature of this [4 + 2] cycloaddition is the mild reaction conditions. Usually, the reaction proceeds at room temperature within minutes in solvents such as acetonitrile or dioxane giving good to excellent yields of the products. All of these attributes prompted us to explore the potential of the amidine–1,2,3-triazine cycloaddition in the context of bioorthogonal ligation methods.



Scheme 1. Schematic presentation of the amidine–1,2,3-triazine cycloaddition reaction leading to substituted pyrimidine products.

Results and Discussion

An essential prerequisite for successful reaction of 1,2,3-triazines with amidines is the presence of the amidine in the form of a free base.[4a] Indeed, our pilot experiments confirmed that acetamidine hydrochloride in reaction with 5-phenyl-1,2,3-triazine does not lead to the desired pyrimidine product. Usually, the free base of amidine is generated by treating the salt with a strong base, such as 1-2 N aqueous NaOH, followed by extraction into organic phase and immediate use in the cycloaddition step. These rather harsh conditions not only limit the substrate scope containing the amidine group, but also involve handling of the relatively unstable free base of amidine. Our first goal was therefore to find reaction conditions allowing us to avoid this limitation. An ideal method should enable deprotonation of the amidine group leading to the *in situ* formation of free amidine base, which can then undergo the cycloaddition reaction with 1,2,3-triazines. We used 5-phenyl-1,2,3-triazine **1a** and commercially available acetamidine hydrochloride **2** to optimize the reaction conditions. We first screened several bases for the *in situ* formation of the amidine free base. We used 1,1,3,3-tetramethylguanidine (TMG), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD), diazabicyclo[5.4.0]undec-7-ene (DBU) and Proton Sponge in these experiments. In particular, four equivalents of the corresponding base were mixed with **2** in the respective solvent prior to addition of 5-phenyl-1,2,3-triazine solution. The formation of the desired 2-methyl-5-phenylpyrimidine **3a** was monitored by HPLC-MS analysis by comparing the reaction mixtures to a standard

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compound. The results are summarized in table 1 (for details see Table S1 and Figure S1 in Supporting Information).

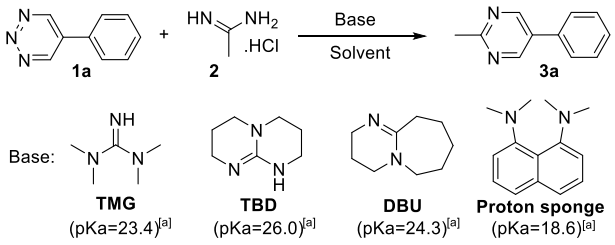
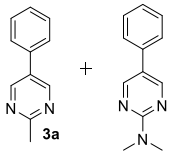
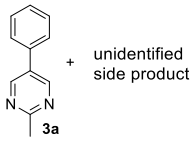
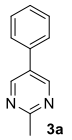
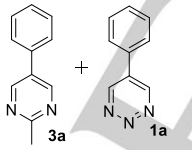
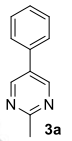
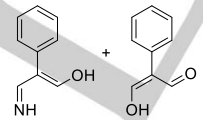
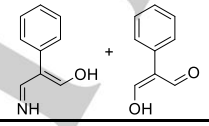


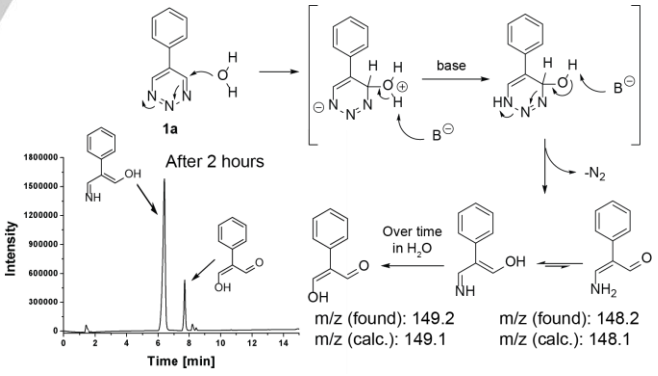
Table 1. Optimization of the amidine – 1,2,3-triazine cycloaddition[b]

Entry	Base	Solvent	Temperature	Results[c]
1	TMG	CH_3CN	r. t.	
2	TBD	CH_3CN	r. t.	 unidentified side product
3	DBU	CH_3CN	r. t.	 formed in 88% isolated yield
4	Proton Sponge	CH_3CN	r. t.	
5	DBU	CH_3CN	37°C	 finished within 8 hours
6	DBU	CH_3CN/ H_2O (1:1)	r. t.	
7	TMG	CH_3CN/ H_2O (1:1)	r. t.	

[a] pKa values in CH_3CN from lit.[5] [b] all reactions were performed using two equivalents of acetamidine and four equivalents of the base. [c] structures of side products are proposed from observed masses during HPLC-MS measurements (for details see Tables S1, S2 and Figures S1, S2 in Supporting Information).

The reaction in CH_3CN using TMG as the base afforded the respective pyrimidine product **3a** accompanied by formation of a side product presumably arising from the cycloaddition reaction of the guanidine base with the phenyl triazine (judged from the observed MS spectrum) (entry 1 in Table 1 and Table S1 and Figure S1 in Supporting Information). Only traces of the desired product **3a** together with unidentified side products were formed in the presence of TBD base (entry 2). The reaction in the presence of Proton Sponge afforded only traces of **3a** and mostly the remaining starting material **1a** (entry 4). This can be attributed to the slightly lower pKa of Proton Sponge (12.3 in water)[6] when compared to acetamidine (12.5 in water)[7] which is unable to efficiently deprotonate the amidine salt. To our delight, the reaction with DBU provided very clean conversion to the desired product **3a** without formation of any side products. Under the optimized conditions **3a** formed in 88% isolated yield (entry 3, for details see Supporting Information). As expected, the formation of the product was faster when we performed the reaction at elevated temperature (entry 5).

With the aim to explore the possibility of using the amidine–1,2,3-triazine cycloaddition on biomolecules we also tested the sensitivity of the reaction to aqueous conditions. Unfortunately, the reaction does not tolerate water. By increasing the water content to 50 % the reaction afforded mainly side products (entry 6 and 7 in Table 1, Table S1 and Figure S1 in Supporting Information). To better understand the formation of these side products under aqueous conditions we next performed a series of experiments. We found that 5-phenyl-1,2,3-triazine alone is stable in water (entry 1 in Table S2 and Figure S2 in Supporting Information). **1a** is also stable as a solution in CH_3CN in the presence of DBU (entry 2 in Table S2 and Figure S2 in Supporting Information). However, the triazine **1a** decomposes in water when DBU is present (entry 3 in Table S2 and Figure S2 in Supporting Information). The same we observed by using TMG as the base (entry 4 and 5 in Table S2 and Figure S2 in Supporting Information). A proposed mechanism leading to the formation of side products (or decomposition of **1a**) under these conditions is shown in scheme 2.

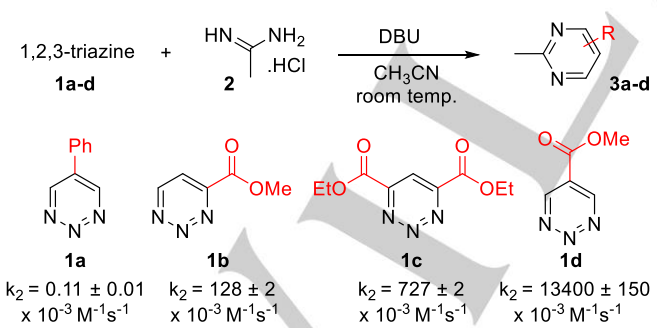


Scheme 2. Proposed reaction mechanism leading to formation of side products from **1a** in CH_3CN/H_2O in the presence of the base. Bottom left is the HPLC chromatogram showing product distribution after 2 hours.

Based on these results we conclude that although strict anhydrous conditions are not required for successful reaction to take place (HPLC grade CH_3CN is sufficient), the requirement for solely organic solvents limits utilization of the method to systems where these conditions are tolerated. Despite this

restriction, our newly developed conditions enabling *in situ* formation of the reactive amidine free bases followed by cycloaddition of the 1,2,3-triazine may find broad utility. They provide an easy-to-perform alternative to the commonly used reaction conditions without the need for additional extraction steps and avoid handling of the unstable free amidine bases.

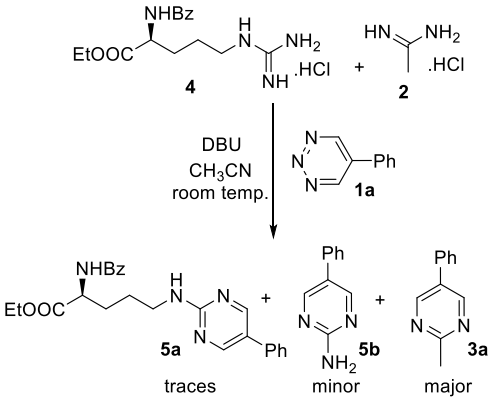
Although the reactivity of various 1,2,3-triazines with amidines was previously experimentally compared,[4a-c] to the best of our knowledge, no quantitative kinetic data are known from the literature. To get better insight into the reactivity of 1,2,3-triazines and to evaluate the generality of our optimized conditions, we next performed reaction kinetic experiments using a series of 1,2,3-triazines shown in scheme 3. The second order rate constants were determined using either HPLC or UV-Vis spectrophotometer (Table S3 in Supporting Information). Our data show that the reactivity of 1,2,3-triazines varies significantly and depends on the structure and substitution pattern. In general, the presence of electron withdrawing substituents increases the reactivity, which is in agreement with the inverse electron-demand nature of the reaction. The most electron poor 1,2,3-triazine **1d** bearing a methyloxycarbonyl substituent at C5 is the most reactive of the series. It exceeds in reactivity the corresponding C4 substituted triazine **1b** by two orders of magnitude and 5-phenyl-1,2,3-triazine **1a** by an impressive six orders of magnitude. The lower reactivity of **1b** when compared to **1d** indicates that the C4 substitution is much less activating even though the substituent is electron withdrawing. 4,6-disubstitution of **1c** increases its reactivity when compared to **1b** however still remains orders of magnitude below that of **1d**. The observed lower reactivity of **1b** and **1c** can be also possibly attributed to increased steric hindrance of substituents at C4 and C6 when compared to substituents at C5. It is known that other effects such as hydrogen bonding ability and the reaction mechanism itself also play an important role and influence the reactivity of 1,2,3-triazines.[8] A more comprehensive computational study would be needed to fully understand and explain the observed experimental data.



Scheme 3. Scheme showing conditions used during measurements of reaction kinetics. The determined second order rate constants for individual 1,2,3-triazines are depicted below the structures.

By comparison to other known bioorthogonal ligations, the determined second order rate constant of 13.4 ± 0.15 M^{-1} s^{-1} (for **1d**) reaches values of the reaction of 1,2,3,4-tetrazines with strained bicyclononyne (BCN).[9] The reaction of the most reactive 1,2,3-triazine **1d** with acetamidine is also an order of magnitude faster than the reaction of 1,2,3,4-tetrazines with norbornenes.[10] Considering the popularity of both of these reactions, the amidine–1,2,3-triazine cycloaddition holds great potential for applications where such exquisite reactivity is desirable.

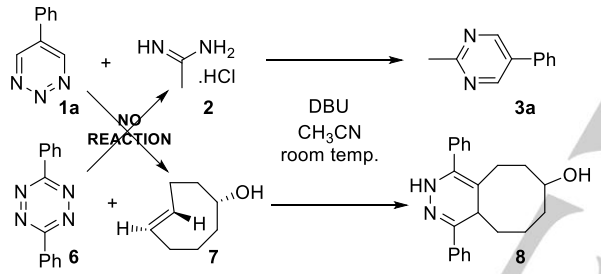
Guanidine is, among other functional groups present in natural amino acids, structurally most similar to the amidine group. As already our reaction optimization experiments using different bases indicated, guanidines can react with 1,2,3-triazines to some extent (TMG base). This would be a potential obstacle for successful use of the 1,2,3-triazines for e.g. selective peptide modification. To gain better insight into this 'side' reaction we mixed N-benzoyl protected ethyl ester of ariginine **4** with triazine **1a** under our optimized conditions (DBU as base, CH_3CN as solvent). Indeed, HPLC-MS analysis of the reaction mixture confirmed the presence of the corresponding cycloaddition product **5a**. In addition, we observed also formation of cycloadduct **5b**, which forms when the amino acid part of the molecule eliminates during the last step of the reaction instead of ammonia (see mechanism in Scheme 1). To further evaluate if this side reactivity represents a serious obstacle for e.g. peptide modification we performed a competitive experiment. We first mixed the ariginine amino acid **4** and acetamidine hydrochloride **2** with an excess of DBU in CH_3CN. These solutions were combined and **1a** was added. Under these conditions, the corresponding 2-methyl-5-phenylpyrimidine **3a** was formed predominantly. However, formation of small traces of **5a** together with **5b**, which both result from the reaction of the triazine with the double bond of the guanidine group, was observed as well (Scheme 4 and Figure S5, S6 in Supporting Information). These experiments confirmed that the amidine–1,2,3-triazine cycloaddition under these conditions competes with cycloaddition to guanidines, however, the latter is slower.



Scheme 4. Competition experiment of the cycloaddition between 5-phenyl-1,2,3-triazine and acetamidine or arginine respectively.

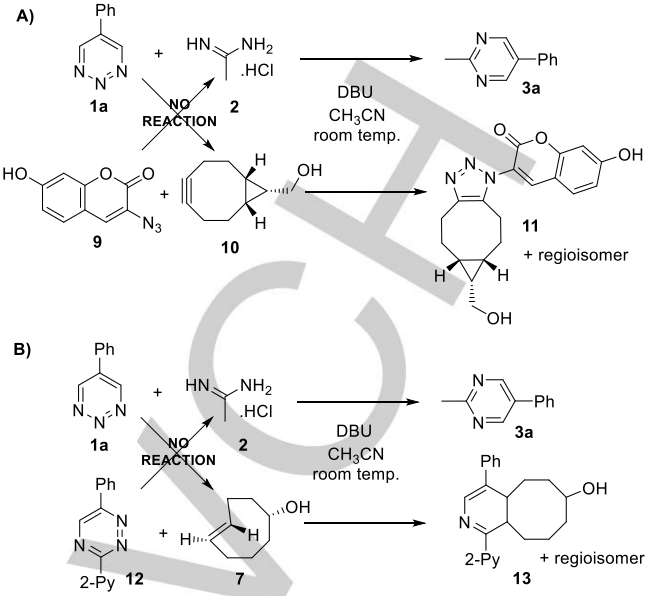
After validating the chemo-selectivity of the reaction toward guanidines we next turned our attention to explore the selectivity

of the reaction in the context of other popular biocompatible ligations. In particular we were interested if the reaction between *trans*-cyclooctenes (TCO), one of the most reactive dienophiles known to date,[11] and 1,2,4,5-tetrazines is orthogonal to the amidine–1,2,3-triazine cycloaddition. To probe the selectivity, we reacted phenyl triazine **1a**, diphenyl-*s*-tetrazine **6**, TCO **7** (we used pure axial isomer) and acetamidine hydrochloride **2** (in both cases two equivalents of the dienophile were used) under our optimized reaction conditions (Scheme 5 and Figure S7 in Supporting Information). We followed the progress of the reaction by HPLC-MS analysis. As expected, the reaction between diphenyl-*s*-tetrazine and TCO was finished already during first analysis and gave the corresponding dihydropyridazine **8**. Most importantly, we found that the amidine–1,2,3-triazine reaction proceeds selectively under these conditions. We did not observe formation of any of the products that would arise from the cross-reaction between the reagents (TCO with 1,2,3-triazine or 1,2,4,5-tetrazine with acetamidine). In other words, the two inverse electron-demand cycloadditions are orthogonal to each other and can be performed in a one pot reaction setup.



Scheme 5. Competition experiment between amidine–1,2,3-triazine vs. TCO–1,2,4,5-tetrazine cycloaddition.

Encouraged by these results, we decided to further probe the selectivity of the amidine–1,2,3-triazine cycloaddition and performed a series of other competition experiments. We first carried out the reaction in the presence of 3-azido-7-hydroxycoumarine **9** and bicyclononyne (BCN) **10** (Scheme 6A), and second, in the presence of 3-(2-pyridyl)-6-phenyl-1,2,4-triazine **12** and TCO **7** (Scheme 6B, for details and additional examples see Supporting Information Scheme S7-S9, Figures S10-S12). We again observed selective formation of the desired products (**3a**, **11** and **13** respectively) in each case without formation of any of the side products which would result from cross reactions between the reagents (Scheme 6, Figure S8 and S9 in Supporting Information). These experiments show that the reaction between amidines and 1,2,3-triazines proceeds with excellent selectivity and that it is orthogonal to other popular cycloadditions such as the strain-promoted azide-alkyne cycloaddition (SPAAC)[12], TCO-1,2,4-triazine[13] and TCO–1,2,4,5-tetrazine cycloaddition.[14] Based on these results, we believe that the amidine–1,2,3-triazine cycloaddition may find application especially in experiments requiring attachment of various moieties to multifunctional scaffolds in a single step.[15]



Scheme 6. Competition experiment between amidine–1,2,3-triazine and A) the strain promoted azide-alkyne cycloaddition or B) TCO–1,2,4,-triazine cycloaddition.

Conclusions

In conclusion, we describe our results from the evaluation of the amidine–1,2,3-triazine cycloaddition as a prospective click ligation method. Our optimized protocol for the *in situ* formation of free amidine bases, which directly participate in cycloaddition with various 1,2,3-triazines, represents simplified and straightforward synthetic route toward modified pyrimidines. Our analysis of the reaction kinetics reveals that the reactivity of 1,2,3-triazines with amidines can vary by orders of magnitude. The most reactive 1,2,3-triazines react with simple amidines with second order rate constants reaching the values of some of the strain-promoted cycloaddition reactions. We show that the amidine–1,2,3-triazine cycloaddition is a powerful ligation method which holds great potential for various applications. Despite observed sensitivity to water, which limits the utility of this chemistry to systems compatible with organic solvents, the reaction proceeds with extraordinary chemo- and regio-selectivity. Under the conditions tested, we show that the reaction is orthogonal to some of the most popular ligation methods including the TCO–1,2,4,5-tetrazine, TCO–1,2,4-triazine as well as the strain-promoted azide-alkyne cycloaddition. We believe that the reported data will serve as valuable guidelines for future studies of the reaction and that the observed and described excellent orthogonality will find utility in multiple tagging/labeling experiments on complex systems.

Experimental Section

Optimization of the amidine–1,2,3-triazine cycloaddition

A 20 mM solution of 5-phenyl-1,2,3-triazine was mixed at a ratio of 1:1 with a 40 mM solution of acetamidine hydrochloride containing 2 eq. of a base (TMG, TBD, DBU or proton sponge). The solutions containing acetamidine hydrochloride and the base were incubated at room temperature for 20 min before mixing with the 5-phenyl-1,2,3-triazine to form the amidine free base. The reactions were performed either in CH_3CN or in CH_3CN/H_2O 1:1. The reaction mixtures were incubated at room temperature or at 37 °C and progress of the reaction was monitored by HPLC-MS analysis.

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Keywords: amidines • click ligation • cycloaddition • Diels-Alder reaction • 1,2,3-triazines

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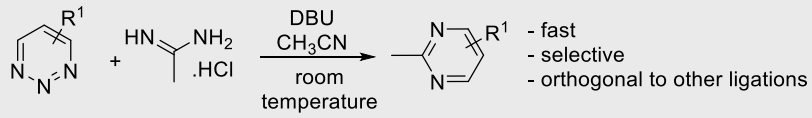
Entry for the Table of Contents

COMMUNICATION



The amidine–1,2,3-triazine cycloaddition was studied as a potential click ligation method. A new protocol for base-promoted *in situ* formation of free amidine bases undergoing cycloaddition with 1,2,3-triazines is reported. The reaction shows excellent orthogonality to other popular ligations such as SPAAC or TCO–1,2,4,5-tetrazine cycloaddition. Limitations and advantages of the reaction are disclosed.

*Ligation chemistry

Ligation chemistry*

*Sebastian Siegl, Milan Vrabel**

Page No. – Page No.

Probing the scope of the amidine–1,2,3-triazine cycloaddition as a prospective click ligation method