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Controlled thioamide *vs.* amide formation in the thioacid–azide reaction under acidic aqueous conditions†

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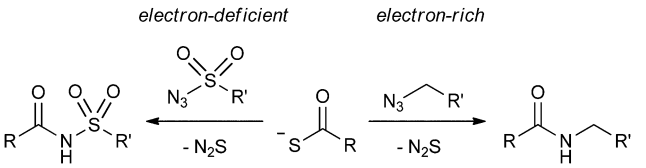
The thioacid–azide reaction and its chemoselectivity were probed with alkyl azides for a potential application to form amide bonds in aqueous solvents. Our results reveal that under acidic conditions thioamides were formed as major reaction products suggesting a competing mechanism, whereas reactions forming amides predominated at slightly higher pH values.

Despite many years of extensive research, amide bond formation still remains a challenging research field. A vast variety of synthetic methods have been developed throughout the last few decades[1] ranging from standard activation and dehydration strategies between carboxylic acids and amines over radical-based to oxidative methods. Other recent approaches include an umpolung strategy between α-bromonitroalkanes and amines[2] or the reaction between acyltrifluoroborates and hydroxylamines.[3] In addition, several chemoselective ligation strategies, such as the native chemical ligation (NCL),[4] the traceless Staudinger ligation (TSL)[5] and the ketoacid–hydroxylamine ligation (KAHA)[6] have been employed to yield the desired amidation product even between polypeptides of synthetic and/or molecular biological origin.[7] However, several limitations, such as solubility and hydrolytic instability[8] (TSL) and the necessity of cysteine (NCL and EPL) or homoserine (KAHA) at the ligation site provoke a demand for further optimization or engineering of reliable chemoselective amidation reactions.

Another promising transformation to yield amide bonds is the thioacid–azide reaction,[9] which is also known as the "Sulfo-Click" reaction (Scheme 1, left).[10] Herein, an electron-deficient sulfonazide reacts with a thioacid through a thiatriazoline intermediate, as postulated by Williams, which then decomposes with the release of sulfur and nitrogen to yield an *N*-acyl sulfonamide.[11]



Scheme 1 Thioacid–azide reactions with electron-deficient azides (left) and electron-rich azides (right).

Several electron-deficient azides have been applied in peptide glycosylations,[12] seleno amidations,[13] the synthesis of resin-bound *N*-peptidyl sulfonamides,[14] short peptide ligations[15] and in kinetic target-guided synthesis.[16] In addition, researchers have shown that the amidation reaction with sulfonazides proceeds rapidly and chemoselectively.[17] In contrast, considerably fewer studies have addressed the performance of less electrophilic alkyl azides for the reaction with thioacids (Scheme 1, right).[11a,18] Several attempts have been made to improve the thioacid–azide reaction probing different reaction temperatures,[11] solvent systems[11,15a] and catalysts such as $RuCl_3$.[18] Despite the apparent lower reactivity of alkyl azides, this reaction is particularly promising for the formation of peptide bonds, which could be applied as a new peptide ligation strategy with orthogonal functional groups to NCL or KAHA. In addition, this reaction could also lead to site-specific post-translational acylations on polypeptides at former azido-Lys residues, which occur in nature as fatty-acylations, acetylations or ubiquitinations.[19]

At the outset of our studies, we intended to probe the reactivity and chemoselectivity of thioacetic acid (**2**) in acetylation reactions on the unprotected and electron-rich ε-azido lysine peptide **1** (Table 1). In the "Sulfo-Click" reaction, best results were often achieved in DMF at room temperature in the presence of lutidine as a base.[11] We thereby employed the previously reported conditions to convert peptide **1**, whose amino acid sequence is derived from the histone protein H4[20] and is rich in the basic amino acids arginine and lysine. For better conversion analysis by HPLC, a fluorescent ε-nitrobenzoxadiazole (NBD) lysine was incorporated into the peptide sequence by standard SPPS (Table 1).

Our initial attempt to perform the amidation reaction in organic solvents under basic conditions revealed that the overall conversion

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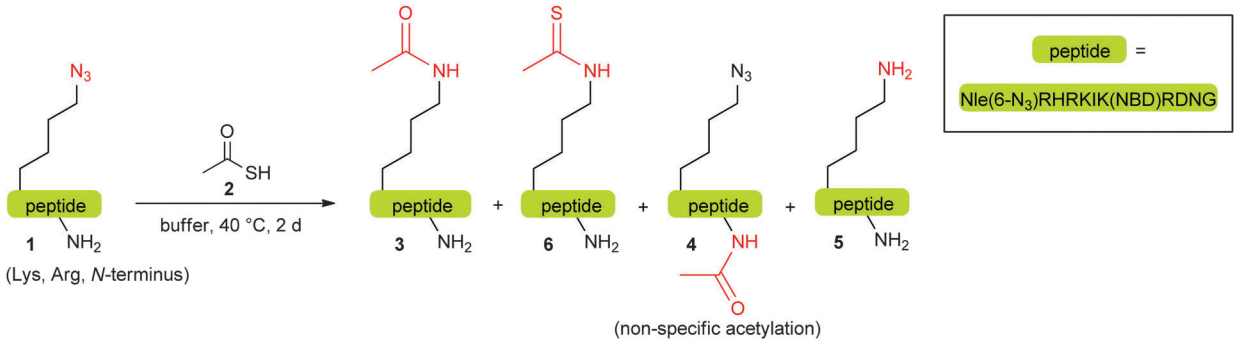
Table 1 Conditions for the reaction of thioacetic acid (**2**) with ε-azido lysine peptide **1** (NBD = ε-nitrobenzoxadiazole, Nle(6-N_3) = 6-azido norleucine)



Entry	c [mM]	TAA[a] [eq.]	pH	Solvent system	Ratio[b] [%] 1	3	6	4
1	3.00	5	—	DMF, 3 mM lutidine	—	2	—	98
2	1.75	5	7.0	Phosphate buffer (100 mM)	38	6	15	38
3	1.75	5	3.0	Citrate buffer (100 mM)	60	6	10	24
4	1.75	5	2.0	Citrate buffer (100 mM)	70	9	5	16
5	3.00	20	2.0	Sodium citrate/HCl buffer (100 mM)	8	25	53	14
6	3.00	100	2.0	Sodium citrate/HCl buffer (100 mM)	—	6	15	79

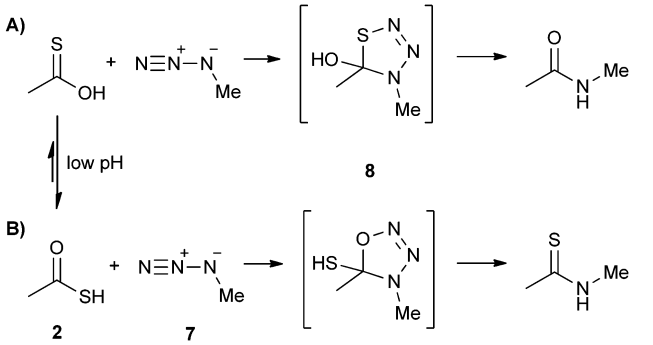
[a] TAA = thioacetic acid (**2**). [b] Determined by LC–Fluorescence peak integration (ex460/em540 nm); detection by HPLC-MS.

of alkyl azides was very low at room temperature and heating to 40 °C was necessary. Additionally, there was hardly any formation of the desired specifically acetylated Lys-peptide **3** and almost full conversion to non-specifically acetylated peptides **4** by a presumably faster acylation of amines with thioacids (Table 1, entry 1). This side reaction has also been described by Kent, who discovered that Cys-[peptide]-thiocarboxylates can undergo direct intramolecular cyclization at neutral pH.[21] When performing the reaction in a phosphate buffer at neutral pH at room temperature, we observed a crucial decrease of non-specific amidation and a slight increase in product formation, though conversion of azide **1** was still incomplete (Table 1, entry 2).

To further suppress non-selective acetylations and lower the nucleophilicity of side chains as well as the N-terminus, the reaction was probed at lower pH (Table 1, entries 3–6). Initial results showed that conversion was very low (Table 1, entries 3 and 4) and the reduced amine peptide **5** was observed as a small side-product. We decided to increase the concentration from 1.75 to 3.00 mM and to add more equivalents of thioacetic acid (**2**). After reaction for 2 d at 40 °C and pH 2 with 20 eq. of thioacetic acid (**2**), the reaction led to almost full conversion of azido peptide **1** and furnished the desired amidation product **3** in 25% conversion (Table 1, entry 5). Although reducing the pH lowered non-selective acetylations to 14%, it could not be completely prevented, showing that in the presence of nucleophilic amino acids like lysine the reaction with electron-rich alkyl azides does not proceed in a chemoselective fashion. A higher amount of thioacetic acid (**2**) only led to an increase in undesired amidation of the starting material and the formed products (Table 1, entry 6).

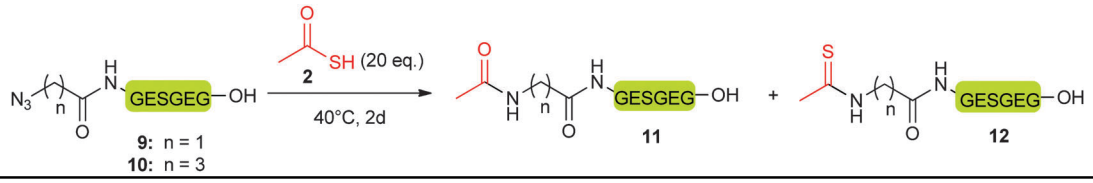
In addition to the desired acetamide **3**, we observed formation of the thioamide-containing peptide **6**. The large amount of thioamide **6** observed at pH 2 indicated that the reaction towards thioamide **6** was faster than the formation of acetamide **3** (Table 1, entry 5). Initially, we assumed that thioamide formation might occur due to traces of dithioacetic acid.[22] However, ^{1}H and ^{13}C NMR analysis of commercial **2** showed only very little dithioacetic acid (<0.5%), which would not be enough to explain thioamide formation in entry 5.

These results led to the assumption that, at lower pH, the reaction might at least partially proceed *via* a different mechanism as the one postulated by Williams in 2006. Based on DFT calculations, Williams proposed the concerted formation of thiatriazolidine **8** starting from a C=S bond for the reaction of methyl azide **7** with thioacetic acid (**2**) (Scheme 2A).[11b] At lower pH, thioacetic acid (**2**) exists in its neutral form to a greater extent with a C=O bond and contains only traces of the C=S tautomer as demonstrated theoretically and experimentally by Liu and Gordy, respectively.[23] Hence, we propose that the concerted cyclization could also include the C=O bond and therefore yield an oxatriazolidine (Scheme 2B), which then delivers the corresponding thioamide (Scheme 2B) as previously proposed by the group of Rademann for the reaction of azides and thioacids in the presence of Lewis acids.[24] Due to the larger covalent radius of sulfur *versus* oxygen and the less efficient overlap of the C_{2p}–S_{3p} π-bond, one might expect the C=S bond to be more reactive than the C=O bond in the cycloaddition step.[25]



Scheme 2 Proposed mechanisms for the thioacid–azide reaction under acidic conditions by (A) Williams[11b] and (B) our group.

Table 2 Conditions for thioacid–azide reactions with **9** and **10**



Entry	*n*	*c* [mM]	Solvent system	Ratio 11 : 12[a]	Conversion[a] [%]
1	1	3	KCl/HCl buffer (100 mM, pH 2.0)	0.08 : 0.92	37
2	3	3	KCl/HCl buffer (100 mM, pH 2.0)	0.32 : 0.68	47
3	1	3	NH_4OAc buffer (100 mM, pH 4.0)	0.62 : 0.38	64
4	3	3	NH_4OAc buffer (100 mM, pH 4.0)	0.47 : 0.53	49
5	1	3	Phosphate buffer (100 mM, pH 7.0)	1 : 0	53
6	3	3	Phosphate buffer (100 mM, pH 7.0)	1 : 0	15
7	1	3	NH_4OAc buffer (100 mM, pH 4.0), 30% DMF (pH 5)	0.88 : 0.12	80
8	3	3	NH_4OAc buffer (100 mM, pH 4.0), 30% DMF (pH 5)	0.64 : 0.36	79
9	1	3	Phosphate buffer (100 mM, pH 9.0), 50% THF	1 : 0	47
10	3	3	Phosphate buffer (100 mM, pH 9.0), 50% THF	1 : 0	14
11	1	3	DMF (dry, 3 mM lutidine)	1 : 0	10
12	3	3	DMF (dry, 3 mM lutidine)	1 : 0	3
13	3	20	KCl/HCl buffer (100 mM, pH 2.0)	0.32 : 0.68	>99

[a] Determined by NMR integration.

However, the low abundance of the C=S bond at lower pH could lead to thioamide formation, if the azide itself is reactive enough to undergo a cycloaddition with the C=O bond.

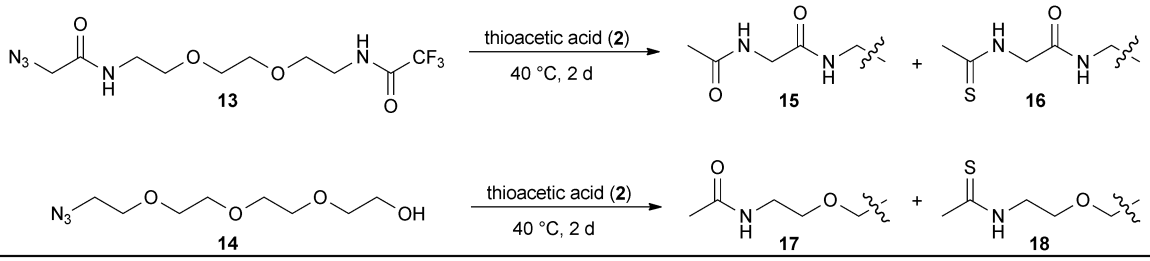
Since it was shown that thioamides are effective quenchers for several fluorophores[26] and partial peak overlapping in the fluorescence chromatograms made qualitative analysis in part difficult, we decided to further investigate thioamide formation for two electronically different azido peptides – namely azido glycine peptide **9** and γ-azido butanoic acid peptide **10** – and the influence of the solvent system in more detail by conducting 1D and 2D NMR experiments (Table 2). In order to focus on the thioamide *vs.* amide formation, peptides **9** and **10** did not possess any nitrogen-containing side chains that could lead to non-selective acetylations.

For better comparison of conversion rates and product ratios, both peptides were reacted with 20 eq. of thioacetic acid (**2**) in different solvent systems with varying pH values for 2 d at 40 °C (Table 2). After removal of excess thioacetic acid (**2**) and all volatiles, each sample was redissolved in D_2O and checked by NMR spectroscopy to determine the various reaction products (for spectra see the ESI†). Notably, formation of thioamide **12** decreased from pH 2 to pH 7 with amide **11** as the sole product at pH 7 and higher (Table 2, entries 5, 6 and 9–12). These results support the previously observed increase in thioamide formation under highly acidic conditions (Table 2, entries 1 and 2). At pH 2, this effect seemed to be much stronger for the modestly electron-poor azido glycine peptide **9** than for the electron-rich γ-azido butanoic acid peptide **10**. Due to the presumably higher reactivity of azido glycine peptide **9**, the azide seems to be reactive enough to undergo oxatriazolidine formation with the C=O bond of thioacetic acid (**2**) yielding a higher amount of thioamide **12**. In contrast, the low reactivity of the electron-rich γ-azido butanoic acid peptide **10** promotes the reaction with the less abundant but more reactive C=S bond. With increasing pH, thioacetic acid (**2**) exists predominantly in its deprotonated form and the reaction seems to increasingly follow the mechanism for thiocarboxylates proposed by Williams and co-workers (Scheme 2A).

Regarding reaction conversions, the results obtained from the NMR spectroscopy confirm previous observations that modestly electron-poor and electron-rich azides tend to react very slowly in DMF with 3 mM 2,6-lutidine (Table 2, entries 11 and 12). In addition, we observed a large increase in conversion around pH 5 (Table 2, entries 7 and 8), which drops again at lower pH values of 2–4 (Table 2, entries 1–4). These findings indicate that careful pH handling can enhance conversion rates with alkyl azides up to 80%. In addition, we could show that by increasing the concentration of the reaction mixture from 3 to 20 mM we could drive the reaction from 47% to almost full conversion without any additives, such as $RuCl_3$. Furthermore, the latter result shows that the thioamide/amide ratio depends solely on the electronic nature of the employed azide and the pH during the thioacid–azide reaction (Table 2, entries 2 and 13).

In the final experiment, we wanted to probe thioacid–azide reactions employing small water-soluble alkyl azido molecules **13** and **14** to determine isolated yields (Table 3). The employed azides **13** and **14** should bear similar electronic properties as azido peptides **9** and **10**, respectively. Thioacid–azide reactions were performed under the reaction conditions used for the previously reported highest azide conversion (Table 2, entry 7) and the highest thioamide formation (Table 2, entry 1). In addition, the concentration was set to 20 mM for all subsequent reactions to achieve higher conversion rates. As a result, all reactions showed higher azide conversion (>80%) compared to the previous results with one exception of azide **14** at pH 5 (Table 3, entry 4), which might be due to its slightly different electronic properties than the previously applied azides **9** and **10**. The preference for thioamide or amide formation does not change for azide **13** and **14** though the ratio is not completely transferable from azido glycine peptide **9** to azido glycine derivative **13** under acidic conditions (Tables 2 and 3, entry 1) underlining the strong influence of the electronic nature of the azide on the reaction outcome. We could thereby isolate the desired thioamides at pH 2 in about 30% yield (Table 3, entries 1 and 2) and the corresponding amides at higher pH in moderate to good yields (20–50%)

Table 3 Thioacid–azide reactions with **13** and **14** (20 mM, 10 eq. thioacetic acid (**2**))



Entry	Azide	Solvent system	Ratio (amide : thioamide)[a]	Conversion[a] [%]	Yield[b] [%]
1	**13**	KCl/HCl buffer (100 mM, pH 2.0)	0.29 : 0.71	80	30 (**16**)
2	**14**	KCl/HCl buffer (100 mM, pH 2.0)	0.40 : 0.60	87	32 (**18**)
3	**13**	NH_4OAc buffer (100 mM, pH 4.0), 30% DMF	0.93 : 0.07	100	50 (**15**)
4	**14**	NH_4OAc buffer (100 mM, pH 4.0), 30% DMF	0.61 : 0.39	61	18 (**17**)

[a] Determined by NMR integration. [b] Main product.

(Table 3, entries 3 and 4). One should note that the conversions in entries 1–3 are excellent and that the drop in the isolated yield was partially due to difficult separation of the two products by HPLC.

In summary, we have shown that at lower pH values the thioacid–azide reaction with electron-rich and modestly electron-poor azides proceeds with high conversion rates and without any additives. With the exception of very basic amino acid side chains such as lysine, the reaction is highly selective in the presence of other functional groups. In addition, we observed an increased formation of thioamides at a pH < 7. We could show that the thioamide/amide ratio can be controlled by varying pH with an increase of up to 92% thioamide conversion for an azido glycine peptide and 68% thioamide conversion for a γ-azido butanoic acid peptide at pH 2. As this effect seems to be stronger for modestly electron-poor azides, such as azido glycine peptide **9**, it would be interesting to see if the employment of highly electron-deficient azides, *e.g.*, sulfonyl azides, might even lead to complete thioamide formation under strong acidic conditions. During the last few decades, thioamides have gained much importance, *e.g.*, as a new class of drugs,[27] as amide isosteres in peptides,[28] and as quenching units in fluorescent proteins to study conformational changes.[29] A new strategy for their selective synthesis might enrich the field of thioamide containing probes.

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