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Bioorganic Chemistry

A Tripeptide Approach to the Solid-Phase Synthesis of Peptide Thioacids and N-Glycopeptides

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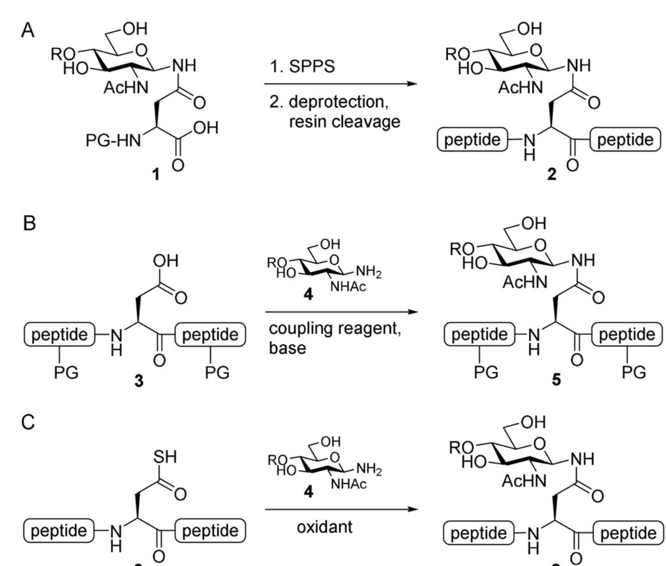
Abstract: A general and robust method for the incorporation of aspartates with a thioacid side chain into peptides has been developed. Pseudoproline tripeptides served as building blocks for the efficient fluorenylmethyloxycarbonyl (Fmoc) solid-phase synthesis of thioacid-containing peptides. These peptides were readily converted to complex N-glycopeptides by using a fast and chemoselective one-pot deprotection/ligation procedure. Furthermore, a novel side reaction that can lead to site-selective peptide cleavage using thioacids (CUT) was discovered and studied in detail.

Protein N-glycosylation is an important posttranslational modification involved in many regulatory events.[1] In this process, an oligosaccharide is transferred to asparagine within the consensus sequence Asn-X-Ser/Thr. Studying the biological functions of this modification usually requires an efficient synthetic access to N-glycopeptides.[2] To achieve this goal, two different approaches are commonly applied. In the linear approach (Scheme 1 A) a pre-glycosylated aspartic acid derivative **1** is applied in solid-phase peptide synthesis (SPPS).[3] Larger glycopeptides are accessible by a convergent approach in which the peptide **3** is glycosylated after SPPS (Scheme 1 B). Pioneered by Lansbury and co-workers in the early 1990s,[4] a distinct aspartic acid residue is selectively deprotected, activated, and coupled to a glycosyl amine **4** under basic conditions. The main drawback of this approach is the formation of aspartimides that can be efficiently suppressed by the introduction of a pseudoproline at the Ser/Thr residue within the consensus sequence.[5] Due to the lack of chemoselectivity, however, the use of protected peptides is essential.



Scheme 1. A) Linear, B) convergent, and C) thioacid-mediated synthesis of N-glycopeptides. PG = protecting group, R = H or glycan.

The application of peptides **6** in which the side-chain carboxy group of aspartic acid is replaced with a thiocarboxylic acid (thioacid) promises a highly chemoselective access to N-glycopeptides employing unprotected peptides and glycans thus preventing laborious late-stage protecting-group manipulations (Scheme 1 C). It has already been shown that thioacid-containing peptides can react with glycosyl amines **4** to give N-glycopeptides under oxidative conditions without concomitant aspartimide (Asi) formation.[6] However, despite these clear benefits, thioacid-containing peptides have not found further application. The major challenge of this approach is the incorporation of aspartic thioacids into peptides by fluorenylmethyloxycarbonyl (Fmoc)-SPPS[7] because thioesters (the common protected form of thioacids) are highly susceptible to nucleophilic attack in particular during Fmoc deprotection. Herein, we report a new approach for the incorporation of aspartic thioacid into peptides in high yields. Using a tripeptide building block, we successfully synthesized a series of decapeptides. After deprotection, the peptides were chemoselectively ligated with mono- and oligosaccharide-derived glycosyl amines forming N-glycopeptides. During our investigation, we discovered a thioacid-mediated side reaction leading to peptide cleavage thus offering new applications of thioacid-containing peptides. Depending on the reaction conditions, this side reaction can be either minimized or used for a quantitative, site-specific peptide cleavage.

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To achieve N-glycopeptide synthesis via thioacids according to Scheme 1 C, we envisioned a building block for Fmoc-SPPS containing a protected thioacid which survives the conditions of peptide synthesis (especially Fmoc deprotection under basic conditions as well as coupling conditions) and is preferably removed during global deprotection under acidic conditions. Previously, trityl (Trt) thioesters were shown to be suitable precursors for thioacids.[8] However, their applicability in SPPS is limited due to their low stability.[7] 2,4,6-Trimethoxybenzyl (Tmob) thioesters were reported to resist Fmoc deprotection conditions (20 % piperidine in DMF).[9]

To confirm the advantage of Tmob over Trt thioesters, we set up a simple model system. Phenylacetic acid Trt and Tmob thioesters were tested for their stability against 20 % piperidine in DMF (Figure S1, Supporting Information). While the Trt thioester decomposed within minutes, more than 95 % of the Tmob derivative were intact after six hours. However, Fmoc-Asp(STmob)-OH (**7**) (Figure 1) was not a suitable building block for SPPS and gave only low coupling yields, probably due to degradation via cyclic anhydride formation. Dipeptide building blocks Fmoc-Asp(STmob)-Xaa-OH (**8**) (Figure 1) solved the problem of decomposition but suffered from C-terminal racemization during fragment coupling. The only way to circumvent this racemization is the use of glycine, proline,[10] or a pseudoproline-protected ($\Psi^{me,me}$pro) Ser or Thr residue at the C-terminus,[11] limiting the scope of the approach significantly. Since N-glycosylation requires the consensus sequence Asn-Xaa-Ser/Thr, we considered pseudoproline tripeptides Fmoc-Asp(STmob)-Xaa-Ser/Thr($\Psi^{me,me}$pro)-OH (**9**) (Figure 1) as building blocks for SPPS. The C-terminal pseudoproline not only prevents racemization of the Ser/Thr residue during fragment coupling[11b] but is also known to efficiently suppress Asi formation during SPPS.[5] Since both the Tmob group and the pseudoproline are removed during global deprotection under acidic conditions, tripeptides of type **9** should meet all requirements for application in Fmoc-SPPS.

Figure 1. Potential building blocks for the incorporation of aspartic thioacid by Fmoc-SPPS.

As a first tripeptide building block we synthesized Fmoc-Asp(STmob)-Ala-Thr($\Psi^{me,me}$pro)-OH (**18 a**) (Scheme 2). Fmoc-Thr-OH (**10**) was reacted with trichloroacetimidate **11**[12] yielding 2-phenylisopropyl ester **12**. Treatment of **12** with piperidine to remove the Fmoc group and subsequent coupling with Fmoc-Ala-OH gave dipeptide **13 a**. Another peptide coupling cycle with Fmoc-Asp(OBn)-OH led to tripeptide **14 a**. We next selectively hydrogenolyzed the benzyl ester in presence of the 2-phenylisopropyl ester and the Fmoc group yielding acid **15 a**. The formation of the pseudoproline was achieved under slightly acidic conditions with pyridinium *p*-toluenesulfonate (PPTS) leaving the acid-labile 2-phenylisopropyl ester untouched. To keep the amount of aspartimide low, it was crucial to introduce the pseudoproline prior to the thioesterification. The latter was achieved with PyBOP, DIPEA and TmobSH at low temperatures (−15 to 0 °C) and led to tripeptide thioes-

Scheme 2. Synthesis of aspartic thioacid-containing tripeptide building block **18 a**. Pip = piperidine, HBTU = *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = *N,N*-diisopropylethylamine, pyr = pyridine, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

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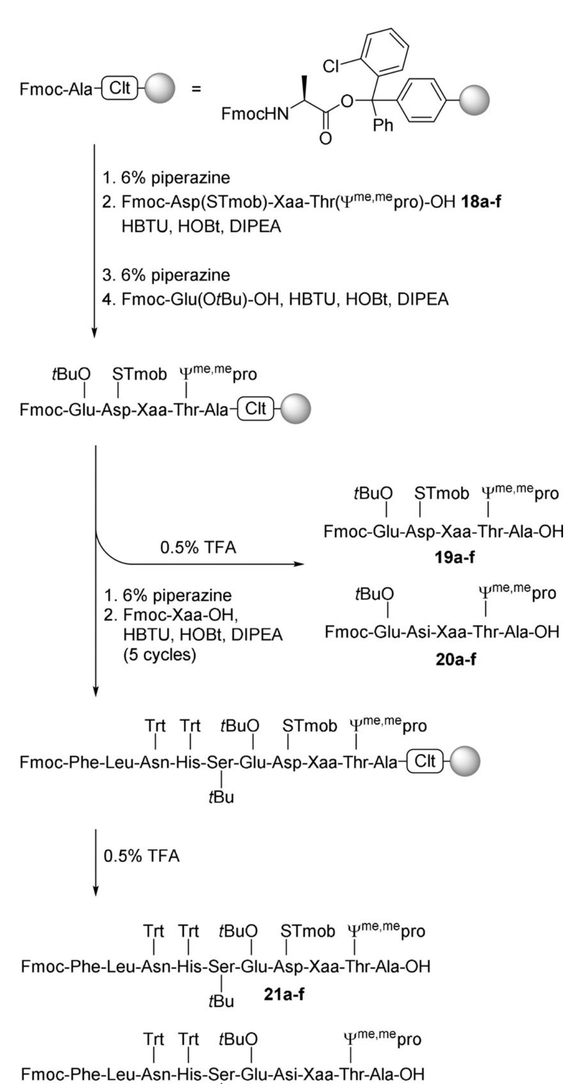
ter **17a** in very good yields. Finally, the C-terminus was deprotected with dilute trifluoroacetic acid (TFA) and the pseudoproline was reinstalled due to partial deprotection to yield the desired tripeptide building block **18a**. Following the same synthetic route, we synthesized five further tripeptide building blocks Fmoc-Asp(STmob)-Xaa-Thr($\Psi^{me,me}$pro)-OH **18b–f** with different amino acids Xaa in position $n+1$ (**18b**: Asp(O*t*Bu), **18c**: Lys(Boc), **18d**: Ser(*t*Bu), **18e**: Trp(Boc), **18f**: Gly) (Scheme S1, Supporting Information).

With the six different tripeptides **18a–f** in hand we performed Fmoc-SPPS (Scheme 3). As a model peptide we chose fragment 205–214 (Phe-Leu-Asn-His-Ser-Glu-Asn-Ala-Thr-Ala) of human haptoglobin, which contains the N-glycosylated Asn-Ala-Thr motif.[13] We started from Fmoc-alanine-loaded 2-chlorotrityl-modified (Clt) polystyrene resin.[14] The Fmoc group was deprotected with piperazine and subsequently the tripeptide building block Fmoc-Asp(STmob)-Ala-Thr($\Psi^{me,me}$pro)-OH **18a** was coupled. This was readily achieved by using five equivalents of tripeptide **18a** along with HBTU/HOBt and DIPEA for three hours. Complete coupling was confirmed by the absence of free amino groups in the Kaiser test.[15] After coupling of the tripeptide building block, another deprotection-coupling cycle with Fmoc-Glu(O*t*Bu)-OH was performed. To reduce the possible formation of aspartimide to a minimum, we consistently relied on piperazine for Fmoc deprotection.[16] At that stage (pentapeptide), we analyzed the synthetic outcome by cleavage of the product from a small resin sample with dilute TFA. The 2-chlorotrityl linker allowed the release of fully protected pentapeptide **19a** from the resin for straightforward product analysis by LCMS. The chromatogram revealed highly pure Ala-pentapeptide **19a** (Figure 2A). Virtually no aspartimide **20a** could be detected.



Scheme 3. Application of tripeptide building blocks **18a–f** in Fmoc-SPPS. Xaa = (**a**) Ala, (**b**) Asp(O*t*Bu), (**c**) Lys(Boc), (**d**) Ser(*t*Bu), (**e**) Trp(Boc), (**f**) Gly.

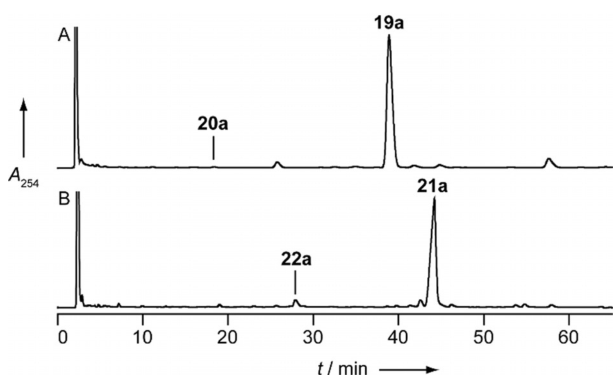


Figure 2. LCMS chromatogram (254 nm) of A) Ala-pentapeptide **19a** and B) Ala-decapeptide **21a** after analytical resin cleavage.

Encouraged by these first results, we further elongated the peptide. After five additional deprotection–coupling cycles, the resulting Ala-decapeptide **21a** was analyzed as described above. Again, the LCMS chromatogram showed highly pure peptide (Figure 2B). Only small amounts of aspartimide **22a** were found. The ratio of desired Ala-decapeptide **21a** to aspartimide **22a** was 96:4 as determined by integration of the corresponding peaks recorded at 254 nm (Table 1, entry 1). These findings demonstrated that the tripeptide approach is indeed highly suitable for the direct incorporation of aspartic thioacids into peptides via Fmoc-SPPS.

Table 1. Ratio of desired peptide to aspartimide at the penta- and decapeptide stages as determined by integration of the corresponding peaks in the LCMS chromatogram (254 nm). Isolated yields of decapeptides **21a–e**.

Entry	Xaa	19/20	21/22	Yield (21) [%]
1	**a**: Ala	>99:1	96:4	59
2	**b**: Asp(O*t*Bu)	99:1	89:11	66
3	**c**: Lys(Boc)	>99:1	99:1	72
4	**d**: Ser(*t*Bu)	>99:1	95:5	62
5	**e**: Trp(Boc)	>99:1	94:6	52
6	**f**: Gly	96:4	12:88	–[a]

[a] Not determined.

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Next, we applied the tripeptide building blocks **18b**–**f** in Fmoc-SPPS (Scheme 3). In all cases, the desired decapeptides **21b**–**f** were obtained. The ratio of decapeptide to aspartimide was mostly between 94:6 (Trp-decapeptide **21e**/**22e**; Table 1, entry 5) and 99:1 (Lys-decapeptide **21c**/**22c**; entry 3). For the Asp-decapeptide the ratio was 89:11 (**21b**/**22b**; entry 2). Only the glycine sequence gave high amounts of aspartimide for the decapeptide (ratio **21f**/**22f** = 12:88; entry 6) although the ratio was acceptable for the tetrapeptide (**19f**/**20f** = 96:4). Glycine in position $n+1$ of aspartic acid derivatives is known to be highly prone to aspartimide formation.[17] The decapeptides **21a**–**e** were purified by flash chromatography and isolated in yields ranging from 52 to 72% (Table 1).

Subsequently, we converted the protected thioaspartic peptides **21a**–**e** into substrates for the synthesis of N-glycopeptides (Scheme 4). For deprotection the decapeptides **21a**–**e** were treated with TFA/TIS/H_2O (95:2.5:2.5). During optimization of the reaction conditions, we found a deprotection time of 50–60 minutes to be optimal. Interestingly, longer reaction times led to the formation of a side product which we identified as the heptapeptide **31** bearing a C-terminal cyclic thioanhydride (Figure 3A). Its formation can be explained by the intramolecular nucleophilic attack of the side-chain thioacid within **29a**–**e** to the protonated backbone amide. Upon thioanhydride formation, the C-terminal tripeptides **30a**–**e** are released.

By comparing several peptide sequences, we found that the efficiency of this peptide cleavage using thioacids (peptide CUT) is to some extent dependent on the amino acid at the $n+1$ position (Table S1, Supporting Information). Most remarkably, the CUT can be highly efficient; prolonging the reaction time to several hours resulted in complete cleavage (Figure 3B, 21 h). Furthermore, by treatment of **31** with aqueous acid, the thioanhydride was hydrolyzed to the aspartic acid derivative **32**. Since aspartimide formation is often associated with isomerization of the Asp residue, we investigated whether this is also the case for the C-terminal aspartic acid of **32**. Comparison of the analytical data of **32** with those of synthetic reference compounds containing either L- or D-Asp clearly demonstrated that the cleavage process occurs without isomerization of the Asp residue (Supporting Information). Thus, peptide CUT marks a unique new way for the highly efficient and site-selective cleavage of peptide bonds. It also provides easy access to peptide thioanhydrides that are valuable synthetic in-

21a-e → 1. TFA/H_2O/TIS (95:2.5:2.5); 2. 23, Cu(OAc)$_2$•H_2O, HOBt, DMF; 3. RP-HPLC → Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Xaa-Thr-Ala-OH 24a-e

Scheme 4. One-pot deprotection/N-glycosylation of aspartic thioacid-containing decapeptide **21a**–**e**. TIS = triisopropylsilane.

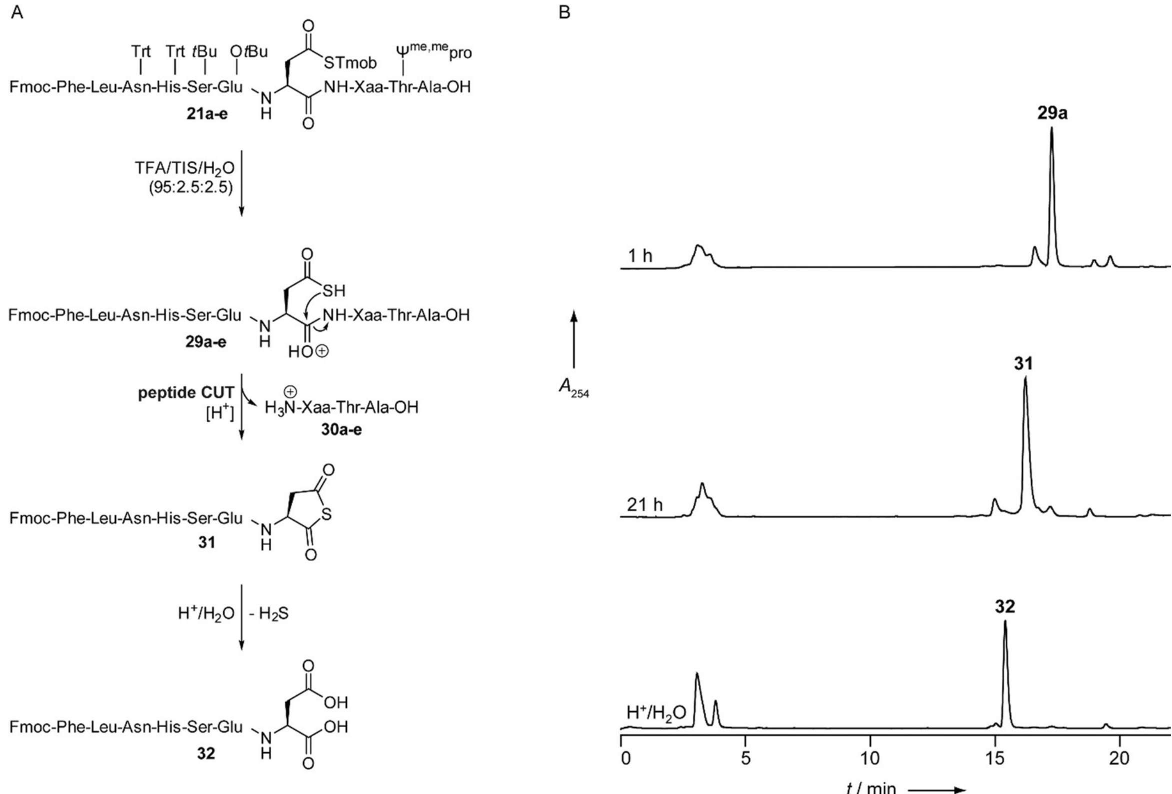

Figure 3. A) Peptide CUT of decapeptides **21a**–**e** during treatment with TFA/TIS/H_2O (95:2.5:2.5) and subsequent hydrolysis of thioanhydride **31** with aqueous acid. B) LCMS chromatograms (254 nm) from the reaction mixture of Ala-decapeptide **21a** with TFA/TIS/H_2O after 1 and 21 h and after subsequent hydrolysis with 1% formic acid (aq.)/DMF (1:1).

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termediates.[18] Nevertheless, these findings do not diminish the access to thioaspartic peptides since the outcome of the deprotection reaction—thioacid deprotection versus peptide cleavage—can be easily controlled by the reaction time.

For the synthesis of N-glycopeptides, we developed a one-pot global deprotection/N-glycosylation protocol to minimize hydrolysis and other decomposition pathways of the free aspartic thioacids (Scheme 4). Thus, the peptides **21 a**–**e** were first treated with a mixture of TFA, TIS, and water (95:2.5:2.5). After one hour, the deprotection cocktail was removed and the peptides **29 a**–**e** were reacted with glycosylamine **23** in a copper-promoted thioacid–amine-ligation.[6b] Insoluble copper sulfide[19] was removed by centrifugation and the reaction mixture was subjected to RP-HPLC. The desired N-glycopeptides

Table 2. Ligation of aspartic thioacid-containing decapeptides **21 a**–**e** with GlcNAc **23**, chitobiose **25** and nonasaccharide **26**.

Entry	Glycan	Peptide	Product	Isolated yield [%]
1	**23**	**21 a**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Ala-Thr-Ala-OH **24a**	58
2	**23**	**21 b**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Asp-Thr-Ala-OH **24b**	52
3	**23**	**21 c**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Lys-Thr-Ala-OH **24c**	61
4	**23**	**21 d**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Ser-Thr-Ala-OH **24d**	51
5	**23**	**21 e**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Trp-Thr-Ala-OH **24e**	77
6	**25**	**21 a**	Fmoc-Phe-Leu-Asn-His-Ser-Glu-Asn-Ala-Thr-Ala-OH **27a**	56
7	**26**	**21 a**	**28a**	21[a]

[a] Solvent: DMSO.

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24 a–**e** were obtained in yields of 51–77% over two steps (Table 2, entries 1–5) besides some hydrolyzed thioacids as major side products which were also observed by Garner and co-workers.[6b]

Next, we investigated the synthesis of more complex N-glycopeptides using larger glycans. Much to our delight, the application of the glycosylamine **25** derived from chitobiose (Supporting Information) led to the smooth formation of glycopeptide **27 a** (56%, entry 6). Use of the complex-type nonasaccharide amine **26**, which we synthesized from the corresponding glycosyl azide[20] (Supporting Information) was also successful and delivered complex oligosaccharyl decapeptide **28 a** in a yield of 21% after HPLC purification (entry 7). These results emphasize the general feasibility of the one-pot deprotection/N-glycosylation protocol for chemoselective synthesis of N-glycopeptides from aspartic thioacid-containing peptides. The approach does not only give good yields but is also fast and non-laborious.

In conclusion, we have developed a tripeptide building block approach for the synthesis of aspartic thioacid-containing peptides via Fmoc-SPPS. These peptides were successfully converted to N-glycopeptides through a chemoselective thioacid–glycosylamine ligation. The high chemoselectivity of the N-glycopeptide formation provides an alternative to the tedious protecting group manipulations of protected full-length peptides. Furthermore, we discovered a new thioacid-mediated site-selective and highly efficient peptide bond cleavage reaction. These findings give interesting new insights into the chemistry and application of peptide thioacids leading to new perspectives in peptide chemistry.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioorganic chemistry · glycopeptides · ligation · solid-phase peptide synthesis · thioacids

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