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Late-Stage Solubilization of Poorly Soluble Peptides Using Hydrazide Chemistry

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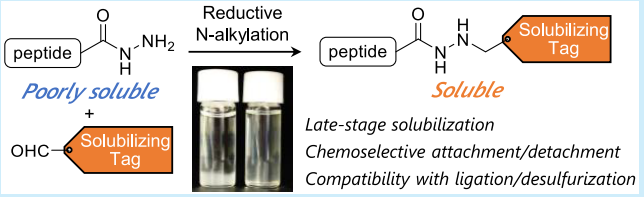
ABSTRACT: A novel late-stage solubilization of peptides using hydrazides is described. A solubilizing tag was attached through a selective N-alkylation at a hydrazide moiety with the aid of a 2-picoline−borane complex in 50% acetic acid−hexafluoro-2-propanol. The tag, which tolerates ligation and desulfurization conditions, can be detached by a Cu-mediated selective oxidative hydrolysis of the *N*-alkyl hydrazide. This new method was validated through the synthesis of HIV-1 protease.



Recent developments in protein research include the use of synthetic protein probes such as precisely labeled proteins, proteins with novel topologies, and mirror image proteins.[1] A strategy for chemical protein synthesis begins with solid-phase peptide synthesis (SPPS) to prepare peptide segments followed by chemoselective assembly of the segments to construct a backbone of the full length of a protein. Among the techniques to assemble peptides, native chemical ligation (NCL), in particular, has been widely used.[2,3] Although modern synthetic chemistry enables the total synthesis of proteins consisting of more than 400 residues,[4] total chemical synthesis of proteins is challenging when dealing with poorly soluble peptides.[5] Consequently, a versatile method that can enhance the solubility of peptides is needed.

The temporary attachment of hydrophilic tags onto poorly soluble peptides to improve their aqueous solubility has been reported.[6] In an early publication, the installation of poly-Arg at the C-terminus of the peptide thioester was developed (Scheme 1A).[7] In this method, the peptide of interest, with the solubilizing tag on a thioester leaving group, is synthesized. However, this solubilizing tag is eliminated during the ligation, so the enhanced solubility cannot be retained during the ligation and purification steps. To overcome this problem, many semipermanent solubilizing tags attached to side chains or backbone amides were developed (Scheme 1B).[8] In these strategies, the tag can be retained during the ligation and subsequent purification steps, enabling the improved handling of the peptide materials. The tags can be removed after synthesis of the entire structure of the desired protein. Despite these advantages, the method has a potential limitation because the tag moiety must be introduced during SPPS, and thus the peptide segments with the solubilizing tag must be resynthesized if the solubility-enhancing property is insufficient.

We have recently developed a trityl-type tag that enables the late-stage solubilization of poorly soluble peptides (Scheme 1C).[9] In this method, the trityl-type tag was attached to the side chain of Cys residues or other thiol groups in an on-demand manner after preparation of the peptide by SPPS or NCL. This strategy enables a choice of types of solubilizing tags such as poly-Arg, poly-Lys, or poly trimethyllysine,[10] even after the synthesis of peptides of interest, but thiol groups at which to install the tag must be present in the peptide. This late-stage solubilization should be useful in the challenging syntheses of poorly soluble peptides or proteins.

We took advantage of hydrazide chemistry to achieve the late-stage solubilization of poorly soluble peptides. Reductive alkylation between hydrazides and aldehydes is expected to be potentially compatible with unprotected peptides.[11] Seitz et al. immobilized an unprotected peptide hydrazide on an aldehyde-modified plate surface through hydrazone formation followed by reduction, yielding the *N*-alkyl hydrazide, which is stable under the conditions of NCL. The resulting peptides were successfully used for on-surface NCL followed by desulfurization.[12] In addition, alkyl hydrazides can be hydrolyzed in the presence of a Cu(II) salt to give the corresponding carboxylic acid.[13] On the basis of these reports, we developed the reaction sequence in Scheme 1D for the late-stage solubilization and removal of solubilizing tags using hydrazide chemistry. This strategy is based on (1) installation of the solubilizing tag, which converts the aldehyde moiety to the

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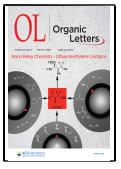

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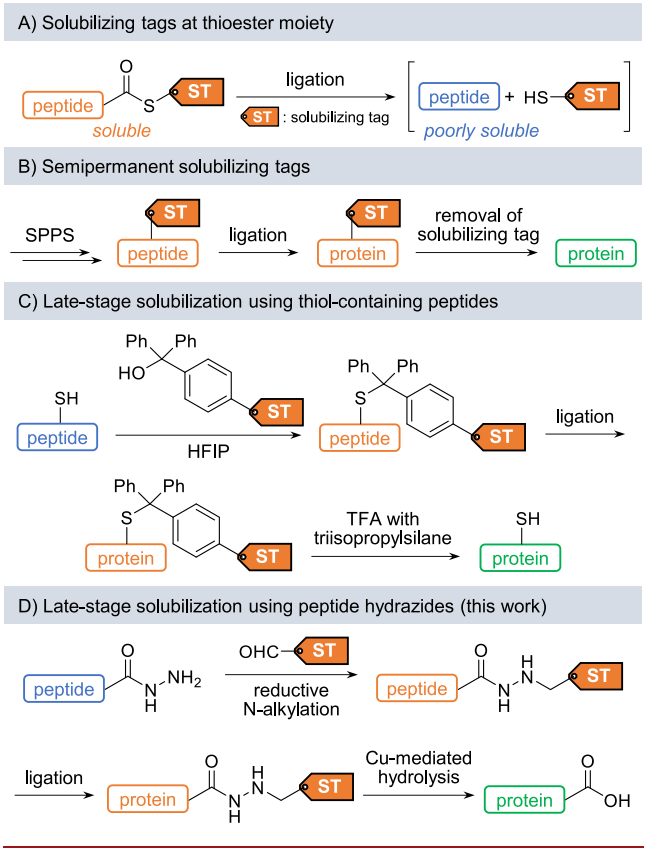
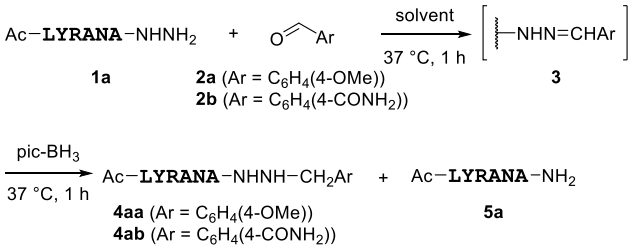
Scheme 1. Solubilization Strategies for Poorly Soluble Peptides



peptide hydrazide by a selective reductive N-alkylation, (2) assembly of peptide segments by ligation with the tag-installed soluble peptide, and (3) Cu(II)-mediated selective oxidative hydrolysis of the N-alkylated hydrazide to produce the corresponding carboxylic acid with the temporarily introduced solubilizing tags removed. In this Letter, we used C-terminal peptide hydrazides to evaluate the applicability of this hydrazide chemistry to the solubilization of poorly soluble peptides.

In our initial attempts to complete the envisioned reactions, the peptide hydrazide Ac-LYRANA-$NHNH_2$ (**1a**) was used as a substrate,[14] and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was selected as the solvent for the installation of the solubilizing tag. HFIP has the ability to assist in dissolving poorly soluble peptides in other solvents,[15] which is necessary because this strategy is to be applied to poorly soluble compounds. The peptide hydrazide (**1a**) and 4-anisaldehyde (**2a**), which has an electron-donating group, were reacted in HFIP for 1 h; then, 20 equiv of 2-picoline−borane complex (pic-BH_3)[16] was added to the reaction mixture, affording the corresponding *N*-alkyl hydrazide (**4aa**) in 42% yield (Table 1 entry 1). The increase in the reaction time to 24 h for the reduction slightly improved the conversion, but the reaction was incomplete (entry 2). The use of AcOH as a cosolvent with HFIP (50% (*v*/*v*)) accelerated the reductive alkylation (entries 3−5).[17] After the reduction for 1 h followed by exposure to an air-stream and subsequent trituration with Et_2O to remove the solvent and excess reagents, the N-alkylated hydrazide was obtained in 87% yield together with a small amount of the peptide amide (**5a**), which was probably generated by reductive cleavage of the hydrazide moiety. A

Table 1. Reductive N-Alkylation of Peptide Hydrazides[a]



			HPLC purity (%)[b]	
entry	aldehyde	solvent	**4**	**5a**
1	**2a**	HFIP	42	<1
2[c]	**2a**	HFIP	50	2
3	**2a**	50% AcOH−HFIP	87	6
4[d]	**2a**	50% AcOH−HFIP	86	<1
5	**2b**	50% AcOH−HFIP	68	4

[a]Reactions were performed as follows: The peptide hydrazide **1a** (1 mM) and aldehyde **2** (1.5 mM) were dissolved in the solvent and then incubated at 37 °C for 1 h. After the addition of pic-BH_3 (20 equiv) to the mixture, the reduction was performed at 37 °C for 1 h. After removal of the solvent by air blowing, the crude material was washed with Et_2O. The resulting precipitate was dissolved in 20% CH_3CN−H_2O and analyzed by HPLC. [b]Detected at 220 nm. [c]Reduction was performed for 24 h. [d]Reduction was performed for 10 min.

short reduction time (10 min) resulted in the suppression of peptide amide formation (entry 4). Using 4-formylbenzamide (**2b**) bearing an electron-withdrawing group, lower reactivity to the reduction of the hydrazone (entry 5) was observed.[18] In light of these results, 4-anisaldehyde was used in the subsequent experiments.

We then examined the Cu-mediated oxidative hydrolysis of the *N*-alkyl hydrazide. The crude material prepared by reductive alkylation of the hydrazide (**1a**) with 4-anisaldehyde reacted with $CuSO_4 \cdot 5H_2O$ (20 equiv) in 20% CH_3CN-containing aqueous solution, affording the corresponding carboxylic acid (**6a**) in a full conversion within 1 h (Table 2, entry 1). The hydrolysis also proceeded with decreased amounts of $CuSO_4$ (2.0 equiv) or even only catalytic amounts of copper salt (0.1 equiv) under an oxygen atmosphere (entries 2 and 3). The benzyl alcohol (**7**) and 4-anisaldehyde (**2a**) were observed simultaneously as coproducts during the hydrolysis. The possible role of the Cu(II) salt would be as an oxidant to form an acyl diazene intermediate from the *N*-alkyl hydrazide, yielding a carboxylic acid and a diazene derivative (**8**) (Scheme 2). The diazene would afford benzyl alcohol and benzaldehyde through further oxidation or tautomerization, respectively.[19]

The behavior of eight C-terminal amino acids (Ala, Val, Phe, Ser, Thr, His, Lys, and Arg) was also evaluated. As summarized in Table 2, the desired carboxylic acids (**6a**−**6h**) were obtained with moderate to high purity (62−91%). The epimerization of C-terminal amino acids (Ala and Val) was suppressed (epimer ratio >99:1, Figure S19). The peptide consisting of oxidative-prone residues, H-WCLYRAM-NH_2, was stable under the Cu-mediated oxidative hydrolysis conditions for at least 5 h while the disulfide dimer was immediately formed (Figure S20).

A synthetic application of our strategy was confirmed through the synthesis of human immunodeficiency virus type 1 (HIV-1) protease, inhibitors of which have been used for the treatment of acquired immunodeficiency syndrome.[20] Total chemical synthesis of this enzyme was reported by Kent et al.,

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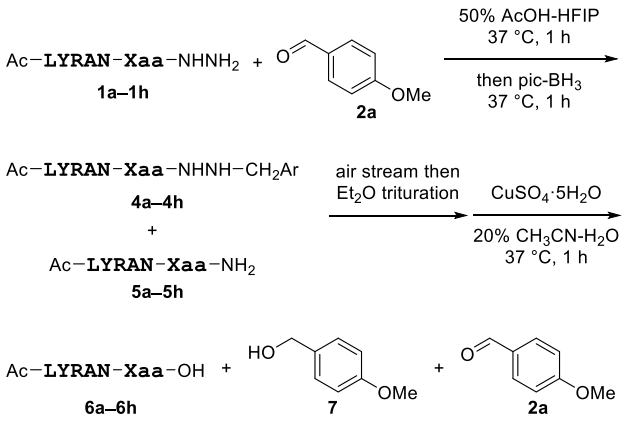
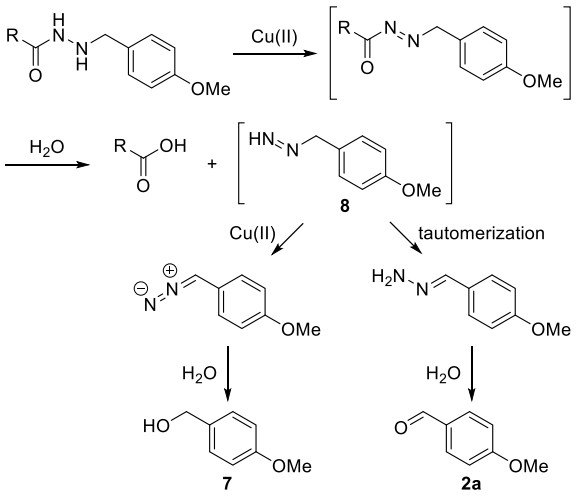
Table 2. Cu-Mediated Oxidative Hydrolysis of *N*-Alkyl Hydrazides and Scope of C-Terminal Amino Acids[a]



			HPLC purity (%)[b]		
entry	Xaa	product	4	5	6
1	Ala	**6a**	87	6	62
2[c]	Ala	**6a**			55
3[d]	Ala	**6a**			62
4	Val	**6b**	91	n.d.[e]	83
5	Phe	**6c**	80	4	80
6	Ser	**6d**	88	3	91
7	Thr	**6e**	88	3	73
8	His	**6f**	82	3	88
9	Lys	**6g**	90	n.d.	91
10	Arg	**6h**	93	n.d.	83

[a]All reactions were performed as follows: After reductive alkylation, as described in Table 1, the peptide was subjected to hydrolysis with $CuSO_4·5H_2O$ (20 equiv) in 20% CH_3CN-H_2O at 37 °C in an air atmosphere. After 1 h, the reaction was quenched with DTT (200 equiv) in 0.5 M HEPES buffer at 37 °C for 15 min. The product was analyzed by HPLC. [b]Detected at 220 nm. [c]Hydrolysis was performed with 2 equiv of $CuSO_4·5H_2O$. [d]Hydrolysis was performed with 0.1 equiv of $CuSO_4·5H_2O$ in an oxygen atmosphere for 19 h. [e]n.d., not detected.

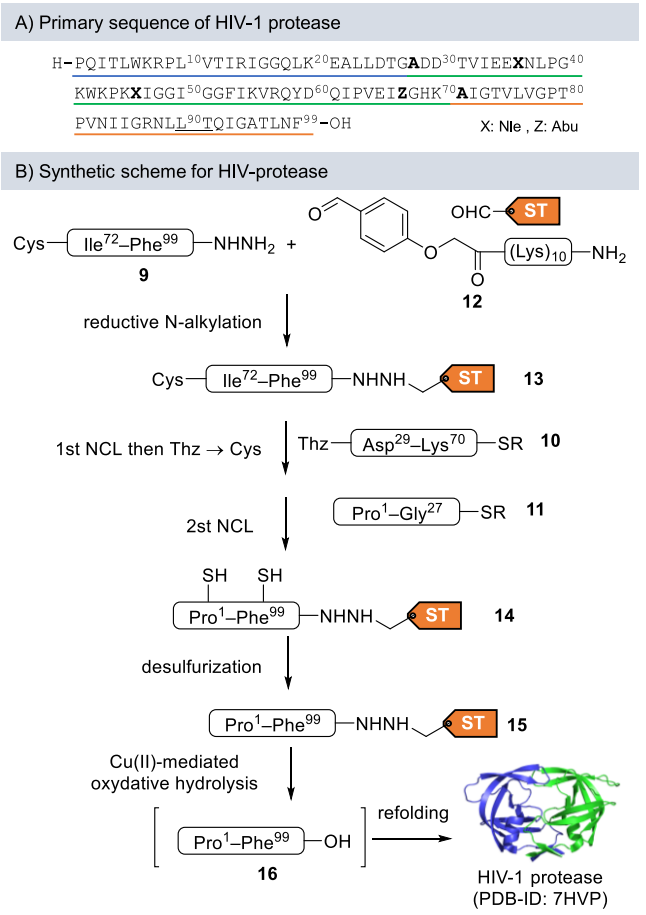
Scheme 2. Possible Pathway for the Generation of Benzyl Alcohol and Benzaldehyde during Cu-Mediated Oxidative Hydrolysis



who used the solubility-enhancing properties of the C-terminal Arg 10-mer tag to overcome the difficulty stemming from the insolubility of the intermediate peptide products.[21] The solubilizing tag was removed by the autoprocessing property of Gag–Pol polyprotein that occurs during maturation of the HIV-1 virus *in vivo*. Being used with HIV-1 protease, which can be matured by itself, the synthetic design does not cover other proteins that have no such autoprocessing property.

Our synthetic plan is shown in Scheme 3. The entire sequence of HIV-1 protease was divided into three peptide

Scheme 3. Synthetic Pathway to HIV-1 Protease



segments (**9**–**11**) with the replacement of Ala at the ligation junctions by Cys or 1,3-thiazolidine-4-carboxo (Thz), a protected Cys.[22] The C-terminal segment (**9**) is known to be insoluble in aqueous media,[21] and its direct synthesis by SPPS is difficult due to the on-resin aggregation of an elongated peptide.[23] We therefore planned to introduce the solubilizing tag into the insoluble peptide (**9**) and to apply a pseudoproline strategy, which can suppress the on-resin aggregation of an elongated peptide, to the synthesis of the peptide segment.[24]

All of the segments were synthesized as hydrazides, and the thioester segments (**10** and **11**) were produced from the corresponding hydrazides by a method developed by our group.[25] Using pseudoproline units at the Leu[90]–Thr[91] sites, the peptide (**9**) was elongated with no synthetic difficulties. The aldehyde-incorporated Lys 10-mer (**12**) as a solubilizing tag was prepared through conventional SPPS. The reductive N-alkylation of hydrazide **9** with the solubilizing tag **12** was performed in 50% AcOH–HFIP with the help of pic-BH_3 for 10 min to suppress the excess installation of **12** on the N-terminal Cys through the thiazolidine formation. This

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successfully produced the desired segment (**13**) without any protections on the Cys residue (Figure 1A).[26] The resulting

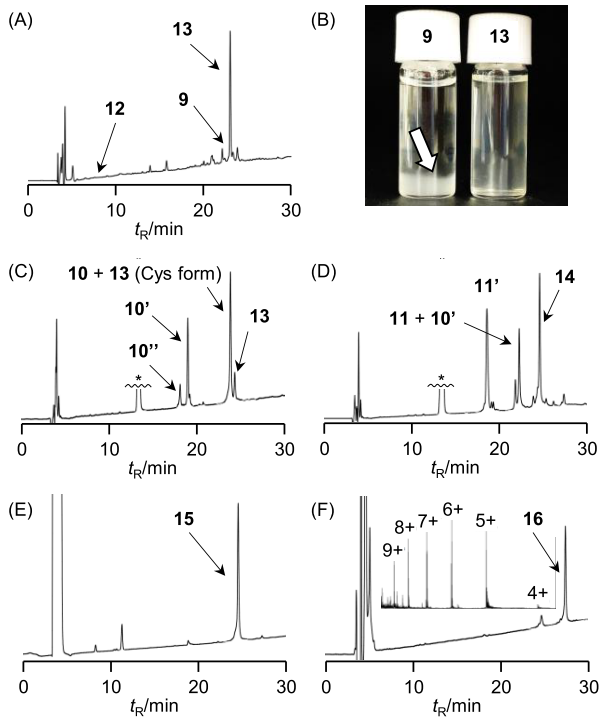

Figure 1. Enhanced solubility of peptide **9** and HPLC analyses of reactions for HIV-1 protease. (A) Reductive *N*-alkylation of **9** with **12** (t = 10 min). (B) 9 μmol of peptide **9** (left) or peptide **13** (right) in 2 mL of 6 M guanidine·HCl−0.1% TFA aq. (C) Ligation between **13** and **10** for 3 h followed by deprotection of Thz (t = 12 h). (D) Ligation between **10** + **13** and **11** (t = 10 h). (E) Desulfurization of **14** (t = 13 h). (F) Cu(II)-mediated oxidative hydrolysis of **15** (t = 1 h). The calculated mass is 10703.69 Da (average isotopes). Deconvolution of the mass spectrum yielded an observed mass of 10 704.28 Da as a proton adduct. *4-Mercaptophenylacetic acid. **10′**, lactam form of **10** at the C-terminal Lys; **10″**, hydrolyzed **10**; **11′**, methoxyamide form of **11**. For reaction and HPLC conditions, see the Supporting Information.

peptide (**13**) showed improved solubility in an aqueous solution compared with the C-terminal segment (**9**), which lacks the solubilizing tag, enabling the purification of the segment (Figure 1B). Ligation between **13** and **10** followed by ring-opening of Thz by the addition of CH_3ONH_2·HCl and the subsequent NCL reaction with **11** were performed in a one-pot manner, yielding the full-length protein (**14**) in 33% isolated yield (Figure 1C,D). After the desulfurization of the resulting protein followed by HPLC purification (70% isolated yield, Figure 1E), the solubilizing tag was smoothly removed with the aid of $CuSO_4$ in CH_3CN−H_2O (Figure 1F). Dialysis of the reaction mixture containing the entire HIV-1 protease sequence (**16**) in the refolding buffer solution decreased the Cu concentration to 1.1 ppm and afforded HIV-1 protease (80%, determined by UV absorbance at 280 nm), which has the enzymatic activity to cleave its substrate peptide (Figure S31).

In summary, we have developed a novel strategy that enables late-stage solubilization of poorly soluble peptides using hydrazide chemistry. The solubilizing tag was attached to peptides bearing a hydrazide moiety by selective, reductive N-alkylation and can be detached by selective Cu(II)-mediated oxidative hydrolysis without significant production of side products. Various aldehyde-containing tags can be used in this protocol. Because the structure of a suitable solubilizing tag (e.g., anionic, cationic, or nonionic) depends on the peptide of interest, such a late-stage attachment strategy is practically preferred over trial-and-error solubilizing studies. Using this chemistry, a His-tag or even a solid support to assist in the purification step could be adopted as the "tag" in the future. Although it was demonstrated through the solubilization of C-terminal segments, our strategy, in principle, should also be applicable to the solubilization of N-terminal or middle segments through the Asp/Glu side-chain hydrazides.[9c] These applications of the developed strategy are in progress in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c00074.

Experimental details of syntheses, charts for the HPLC analyses, and mass spectra of synthesized products (PDF)

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Notes

The authors declare no competing financial interest.

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