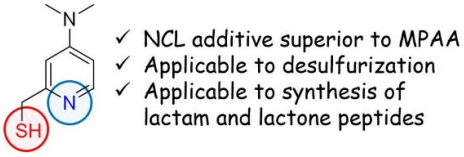
Sulfanylmethyldimethylaminopyridine as a Useful Thiol Additive for Ligation Chemistry in Peptide/Protein Synthesis

Kento Ohkawachi,[†] Daishiro Kobayashi,[†] Kyohei Morimoto,[†] Akira Shigenaga,[†] Masaya Denda,[†] Kenzo Yamatsugu,[‡] Motomu Kanai,[‡] and Akira Otaka[*,†]

[†]Insutitute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University, Tokushima 770-8505, Japan.

[‡]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan.

Supporting Information Placeholder



ABSTRACT: Sulfanylmethyl-installed dimethylaminopyridine, 2-sulfanylmethyl-4-dimthylaminopyridine (SMDMAP) (**2**), has an acidic thiol group comparable to that in aryl thiols due to the formation of a zwitterion consisting of a thiolate anion and a pyridinium cation. It can be used as an additive for native chemical ligation. The alkyl thiol in **2** allows it to be used for the one-pot/NCL–desulfurization protocol in peptide synthesis. The utility of **2** in the synthesis of cyclic peptides is demonstrated.

A variety of thiol additives have been utilized to promote native chemical ligation (NCL) involving the chemoselective condensation between thioesters and peptides with N-terminal cysteine.[1] The moderate reactivity of alkyl thioester peptides generally employed for NCL supports their shelf-stable property, but completion of NCL with the alkyl thioesters requires the use of thiols such as aryl thiols including 4-mercaptophenylacetic acid (MPAA).[2] Thioester exchange generating the aryl thioester enables NCL to proceed to completion in a few hours. To date, MPAA as a water-soluble aryl thiol has been the standard additive for NCL.

Advances in NCL protocols for protein synthesis include a one-pot/sequential use of NCL-related technologies containing the ligation and desulfurization[3] of cysteine to form an alanine residue. Such a one-pot protocol avoids the laborious purification steps necessary for ligation products and improves the overall yield of the final product. Desulfurization of NCL-derived β or γ-sulfanyl amino acid residues has further enhanced the utility of NCL for synthesis of non-cysteine-containing proteins,[4] and as a result, integration of NCLs and subsequent desulfurization in a one-pot operation is desirable. MPAA has served as the most efficient thiol additive in NCL protocols. A limitation of its use, however, is that MPAA inhibits the most frequently used tris(2-carboxyethyl)-phosphine (TCEP)-mediated radical de-sulfurization[5] due to much lower bond dissociation energy of aryl thiols when compared to that of alkyl thiols.[6] Therefore, desulfurization-applicable NCL additives have been sought.[7] In this context, sodium mercaptoethanesulfonate (MESNa), trifluoroethanethiol (TFET) and methyl thioglycolate categorized into alkyl thiols have served as NCL additives compatible with the one-pot/desulfurization operation, but there is much room for improvement in terms of the NCL efficiency and the malodorous character of alkyl thiols. Alternatively, an elegant protocol using a thiol-capturing resin has been introduced.[8]

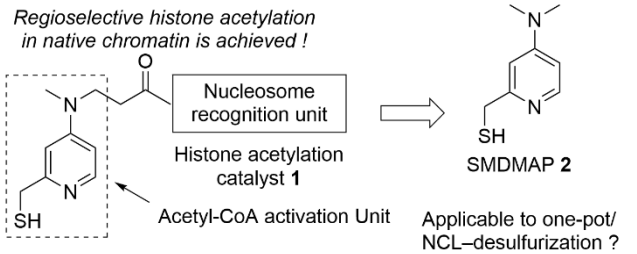
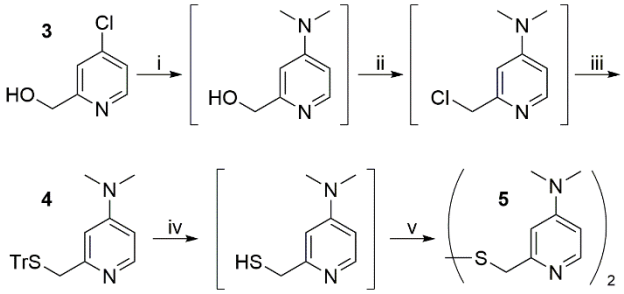


Figure 1. Structure of thiol-containing dimethylaminopyridines as thioester additives.

Recently, Kanai et al. developed a catalyst **1** which activates acetyl-CoA as a relatively stable thioester with an ε-amino group of lysine in histone proteins being regioselectively acetylated.[9] This catalyst **1** includes a thioester activation and a nucleosome recognition unit. Subsequent inter- and intra-molecular S–S and S–N acetyl transfers are thought to be responsible for the activation of acetyl-CoA through the formation of an *N*-acyl pyridinium species. The thioester activation unit in **1** forms a zwitterion consisting of the thiolate anion and a pyridinium cation, and functioning as a relatively acidic thiol.[10] Computational prediction using ACD Labs software[11] indicates that the

pK_a of the thiol in 2-sulfanylmethyl-4-dimethylaminopyridine (SMDMAP) **2** is 6.15 ± 0.6, lower than that of MPAA (6.97 ± 0.4 (predicted value); 6.6 (measured value[2])). Taking into consideration that SMDMAP provably functions as a better leaving group and more preferentially exists as the corresponding highly nucleophilic thiolate anion than MPAA does, we envisioned that at the appropriate pH, SMDMAP should be superior to MPAA as an effective NCL additive. More importantly, due to its alkyl thiol structure, SMDMAP was expected to not interfere with the subsequent desulfurization.

Scheme 1. Synthesis of the disulfide dimer of 2-Sulfanylmethyl-4-dimethylaminopyridine (di-SMDMAP 5)[a]



[a]Reagents and conditions: i) $Me_2NH{\cdot}HCl$, NaOH, H_2O, 155 °C; ii) $SOCl_2$, CH_2Cl_2, rt; iii) Tr-SH, DBU, CH_2Cl_2, rt; iv) TFA, triethylsilane, CH_2Cl_2, rt; v) DMSO-H_2O (9:1), rt.

Synthesis of SMDMAP **2** is shown in Scheme 1 and begins with 4-chloro-2-pyridinemethanol (**3**). Reaction of **3** with dimethylamine followed by chlorination with thionyl chloride and subsequent nucleophilic substitution with triphenylmethanethiol (TrSH) afforded a sulfanyl moiety incorporating DMAP (**4**) in 56% isolated yield over 3 steps. Treatment of **4** in TFA-CH_2Cl_2 with triethylsilane gave a mixture of **2** and the corresponding disulfide (di-SMDMAP **5**). Because these components were difficult to separate, the disulfide (**5**) was isolated as its hydrochloride salt[12] in 28% yield (after recrystallization) through the HCl treatment followed by DMSO-mediated oxidation of the mixture. The resulting disulfide additive **5**, following *in situ* reduction with TCEP, can be used for NCL.

Having the hydrochloride salt of the requisite thiol additive **5**·2HCl as a crystalline and shelf-stable compound, we examined its applicability to NCL using model thioesters (H-**LYRANX**-S-CH_2CH_2CO-**L**-NH_2 (**6**)) and an N-terminal cysteine peptide (H-**CSPGYS**-NH_2 (**7**)). Initially the NCL of an Ala-containing thioester (**X** = Ala (**6a**), 1 mM) with **7** (1 mM) in 6 M guanidine·HCl (Gn·HCl)–0.2 M phosphate buffer in the presence of 50 mM TCEP and 10 mM **5** at pH 6.9, 37 °C for 3 h was found to go almost to completion, affording the ligation product (H-**LYRANA-CSPGYS**-NH_2 (**8a**)) in 94% conversion (isolated yield 85%). (See Figure S1(A) in Supporting Information - SI). At pH 4, no NCL product was observed and the starting materials remained (SI, Figure S1(B)). The reaction at pH 6.0 required more than 3 h for its completion (Figure S1(C)), and the reactions at pH 8 or 10 were accompanied by the formation of hydrolyzed side products **9** (SI, Figure S1(D and E)). No acylation of the ε-amino group of lysine was observed at pH 6.9 in an attempt using a Lys-containing amine component (SI, Figure S2). Accordingly, we employed the NCL at pH 6.9 as optimized conditions for further examination of the substrate scope.

Several thioesters **6** with different C-terminal amino acids (**X** = Ala (**6a**), Leu (**6b**), Val (**6c**), Ser (**6d**) and Lys (**6e**)) were subjected to the reaction with **7** (SI, Figure S3). Ligations at aliphatic amino acid sites (**6a**-**6c**) afforded the desired products without significant side products, but NCL of **6c** required an elevated temperature (50 °C) (SI, Figure S3(A-C)). Although an acceptable amount of epimerization product[13] (< 6%) was detected in the NCL of the Ser thioester **6d** with the hydrolyzed material being generated, more than 90% conversion was achieved (Figure S3 and S4). Reaction of **6e** also gave desired material along with formation of ~8% lysine-related 7-membered ring lactam peptide (SI, Figure S3(D)).

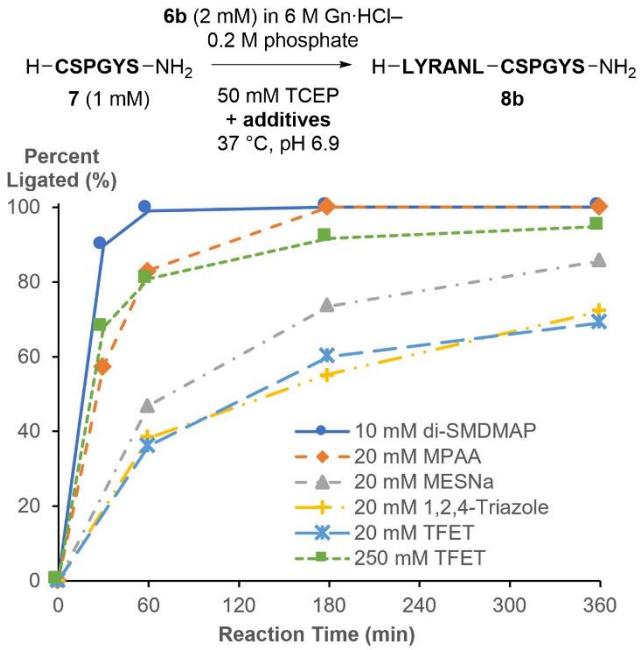


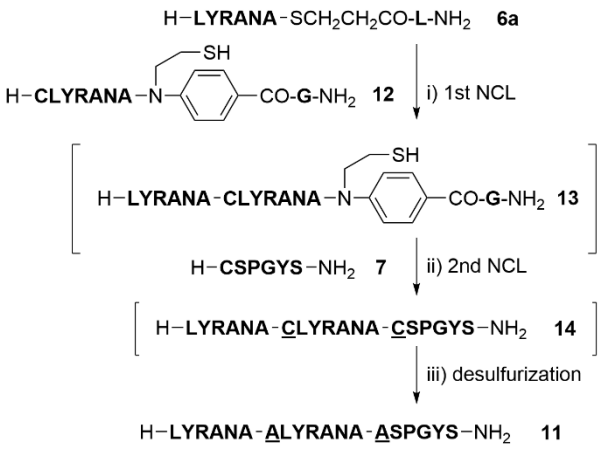
Figure 2. Comparison of SMDMAP (**2**) with other NCL additives. [a]Percentages of ligation were determined by HPLC analyses with UV detection at 220 nm and calculated using the following equation (percent ligated = 100 x (integration (integ.) **8b** / (integ. **7** + integ. **8b**)).

We next compared the catalytic activity of **2** with that of other additives (Figure 2). In the presence of 50 mM TCEP and 20 mM each of active additives, the NCL of **6b** (2 mM) with **7** (1 mM) at pH 6.9 was performed. SMDMAP showed NCL-promoting activity comparable or slightly superior to that of MPAA, as was expected from the pK_a values. In the case of low concentrations (20 mM) of desulfurization-compatible additives, SMDMAP facilitated the NCL more efficiently than MESNa, TFET or 1,2,4-triazole.[14] Although the reaction using high concentration (250 mM) of the volatile TFET (bp 35–37 °C) reached the level comparable to the MPAA or SMDMAP-mediated reaction, careful handling of TFET in a chemical fume hood is necessary. In contrast to TFET, use of the non-malodorous SMDMAP allows for the ligations without ventilating facilities.

We next evaluated the applicability of **2** to desulfurization reactions (SI, Figure S5). NCL of **6a** with **7** in the presence of 50 mM TCEP and 10 mM of **5** at pH 6.9, 37 °C for 3 h, followed by addition of desulfurization buffer containing 200 mM TCEP, 80 mM VA-044 and 80 mM glutathione and subsequent reaction for 3 h at 37 °C, yielded the desulfurization peptide (H-**LYRANA-ASPGYS**-NH_2 (**10**)) in 77% isolated yield. Because additive **2** was proved to be compatible with the one-pot/NCL–desulfurization protocol, we next evaluated the applicability of

2 to one-pot/sequential NCL–desulfurization protocols indispensable for protein chemical synthesis using a model peptide.

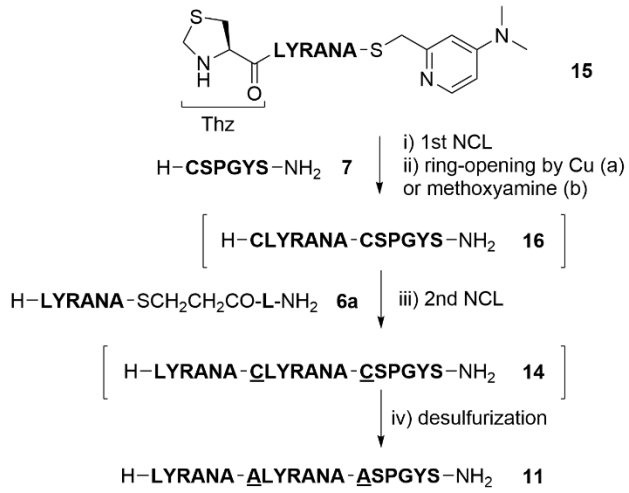
Scheme 2. One-pot/N-to-C directed NCLs-desulfurization protocol[a]



[a]Conditions: i) Peptides (**6a** and **12**, 1 mM each), 6 M Gn· HCl–0.1 M HEPPS, 50 mM TCEP and 10 mM of **5**, pH 6.9, 37 °C, 4 h; ii) Peptide **7**, 0.5 M phosphate, pH 6.9, 37 °C, 7 h; iii) desulfurization buffer (200 mM TCEP, 80 mM VA-044 and 80 mM glutathione), 37 °C, 6 h.

Sequential NCLs are classified as N–to–C- and C–to–N-directed protocols.[15] Compatibility with a one-pot/N–to–C-directed NCLs–desulfurization was initially evaluated through the one-pot synthesis of model peptide (H-**LYRGA-ALYRANA-ASPGYS**-NH_2 (**11**)) (Scheme 2, Figures 3 (A–C), S6 and S7).

Scheme 3. One-pot/C–to–N-directed NCLs-desulfurization protocol[a]



[a]Conditions: i) Peptides (**7** and **15**, 1 mM each), 6 M Gn· HCl–0.1 M HEPPS, 5 mM TCEP, pH 6.9, 37 °C, 3 h; ii-a) $CuSO_4$ (5 mM); ii-b) methoxyamine (200 mM), pH 4.2, 37 °C, 4 h; iii) Peptide **6a** (0.75 mM, 1.5 equiv.), 6 M Gn· HCl–0.2 M phosphate, 50 mM TCEP and 10 mM of **5**, pH 6.9, 37 °C, 2 h; iv) desulfurization buffer (200 mM TCEP, 80 mM VA-044 and 80 mM glutathione), 37 °C, 2 h.

As an N–to–C protocol, we employed the phosphate-controllable *N*-sulfanylethylanilide (SEAlide)-mediated sequential NCLs.[16] Three fragments (thioester **6a**, N-terminal cysteinyl SEAlide peptide, H-**CLYRANA**-SEAlide-**G**-NH_2 (**12**) and **7**) were sequentially ligated as shown in Scheme 2. The first NCL of **6a** with **12** in 6 M Gn·HCl–0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-propanesulfonic acid (HEPPS) in the presence of 50 mM TCEP and 10 mM of **5** at pH 6.9, 37 °C afforded the ligated SEAlide peptide **13** in 4 h. Addition of phosphate solution of **7** to the reaction allowed the SEAlide unit to work as a thioester, resulting in the formation of fully ligated peptide **14** in 6 h. Then, desulfurization buffer was added to the mixture to initiate the TCEP-mediated radical desulfurization. After 10 h at 37 °C, the desired **11** was obtained in 44% isolated overall yield (Figure 3 (A–C).

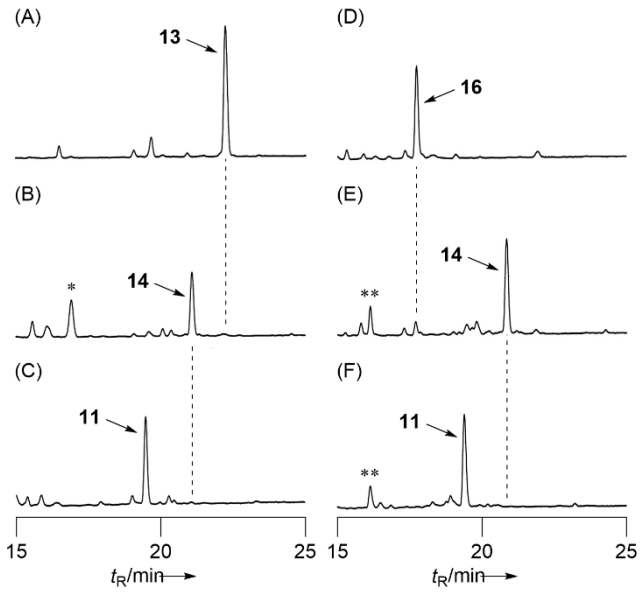


Figure 3. (A–C) HPLC monitoring of one-pot/N–to–C-directed NCLs–desulfurization protocol for the synthesis of **11**. (A) first NCL (t = 3 h); (B) 2nd NCL (t = 9 h); (C) one-pot desulfurization (t = 3 h). *Non-peptide impurity. (D–E) HPLC monitoring of one-pot/C–to–N-directed NCLs–desulfurization protocol using the ring-opening by methoxyamine. (D) ring-opening by methoxyamine (t = 4 h); (E) 2nd NCL (t = 2 h); (F) one-pot desulfurization (t = 2 h). **Remaining SMDMAP thioester of **6a**.

The C–to–N-operation using the SMDMAP thioester **15** of a thiazolidine (Thz) peptide[17,18] is summarized in Scheme 3. The first NCL of **15** with **7** in 6 M Gn·HCl–0.1 M HEPPS in the presence of 5 mM TCEP under well-degassed conditions gave a ligated product which was converted to the ring-opening product **16** by the action of $CuSO_4$.[19] Subsequent NCL with a thioester **6a** added to the mixture proceeded successfully to give a ligated peptide **14** but the attempted one-pot desulfurization failed to proceed to completion (SI, Figures S8 and S9).[20] An alternative protocol using 200 mM methoxyamine as a ring-opening reagent at pH 4.2,[7b,16] followed by the second NCL with **6a** and subsequent desulfurization in a one-pot reaction, yielded the desired product **11** in 41% isolated yield after HPLC purification. The progress of the sequence of reactions including the methoxyamine-mediated ring opening is shown in Figures 3 (D–F) and S7.

Finally, another potential utility of SMDMAP in ligation chemistry was explored. Expecting that additive **2** should be applicable to the synthesis of lactam or lactone peptides since the parent catalyst **1** promotes the acylation of histone proteins, we attempted to use SMDMAP for the synthesis of cyclic peptides.

Suitable conditions for lactam formation were explored by using the substrate peptide (H-**GALYRGFA**-SCH_2CH_2-CO-**G**-NH_2 (**17**)). Cyclization of **17** (0.5 mM) proceeded quantitatively in the presence of 20 mM of **5**, 30 mM TCEP and 170 mM diisopropylethylamine (DIPEA) in *N*-methyl-2-pyrolidone (NMP)/H_2O (4:1)[21] to afford the cyclic peptide ((cyclo(-**GALYRGFA**-) (**18**)) in 98% isolated yield without epimerization (Figure 4 (A–B), SI, Figure S10, for reaction optimization see Table S1). Optimized conditions were applicable to the cyclization of other linear peptides with chains of different lengths and ligation junctions (SI, Table S2). The presence of thiol and pyridine units in the molecule was proved to be critical through a comparative study using other additives (SI, Figure S11). In addition, lactone formation of the serine-containing thioester (Ac-**SAFYG**-S-CH_2CH_2-CO-**L**-NH_2 (**19**)) almost reached completion in NMP with 40 mM of **5**, 40 mM TCEP and 80 mM DIPEA at 50 °C to give the corresponding lactone peptide **20** with 95% conversion and 86% isolated yield (Figure 4 (C–D), SI, Table S3).

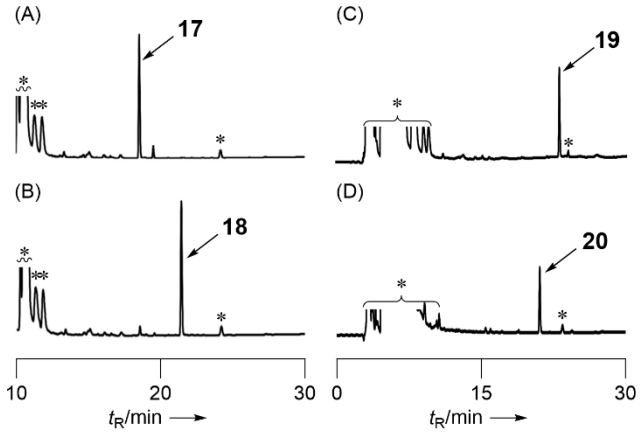


Figure 4. HPLC monitoring of cyclization reaction for lactam (A–B) and for lactone peptide (C–D). (A) $t < 5$ min; (B) $t = 3$ h; (C) $t < 5$ min; (D) $t = 12$ h. *Non-peptide impurity.

In conclusion, in addition to NCL-accelerating activity comparable to that of MPAA, SMDMAP is also compatible with radical desulfurization and is thus applicable to the one-pot/sequential NCL–desulfurization protocol that is central to modern protein chemical synthesis. In addition, syntheses of lactam or lactone peptides from peptide thioesters using SMDMAP were achieved.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures and charts for the HPLC analyses of the attempted reactions (PDF)

AUTHOR INFORMATION

Corresponding Author

*aotaka@tokushima-u.ac.jp

ACKNOWLEDGMENT

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) ant The Canon Foundation. A.S. is grateful for support from the Research Foundation for Pharmaceutical Sciences.

REFERENCES

1. (a) Dawson, P.; Muir, T.; Clark-Lewis, I.; Kent, S., Synthesis of proteins by native chemical ligation. *Science* **1994**, *266*, 776-779. (b) Kent, S. B. H., Total chemical synthesis of proteins. *Chem. Soc. Rev*. **2009**, *38*, 338-351.
2. Johnson, E. C. B.; Kent, S. B. H., Insights into the Mechanism and Catalysis of the Native Chemical Ligation Reaction. *J. Am. Chem. Soc*. **2006**, *128*, 6640-6646.
3. Agouridas, V.; El Mahdi, O.; Diemer, V.; Cargoët, M.; Monbaliu, J.-C. M.; Melnyk, O., Native Chemical Ligation and Extended Methods: Mechanisms, Catalysis, Scope, and Limitations. *Chem. Rev*. **2019**, *119*, 7328-7443.
4. Malins, L. R.; Payne, R. J., Modern Extensions of Native Chemical Ligation for Chemical Protein Synthesis. In *Protein Ligation and Total Synthesis I*, Liu, L., Ed.; Topics in Current Chemistry 362; Springer International Publishing, **2015**; pp 27-87.
5. Wan, Q.; Danishefsky, S. J., Free-Radical-Based, Specific Desulfurization of Cysteine: A Powerful Advance in the Synthesis of Polypeptides and Glycopolypeptides. *Angew. Chem. Int. Ed*. **2007**, *46*, 9248-9252.
6. Rohde, H.; Schmalisch, J.; Harpaz, Z.; Diezmann, F.; Seitz, O., Ascorbate as an Alternative to Thiol Additives in Native Chemical Ligation. *ChemBioChem* **2011**, *12*, 1396-1400.
7. (a) Siman, P.; Blatt, O.; Moyal, T.; Danieli, T.; Lebendiker, M.; Lashuel, H. A.; Friedler, A.; Brik, A., Chemical Synthesis and Expression of the HIV-1 Rev Protein. *ChemBioChem* **2011**, *12*, 1097-1104. (b) Thompson, R. E.; Liu, X.; Alonso-García, N.; Pereira, P. J. B.; Jolliffe, K. A.; Payne, R. J., Trifluoroethanethiol: An Additive for Efficient One-Pot Peptide Ligation−Desulfurization Chemistry. *J. Am. Chem. Soc*. **2014**, *136*, 8161-8164. (c) Huang , Y.-C.; Chen , C.-C.; Gao, S.; Wang, Y.-H.; Xiao, H.; Wang, F.; Tian, C.-L.; Li, Y.-M., Synthesis of l- and d-Ubiquitin by One-Pot Ligation and Metal-Free Desulfurization. *Chemistry – A Euro. J*. **2016**, *22*, 7623-7628.
8. Moyal, T.; Hemantha, H. P.; Siman, P.; Refua, M.; Brik, A., Highly efficient one-pot ligation and desulfurization. *Chem. Sci.* **2013,** *4*, 2496-2501.
9. Amamoto, Y.; Aoi, Y.; Nagashima, N.; Suto, H.; Yoshidome, D.; Arimura, Y.; Osakabe, A.; Kato, D.; Kurumizaka, H.; Kawashima, S. A.; Yamatsugu, K.; Kanai, M., Synthetic Posttranslational Modifications: Chemical Catalyst-Driven Regioselective Histone Acylation of Native Chromatin. *J. Am. Chem. Soc*. **2017**, *139*, 7568-7576.
10. Yamatsugu, K.; Furuta, M.; Xi, S.; Amamoto, Y.; Liu, J.; Kawashima, S. A.; Kanai, M., Kinetic analyses and structure-activity relationship studies of synthetic lysine acetylation catalysts. *Bioorg. Med. Chem*. **2018**, *26*, 5359-5367.
11. Meloun, M.; Bordovská, S., Benchmarking and validating algorithms that estimate pK_a values of drugs based on their molecular structures. *Anal. Bioanal. Chem*. **2007**, *389*, 1267-1281.
12. Liu, Z.; Ma, Q.; Liu, Y.; Wang, Q., 4-(*N*,*N*-Dimethylamino)pyridine Hydrochloride as a Recyclable Catalyst for Acylation of Inert Alcohols: Substrate Scope and Reaction Mechanism. *Org. Lett*. **2014,** *16*, 236-239.
13. Kawakami, T.; Aimoto, S., The use of a cysteinyl prolyl ester (CPE) autoactivating unit in peptide ligation reactions. *Tetrahedron* **2009**, *65*, 3871-3877.
14. Sakamoto, K.; Tsuda, S.; Nishio, H.; Yoshiya, T., 1,2,4-Triazole-aided native chemical ligation between peptide-N-acyl-N′-methyl-benzimidazolinone and cysteinyl peptide. *Chem. Commun*. **2017**, *53*, 12236-12239.
15. Raibaut, L.; Ollivier, N.; Melnyk, O., Sequential native peptide ligation strategies for total chemical protein synthesis. *Chem. Soc. Rev*. **2012**, *41*, 7001-7015.
16. (a) Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Sumikawa, Y.; Sakamoto, K.; Otaka, A., N-Sulfanylethylanilide Peptide as a Crypto-Thioester Peptide. *ChemBioChem* **2011**, *12*, 1840-1844.

(b) Sato, K.; Shigenaga, A.; Kitakaze, K.; Sakamoto, K.; Tsuji, D.; Itoh, K.; Otaka, A., Chemical Synthesis of Biologically Active Monoglycosylated GM2-Activator Protein Analogue Using N-Sulfanylethylanilide Peptide. *Angew. Chem. Int. Ed*. **2013**, *52*, 7855-7859.

17. Bang, D.; Kent, S. B. H., A One-Pot Total Synthesis of Crambin. *Angew. Chem. Int. Ed*. **2004**, *43*, 2534-2538.
18. The SMDMAP thioester **15** was readily prepared by the reaction of the precursor peptide azide with **2** formed *in situ* by partial reduction of **5** with less stoichiometric amount of TCEP. And the synthesized SMDMAP **15** remains intact for several weeks under refrigeration at 4 °C.
19. (a) Naruse, N.; Kobayashi, D.; Ohkawachi, K.; Shigenaga, A.; Otaka, A., Copper-Mediated Deprotection of Thiazolidine and Selenazolidine Derivatives Applied to Native Chemical Ligation. *J. Org. Chem*. **2020**, *85*, 1425-1433. (b) Zhao, Z.; Metanis, N., Copper-Mediated Selenazolidine Deprotection Enables One-Pot Chemical Synthesis of Challenging Proteins. *Angew. Chem. Int. Ed*. **2019**, *58*, 14610-14614.
20. The reason for incompletion of the desulfurization in the one-pot protocol including Cu-mediated ring opening of thiazolidine is probably due to the presence of Cu ions in the reaction. However, co-existence of reagents including thiols, EDTA, DTT and diethyldithiocarbamate (DTC)[22] which could trap the Cu ions did not improve the reaction (Figure S9).
21. (a) Payne, R. J.; Ficht, S.; Greenberg, W. A.; Wong, C.-H., Cysteine-Free Peptide and Glycopeptide Ligation by Direct Aminolysis. *Angew. Chem. Int. Ed.* **2008,** *47*, 4411-4415. (b) Thomas, G. L.; Hsieh, Y. S. Y.; Chun, C. K. Y.; Cai, Z.-L.; Reimers, J. R.; Payne, R. J., Peptide Ligations Accelerated by N-Terminal Aspartate and Glutamate Residues. *Org. Lett*. **2011**, *13*, 4770-4773.
22. Laps, S.; Sun, H.; Kamnesky, G.; Brik, A., Palladium-Mediated Direct Disulfide Bond Formation in Proteins Containing S-Acetamidomethyl-cysteine under Aqueous Conditions. *Angew. Chem. Int. Ed.* **2019,** *58*, 5729-5733.