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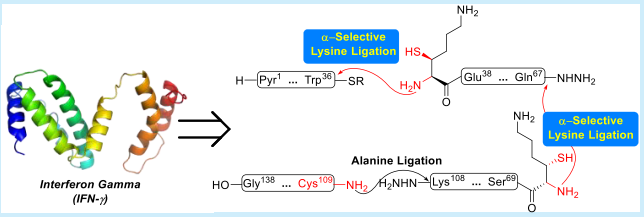
α-Selective Lysine Ligation and Application in Chemical Synthesis of Interferon Gamma

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Supporting Information

ABSTRACT: A traceless β-mercaptan-assisted α-selective ligation of *N*-terminal lysine-containing peptides has been developed. In this ligation−desulfurization-based protocol, the ε-amine of lysine is free of protection, thus improving the overall synthetic efficiency and avoiding harsh reactions in preparing large peptides and proteins. The applicability of this methodology has been demonstrated in the synthesis of an acid-labile therapeutic protein, interferon gamma, and the anticancer activity of synthetic protein has also been evaluated.



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In the emerging field of chemical protein synthesis and protein modification, one of the major challenges is the identification of suitable reaction conditions that are biocompatible, avoiding heavy metals and strong acids/bases that may irreversibly denature the proteins and lead to degradation. Representative reactions that perfectly address such issues include native chemical ligation (NCL)[1] and metal-free desulfurization (MFD),[2] both of which are highly chemoselective and can be carried out in aqueous media without any protective groups for the side chains of peptides and proteins.

Aiming to expand the toolbox for protein synthesis under mild conditions and guided by the logic of NCL and postligation desulfurization, a number of mercaptoamino acid based ligation methods have been developed.[3] While most of these protocols retain the protecting group free feature of NCL, the ε-amine of lysine has to be capped when such diamino residues were used as ligation sites at the peptidyl *N*-terminus. For instance, Liu et al. have accomplished peptide ligation at the Xaa-Lys sites using a γ-mercaptolysine derivative with orthogonally protective carboxybenzyl (Cbz) or *o*-nitroveratryloxycarbonyl (NVOC) groups on the ε-amine (Figure 1a).[4] In these cases, following α-peptide formation and subsequent removal of the protecting groups, the crucial γ-thiol can assist a second peptide chain elongation at the ε-position accomplishing dual peptide ligation at the lysine sites.

Despite these important advances, the chemical synthesis of lysine-rich proteins,[5] in particular those with complex and fragile post-translational modifications (PTMs) such as glycosylation and phosphorylation, still calls for a more straightforward protocol and milder conditions that may avoid extra harsh deprotection steps at a late stage, with minimal HPLC purifications. Herein, we report an α-selective peptide ligation at the lysine sites, as well as the application of this protocol to the synthesis of a lysine-rich therapeutic protein interferon gamma (IFN-γ).

Since the discovery of native chemical ligation, various thioamino acid-derived[3] or auxiliary-containing peptides have been demonstrated to undergo a NCL-like process,[6] beginning with a reversible intermolecular trans-thioesterification, followed by an irreversible 1,4- or 1,5- S → N acyl shift in most cases that leads to the formation of a stable amide bond. Besides these successful precedents, a couple examples of 1,6- S → N acyl shifts have been reported to furnish ligations at the Xaa-Ser/Thr[7] or Xaa-Trp sites.[8] These ligations proceed at a much slower rate and require elevated reaction temperatures that result in side reactions, including hydrolysis of the starting peptides, and structural modification of tryptophan. We envisioned that a β-thiolysine-containing peptide **2** may react with peptidyl thioester **1** to form the *S*-isopeptide **3** (Figure 1b), which would preferentially undergo 1,4- S → N acyl transfer to generate the α-peptide **4**, while the 1,6- S → N acyl transfer might be prohibited due to the significantly higher activation energy required for the seven-membered ring intermediate. Eventually, the mercaptan group could be removed under metal-free desulfurization conditions to reveal the native lysine-containing peptide sequences.[2]

We initiated the study by synthesizing the β-thiolysine derivatives **8** (Scheme 1a),[9] based on a one-step biocatalytic transformation of lysine to β-hydroxyllysine (**7**) reported by Renata et al.,[10] and then incorporated these amino acids to the *N*-termini of thiolysine-containing peptides **14** using standard solid-phase peptide synthesis (SPPS) conditions (Scheme 1b).[11] Notably, the stereochemistry of the obtained thiolysine derivative (2*S*,3*S*) coincides with a previously reported stereoisomer of β-thioaspartate used in a ligation reaction.[12] On the other hand, the thioester peptide **11**, bearing different *C*-terminal residues, was synthesized following a Fmoc-based

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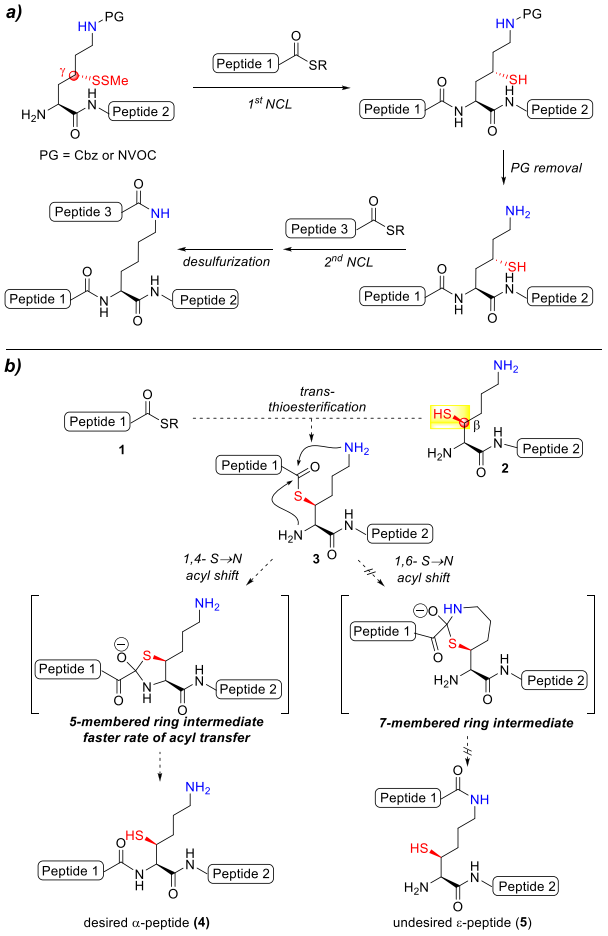
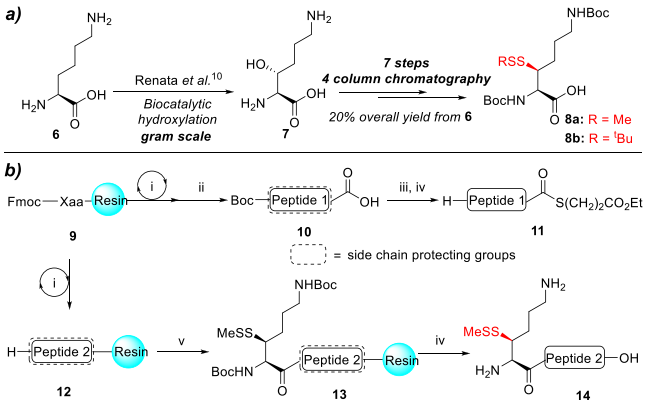


Figure 1. (a) γ-Thiolysine-assisted dual peptide ligation. (b) Proposed α-selective lysine ligation assisted by β-mercaptan.

Scheme 1. Synthesis of β-Thiolysine Derivatives 8 and Peptide Segments 11 and 14[a]



[a]Reagents and conditions: (i) Fmoc-based SPPS; (ii) DCM/TFE/AcOH (3:1:1, v/v/v); (iii) ethyl 3-mercaptopropionate, PyClock, DIEA, DMF, −20 °C, 2 h; (iv) TFA/TIS/H_2O (95:2.5:2.5, v/v/v), 2 h; (v) **8a**, HATU, DIEA, DMF, 30 min.

SPPS protocol and thioesterification procedures.[9,13] Notably, use of PyClock as the coupling reagent in preparing the peptidyl thioesters was necessary to avoid the pyrrolidine-coupled byproducts generated when using PyBOP as reported in the original protocol.[14] Next, the obtained pentapeptides **11a** and **14a** (Figure 2a), derived from a fragment of human

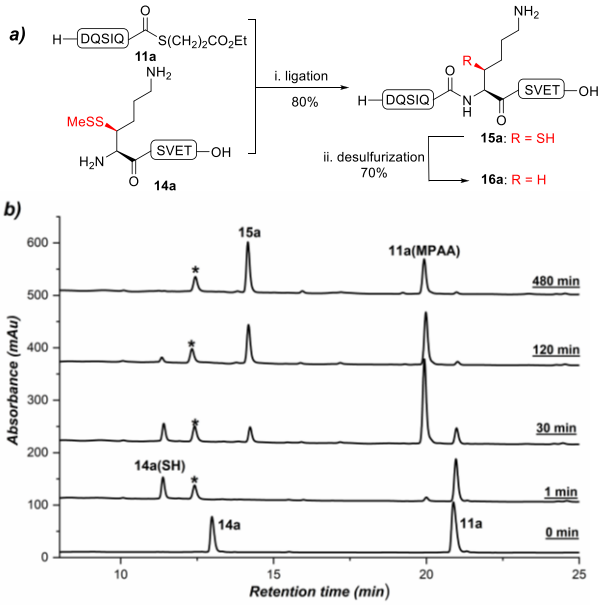


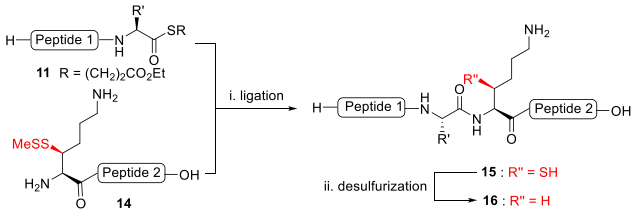
Figure 2. (a) Ligation reaction between **11a** and **14a** and postligation desulfurization on thiolysine-containing peptide. (b) UV traces from HPLC−MS analysis of ligation between **11a** and **14a**. *Denotes the peaks of UV signal generated from ligation buffer.

IFN-γ (vide infra), were evaluated for the proposed β-thiolysine-mediated ligation. Following a typical MPAA-promoted ligation protocol,[15] the reaction under buffered conditions proceeded smoothly, generating a ligated peptide **15a** in almost full conversion after 8 h (Figure 2b) and 80% isolated yield after HPLC purification. Desulfurization of **15a** effectively afforded native lysine-containing peptide **16a**, which was unambiguously assigned as the α-peptide by comparing with two synthetic authentic samples **16a-*α*** and **16a-*ε***(Figure S23).[9] The addition of excess peptidyl thioester **11a** (2.2 equiv), or conducting reactions at an elevated temperature of 37 °C, failed to result any ε-acylated products,[9] suggesting the distinct reactivity of β-thiolysine comparing to the γ- or δ-thiolysines studied by Brik, Ovaa, and Liu.[4,16] Moreover, ligation between peptides **11b** (H-ILKNW-S$(CH_2)_2CO_2$Et) and **14b** (H-K$^{\beta\text{-SSMe}}$EESD-OH) has also been evaluated (Table 1, entry 2), where the obtained final product was also verified to be the desired α-peptide (Figure S30),[9] further confirming the high regioselectivity of this β-thiolysine-based ligation.

We evaluated the efficiency of this α-selective lysine ligation in a number of peptidyl thioesters bearing various *C*-terminal residues (Table 1). In addition to the aforementioned peptide segments **11a** and **11b**, which contain *C*-terminal Gln and Trp residues, respectively (entries 1 and 2), peptidyl thioesters containing *C*-terminal Gly and Phe also reacted efficiently with the corresponding β-thiolysine-containing peptides, including a mannosylated peptide **14c**, to generate ligation products in good to excellent yields (entries 3 and 4). Reactions with more sterically demanding amino acid residues at the *C*-terminus of peptide thioesters, such as Leu (entry 5) and Val (entry 6), proceeded at a slower rate, and elevated temperature or extended reaction time was required to obtain moderate to good yields of products. All 20 proteogenic amino acid residues, as well as several representative PTMs including phosphorylation (entry 6) and glycosylations (entries 4 and 7), were found to be compatible in the ligation and the subsequent metal-free desulfurization, showing the potential applicability

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Table 1. Scope and Limitations of α-Selective Lysine Ligation[a]



Entry	peptide thioesters (**11**)	thiolysine-containing peptides (**14**)	ligation yield[b]	desulfurization yield[b]
1	H–DQSI–N(H)–C(=O)–SR (**11a**)	MeSS–SVET–OH (**14a**)	80%	70%
2	H–ILKN–N(H)–C(=O)–SR (**11b**)	MeSS–EESD–OH (**14b**)	77%	76%
3	H–LRLRG–N(H)–C(=O)–SR (**11c**)	MeSS–SVET–OH (**14a**)	95%	70%
4	H–TRQC(Acm)–N(H)–C(=O)–SR (**11d**)	MeSS–SMYK–OH (**14c**)	73%	88%
5	H–DQSI–N(H)–C(=O)–SR (**11e**)	MeSS–EESD–OH (**14b**)	71%[c]	78%
6	H–AHTD(PO3H2)–N(H)–C(=O)–SR (**11f**)	MeSS–EESD–OH (**14b**)	44%[d]	76%
7	H–DNGTR–N(H)–C(=O)–SR (**11g**)	MeSS–SMYK–OH (**14c**)	85%	72%
8[e]	H–WASKD–N(H)–C(=O)–SR (**11h**)	MeSS–SVET–OH (**14a**)	NA	59%

[a]Reagents and conditions: (i) **11** (3 mM), **14** (1.1 equiv), MPAA buffer, pH 7.0−7.2, rt, 8 h; (ii) guanidine buffer, TCEP solution, 2-methyl-2-propanethiol, VA-044, 37 °C, 1 h. [b]Isolated yield after HPLC purification. [c]Conducted at 37 °C for 8 h. [d]Conducted at 37 °C for 56 h. [e]One-pot ligation desulfurization: guanidine buffer, rt, 8 h, then TCEP solution, 2-methyl-2-propanethiol, VA-044, 37 °C, 1 h.

of this protocol to the synthesis of large phosphorylated proteins and glycoproteins. Notably, in combination with our previously reported 4-mercaptoprolyl thioester-based internal activation strategy,[17] efficient ligation at the native Pro-Lys sites can be accomplished via one-pot ligation−desulfurization,[18] an advantageous feature in terms of overall synthetic efficiency, as minimal HPLC purification is required.

To further evaluate the applicability of the α-selective lysine ligation protocol, chemical synthesis of a human cytokine, interferon gamma, was attempted. Also known as immune interferon or type-II interferon, IFN-γ is produced by T-lymphocytes and natural killer cells and is involved in a wide range of immunoregulatory activities in the human body.[19] Researchers have shown that IFN-γ exhibits antiviral and anticancer activities.[19] Moreover, recombinant IFN-γ and its several isoforms, such as IFN-γ-1b (Actimmune) and IFN-γ-1a (Imunomax), have been applied clinically in treating a number of diseases, including chronic granulomatous disease (CGD), severe malignant osteopetrosis (SMO), renal carcinoma, and several infectious diseases. As several studies indicated that in the treatment of cancers immune therapy failed when IFN-γ was not included in the treatment,[20] clinical trials have also been conducted to utilize IFN-γ in combination with immune checkpoint inhibitors to treat tumors. The intriguing bioactivity and prominent therapeutic potential of IFN-γ have attracted many scientific studies, including a synthetic effort attempted by Kajihara et al.[21]

The native sequence of mature IFN-γ consists of 138 amino acid residues (Figure 3),[22] including one pyroglutamate (Pyr)

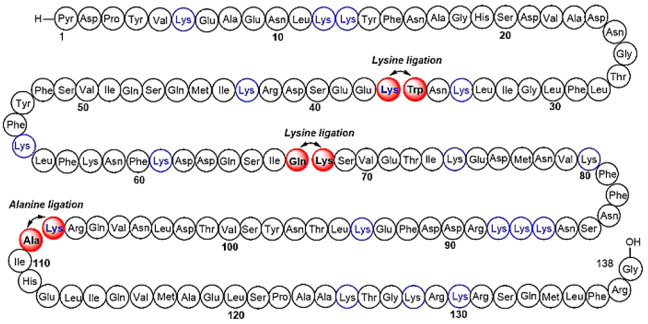


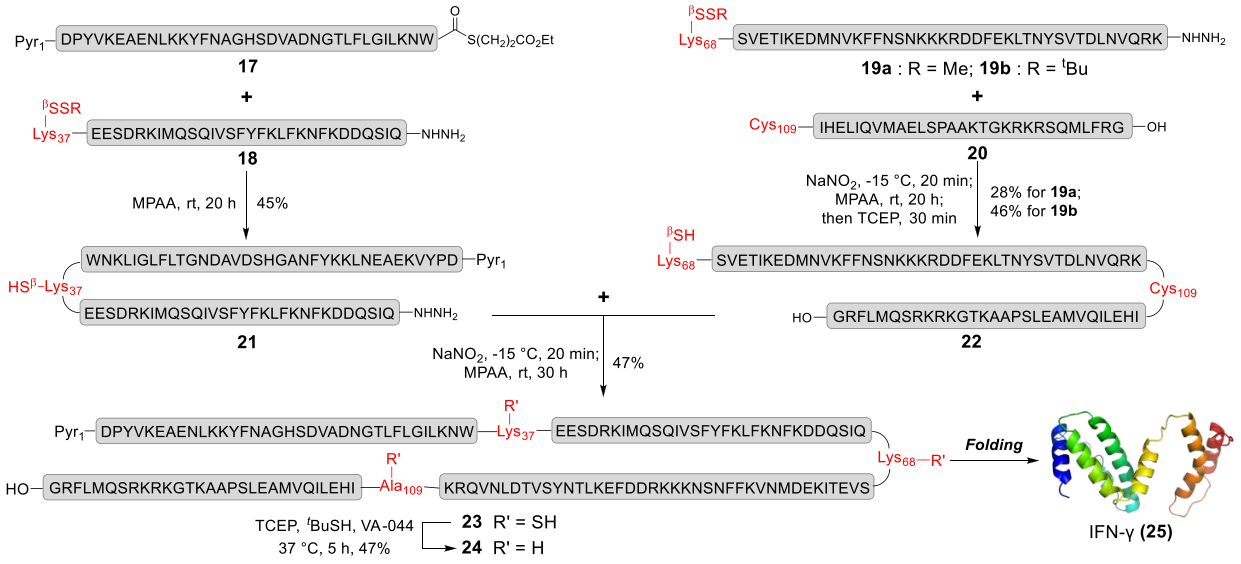
Figure 3. Native sequence of interferon gamma (IFN-γ). Proposed ligation sites depicted in red.

at the *N*-terminus, lysine residues within the primary structure, only seven alanines that mostly occur in two terminal regions, and no cysteines. Considering the high frequency of lysine in IFN-γ, a synthetic strategy based on the α-selective lysine ligation affords flexibility in choosing dissection sites for connecting the corresponding peptide fragments. Moreover, as IFN-γ has been reported to be acid-labile and tends to aggregate at low pH,[23] the ε-amine-free lysine ligation strategy becomes particularly advantageous. Accordingly, the full sequence may be disconnected into four segments, Pyr_1-Trp_{36} (**17**), Lys_{37}-Gln_{67} (**18**), Lys_{68}-Lys_{108} (**19**), and Ala_{109}-Gly_{138} (**20**), which are then assembled in a convergent manner through two lysine ligations and one alanine ligation.

We carried out the forward synthesis by first preparing peptide segments **17**−**20** utilizing Fmoc-based SPPS and literature-reported derivatization protocols for peptidyl thioesters[24] and peptidyl hydrazides.[9,25] In particular, as the *N*-terminus of mature IFN-γ is a pyroglutamate residue,[22] Pyr was utilized to the synthesis of segment **17**, similar to the reported syntheses of monocyte chemotactic protein-3 (MCP-3)[26] and chimadanin.[27] To assemble the full sequence of IFN-γ, segments **17** and **18** were ligated under MPAA-promoted NCL conditions,[15] which generated segment **21** in a decent isolated yield of 45% (Scheme 2). The attempted ligation of segments **19a** and **20** turned out to be problematic, as **19a**

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Scheme 2. Synthesis of Interferon Gamma (IFN-γ)[9]



preferentially underwent self-ligation to generate cyclic peptide as the predominant byproduct.[9] Considering the potential lability of −SMe as the protecting group, we evaluated the more stable −S^{t}Bu-protected thiolysine-derived peptide **19b**. As hoped, undesired formation of cyclopeptide byproduct was largely suppressed with the use of peptide **19b** as a coupling partner,[9] and the ligated product was obtained in a good yield. Such ^{t}Bu-protected β-thiolysine peptides may be utilized in kinetically controlled ligations, thus broadening the scope of thiolysine-based ligations. Next, peptidyl hydrazide-based ligation of **21** and **22**, followed by global desulfurization to remove three extraneous mercaptan groups on Lys_{37}, Lys_{68}, and Ala_{109}, generated product **24**, representing the full native sequence of IFN-γ.

Folding of interferon gamma was conducted following a dialysis protocol[28] that afforded the protein with the correct mass (Figures 4a,b). Furthermore, circular dichroism (CD) spectroscopy indicated that the secondary structure of the synthetic sample aligned well with a purchased recombinant wild-type IFN-γ that contains an extra Met at the *N*-terminus and five more residues at the *C*-terminus representing the propeptide region of the natural form (Figures S59 and S60).[9] SDS−PAGE analysis of the synthetic protein displayed a single band, confirming the purity and approximate molecular weight (Figure S60). Drawing inspiration from the reported anticancer activities of IFN-γ,[29] the biological performance of the synthetic sample was further evaluated. The folded protein was tested in an antiproliferation assay using HeLa cell lines, with the aforementioned recombinant IFN-γ as a comparison. After incubation for 48 h, detection using CCK-8 assay indicated that the synthetic sample exhibited comparable antiproliferation activity (Figure 4c), confirming the antitumor activity in vitro, albeit in a moderate efficiency similar as the reported case in literature.[29b] The results from this biological evaluation also suggest that the synthetic IFN-γ possesses the correct tertiary structure, similar to the natural protein.

Chemical protein synthesis has emerged as an indispensable tool to produce homogeneous proteins with structurally well-defined PTMs[30] as well as to engineer proteins with unnatural modifications.[31] The β-thiolysine-mediated ligation method described herein not only affords an effective potential solution

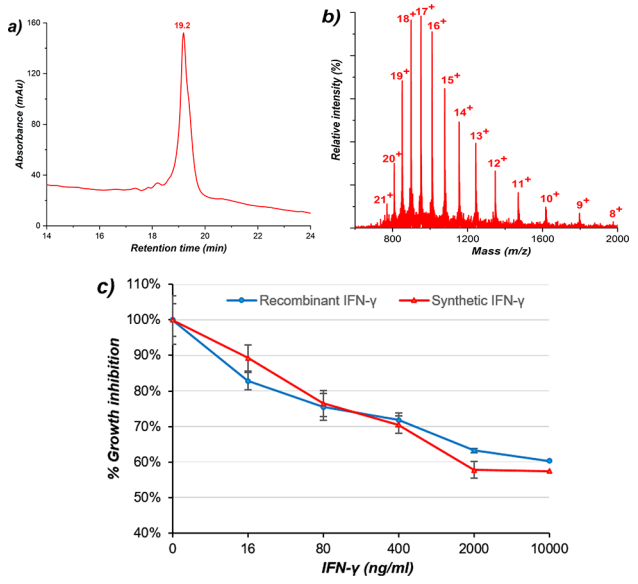


Figure 4. Characterization of synthetic IFN-γ (**25**). (a) UV traces of the synthetic IFN-γ. (b) ESI-MS analysis of the synthetic IFN-γ. (c) Antiproliferation assay of IFN-γ in HeLa cell lines. HeLa cell lines were treated with different doses of recombinant IFN-γ (●) and synthetic IFN-γ (▲) for 48 h. The percentage of inhibition of cell proliferation was calculated taking cell growth in untreated cultures as 100% proliferation. Values are the means of three separate experiments with SD < 10%.

to the synthesis of lysine-rich proteins, such as histones,[5c] and high-mobility group B1 (HMGB1)[5a] but also offers a representative strategy for the incorporation of unnatural thiol-containing amino acids, which potentially could be utilized in the preparation of protein analogues, such as disulfide-engineered proteins.[32] The successful synthesis of interferon gamma using an α-selective lysine ligation−desulfurization strategy has established viable access to this therapeutically promising protein, which would be of benefit for further studies on generating engineered IFN-γ, such as glycosylated versions, with potentially improved efficacy and pharmacokinetic properties. Another important lesson learned

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from this study is that properly designed small molecules may well effect selective and efficient transformation on large biomolecules including polypeptides and proteins, where the principles of chemistry still stand.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b00980.

Experimental procedure and spectroscopic data for all compounds (PDF)

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Notes

The authors declare no competing financial interest.

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