Glycoproteins

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Chemical Synthesis of O-Glycosylated Human Interleukin-2 by the Reverse Polarity Protection Strategy**

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Abstract: *The chemical synthesis of human interleukin-2 (IL-2), having a core 1 sugar, by a ligation method is reported. Although IL-2 is a globular glycoprotein, its C-terminal region, in particular (99-133), is extremely insoluble when synthesized by solid-phase method. To overcome this problem, the side-chain carboxylic acid of the Glu residues was protected by a picolyl ester, thus reversing its polarity from negative to positive. This reverse polarity protection significantly increased the isoelectric point of the peptide segment and made it positive under acidic conditions and facilitated the purification. An efficient method to prepare the prolyl peptide thioester required for the synthesis of the (28-65) segment was also developed. These efforts resulted in the total synthesis of the glycosylated IL-2 having full biological activity.*

Ligation chemistries, such as the native chemical ligation (NCL),[1] as well as the thioester method,[2] have been highly advanced so that (glyco)proteins of over 100 amino acid residues can be routinely synthesized. By using synthetic (glyco)proteins, various functional and structural studies have been achieved.[3] One of the advantages of the chemical methods is that they can realize the synthesis of proteins which are difficult to make by recombinant DNA technology, such as D-proteins known to facilitate the crystallization as a cocrystal with the corresponding L-protein.[4] However, for ligation chemistry to become a more general method for (glyco)protein synthesis, further developments are still required. We often encounter serious solubility problems during the ligation reaction, even in the synthesis of globular (glyco)proteins. For example, during the synthesis of saposin C[5] by the NCL method, the N-terminal thioester was extremely insoluble during the reversed-phase high-performance liquid chromatography (RPHPLC) purification, as well as the ligation reaction, precluding its synthesis. Thus, we introduced the O-acylisopeptide method,[6] developed by Sohma et al., for the N-terminal thioester preparation, leading to the successful synthesis of saposin C.

For more than fifteen years, we have been attempting to synthesize human interleukin-2 (IL-2)[7] as a suitable model for O-glycoprotein synthesis. We attempted its synthesis by using the thioester method,[2] which utilizes the direct aminolysis of the thioester group by the terminal amino group of the N- and S-protected peptide segment, dividing the sequence at the arrows shown in Figure 1. However, the synthesis failed because of the following problems: 1) The extremely low solubility of the C-terminal part, especially (99-133), even in its protection-free form, thus prohibiting purification and the ligation reaction. The reason for the low solubility of this segment, in spite of IL-2 itself being a soluble protein, seems to arise from the fact that this region forms a relatively nonpolar helix in the folded state which contains a high content of hydrophobic amino acids.[8] 2) The requirement of an efficient synthetic route of the prolyl thioester needed to synthesize the (28-65) segment. In this study, we found solutions to these problems and realized the efficient synthesis of IL-2, carrying the core 1 O-linked sugar **1** (Figure 1), with full biological activity.

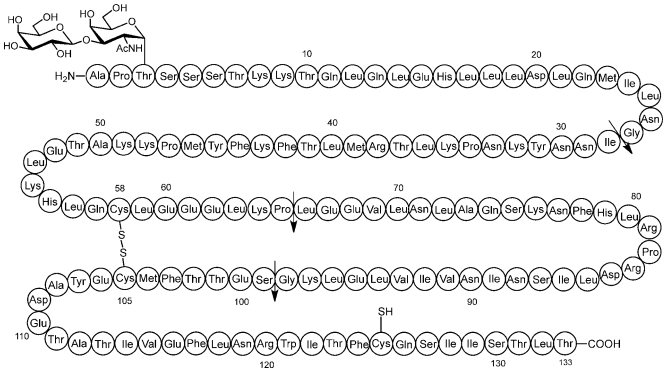

Figure 1. The structure of the human interleukin-2 (IL-2) **1**. The arrows show the points of ligation.

To start the synthesis by overcoming the solubility problem of the C-terminal region. The entire sequence was divided into the same segments as in the previous trial (Figure 1). As already mentioned, the C-terminal segment (99-133), having no protecting group except for the acetamidomethyl (Acm) groups, retains an extremely low solubility

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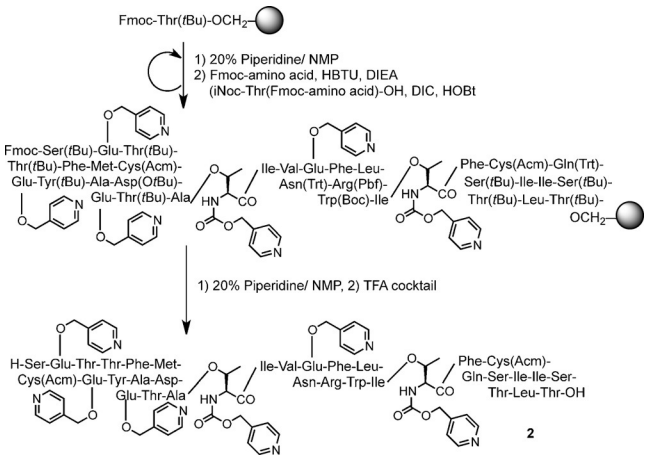
and could not be isolated by RPHPLC. The use of aqueous isopropanol instead of acetonitrile as an eluent did not facilitate the purification. We then examined the O-acylisopeptide method[6] following our previous synthesis of saposin C. Two isopeptide structures at Ala^{112}-Thr^{113} and Ile^{122}-Thr^{123} were introduced during the resynthesis of the C-terminal peptide (see Scheme SI1 in the Supporting Information). As a result, the peptide **2′** (Scheme SI1) was partially soluble in aqueous acetonitrile containing 6 M guanidine hydrochloride (Gu·HCl). However, isolation of the product failed as the by-products could not be removed (see Figure SI1 in the Supporting Information), indicating that the segment still has a strong tendency to aggregate.

We then examined the introduction of positive charges to this segment by protecting the carboxylic acid as a picolyl (Pic) ester as shown in Scheme 1. The Pic group was previously used to increase the solubility of short protected peptides.[9] Since then, no practical peptide synthesis using this group has been reported. This reverse polarity protection of the functional group has a significant effect on the increase in the isoelectric point of the peptide, and facilitates the



Scheme 1. Synthetic route of IL-2 (99-133) containing a Pic ester and the isopeptide structure **2**.

HPLC purification under acidic conditions (e.g., in the presence of 0.1% TFA). The solid-phase peptide synthesis (SPPS) was carried out by the 9-fluorenylmethoxycarbonyl (Fmoc) method incorporating Fmoc-Glu(OPic)-OH at Glu^{100,106,110,116}. O-acylisodipeptides at Ala^{112}-Thr^{113} and Ile^{122}-Thr^{123} were also introduced to enhance the solubility of the segment even after the final deprotection. The problem in this resynthesis was that after the introduction of Fmoc-Glu-(OPic)-OH and subsequent Fmoc removal, a part of the Glu residue was converted into the pyroglutamic acid residue, which terminated additional chain elongation (see Figure SI2 in the Supporting Information). This side reaction is sequence dependent, occuring at Glu^{106} and Glu^{116}. The addition of 1-hydroxybenzotriazole (HOBt) or other additives to the piperidine during the Fmoc removal[10] did not completely suppress this side reaction. Further studies are required to overcome this problem. However, as a result of introducing four Pic esters, the solubility was significantly increased and **2** was easily purified by RPHPLC using an aqueous acetonitrile eluent containing 0.1% TFA, and obtained in a yield of 3.4% based on the starting Thr residue on the resin.

As segment (66-98) was intended to be converted into a thioester using the *N*-alkylcysteine (NAC) assisted thioesterification method,[11] Fmoc-[Lys(*i*Noc)^{76,97}]-IL2(66-98)-(Et)Cys-Lys$_2$-NH$_2$ (**3′a,b**; *i*Noc: 4-pyridylmethoxycarbonyl)[12,13] was prepared by the Fmoc method (see Scheme SI2 in the Supporting Information). Although this peptide was soluble in aqueous acetonitrile and observed on RPHPLC, no major peak appeared after the thioester exchange reaction by 3-hydroxythiophenol, indicating that the solubility of the peptide decreased because of the loss of the diLys unit with the NAC moiety (see Figure SI3 in the Supporting Information). Thus, the segment was resynthesized by introducing two Pic esters at Glu^{67,68} (see Scheme SI3). In the synthesis of this peptide, the formation of pyroglutamic acid during the Fmoc removal was effectively suppressed by adding 1 M HOBt to 20% piperidine-1-methyl-2-pyrrolidinone (NMP). The obtained peptide was then converted into the thioester by 3-hydoxythiophenol, and as a result, even after the thioesterification reaction, the peptide retained a good solubility and was easily purified by RPHPLC (see Figure SI4 in the Supporting Information). Fmoc-[Glu-(OPic)^{67,68}, Lys(*i*Noc)^{76,97}]-IL2(66-98)-SC$_6$H$_4$-*m*-OH (**3**) was successfully obtained in 4.9% yield based on the Gly content of the initial resin.

The synthesis of the prolyl thioester by the prevalent N-to-S acyl shift reaction is known to be less efficient than other amino acid residues.[14] The reason for this problem seems to be the slow N-to-S acyl shift at the C-terminus of the Pro residue, and it might also be the case in the NAC method. In fact, the preliminary thioester exchange experiment using Fmoc-Asn-Phe-His-Leu-Arg-Pro-(Et)Cys-X (**17**; X = Lys-NH$_2$) by sodium 2-mercaptoethanesulfonate (MESNa) in the presence of AcOH showed that the reaction seldom occurred (see Figure SI5 in the Supporting Information). We recently reported that the peptidyl NAC with a carboxylic acid efficiently provided the peptide thioester by the thioester exchange reaction. The carboxylic acid seems to participate in the N-to-S acyl shift reaction and enhance the overall rate of thioesterification reaction.[15] Thus, the same hexapeptidyl NAC with a carboxylic acid (**18**; X = OH) was synthesized and the thioesterification reaction was attempted. As a result, the desired peptide thioester with MESNa was successfully obtained (see Figure SI6 in the Supporting Information). Based on these data, the (28-65) segment **4**, having the prolyl thioester, was prepared starting from the Fmoc-Pro-(Et)Cys-(Trt)-O-(2Cl)Trt-resin as shown in Scheme 2. After the completion of the synthesis, the peptide was deprotected and converted into the thioester by MESNa. As a result, the reaction efficiently proceeded within four days (see Figure SI7 in the Supporting Information) and the desired peptide thioester **4** was obtained in 18% yield based on the initial resin. The N-terminal aryl thioester (1-27) carrying the disaccharide **5** was synthesized using the NAC method as previously reported[16] in a yield of 11% (see Scheme SI4 and Figure SI8 in the Supporting Information).

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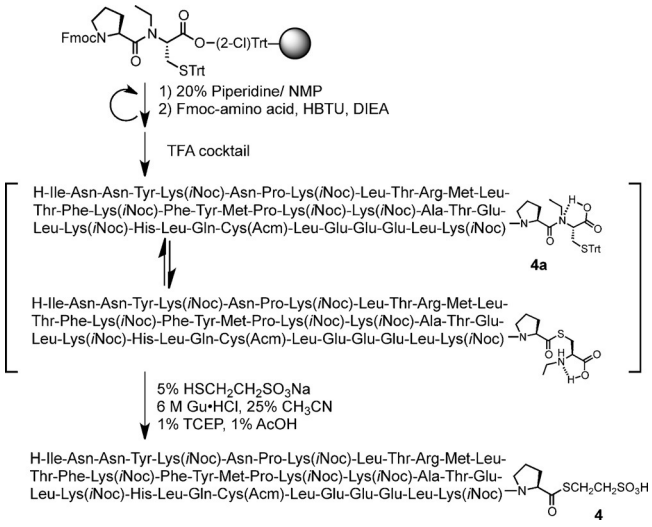
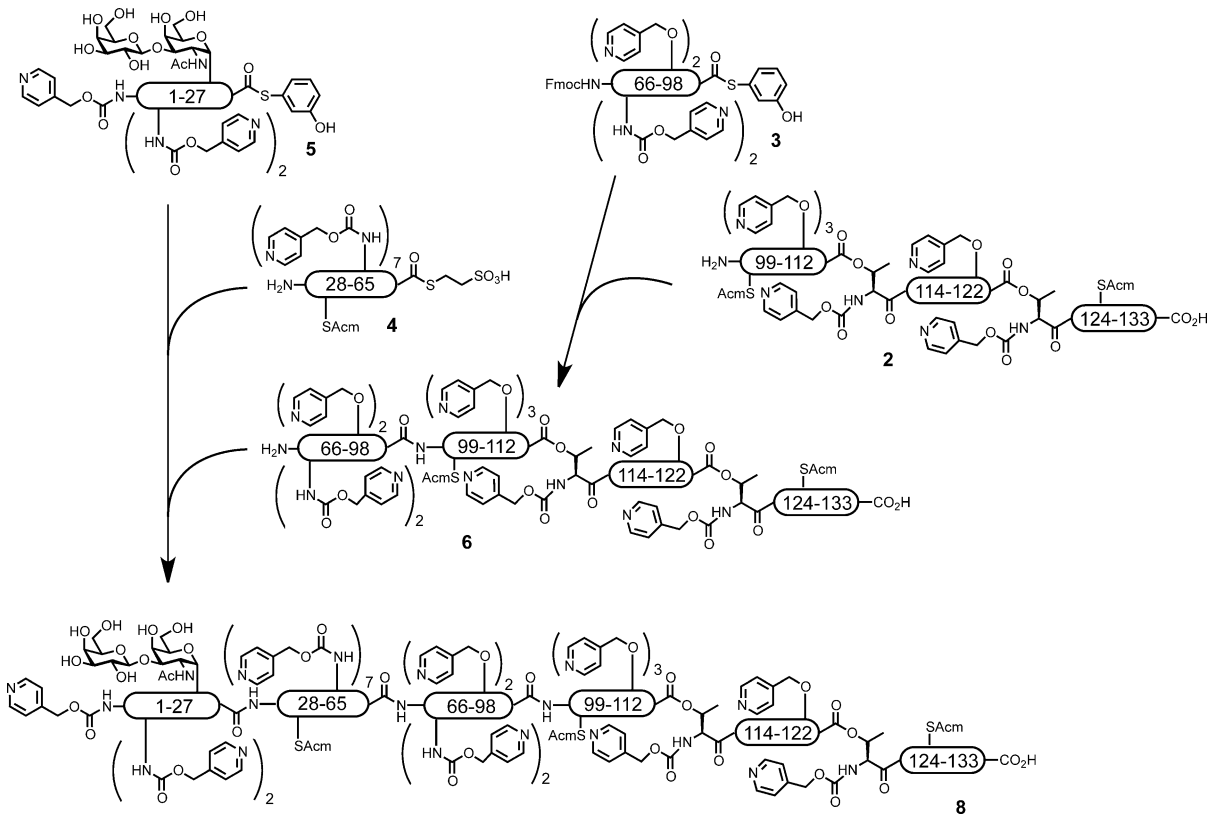


Scheme 2. Synthesis of the prolyl thioester **4** using NAC which has a carboxylic acid.

Having all the required segments in hand, we next performed the ligation reaction as shown in Scheme 3. We previously developed a sequential ligation method for three segments by using the thioester method, taking advantage of the reactivity difference between the aryl and alkyl thioesters.[13] To apply the method, we first condensed the two C-terminal segments **2** and **3** using the Ag^{+}-free thioester method. Because of the presence of hydrophilic protecting groups (O-Pic ester and N-*i*Noc) and isopeptide structures, the segments were highly soluble in DMSO and the reaction proceeded efficiently (see Figure SI9 in the Supporting Information). After an overnight reaction, the Fmoc group was removed by adding piperidine to the solution and the desired product **6** was isolated by RPHPLC in 56% yield. The sequential ligation was then achieved using the peptides **5**, **4**, and **6**. The aryl thioester **5** and the alkyl thioester **4** were dissolved in DMSO containing 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) and *N*,*N*-diisopropylethylamine (DIEA). The ligation efficiently proceeded and the intermediate peptide (1-65) containing the alkyl thioester **7** was successfully obtained (Figure 2). After 24 hours, **6** and AgCl were added to initiate the second ligation which was performed at the Pro-Leu site. The NCL reaction at the prolyl site usually does not proceed to completion because of the electron donation from the carbonyl oxygen atom of the penultimate amino acid to the carbonyl carbon atom of the Pro residue, thus lowering the reactivity of the thioester.[17a] In addition, the reaction is usually accompanied by the formation of a deletion product.[14,17b] In this synthesis which employs the thioester method, the reaction efficiently proceeded without serious side reactions to give the glycopolypeptide **8** comprising the entire sequence of IL-2. This glycopolypeptide was easily solubilized in aqueous acetonitrile and isolated in 57% overall yield for the two ligation reactions.



Scheme 3. Synthesis of polypeptide chain of IL-2 by the sequential thioester method. Reaction conditions: a) 1). HOOBt, DIEA, DMSO, RT, 18 h; 2. 20% piperidine/DMSO, RT, 5 min, 56%; b) 1. **4**, **5**, HOOBt, DIEA, DMSO, RT, 24 h; 2. **6**, AgCl, 50 °C, 2 d, 57% (overall yield of sequential ligation). DMSO = dimethylsulfoxide.

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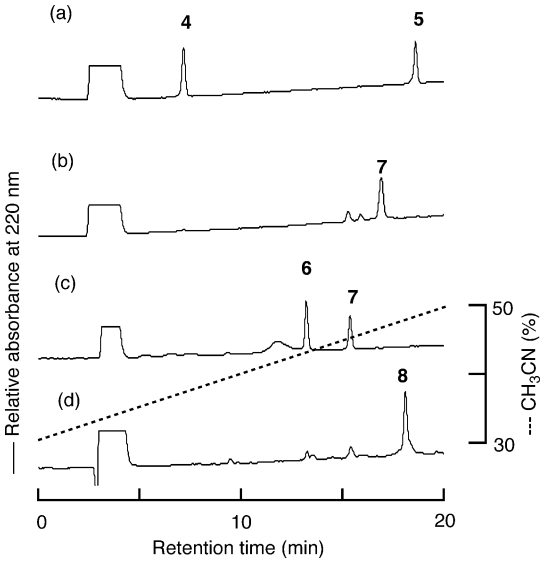


Figure 2. RPHPLC profile of the ligation progress. Ligation of **5** with **4** at 0 h (a) and at 24 h (b). Ligation of **7** with **6** at 0 d (c) and at 2 d (d). A linear gradient starting from 30% to 50% CH_3CN for 20 min was applied. Column used in (a) and (b): Mightysil RP-18 SP2. Column used in (c) and (d): YMC-pack Protein-RP.

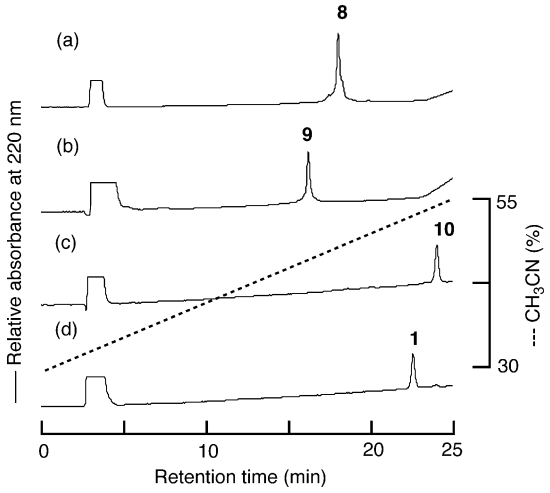
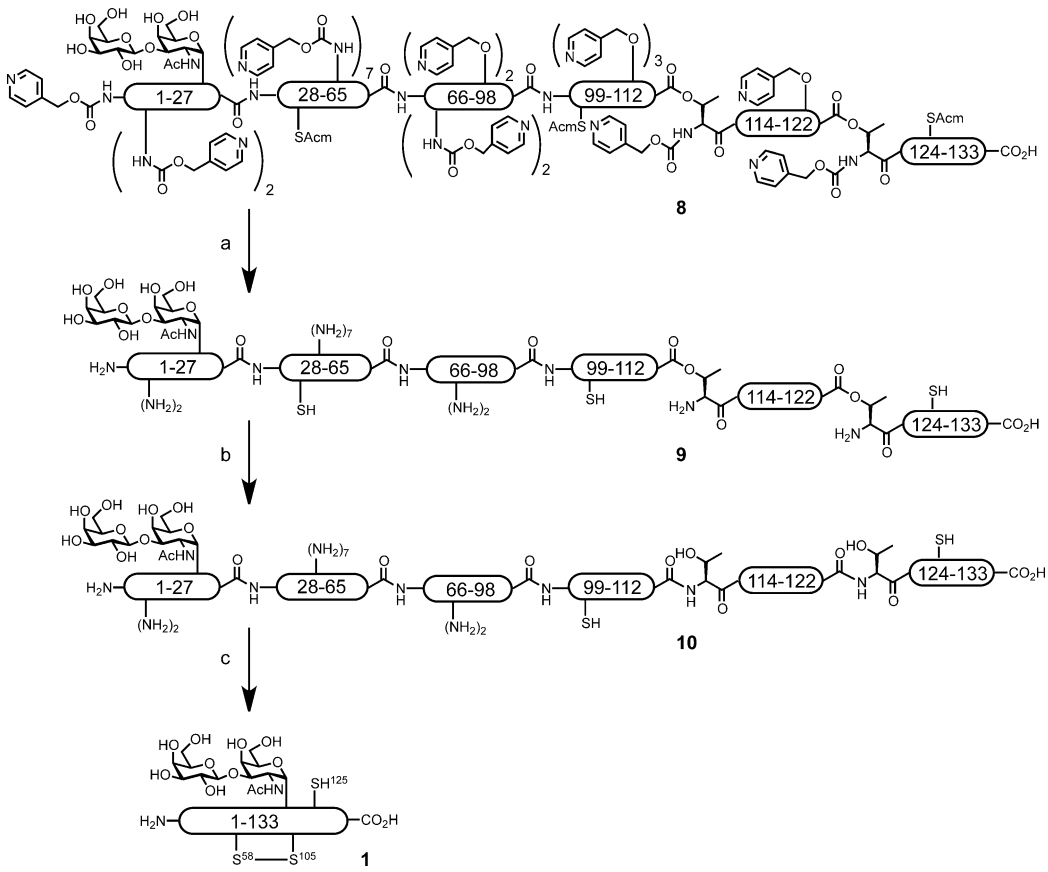


Figure 3. RPHPLC profile of deprotection and folding. a) Isolated peptide **8**. b) Crude peptide **9**. c) Acyl migration reaction of **9** after 1 h. d) Folding of **10** after 24 h. A linear gradient starting from 30 to 55% CH_3CN for 25 min was applied. Column: YMC-pack Protein-RP.

The deprotection of **8** was first performed by AgOAc treatment in 50% aq. AcOH for the Acm removal (Scheme 4). The silver ions were then precipitated by adding 3-mercaptopropionic acid (MPA) and removed by centrifugation. Without purification, the supernatant was treated with zinc dust in the presence of MPA for 30 minutes for the *i*Noc and Pic ester removal. These reactions efficiently proceeded and gave the completely deprotected IL-2 **9** having two isopeptide structures (Figure 3). Prior to the folding reaction, the isopeptide structures were converted into the native peptide bond. This conversion was performed in a buffer containing 6 M Gu·HCl, 0.1 M Tris, and 30 mM reduced glutathione (pH 8). The peak in the HPLC trace for **9** completely disappeared and a new peak, with a longer retention time and the same mass number, appeared for **10**, indicating that the conversion of the isopeptide into the peptide bond was



Scheme 4. Deprotection and folding of IL-2. Reaction conditions: a) 1. AgOAc, 50% AcOH aq., 50 °C, 2 h; 2. Zn, MPA, 6 M Gu·HCl aq., RT, 30 min, 63%; b) 30 mM reduced glutathione, 6 M Gu·HCl in 0.1 M Tris buffer, pH 8.0, 50 °C, 1 h; c) then diluted with 1.5 mM oxidized glutathione in 0.1 M Tris buffer, pH 8.0, RT, 24 h, 50%.

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complete (Figure 3). The solution was then diluted with 0.1M Tris containing oxidized glutathione and the resulting solution was kept for 24 hours at room temperature.[18] The original peak was converted into a more hydrophilic one, indicating that the folding completed successfully to give IL-2 carrying the core 1 O-linked sugar **1**. The successful folding was also supported by the CD spectrum measurement, which showed the structure to be rich in α-helix (see Figure SI10 in the Supporting Information).

The T cell proliferation and INF-γ production activity in response to IL-2 stimulation was then analyzed. We first examined $CD4^+$ T cell activity in response to the synthesized IL-2 ex vivo (see Figure SI12 in the Supporting Information). When we stimulated purified $CD4^+$ T cells with the synthesized IL-2 together with anti-CD3 antibody, these cells proliferated normally in response to IL-2. In accordance with this result, $CD4^+$ T cells produced INF-γ in response to IL-2 stimulation with anti-CD3 antibody. Collectively, these results indicate that synthesized IL-2 has full biological activity.

In conclusion, we report the efficient synthesis of IL-2, carrying the core 1 O-linked sugar at Thr^3, by using the reverse polarity protection of the carboxylic acid along with the O-acylisopeptide method to increase solubility during HPLC purification and the ligation reaction. All the protecting groups were easily removed by a sequential deprotection reaction. The obtained, completely deprotected IL-2, having two isopeptide structures, retained good solubility and efficiently folded into the native structure, which was proven by the biological activity and CD spectrum measurements. This strategy would be useful for the synthesis of other globular (glyco)proteins, which have a low solubility until the entire chain is assembled, and even for membrane proteins. Additional syntheses will be pursued along these lines.

Keywords: glycoprotein · ligation · peptides · synthesis design · synthetic methods

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