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Chemical synthesis of human adiponectin(19–107) bearing post-translational glycosylation

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ABSTRACT

Human adiponectin(19–107), which consists of the variable region and the collagenous domain bearing post-translational modifications including glycosylation, was chemically synthesized for the first time. A glycoside of 5-hydroxylysine (Hyl) was incorporated using an α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl/Hyl-Gly building block in a benzyl-protected form by solid-phase peptide synthesis (SPPS). The molecule was assembled from four segments prepared by SPPS via native chemical ligation (NCL) and thioester methods.

Adiponectin is an insulin-sensitizing adipokine with antidiabetic, anti-atherogenic, anti-inflammatory and cardioprotective properties.[1] In recent years, adiponectin has attracted much attention because of its multiple beneficial effects on a cluster of obesity-related metabolic and cardiovascular dysfunctions.[2] Its human form is composed of 244 amino acid residues divided into four structurally distinct domains, that is, a signal peptide, a variable region, a collagenous domain and a globular domain that binds to the adiponectin receptors. It is post-translationally modified with glycosylation on several conserved Lys residues and hydroxylation on Pro residues within its collagenous domain. In circulating blood, adiponectin exists as three isoforms including trimeric, hexameric and high-molecular-weight (HMW, at least 18 protomers) oligomeric forms, and its monomeric form has never been detected under native conditions. Thus, oligomerization may represent a key mechanism regulating the multiple biological activities of adiponectin.[3] Trimers are generated when a triple helix is formed by noncovalent interactions between the collagenous domains with the aid of hydrophobic interactions between the globular domains. Hydroxylation of Pro within the collagenous domain contributes to stabilizing the triple helix structure,[4] and disulfide formation involving Cys36 in the variable region is critical for the production of hexamers and HMW oligomers. On the other hand, modification of the four conserved Lys within the collagenous domain, that is, hydroxylation followed by subsequent glycosylation, is demonstrated to facilitate efficient formation and secretion of the HMW oligomers.[5] However, the mechanism of oligomerization remains to be addressed. In the present study, we synthesized human adiponectin(19–107) consisting of the variable region and the collagenous domain, the latter bearing post-translational modifications, in order to understand the role of glycosylation in forming the HMW structures.

Our synthetic strategy is shown in Figure 1. The molecule was divided into four segments I, II, III, and IV corresponding to (19–35), (36–63), (64–87), and (88–107), respectively. The two *N*-terminal segments were synthesized by *t*-butoxycarbonyl (Boc)-based solid-phase peptide synthesis (SPPS) and the two *C*-terminal segments with the glycosylated 5-hydroxylysine (Hyl) residue(s) were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc)-based SPPS. When using the native chemical ligation (NCL) method,[6] the Cys residue is required at the ligation site. In the target molecule of adiponectin(19–107), however, only one Cys residue located at position 36 in the second segment II is available for NCL at Ala35-Cys36 site (Arrow A). To perform the peptide-chain assembly at Gly87-Ala88 site (Arrow C) by Ala ligation involving a ligation–desulfurization approach,[7] a Cys residue was

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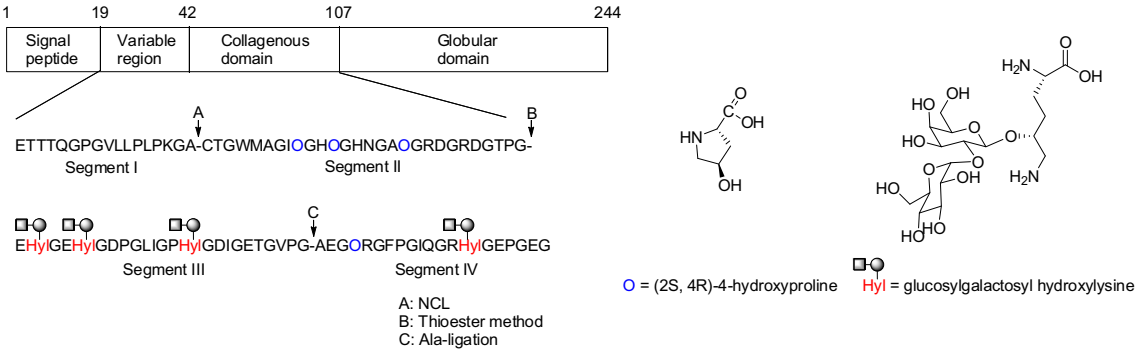
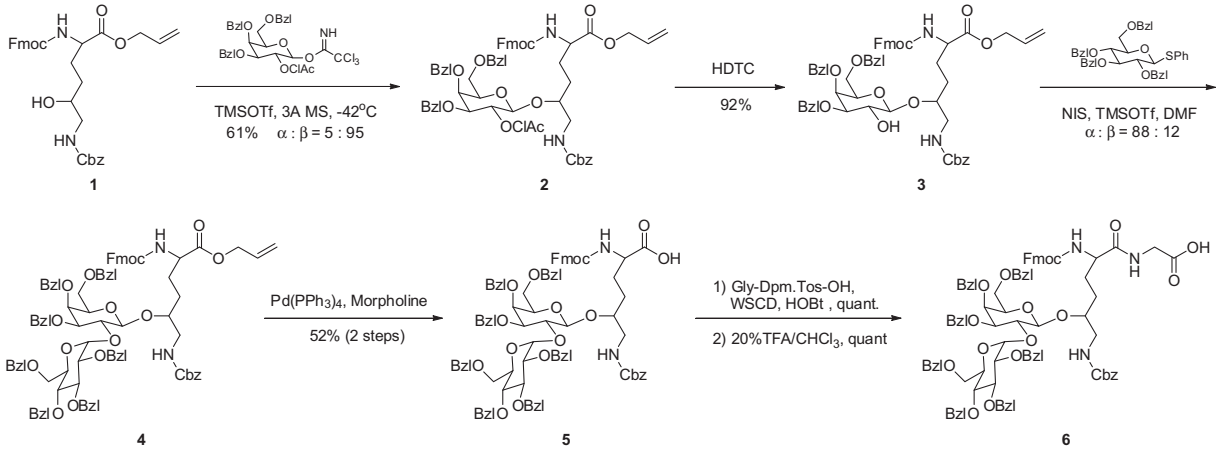


Figure 1. Structural domains of human adiponectin and its post-translational modifications. Arrows indicate the ligation sites.

introduced at position 88 instead of Ala in the *C*-terminal segment IV. The conversion of Cys88 to Ala88 should be carried out prior to NCL at the Ala35-Cys36 site in order to achieve chemoselective desulfurization. For ligation at the Gly63-Glu64 site (Arrow B), we employed the thioester method.[8] Thus, the synthetic route of human adiponectin(19–107) consists of coupling segments II and III via the thioester method, followed by coupling the resulting segment (II + III) successively with segments IV and I in the *C* and *N* direction, respectively, to afford the entire molecule via the NCL reaction. Therefore, the thioester moiety of segment III should be preserved during the thioester reaction and should be generated upon the subsequent Ala ligation. Among such thioester-surrogate approaches in Fmoc chemistry, one of the promising methods is employing an *N*–*S* acyl transfer device, such as the cysteinylprolyl ester-mediated method,[9] *N*-alkylcysteine-assisted method,[10] or *N*-sulfanylethylanilide-mediated method.[11] We adopted an *S*-protected *N*-alkylcysteine as a latent thioester functionality in the synthesis of segment III because it is readily available.

The disaccharide moiety, α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl (Glc-Gal), on four (2S,5R)-5-hydroxylysine (Hyl) residues plays a fundamental role in adiponectin bioactivity as its recombinant product containing no glycosylation was functionally inactive. In the first step to synthesize the glucosylgalactosyl Hyl building block **5** (Scheme 1),[12] for a glycosyl donor, we chose galactosyl trichloroacetimidate with the 2-hydroxy group protected by chloroacetate (ClAcO), which participates in the formation of a selective β-*O*-glycoside linkage to the Hyl derivative **1** as a neighboring group. Trimethylsilyl triflate (TMSOTf) promoted the galactosylation of **1** in dichloromethane at −42 °C to afford the β-*O*-galactoside **2** in 61% yield after chromatographic purification on silica gel. Lowering the reaction temperature from 0 °C to −42 °C improved β selectivity from α/β = 11:89 to 5:95, respectively, as well as the yield. After removal of the ClAc group by treatment with hydrazinedithiocarbonate (HDTC) under the neutral condition,[13] the 2-hydroxy group on **3** allowed for the second coupling reaction with the phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside via DMF-modulated glycosylation,[14] which led to α-selectivity (α/β = 88:12), followed by removal of the Allyl group, affording the α-anomer **5** in 52% yield.

The molecule of human adiponectin(19–107) was constructed according to Scheme 2. The two *N*-terminal thioester peptides **7** and **8** were synthesized using an ABI 433A peptide synthesizer on a Boc-Ala-SCH_2CH_2CO-[Arg(Tos)]$_3$-PAM resin and a Boc-Gly-SCH_2CH_2CO-[Arg(Tos)]$_3$-PAM resin, respectively, using in situ neutralization protocols of coupling with Boc-amino acid/1-[bis (dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU)/6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt)/*N*,*N*-diisopropylethylamine (DIEA) (4:4:4:6 equiv) in 1-methyl-2-pyrrolidinone (NMP).[15] The peptide resins were treated by HF in the presence of *p*-cresol at −2 °C to −5 °C for 1 h to remove all the protecting groups except for the acetamidomethyl (Acm) group on the *N*-terminal Cys residue in peptide **8**. The crude products were purified by RP-HPLC to give highly pure thioester peptides in 60% yield for (19–35)-thioester **7** and 16% yield for (36–63)-thioester **8**. The latter was subjected to a reaction involving introduction of a Boc group using Boc-OSu into the α-amino function to quantitatively produce peptide **9**. The two *C*-terminal peptides **10** and **13** were synthesized by the Fmoc-SPPS method on an Fmoc-NH-SAL-PEG resin and an Fmoc-Gly-Wang-PEG resin, respectively. To



Scheme 1. Synthesis of glucosylgalactosyl Hyl (**5**) and glucosylgalactosyl Hyl-Gly (**6**) building blocks.