The First Synthesis of *N*-Man-Trp: Alternative Mannosylation Modification of Protein

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Abstract: A novel protein modification, *N*-mannosyl tryptophan (*N*-Man-Trp) was synthesized in a stereoselective manner.

Key words: carbohydrate, glycopeptides, glycoamino acid, mannosyl tryptophan, total synthesis

Glycosylation is the most widely recognized protein post-translational modification and it affects various functions.[1] There are two major classifications based on the structure of the linkage between the sugar and protein parts; O-linked oligosaccharides and N-linked oligosaccharides.[2] In O-glycosylation, the sugar is attached to the hydroxy group of serine or threonine, while in N-glycosylation, sugar is attached to the protein via the side-chain amide group of aspargine in the recognition sequence of Asn-X-Ser/Thr, where X can be any amino acid except proline.

However, a novel type of protein glycosylation pattern involving mannosylation was recently reported. The C-mannosylation was first identified as a mannosyl modification in RNase.[3] The mannose residue was connected to the indole of tryptophan via carbon–carbon linkage (Figure 1). C-Mannosyl tryptophan **1** (*C*-Man-Trp) has been found in a number of mammalian proteins, including interleukin-12 expressed in Chinese hamster ovary cells, complements, properdin, thrombospondin, mucins, and a bovine lens protein, as well as in marine ascidians and the Ebola virus.[4–6] The sequence *Trp*-X-X-Trp (Italic *Trp* is mannosylated) appears to be the recognition motif, but some exceptions have also been identified.[7]

We previously succeeded in the first total synthesis of C-mannosylated tryptophan and related peptides.[8,9] Using an antibody against *C*-Man-Trp and derivatives prepared from chemically synthesized compounds, we were able to investigate the relationship between diabetes and C-mannosylation.[10] We also found that *C*-Man-Trp-containing peptides enhance cytotoxicity together with lipopolysaccharide via the upregulation of tumor necrosis factor-α.[11] In addition, Pallavicini suggested a relationship between breast cancer and C-mannosylation using high throughput mass spectrometry techniques.[12]

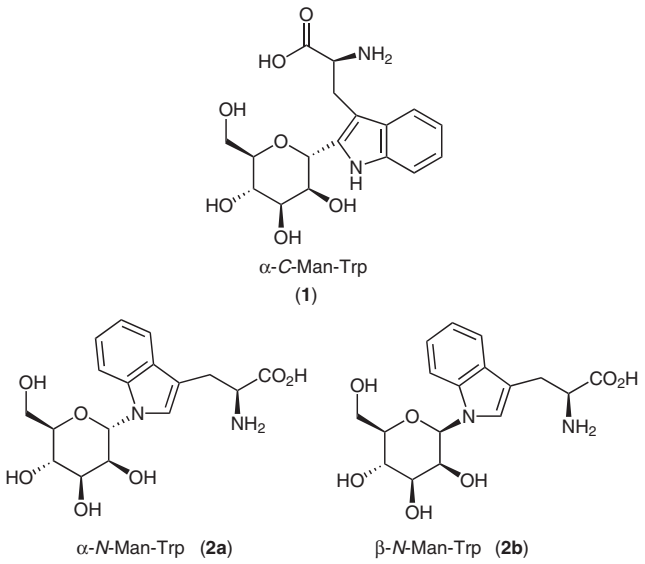

Figure 1 The structure of mannosylated tryptophan as protein post-translational modification

In 2005, Li et al. identified another mannosylation modification, N-linked mannosylation **2**, in *Asdes aegypti* chorion peroxidase.[13] The structure was identified by electrospray ionization/tandem mass spectrometry and peptide-*N*4(*N*-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase) digestion. On the mass spectra, a 162-Da subsistent was seen in each Trp residue, and abundant fragmentation at $m/z = 163$ without the loss of 120 Da was observed. Because of limitations in sample quantity, the stereochemistry at the anomeric center was not determined rigorously. PNGase was reported able to hydrolyze the C–N linkage between indole and mannose. But, since PNGase hydrolyzes the β-amide bond between the aspargine side chain and sugar moiety, and it has very high substrate specificity,[14,15] the ability to hydrolyze the indole sugar is critical to the biochemical mechanisms (Scheme 1).

In order to clarify this question, we planned to synthesize the N-linked mannosyl tryptophan in a stereoselective manner. We report here the first total synthesis of α-N-linked mannosyl tryptophan **2a**. The indole glycosylation reaction is known to be difficult.[16] Indole is not stable under Lewis acidic conditions, and the glycosylation reaction causes numerous side reactions including orthoester-type 1,2-*O*-(indol-1-yl)ethylidene compound formation. Unverzagt reported a β-Glc-Trp synthesis using a Lewis

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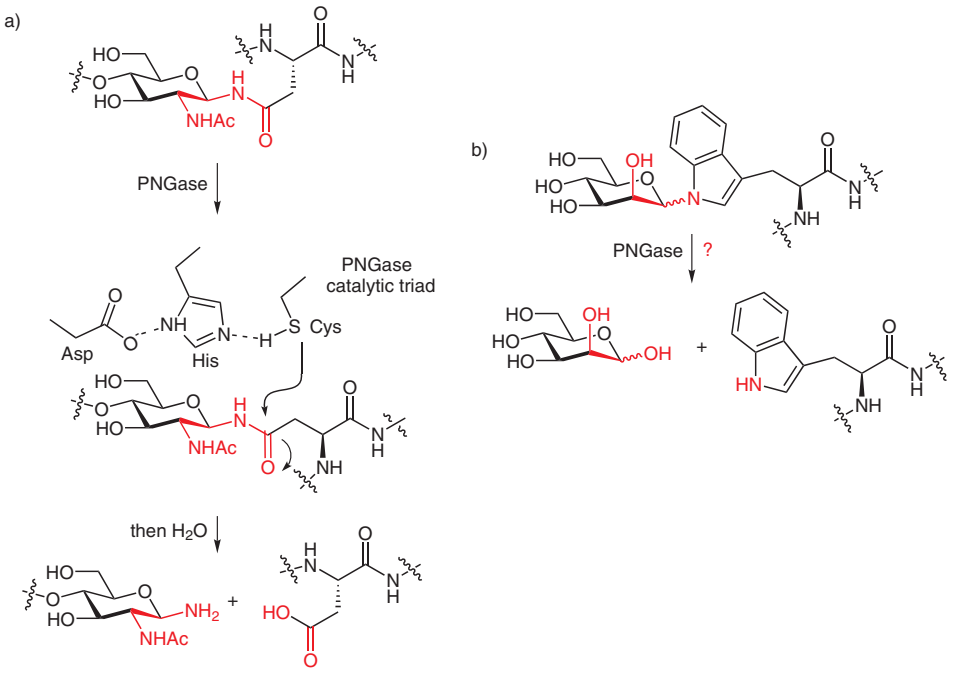
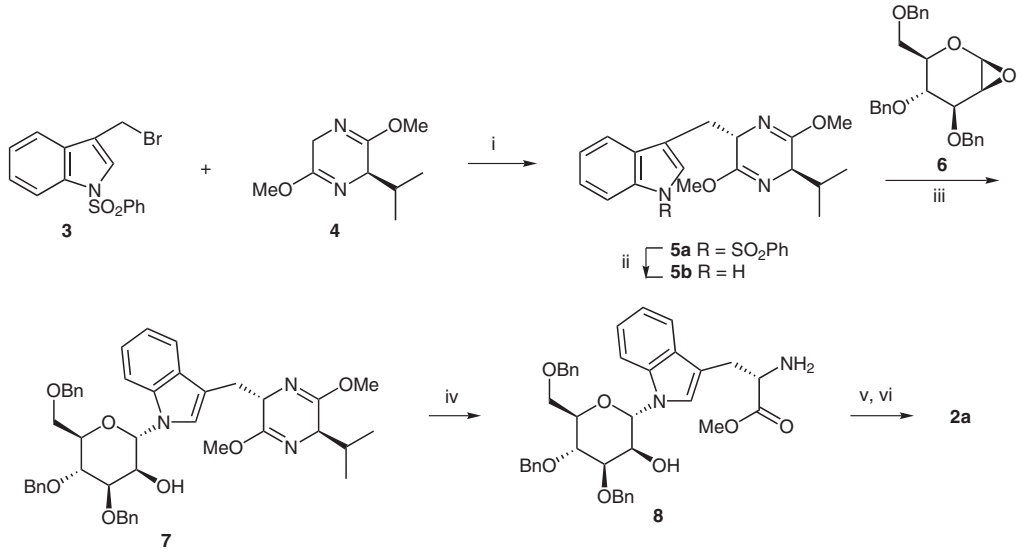


Scheme 1 a) Proposed mechanism of hydrolysis of amide bond by PNGase; b) hydrolysis of anomeric carbon–indole bond.



Scheme 2 *Reagents and conditions*; i) BuLi, THF, 64%; ii) Mg, EtOH, 98%; iii) KHMDS, THF, 49%, iv) HCl, dioxane, H_2O, 71%; v) H_2, $Pd(OH)_2/C$, AcOH, H_2O; vi) $LiOH{\cdot}H_2O$, H_2O, 75%.

acidic glycosylation reaction, but the yield was not sufficiently high and a bulky pivaloyl group was necessary at the 2-position to prevent side reactions.[17] To avoid Lewis acidic conditions, we planned to make the anomeric carbon–indole nitrogen connection via nucleophilic attack under basic conditions.

The amino acid moiety of the tryptophan was therefore masked as a bislactim structure, which is stable under basic conditions. The tryptophan equivalent bislactim structure was prepared from indolyl bromide **3** and Schöllkopf chiral auxiliary **4**[18] (Scheme 2). The newly formed chiral center was stereochemically pure based on 400 MHz ^{1}H NMR analysis. The sulfonamide group at indole **5a** was then removed by Mg in EtOH without racemization at the chiral centers of bislactim moiety in 98% yield.[19] The indole anion generated from **5b** attacked the anomeric position of the epoxide[20] **6** in S_N2 fashion to give adduct **7** in 49% yield. After cleaving the bislactim group under acidic conditions, the benzyl group was removed under typical hydrogenolysis conditions using $Pd(OH)_2/C$ as a catalyst. After hydrolysis of the methyl ester by LiOH, α-*N*-Man-Trp (**2a**) was obtained in 75% yield (two steps) after purification by reverse-phase silica gel column chromatography. The ^{1}H NMR coupling constant of anomeric position is 4.5 Hz.[21] This large coupling constant at anomeric position suggests that the pyranose ring conformation is dis-

torted, probably due to the lack of anomeric effects and steric hindrance of the indole ring. An antibody against α-*N*-Man-Trp is now being prepared using the chemically synthesized α-*N*-Man-Trp derivatives, and biological studies will be reported. The synthesis of the other stereoisomer, β-*N*-Man-Trp, is also currently under way.

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(21) ^{1}H NMR (600 MHz, D_2O, KH_2PO_4–Na_2HPO_4 buffer pH 7, dioxane 3.75 ppm as a ref., 25 °C): δ = 7.74 (d, *J* = 8.1 Hz, 1 H, C-5), 7.72 (d, *J* = 8.1 Hz, 1 H, C-8), 7.44 (s, 1 H, C-2), 7.36 (dd, *J* = 8.1, 7.1 Hz, 1 H, C-7), 7.27 (dd, *J* = 8.1, 7.1 Hz, 1 H, C-6), 6.03 (d, *J* = 4.5 Hz, 1 H, C′-1), 4.68 (dd, *J* = 4.5, 3.5 Hz, 1 H, C′-2), 4.21 (dd, *J* = 6.5, 3.5 Hz, 1 H, C′-3), 4.07 (dd, *J* = 8.1, 5.0 Hz, 1 H, CH of Trp) 4.00 (dd, *J* = 12.6, 7.6 Hz, 1 H, C′-6), 3.92 (dd, *J* = 6.5, 6.5 Hz, 1 H, C′-4), 3.76 (dd, *J* = 12.6, 3.0 Hz, 1 H, C′-6), 3.55 (ddd, *J* = 7.6, 6.5, 3.0 Hz, 1 H, C′-5), 3.47 (dd, *J* = 15.1, 5.0 Hz, 1 H, CH_2 of Trp), 3.31 (dd, *J* = 15.1, 8.1 Hz, 1 H, CH_2 of Trp). ^{13}C NMR (125 MHz, D_2O, KH_2PO_4–Na_2HPO_4 buffer pH 7): δ = 175.0 (C, CO_2H), 137.5 (C, C-9), 128.5 (C, C-4), 123.5 (CH, C-7), 125.8 (CH, C-2), 121.4 (CH, C-6), 119.6 (CH, C-5), 112.3 (CH, C-8), 110.2 (C, C-3), 82.4 (CH, C′-1), 77.5 (CH, C′-5), 71.8 (CH, C′-3), 68.6 (CH, C′-4), 68.3 (CH, C′-2), 60.5 (CH_2, C′-6), 55.4 (CH, CH of Trp), 26.86 (CH_2, CH_2 of Trp); C–N linkage between anomeric carbon and indole nitrogen is confirmed by HMBC analysis; $[\alpha]_D^{24}$ –17 (*c* 0.1, H_2O).