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SYNTHESIS OF A HIGH-MANNOSE-TYPE GLYCOPEPTIDE ANALOG CONTAINING A GLUCOSE-ASPARAGINE LINKAGE

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Abstract: The title compound was prepared by enzymatic transfer of oligosaccharide to a synthetic pentapeptide containing the Glc-Asn linkage. The compound was not hydrolyzed by glycoamidases from plant and bacterial sources, but it inhibited both enzymes in the micromolar range. Its activity is compared to other potential inhibitors.

Glycoamidase (peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)-asparagine amidase, PNGase, GA) is found in a number of organisms across different kingdoms.[1-3] The enzyme catalyzes the release of intact oligosaccharides from *N*-linked glycopeptides and glycoproteins via hydrolysis of the β-amide of the linking asparagine. To probe the mechanism and substrate requirements for glycoamidases, a number of natural and unnatural mono- and disaccharide glycopeptides were synthesized and tested for substrate activity. Cellobiose and lactose glycopeptides, in which GlcNAc-GlcNAc linked to Asn is replaced by a disaccharide of non-aminosugars, are not substrates for glycoamidases.[4] However, we wanted to examine the effects of changing only the innermost sugar, while retaining the second aminosugar and mannosyl residues, of a native substrate. Thus, we synthesized **1**, a substrate analog in which the linking sugar, normally GlcNAc, is replaced with Glc.

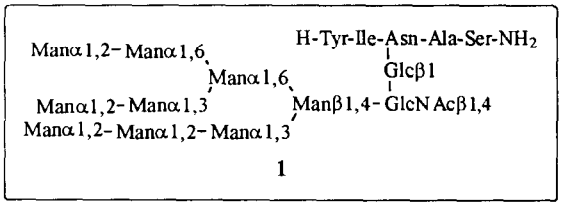

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Figure 1. Structure of title compound.

The unusual Glc-Asn linkage has been found in nature, notably in *Archaebacteria*[5] and laminin,[6] although the structure of the oligosaccharide beyond the linking Glc has not yet been elucidated. Additionally, Glc-Asn in the α linkage is found in a nephritogenic glycopeptide.[7] Our compound serves further purpose as a model to study this class of glycoconjugate.

Total organic synthesis of the target compound would be laborious, so instead we combined chemical and enzymatic methods to prepare the glycopeptide. The synthesis is based on the effective transglycosylation

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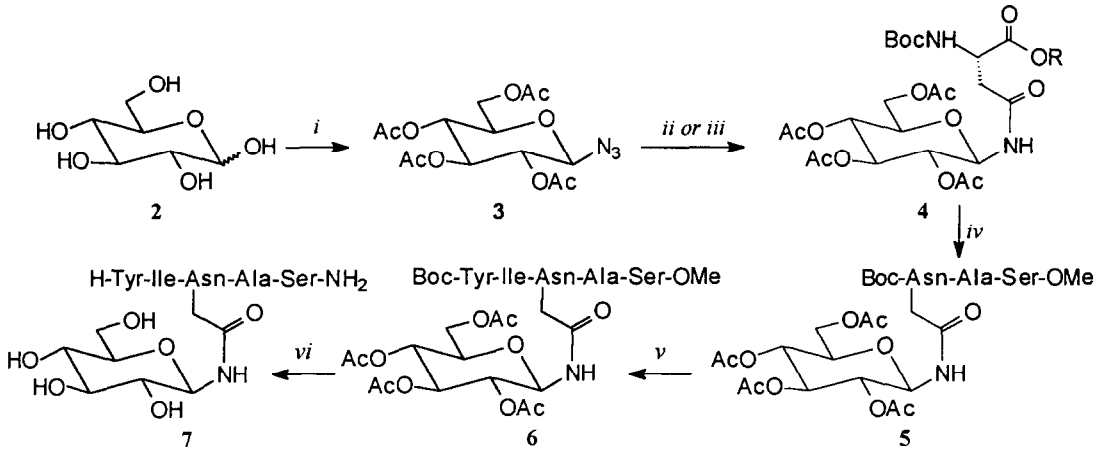
activity of endo-*N*-acetyl-β-D-glucosaminidase from *Arthrobacter protophormiae* (Endo-A), normally acting as an endoglycosidase, cleaving between the two GlcNAc residues of high-mannose-type *N*-glycans. In some aqueous organic solvents, however, the enzyme can mediate transfer of $Man_{5-9}GlcNAc$ to the equatorial 4-OH of an acceptor molecule.[8] In our case, $Man_9GlcNAc_2Asn$, isolated from soybean agglutinin,[9] served as the donor and glucosyl pentapeptide (7) served as the acceptor.

Synthesis of the enzymatic acceptor 7 is summarized in Scheme 1. Glucosyl azide tetra-*O*-acetate (**3**) was readily prepared from D-glucose,[10] and protected glucosyl asparagine (**4**) could be prepared by two methods from **3**. The first option was reduction of the azide to amine, followed by coupling with Boc-Asp-OBn.[11,12] Alternatively, **4** was prepared in one step from **3** and Boc-Asp-OBn via triethylphosphine.[13] Elongation to the pentapeptide[14] followed. First, **4** was debenzylated by hydrogenolysis with subsequent coupling with H-Ala-Ser-OMe. The resultant tripeptide **5** was deprotected at the amino terminus under acidic conditions, followed by coupling with Boc-Tyr-Ile-OH. The pentapeptide **6** was then deprotected by sequential ammonolysis and acidolysis to yield 7.

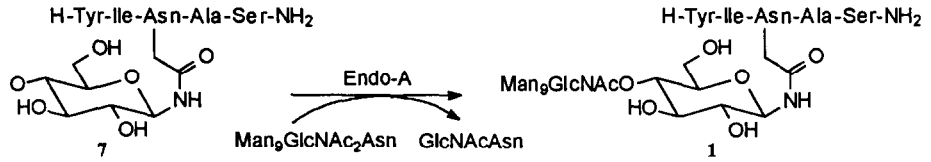


i: (a) Ac_2O, HBr-HOAc, rt, 2 h; (b) NaN_3, DMF, rt, 16 h, 81%. *ii:* (a) H_2, PtO_2, MeOH, rt, 16 h; (b) Boc-Asp-OBn, DCC, HOBt, CH_2Cl_2-DMF, rt, 2 h, 52%. *iii:* Boc-Asp-OBn, Et_3P, CH_2Cl_2, rt, 16 h, 54%. *iv:* (a) H_2, Pd/C, EtOH, rt, 6 h; (b) H-Ala-Ser-OMe, DCC, HOBt, DMF, rt, 16 h, 58%. *v:* (a) 55% TFA, 2% PhOH, CH_2Cl_2, rt, 15 min; (b) Boc-Tyr-Ile-OH, DCC, HOBt, DMF, rt, 16 h, 58%. *vi:* (a) NH_3, THF-MeOH, rt, 2 d; (b) 55% TFA, 1% PhOH, H_2O, rt, 15 min, 30%.

Scheme 1. Synthesis of glucosyl pentapeptide acceptor.

Enzymatic transfer of the $Man_9GlcNAc$ structure to 7 was performed according to literature procedure.[8] Briefly, $Man_9GlcNAc_2Asn$, 7, and Endo-A, in ammonium acetate, pH 6, containing 35% acetone, were incubated at 37 °C for 20 min and boiled for 3 min to stop the reaction. The product was purified by HPLC on a Spherisorb S5 ODS semiprep column (1 x 25 cm) with 9% aqueous acetonitrile containing 0.05% trifluoroacetic acid (3.5 mL/min) as eluant. The product eluted at 9.5 min, and excess acceptor eluted at 17.9 min. Both

product and acceptor were collected and recovered. Eluant containing product was lyophilized to yield the desired product as a white solid in 39% yield.



Scheme 2. Enzymatic transfer of $Man_9GlcNAc$ to glucosyl pentapeptide acceptor.

^1H NMR and amino acid and monosaccharide composition analyses were consistent with the expected structure of the product.[15] The ^1H NMR chemical shift values of the anomeric protons beyond the linking Glc were very similar to those reported for known glycopeptides containing the $Man_9GlcNAc$ structure.[16]

The synthetic product **1** was tested as a substrate for commercially available glycoamidases from almond (GAA) or from *Flavobacterium meningosepticum* (GAF). **1** was incubated overnight with either enzyme in ammonium acetate buffer and analyzed by HPLC. No change was observed by HPLC in the presence or absence of either enzyme.

1 was then tested as an inhibitor for glycoamidases. Using glycopeptide isolated from thermolysin digest of bovine asialofetuin as substrate, a mixture containing 10 mM ammonium acetate (pH 5 and 8 for GAA and GAF, respectively), 12.5 μM glycopeptide, 0.5-1000 μM **1**, and glycoamidase was incubated at 37 °C for 10 min, and the reaction was stopped by boiling for 3 min. The mixture was then analyzed by HPLC on a Shimadzu CLC-ODS column (60 x 150 mm) with 6% aqueous acetonitrile containing 0.05% trifluoroacetic acid (1 mL/min) as eluant. Substrate, hydrolysis product, and inhibitor eluted at 6.7, 9.8, and 13.1 min, respectively. Peak area of the product was measured and compared to a control containing no inhibitor to determine relative activity. Results in the figure show an IC_{50} of 8 μM for GAA and 62 μM for GAF. The K_m values are 4 μM and 75 μM for GAA and GAF, respectively. Correlating IC_{50} and K_m with K_i,[17] the K_i values for **1** were determined to be 2 μM for GAA and 53 μM for GAF. The lack of turnover and the inhibition results suggest that the acetamido group of the innermost sugar plays an important role in recognition and cleavage of the natural GlcNAc-Asn bond.

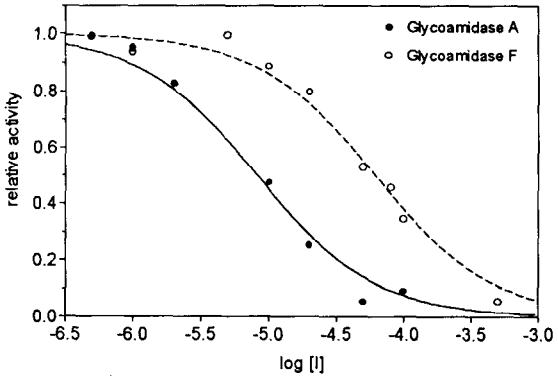


Figure 2. Studies of inhibition of glycoamidases by **1**.

The inhibition by **1** of glycoamidases is comparable to a *C*-glycopeptide analog, in which a methylene bridge is inserted between the high-mannose-type glycan and asparagine β-amide of a pentapeptide (1 and 43 μM for GAA and GAF, respectively).[14] Initial inhibition studies by other nonsubstrate compounds for GAA or GAF demonstrated different activities. $Man_9GlcNAc_2Asn$, $Man_9GlcNAcGlcAsn$ (a truncated form of **1**), and the glycan from fetuin glycopeptide inhibited GAF at 1000 μM. On the other hand, the same compounds showed no inhibition of GAA. Furthermore, neither enzyme was inhibited by 500 μM **7**, demonstrating a role for the oligosaccharide chain for inhibition.

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15. ^1H NMR shifts of anomeric protons (300 MHz) of **1** and reported chemical shifts of anomeric protons of $Man_9GlcNAc_2Asn$-XX (500 MHz) from soybean agglutinin (ref. 16) in parentheses: 5.409 (5.404), 5.341 (5.334), 5.316 (5.308), 5.148 (5.143), 5.042 (3H, 5.061, 5.049, 5.042), 4.868 (4.869, α-Man H-1), 4.949 (J = 9.20 Hz, Glc H-1), 4.771 (~4.77, β-Man H-1), 4.567 (4.610, GlcNAc H-1).
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