JOURNAL OF THE CHINESE CHEMICAL SOCIETY

Communication

Synthesis of a Versatile Probe for Analysis of Cytoplasmic Peptide-*N*-Glycanase

Mizuho Masuda,[a] Shinpei Miyazawa,[a] Yukishige Ito[b] and Ichiro Matsuo[a]*
[a]*Department of Chemistry and Chemical Biology, Graduate School of Engineering, Gunma University, 1-5-1 Tenjin, Kiryu, Gunma 376-8515, Japan*
[b]*RIKEN Advanced Science Institute and ERATO Japan Science and Technology Agency, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

(Received: Oct. 27, 2011; Accepted: Nov. 28, 2011; Published Online: Dec. 20, 2011; DOI: 10.1002/jccs.201100631)

A clickable alkyne tagged chloroacetamidyl chitobiose derivative was synthesized as a potential inhibitor of cytoplasmic peptide-*N*-glycanase (PNGase). Construction of a chitobiose structure containing both alkyne and azide functional groups was performed using a thioglycoside donor having a triisopropylsilyl (TIPS) protected alkyne group and a glycosyl azide acceptor. The resultant chitobiosyl azide derivative was reduced to the corresponding glycosyl amine and a chloroacetyl group was introduced. Finally, removal of the TIPS group using tetrabutylammonium fluoride (TBAF) gave the target compound. We conjugated the dansyl derivative, using click conditions and obtained the fluorescent labeled disaccharide derivative, selectively. Our probe can easily lead to a variety of compounds and should help in the understanding of the biological functions of cytoplasmic PNGase.

Keywords: PNGase; Synthesis; Inhibitor; Click coupling.

INTRODUCTION

Protein quality control in the endoplasmic reticulum (ER) is a cellular process during which terminally misfolded proteins in the lumen of the ER are recognized and targeted for ER-associated degradation (ERAD).[1] Recent studies have revealed that the *N*-glycan plays a regulatory role in determining the fate of newly synthesized glycoproteins.[2] Misfolded proteins are retrotranslocated into the cytosol by the action of lectin-like proteins and eventually degraded by proteasomes.[3] During degradation, cytoplasmic peptide-*N*-glycanase (PNGase) plays an important role, cleaving the *N*-glycans from the misfolded glycoproteins in the cytosol, after which the misfolded proteins are effectively scavenged by 20S proteasomes.[4] Because the barrel shaped proteasome has its active site in the interior of the pore, removal of bulky *N*-glycans from glycoproteins is necessary in order to insert the peptide chain into its degrading chamber.[5] Therefore, cytoplasmic PNGase is not merely a deglycosylating enzyme, but is even more important as the key enzyme in the glycoprotein degradation machinery. Furthermore, deglycosylation independent biological functions of PNGase have been described in various organisms.[6] Functional diversification of this protein should be conducted in order to clarify its interactions at the molecular level.

In order to investigate cytoplasmic PNGase, chemical probes such as specific inhibitors would be powerful tools, but only few inhibitors are currently available.[7] Recently we developed a specific inhibitor for PNGase based on an oligosaccharide structure having a thiol-reactive haloacetoamidyl group at the reducing end. These probes covalently modify the cysteine residue of the catalytic triad.[8] We succeeded in inhibiting the intracellular activity of PNGase.[9] Further, the chloroacetamidyl chitobiose derivative was analyzed by X-ray crystallography of the PNGase and this revealed the catalytic site of Cys residue at the atomic level.[10]

To better understand the biological function of PNGase at the molecular level, a diverse compound library is needed. We decided to synthesize a chloroacetamidyl chitobiose derivative bearing an alkyne group on the non-reducing end. This allows for a variety of functional groups to be introduced such as detection and purification tags easily using a Cu(I)-catalyzed[3,2]cycloaddition reaction between the alkyne group and an azide group, so-called "click chemistry".[11] In this paper, we report a synthetic study of the clickable probe as a versatile tool for the analysis of PNGase and demonstrate attachment of a fluorescent tag.

RESULTS AND DISCUSSION

Synthetic plan for the clickable probe is shown in Fig. 1. The reducing end *N*-acetylglucosamine was designed as

Dedicated to the memory of Professor Yung-Son Hon (1955–2011).

* Corresponding author. E-mail: matsuo@gunma-u.ac.jp

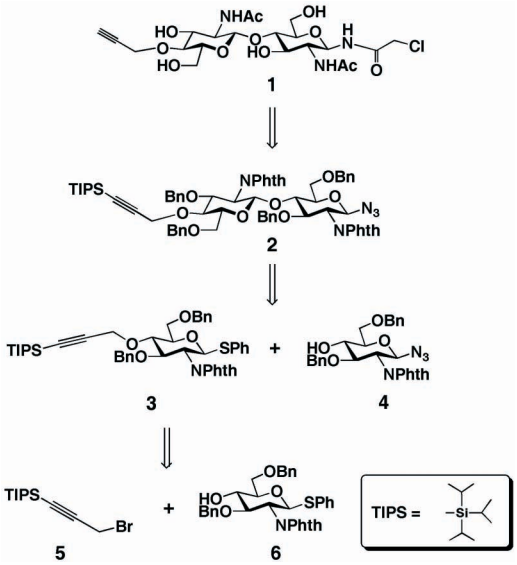
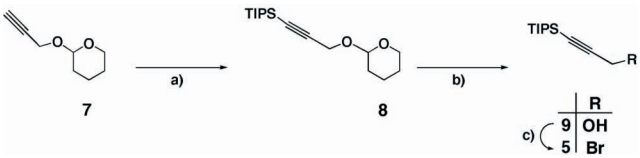


Fig. 1. Synthetic plan for clickable probe **1**.

a glycosyl azide **4** so that late stage coupling of chloroacetic acid could be performed via glycosylamine. Coupling of **4** with the glucosamine donor **3** having TIPS protected alkynyl group should be possible without interference of azide and alkyne groups.[12] Compound **3**, in turn, was designed to be prepared from thioglycoside **6** and the propargyl bromide derivative **5**.

Preparation of the propargyl bromide derivative **5** proceeded in three steps starting from commercially available compound **7** which was first treated with *n*-butyllithium and triisopropylsilyl chloride to afford **8**. Subsequent deprotection of the tetrahydropyranyl ether using Hon's conditions[13] and conversion of the resultant alcohol **9** into a bromide to gave **5**[14] (Scheme I).

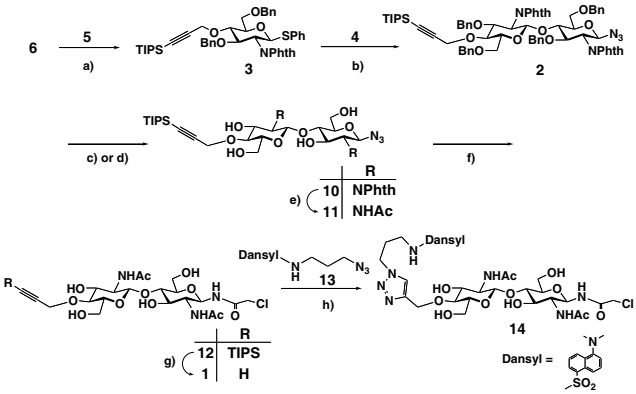
Scheme I Synthesis of propargyl bromide derivative **5**



Reagents and conditions: (a) TIPSCl, BuLi, THF, 84%; (b) Acetonyltriphenylphoshonium Bromide, MeOH, 90%; (c) Ph_3P, CBr_4, CH_2Cl_2, quant.

Introduction of the alkyne group into compound **6**[15] using **5** with NaH in DMF gave thioglycoside donor **3** in 51% yield. Coupling of donor **3** and glycosyl azide acceptor **4**[16] using NIS-AgOTf as a promoter,[17] resulted in the chitobiose derivative **2** in 83% yield. In order to remove the benzyl groups, without affecting the azide and alkyne groups, we employed oxidative debenzylation using 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ).[18] Removal of the benzyl groups of **2** was cleanly achieved using 20 equivalents of DDQ in aq. CH_2Cl_2 to afford **10** in 95% yield. In addition, to reduce the amount of DDQ, we examined regeneration of DDQ from dihydroxybenzoquinone using MnO_2 as a co-oxidizing agent.[19] This improved condition allowed us to decrease the amount of DDQ by 1/2 resulting in **10** in quantitative yield. Subsequent dephthaloylation of **10** was performed under Kanie's conditions,[20] which was followed by the acetylation of the resulting amine, and deacetylation of hydroxyl groups providing compound **11**. Reduction of **11** was carried out using propanedithiol and the resultant glycosyl amine was chloroacetylated to give **12**. Removal of the TIPS group from **12** using 2 eq. of TBAF and 4 eq. of acetic acid in THF at 40 °C gave the target compound **1** in 69% yield (Scheme II).

Scheme II Synthesis of clickable probe **1**



Reagents and conditions: (a) NaH, DMF, 51%; (b) NIS, AgOTf, CH_2Cl_2, 83%; (c) DDQ, 10 eq. H_2O, CH_2Cl_2, 95%; (d) DDQ, MnO_2, 8 eq. MeOH, $(CH_2Cl)_2$, quant.; (e) 1. 1,2-diaminoethane, n-BuOH, 2. Ac_2O, DMAP, 3. NaOMe, MeOH/THF, 74%; (f) 1. 1,3-propanedithiol, DIEA, MeOH, 2. $ClAc_2O$, MeOH/CH_2Cl_2, 79%; (g) 2 eq. TBAF, 4 eq. AcOH, THF, 69%; (h) CuI, 2,6-lutidine, DIPEA, CH_3CN/H_2O (1/1), 78%.

Target compound in hand, we carried out a Cu(I)-catalyzed[3+2]cycloaddition reaction between **1** and the dansyl derivative **13** bearing azide group as shown in Scheme II. Coupling of **1** and **13** using CuI in CH_3CN/H_2O (1/1) gave **14** as a single product in 78% yield.[21] The ^{1}H-NMR spectrum of compound clearly reveals the presence of two anomeric protons, chloroacetamide methylene protons, the

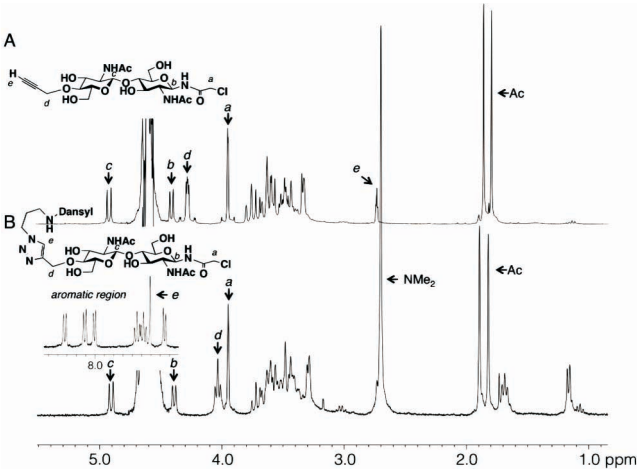


Fig. 2. ^{1}H-NMR spectra (300 MHz, D_2O, 25 ºC, referenced to HOD adjusted to 4.65 ppm) of clickable probe **1** (A) and fluorescent labeled probe **14** (B).

aromatic protons of dansyl group, and a single triazol proton (Fig. 2).

As described above, we synthesized the clickable alkyne tagged chloroacetamidyl chitobiose derivative and demonstrated the introduction of a fluorescent tag by click chemistry. This versatile compound should be extremely useful in the preparation of a variety of probes using click chemistry, enabling the measurement of enzyme activity, fluorescent labeling, the exploration of a novel PNGase, localization analysis, and functional analysis of PNGase in cells. It should also be useful to determining the exact function of PNGase. Our current research is focused on the investigation of a novel PNGase from a natural source using probes with conjugated affinity tags.

ACKNOWLEDGEMENTS

A part of this work was financially supported by a Grant-in-Aid for Scientific Research (21580410) from Ministry of Education, Culture, Sports, Science, and Technology of Japan and ERATO JST. We gratefully thank Dr. R. Walton for his helpful discussions in completing this article, and Mr. K. Iino and Ms. K. Kobayashi for technical assistance.

REFERENCES

1. (a) Hebert, D. N.; Bernasconi, R.; Molinari, M. *Semi. Cell Dev. Biol.* **2010**, *21*, 526-532. (b) Stolz, A.; Wolf, D. H. *Biochem. Biopys. Acta* **2010**, *1803*, 694-705.
2. Aebi, M.; Bernasconi, R.; Clerc, S.; Molinari, M. *Trends Biochem. Sci*. **2010**, *35*, 74-82.
3. Christianson, J. C.; Shaler, T. A.; Tyler, R. E.; Kopito, R. R. *Nat. Cell Biol.* **2008**, *10*, 272-282.
4. (a) Suzuki, T.; Seko, A.; Kitajima, K.; Inoue, Y.; Inoue, S. *J. Biol. Chem.* **1994**, *269*, 17611-17618. (b) Suzuki, T. *Semi. Cell Dev. Biol.* **2007**, *18*, 762-769. (c) Hagihara, S.; Goda, K.; Matsuo, I.; Ito, Y. *Biochem. Biophys. Res. Commun.* **2007**, *360*, 357-362.
5. Hirsch, C.; Blom, D.; Ploegh, H. L. *EMBO J.* **2003**, *22*, 1036-1046.
6. (a) Funakoshi, Y.; Negishi, Y.; Gergen, J. P.; Seino, J.; Ishii, K.; Lennarz, W. J.; Matsuo, I.; Ito, Y.; Taniguchi, N.; Suzuki, T. *PLoS One* **2010**, *5*, e10545. (b) Suzuki, T.; Park, H.; Lennarz, W. J. *FASEB J.* **2002**, *16*, 635-641.
7. (a) Witte, M. D.; Descals, C. V.; De Lavoir, S. V. P.; Florea, B. I.; Van der Marel, G. A.; Overkleeft, H. S. *Org. Biomol. Chem*. **2007**, *5*, 3690-3697. (b) Witte, M. D.; Horst, D.; Wiertz, E. J. H. J.; Van der Marel, G. A.; Overkleeft, H. S. *J. Org. Chem*. **2009**, *74*, 605-616. (c) Misaghi, S.; Pacold, M. E.; Blom, B.; Ploegh, H. L.; Korbel, G. A. *Chem. Biol.* **2004**, *11*, 1677-1687.
8. (a) Miyazaki, A.; Matsuo, I.; Hagihara, S.; Kakegawa, A.; Suzuki, T.; Ito, Y. *Glycoconj. J.* **2009**, *26*, 133-140. (b) Suzuki, T.; Hara, I.; Nakano, M.; Zhao, G.; Lennarz, W. J.; Schindelin, H. *J. Biol. Chem.* **2006**, *281*, 22152-22160.
9. Hagihara, S.; Miyazaki, A.; Matsuo, I.; Tatami, A.; Suzuki, T.; Ito, Y. *Glycobiology* **2007**, *17*, 1070-1076.
10. Zhao, G.; Zhou, G. L. X.; Matsuo, I.; Ito, Y.; Suzuki, T.; Lennarz, W. J.; Schindelin, H. *Glycobiology* **2009**, *19*, 118-125.
11. (a) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057-3064. (b) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596-2599. (c) Himo, F.; Lovell, T.; Hilgraf, R.; Rostovtsev, V. V.; Noodleman, L.; Sharpless, K. B.; Fokin, V. V. *J. Am. Chem. Soc.* **2005**, *127*, 210-216.
12. Valverde, I. E.; Delmas, A. F.; Aucagne, V. *Tetrahedron* **2009**, *65*, 7597-7602.
13. (a) Hon, Y. S.; Lee, C. F. *Tetrahedron Lett*. **1999**, *40*, 2389-2392. (b) Hon, Y. S.; Lee, C. F. *Tetrahedron* **2001**, *57*, 6181-6188. (c) Hon, Y. S.; Lee, C. F.; Chen, R. J.; Szu, P. H. *Tetrahedron* **2001**, *57*, 5991-6001.
14. Physical data for new compounds given below. ^{1}H-NMR spectra were measured on a JEOL AL-300 spectrometer. Analytical TLC was performed on aluminium sheets coated with silica gel 60 F254 (Merck). MALDI-TOF MS spectra were recorded in the positive ion mode on an AXIMA CFR Kompact MALDI (Shimazu/KRATOS) equipped with nitrogen laser with an emission wavelength of 337 nm. **5**: ^{1}H NMR ($CDCl_3$): δ 3.94 (s, 2H), 1.07 (s, 21H); R_f 0.80 (hexane) MS (MALDI-TOF) calcd for $C_{12}H_{23}Br_1Na$ $(M+Na)^+$ *m/z* 297.1, found 296.8. **3**: ^{1}H NMR ($CDCl_3$): δ 7.82-6.85 (arom. 15H), 5.52 (d, *J* = 10.2 Hz, 1H), 4.81-4.37 (m, 6H), 4.34 (dd, *J* = 8.1 Hz, *J* = 9.9 Hz, 1H), 4.21 (t, *J* = 10.2 Hz, 1H), 3.97 (d, *J* = 10.8 Hz, 1H), 3.80 (dd, *J* = 5.1 Hz, *J* = 10.8 Hz, 1H), 3.97

(m, 1 H), 3.64 (m, 1H), 1.06 (s, 21H); R_f 0.41 (hexane:EtOAc, 2:1); MS (MALDI-TOF) calcd for $C_{46}H_{53}N_1O_6S_1Si_1Na$ $(M+Na)^+$ *m/z* 798.3, found 799.2. **2:** ^{1}H NMR ($CDCl_3$): δ 7.66-6.75 (arom. 28H), 5.52 (d, *J* = 10.2 Hz, 1H), 4.86-4.11 (m, 16H), 4.03 (t, *J* = 9.6 Hz, 1H), 3.85-3.37 (m, 7H), 1.05 (s, 21H); R_f 0.36 (hexane:EtOAc, 2:1), MS (MALDI-TOF) calcd for $C_{68}H_{75}N_5O_{12}Si_1Na$ $(M+Na)^+$ *m/z* 1209.5, found 1209.5. **10**: ^{1}H NMR ($CDCl_3$): δ 7.89-7.74 (arom. 8H) 5.39 (d, *J* = 8.2 Hz, 1H), 5.38 (d, *J* = 9.3 Hz, 1H), 4.53-3.47 (m, 11H), 3.23 (m, 1H), 1.04 (s, 21H); R_f 0.41 ($CHCl_3$:MeOH, 10:1), MS (MALDI-TOF) calcd for $C_{40}H_{49}N_5O_{12}Si_1Na$ $(M+Na)^+$ *m/z* 842.3, found 842.3. **11**: ^{1}H NMR (CD_3OD): δ 4.93 (d, *J* = 9.3 Hz, 1H), 4.50 (s, 2H), 4.49 (d, *J* = 9.3 Hz, 1H), 4.03-3.30 (m, 12H), 2.00 (s, 3H), 1.94 (s, 3H), 1.09 (s, 21H); R_f 0.39 ($CHCl_3$:MeOH, 4:1), MS (MALDI-TOF) calcd for $C_{28}H_{49}N_5O_{10}Si_1Na$ $(M+Na)^+$ *m/z* 666.3, found 667.0. **12**: ^{1}H NMR (CD_3OD): δ 4.93 (d, *J* = 7.2 Hz, 1H), 4.50 (s, 2H), 4.49 (d, *J* = 6.3 Hz, 1H), 4.03 (s, 2H), 4.03-3.30 (m, 12H), 2.00 (s, 3H), 1.94 (s, 3H), 1.09 (s, 21H); R_f 0.80 ($CHCl_3$:MeOH, 3:1), MS (MALDI-TOF) calcd for $C_{30}H_{52}N_3O_{11}Si_1Cl_1Na$ $(M+Na)^+$ *m/z* 716.3, found 717.4. **1**: ^{1}H NMR (D_2O): δ 4.96 (d, *J* = 9.3 Hz, 1H), 4.45 (d, *J* = 8.1 Hz, 1H), 4.32 (m 2H), 3.99 (m, 2H), 3.85-3.39 (m, 12H), 2.80 (m, 1H), 1.94 (s, 3H), 1.87 (s, 3H); R_f 0.40 ($CHCl_3$:MeOH, 3:1), MS (MALDI-TOF) calcd for $C_{21}H_{32}N_3O_{11}Cl_1Na$ $(M+Na)^+$ *m/z* 560.2, found 560.9. **14**: ^{1}H NMR (D_2O) MS (MALDI-TOF) calcd for $C_{36}H_{51}N_8O_{13}S_1Cl_1Na$ $(M+Na)^+$ *m/z* 893.3, found 894.4.

15. Matsuo, I.; Isomura, M.; Ajisaka, K. *Tetrahedron Lett.* **1996**, *37*, 8795-8798.
16. Matsuo, I.; Nakahara, Y.; Ito, Y.; Nukada, T.; Nakahara, Y.; Ogawa, T. *Bioorg. Med. Chem*. **1995**, *3*, 1455-1463.
17. Kanie, O.; Ito, Y.; Ogawa, T. *Tetrahedron Lett*. **1996**, *37*, 4551-4554.
18. (a) Ikemoto, N.; Schreiber, S. L. *J. Am. Chem. Soc.* **1992**, *114*, 2524–2536. (b) Baek, S.; Jo, H.; Kim, H.; Kim, H.; Kim, S.; Kim, D. *Org. Lett*. **2005**, *7*, 75-77.
19. Liu, L.; Floreancig, P. E. *Org. Lett.* **2010**, *12*, 4686-4689.
20. Kanie, O.; Crawley, S. C.; Palcic, M. M.; Hindsgaul, O. *Carbohydr. Res*. **1993**, *243*, 139-164.
21. Bernard, S.; Defoy, D.; Dory, Y. L.; Klarskov, K. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6127-6130.

