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Sortase-Catalyzed Peptide−Glycosylphosphatidylinositol Analogue Ligation

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Glycosylphosphatidylinositol (GPI) anchoring of cell surface proteins and glycoproteins onto cell membranes is ubiquitous in eukaryotic species,[1] and GPI-anchored proteins and glycoproteins play a vital role in various biological processes.[2] It has been well-established that GPI-anchored proteins and glycoproteins cannot function properly without the GPI anchor. On the other hand, linking naturally non-GPI-anchored proteins to GPI anchors may assist in the targeting, trafficking, and shedding of the proteins and help tune their biological function. For instance, Cathepsin D is a non-GPI-anchored protein that has no affinity for the pore-forming toxin aerolysin, but when Cathepsin D was expressed in GPI-anchored form via bioengineering, it was converted to an aerolysin-binding variant.[3] Consequently, methods to access GPI-anchored proteins and glycoproteins and related structures are valuable for studying GPI anchoring and developing novel biotechnologies.[4]

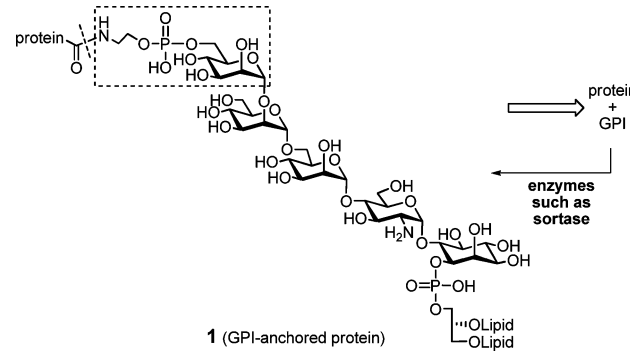
Because of the problem of microheterogeneity, it is particularly difficult to obtain homogeneous GPI-linked proteins/glycoproteins from biological resources. Meanwhile, the chemical synthesis of GPI-anchored proteins currently remains a significant challenge. Though GPI conjugates containing short peptides have been prepared chemically by total synthesis,[5−7] the strategy is not well-suited for GPI conjugates containing full-sized proteins. The only described synthesis of any well-defined GPI-anchored protein was based on native ligation,[8] but this synthetic strategy requires Cys-modified GPI anchors and C-terminus-modified proteins, both of which are difficult to acquire.

To develop a practical and widely applicable method for the synthesis of GPI-anchored proteins (**1**), we became interested in a biomimetic approach. GPI addition to proteins is a post-translational process mediated by GPI transamidase.[9] All proteins are attached to GPIs at the nonreducing-end mannose 6-O position of the GPI glycan core through the peptidic C-terminus and a phosphoethanolamine bridge (dashed box in Scheme 1). Therefore, we envisioned a strategy for ligating synthetic GPIs to synthetic or recombinant peptides/proteins by enzymatic methods (Scheme 1). In this regard, sortases, a group of bacterial transpeptidases that catalyze surface protein anchoring to bacterial cell walls by mechanisms similar to that of GPI transamidase,[10,11] appeared to be extremely attractive.

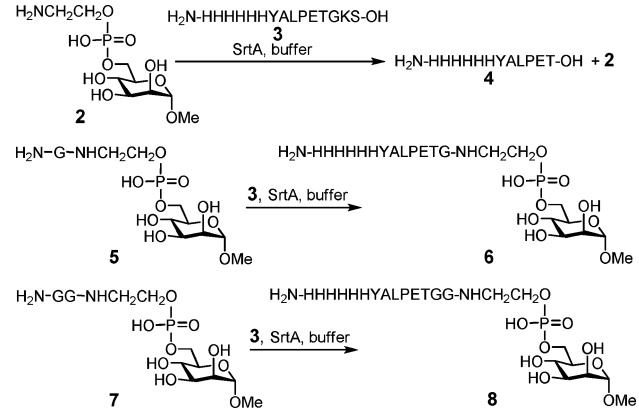
Sortase A (SrtA) from *Staphylococcus aureus* recognizes a pentapeptide LPXTG sequence near the C-terminus of a protein,[12] breaks the peptide bond between T and G of LPXTG, and links the new C-terminus of the target protein to the amino group of the N-terminal glycine of a peptidoglycan.[10] SrtA is rather substrate promiscuous, and therefore, it has been utilized for site-specific attachment of peptides and proteins to synthetic structures that contain one or multiple glycine residues[13−18] and to carbohydrate derivatives directly.[19] Accordingly, we expected that SrtA might be able to link peptides and proteins to GPI anchors and thus be used to prepare GPI-anchored peptides and proteins.

To explore the above hypothesis, we synthesized **2** as a simple GPI analogue and studied its reaction with peptide **3** in the presence

Scheme 1



Scheme 2



of SrtA. Compound **2** has the same anomeric configuration as the mannose in natural GPIs, while the methyl group is a useful diagnostic signal for NMR analysis. SrtA used in the research was a truncated, water-soluble form of the natural enzyme containing the extracellular but not the transmembrane sequence, and it was prepared in our laboratory by a standard recombinant technique. Peptide **3** had LPETG linked to the hexahistidine tag commonly used in recombinant techniques to facilitate protein purification. The reaction was performed at 37 °C in 0.3 M Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$, 2 mM 2-mercaptoethanol, and ∼6.8 μM SrtA, and its progress was monitored by MALDI−TOF mass spectrometry (MS) and reversed-phased (RP) HPLC. The reaction did slowly yield a new compound that was not the desired product but proved to be the hydrolyzed peptide **4** (MS *m*/*z*: calcd, 1514.7; found, 1515.8 $[M + H]^+$) (Scheme 2). This result indicated that **3** was indeed activated by SrtA but that **2** is not a sufficiently active nucleophile to react with the reactive enzyme−peptide complex; consequently, the activated peptide slowly hydrolyzed to afford **4**. We believed that the poor nucleophilicity of **2** was not likely due to steric hindrance, since much more sterically demanding sugar derivatives are easily accom-

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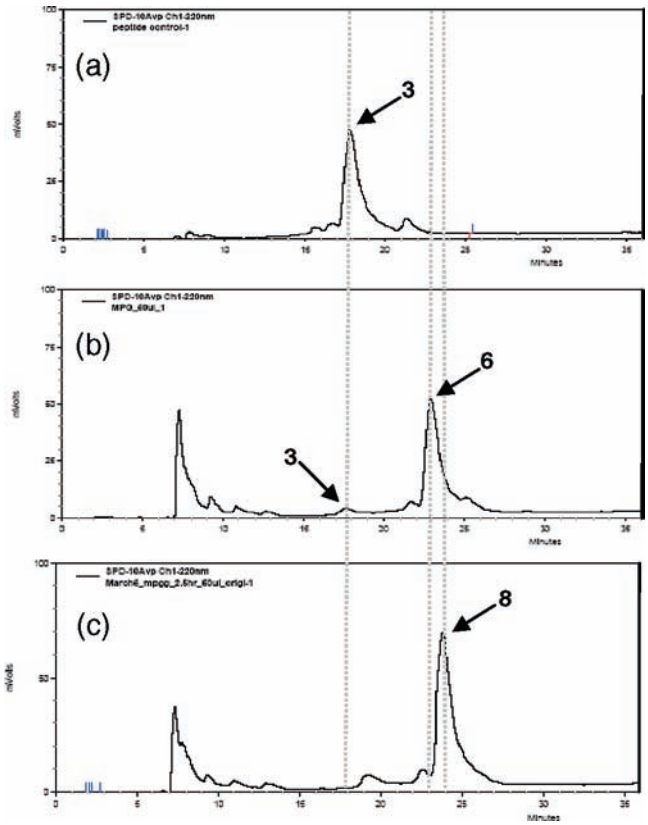

Figure 1. HPLC traces for (a) peptide **3**, (b) the reaction of **3** and **5**, and (c) the reaction of **3** and **7** (C18 column, gradient eluent using CH_3CN/H_2O).

modated by SrtA.[19] Most probably, the nearby phosphate functionality had a negative impact on the nucleophilicity of the amino group of **2**.

To establish the GPI forms acceptable for SrtA, we introduced a glycine amino acid to the phosphoethanolamine group to obtain **5** and investigated its reaction with **3** in the presence of SrtA. To our satisfaction, SrtA did catalyze an efficient coupling between **3** and **5** to afford **6** under the reaction conditions described above. After ~36 h of incubation at 37 °C, the reaction was quenched, and purification by RP HPLC (see the Supporting Information) afforded a 45% isolated yield of the product **6**, which was characterized by both NMR and MS analyses (MS *m/z*: calcd, 1870.8; found, 1871.8 $[M + H]^+$, 1893.9 $[M + Na]^+$).

To inspect whether elongating the peptide chain linked to the GPI analogue would have a further impact on the enzymatic reaction, we introduced another glycine residue onto **5** to obtain **7**. The reaction between **3** and **7** in the presence of SrtA afforded **8** (MS *m/z*: calcd, 1927.8; found, 1928.8 $[M + H]^+$), and its rate and efficiency were similar to those of the reaction between **3** and **5**, suggesting that incorporation of a single glycine residue onto the phosphoethanolamine moiety at the nonreducing end of the GPI is probably sufficient for SrtA-catalyzed ligations between GPIs and peptides or proteins.

In the above experiments, we found that the reactions reached the maximal yield of desired conjugate products after ~24 h of incubation. Thereafter, the remaining substrate peptide **3** could not be transformed into GPI conjugate, but it was not hydrolyzed either. This result suggests that SrtA may lose its activity over a long incubation time. Moreover, because these reactions did not give rise to significant hydrolysis products, both **5** and **7** seemed to be sufficiently reactive to suppress the hydrolysis and reverse reactions. Therefore, in theory, complete conversion of **5** and **7** into the desired conjugates should be possible.

To explore the optimal conditions for these transformations, we next examined the reactions between **3** (0.5 mM) and GPI analogues **5** and **7** (2.5 mM) in the presence of an increased concentration (30 μM) of SrtA in the same buffer as described above. It was observed that under these conditions, the rates and efficiencies of the two reactions were significantly improved. HPLC analyses (Figure 1) revealed that after only 2.5 h of incubation at 37 °C, the reactions afforded the desired GPI conjugates **6** and **8** in yields greater than 95%, and no significant hydrolysis was observed.

In conclusion, this work has demonstrated that introducing a glycine residue onto the phosphoethanolamine moiety of the nonreducing-end glycan of GPI analogues can transform them into SrtA-acceptable substrates and that SrtA can be utilized to effectively ligate small peptides and modified GPI analogues. This represents the first chemoenzymatic synthesis of any GPI−peptide conjugate and is also a proof-of-concept for the application of SrtA to the synthesis of GPI-anchored proteins and glycoproteins. Currently, we are examining the SrtA-catalyzed attachment of peptides and full-sized proteins to complex GPI analogues and intact GPIs. On the basis of the present results and literature reports that SrtA accepts large proteins and various sterically demanding nucleophiles,[13−19] we are confident that SrtA can be a powerful tool for coupling proteins and glycoproteins to GPIs for chemoenzymatic syntheses of natively linked GPI-anchored proteins and glycoproteins.

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Supporting Information Available: Preparation of SrtA, **2**, **3**, **5**, and **7**; enzymatic reaction conditions and procedures; HPLC results for the reactions; and MS and NMR spectra of SrtA and **2**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ferguson, M. A. J. *J. Cell Sci.* **1999**, *112*, 2799.
(2) Menon, A. K.; Baumann, N. A.; Rancour, D. M. In *Carbohydrates in Chemistry and Biology*; Hart, G. W., Hart, G., Sinaÿ, P., Ernst, B., Sinaikin, P., Eds.; Wiley-VCH: New York, 2000; Vol. 4, pp 757−769.
(3) Diep, D. B.; Nelson, K. L.; Raja, S. M.; Pleshak, E. N.; Buckley, J. T. *J. Biol. Chem.* **1998**, *273*, 2355.
(4) Mayor, S.; Riezman, H. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 110.
(5) Xue, J.; Shao, N.; Guo, Z. *J. Org. Chem.* **2003**, *68*, 4020.
(6) Shao, N.; Xue, J.; Guo, Z. *Angew. Chem., Int. Ed.* **2004**, *43*, 1569.
(7) Tanaka, Y.; Nakahara, Y.; Hojo, H.; Nakahara, Y. *Tetrahedron* **2003**, *59*, 4059.
(8) Becker, C. F. W.; Liu, X.; Olschewski, D.; Castelli, R.; Seidel, R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2008**, *47*, 8215.
(9) Udenfriend, S.; Kodukula, K. *Annu. Rev. Biochem.* **1995**, *64*, 563.
(10) Marraffini, L. A.; DeDent, A. C.; Schneewind, O. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 192.
(11) Perry, A. M.; Ton-That, H.; Mazmanian, S. K.; Schneewind, O. *J. Biol. Chem.* **2002**, *277*, 16241.
(12) Mazmanian, S. K.; Liu, G.; Ton-That, H.; Schneewind, O. *Science* **1999**, *285*, 760.
(13) Pritz, S.; Kraetke, O.; Klose, A.; Klose, J.; Rothemund, S.; Fechner, K.; Bienert, M.; Beyermann, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 3642.
(14) Tanaka, T.; Yamamoto, T.; Tsukiji, S.; Nagamune, T. *ChemBioChem.* **2008**, *9*, 802.
(15) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjugate Chem.* **2007**, *18*, 469.
(16) Pritz, S.; Wolf, Y.; Kraetke, O.; Klose, J.; Bienert, M.; Beyermann, M. *J. Org. Chem.* **2007**, *72*, 3909.
(17) Mao, H.; Hart, S. A.; Schink, A.; Pollok, B. A. *J. Am. Chem. Soc.* **2004**, *126*, 2670.
(18) Antos, J. M.; Miller, G. M.; Grotenbreg, G. M.; Ploegh, H. L. *J. Am. Chem. Soc.* **2008**, *130*, 16338.
(19) Samantaray, S.; Marathe, U.; Dasgupta, S.; Nandicoori, V. K.; Roy, R. P. *J. Am. Chem. Soc.* **2008**, *130*, 2132.

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