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Sortase A-Catalyzed Transpeptidation of Glycosylphosphatidylinositol Derivatives for Chemoenzymatic Synthesis of GPI-Anchored Proteins

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Abstract: Several peptides/small proteins and glycosylphosphatidylinositol (GPI) derivatives were synthesized and compared as substrates of sortase A (SrtA), a bacterial transpeptidase, for enzymatic coupling. It was observed that peptides containing the LPKTGGS and LPKTGGRS sequences as sorting signals at the peptide C-terminus were effectively coupled to GPI derivatives having one or two glycine residues attached to the phosphoethanolamine group at the nonreducing end. This reaction was employed to prepare several analogues of the human CD52 and CD24 antigens, which are naturally GPI-anchored glycopeptides/glycoproteins. It was further observed that the trisaccharide GPI analogues **5** and **6** were better SrtA substrates than monosaccharide GPI analogue **4**, suggesting that steric hindrance of the GPI analogues does not affect their peptidation reaction mediated by SrtA. Therefore, this synthetic strategy may be useful for the preparation of more complex GPI-anchored peptides, glycopeptides, proteins, and glycoproteins.

Introduction

Many surface proteins and glycoproteins are associated with the cell membrane through glycosylphosphatidylinositol (GPI) anchoring.[1−3] GPI-anchored proteins and glycoproteins play a pivotal role in numerous biological events.[4−8] For example, the human CD52 and CD24 antigens (Figure 1) are representative GPI-anchored glycopeptide/glycoprotein antigens.[9−11] The CD52 antigen is ubiquitously expressed by human lymphocyte and sperm cells[9,10] and has been demonstrated to play an important role in the human immune system and the human reproductive process. The CD24 antigen is expressed by T cells[12] and neurons[13,14] and overexpressed on a number of carcinoma cell lines.[15,16] To study the functions of GPI-anchored proteins and glycoproteins at the molecular level, it is necessary to have access to these molecules and their derivatives in homogeneous forms and sufficient quantities, which is currently difficult to achieve by either biological or chemical means. Therefore, a feasible method for synthesizing GPI-linked peptides/proteins and glycopeptides/glycoproteins is highly desirable.[17−21]

Every naturally GPI-anchored protein or glycoprotein has its polypeptide C-terminus linked to the phosphoethanolamine group at the nonreducing end of the GPI core glycan (Figure 1). Accordingly, a general method for the preparation of various natively linked GPI−protein/glycoprotein conjugates would be feasible once a reaction for site-specific attachment of peptides/proteins to the phosphoethanolamine group of GPIs is established. For this purpose, an enzyme-catalyzed ligation method is particularly appealing, not only because enzymatic reactions are usually highly specific and efficient but also because enzymes are especially suitable for complex structures such as large proteins and intricate GPIs. For this purpose, we became interested in sortases, a class of transpeptidases found in Gram-positive bacteria.[22]

Results and Discussion

Sortases catalyze transpeptidation reactions on the bacterial cell wall, known as the "cell-wall sorting reactions", which anchor surface proteins onto bacterial cell walls.[22] The reaction mechanisms of sortase-catalyzed transpeptidation have been established.[23] For instance, *Staphylococcus aureus* sortase A

(1) Ferguson, M. A. J.; Williams, A. F. *Annu. Rev. Biochem.* **1988**, *57*, 285–320.
(2) Englund, P. T. *Annu. Rev. Biochem.* **1993**, *62*, 121–138.
(3) Hwa, K. *Adv. Exp. Med. Biol.* **2001**, *491*, 207–214.
(4) Robinson, P. J. *Cell Biol. Int. Rep.* **1991**, *15*, 761–767.
(5) Ferguson, M. A. J. *Parasitol. Today* **1994**, *10*, 48–52.
(6) Gaulton, G. N.; Pratt, J. C. *Semin. Immunol.* **1994**, *6*, 97–104.
(7) Kasahara, K.; Sanai, Y. *Glycoconjugate J.* **2000**, *17*, 153–162.
(8) Loertscher, R.; Lavery, P. *Transplant Immunol.* **2002**, *9*, 93–96.
(9) Treumann, A.; Lifely, M. R.; Schneider, P.; Ferguson, M. A. J. *J. Biol. Chem.* **1995**, *270*, 6088–6099.
(10) Schröter, S.; Derr, P.; Conradt, H. S.; Nimtz, M.; Hale, G.; Kirchhoff, C. *J. Biol. Chem.* **1999**, *274*, 29862–29873.
(11) Kay, R.; Rosten., P. M.; Humphries, R. K. *J. Immunol.* **1991**, *147*, 1412–1416.
(12) Williams, L. A.; Hock, B. D.; Hart, D. N. J. *Blood* **1998**, *88*, 3048–3055.
(13) Kadmon, G.; Kowitz, A.; Altevogt, P.; Schachner, M. *J. Cell Biol.* **1992**, *110*, 209–218.
(14) Rougon, G.; Alterman, L. A.; Dennis, K.; Guo, X. J.; Kinnon, C. *Eur. J. Immunol.* **1991**, *21*, 1397–1402.
(15) Jackson, D.; Waibel, R.; Weber, E.; Bell, J.; Stahel, R. A. *Cancer Res.* **1992**, *52*, 5264–5270.
(16) Lim, S.; Oh, S. *Pathol. Res. Pract.* **2005**, *201*, 479–486.
(17) Tanaka, Y.; Nakahara, Y.; Hojo, H.; Nakahara, Y. *Tetrahedron* **2003**, *59*, 4059–4067.
(18) Xue, J.; Shao, N.; Guo, Z. *J. Org. Chem.* **2003**, *68*, 4020–4029.
(19) Shao, N.; Xue, J.; Guo, Z. *Angew. Chem., Int. Ed.* **2004**, *43*, 1569–1573.
(20) Paulick, M. G.; Wise, A. R.; Forstner, M. B.; Groves, J. T.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2007**, *129*, 11543–11550.
(21) Becker, C. F. W.; Liu, X.; Olschewski, D.; Castelli, R.; Seidel, R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2008**, *47*, 8215–8219.
(22) Marraffini, L. A.; DeDent, A. C.; Schneewind, O. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 192–221.

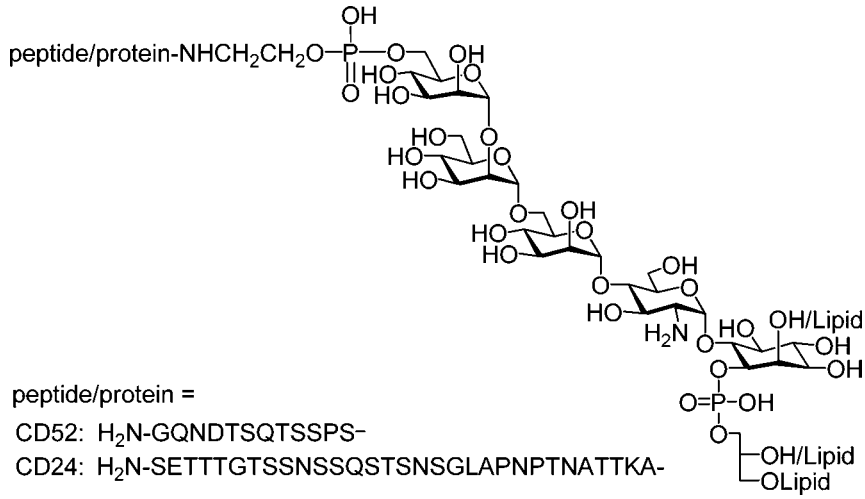


Figure 1. Generic structure of the human CD52 and CD24 antigens.

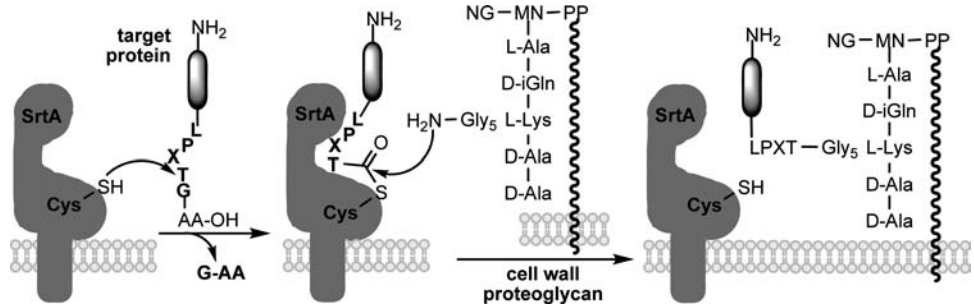
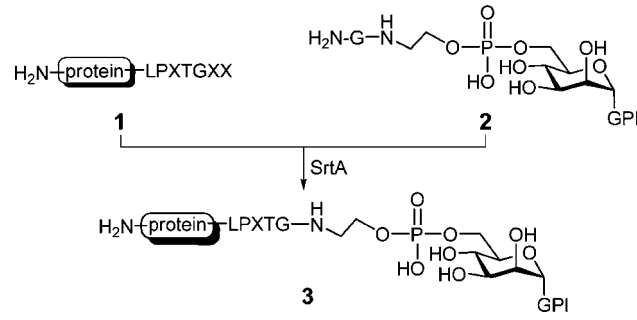


Figure 2. Anchorage of surface proteins to cell-wall proteoglycans by SrtA in *S. aureus*. SrtA recognizes LPXTG near the C-terminus of a target protein. The Cys^{184} of SrtA performs a nucleophilic attack at the peptide bond between T and G in LPXTG, resulting in a thioester intermediate with the carboxyl group of the C-terminal T linked to Cys^{184}. This reactive intermediate reacts with the cross-bridge N-terminus of a cell-wall proteoglycan to anchor the target protein to the cell-wall peptidoglycan.

(SrtA) was found to recognize a pentapeptide LPXTG (known as the sorting signal) near the substrate protein C-terminus, break the peptide bond between T and G in the sorting signal to form a reactive SrtA−substrate protein complex, and then link the substrate protein to cell-wall peptidoglycans (Figure 2).[22] Specifically, the thiol group of Cys^{184} at the active center of SrtA reacts with LPXTG to form a thioester,[24] which then transfers the acyl group to the N-terminus of the cross-bridge of the cell-wall proteoglycan.[23]

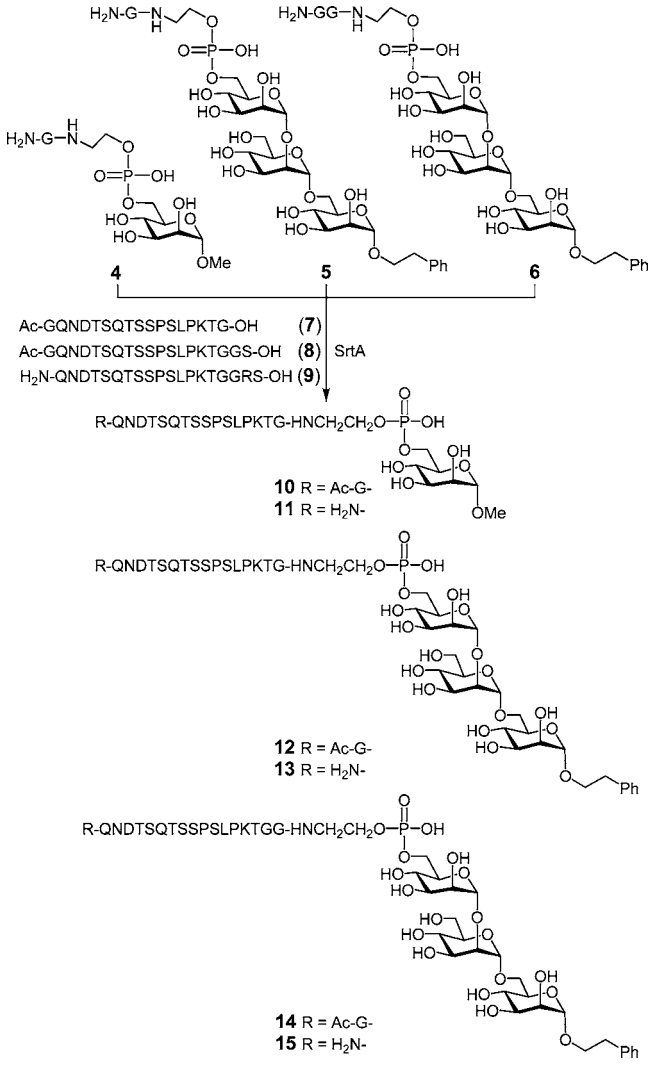
SrtA has been shown to accept various peptide/protein substrates, so long as they bear the sorting signal LPXTG, and a range of amino nucleophiles. SrtA has thus been utilized as a powerful tool[25,26] for ligating peptides and proteins;[27−30] linking peptides/proteins to nucleic acids,[31] lipids,[32] and carbohydrates;[33] and labeling proteins on living cell surfaces.[34−36] On the basis of these findings, we envisioned that SrtA might be used to couple proteins and glycoproteins to GPIs for the synthesis of GPI conjugates, as outlined in Scheme 1, provided that the substrate protein **1** contains a sorting signal at its C-terminus and that GPI **2** has a Gly residue linked to the

Scheme 1. Synthesis of GPI-Anchored Proteins via SrtA-Catalyzed Transpeptidation of GPIs



(23) Huang, X.; Aulabaugh, A.; Ding, W.; Kapoor, B.; Alksne, L.; Tabei, K.; Ellestad, G. *Biochemistry* **2003**, *42*, 11307–11315.
(24) Ton-That, H.; Mazmanian, S. K.; Alksne, L.; Schneewind, O. *J. Biol. Chem.* **2002**, *277*, 7447–7452.
(25) Budisa, N. *ChemBioChem* **2004**, *5*, 1176–1179.
(26) Tsukiji, S.; Nagamune, T. *ChemBioChem* **2009**, *10*, 787–798.
(27) Mao, H.; Hart, S. A.; Schink, A.; Pollok, B. A. *J. Am. Chem. Soc.* **2004**, *126*, 2670–2671.
(28) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjugate Chem.* **2007**, *18*, 469–476.
(29) Pritz, S.; Kraetke, O.; Klose, A.; Klose, J.; Rothemund, S.; Fechner, K.; Bienert, M.; Beyermann, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 3642–3645.
(30) Kobashigawa, Y.; Kumeta, H.; Ogura, K.; Inagaki, F. *J. Biomol. NMR* **2009**, *43*, 145–150.
(31) Pritz, S.; Wolf, Y.; Kraetke, O.; Klose, J.; Bienert, M.; Beyermann, M. *J. Org. Chem.* **2007**, *72*, 3909–3912.
(32) Antos, J. M.; Miller, G. M.; Grotenbreg, G. M.; Ploegh, H. L. *J. Am. Chem. Soc.* **2008**, *130*, 16338–16343.
(33) Samantaray, S.; Marathe, U.; Dasgupta, S.; Nandicoori, V. K.; Roy, R. P. *J. Am. Chem. Soc.* **2008**, *130*, 2132–2133.
(34) Popp, M. W.; Antos, J. M.; Grotenbreg, G. M.; Spooner, E.; Ploegh, H. L. *Nat. Chem. Biol.* **2007**, *3*, 707–708.
(35) Tanaka, T.; Yamamoto, T.; Tsukiji, S.; Nagamune, T. *ChemBioChem* **2008**, *9*, 802–807.
(36) Yamamoto, T.; Nagamune, T. *Chem. Commun.* **2009**, 1022–1023.

Scheme 2. SrtA-Mediated Chemoenzymatic Synthesis of CD52 Analogues

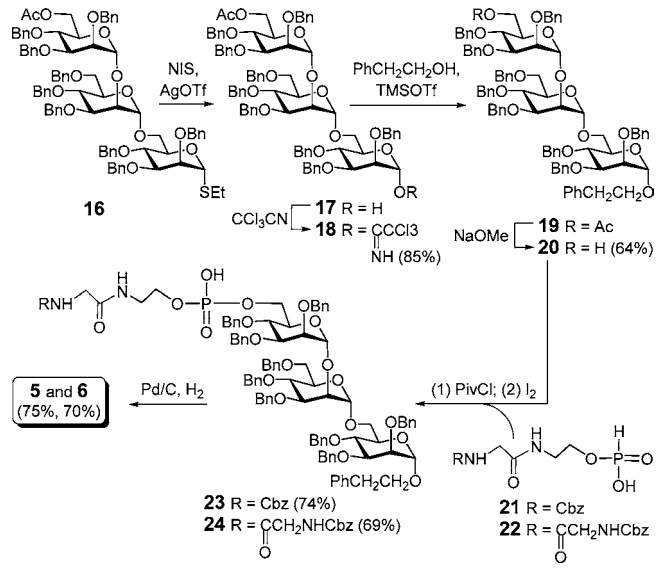


phosphoethanolamine moiety. It is worth noting that the resulting GPI-anchored protein/glycoprotein **3** would differ slightly from the natural products as a result of the insertion of a pentapeptide sequence between the GPI and the native protein. How this minor structural change may affect the biological functions of GPI-anchored proteins and glycoproteins is an interesting question to study. We also expected this synthetic method to be applicable as well to the coupling of GPIs to proteins and glycoproteins that are not GPI-anchored in nature. These non-natural products would be useful for various biological studies and the investigation of GPI-directed targeting of molecules to specific cell or tissue types.[37,38]

The above synthetic strategy was explored in the preparation of analogues of the human CD52 and CD24 antigens. Initially, our investigation was focused on the coupling reaction of GPI analogues **4**, **5**, and **6** with CD52 peptides **7**, **8**, and **9** (Scheme 2). Our previous studies revealed that SrtA accepts **4** as a nucleophilic peptide acceptor,[39] though only a monosaccharide GPI analogue and a very short peptide were involved therein.

(37) Ilangumaran, S.; He, H.-T.; Hoessli, D. C. *Immunol. Today* **2000**, *21*, 2–7.
(38) Trotter, J.; Klein, C.; Kramer, E. *Neuroscientist* **2000**, *6*, 271–284.
(39) Guo, X.; Wang, Q.; Swarts, B. M.; Guo, Z. *J. Am. Chem. Soc.* **2009**, *131*, 9878–9879.

Scheme 3. Synthesis of GPI Analogues **5** and **6**



Nevertheless, **4** was used as a positive control to evaluate SrtA-catalyzed ligation of more complex structures. GPI analogues **5** and **6** containing the GPI core trisaccharide are excellent mimics of natural GPI anchors. Moreover, because **5** and **6** have one and two Gly residues, respectively, linked to the phosphoethanolamine group at the carbohydrate nonreducing end, comparison of these analogues would be able to demonstrate whether the number of Gly residues in the GPI substrate has a major impact on its reactivity as a peptide acceptor. Peptide **7** differs from peptides **8** and **9** mainly in regard to the position of the sorting signal: the sorting signal in **7** is exposed at the C-terminus, while it is only close to the C-terminus in **8** and **9**. According to Pritz,[31] the C-terminal sequence may affect the reaction rate, efficiency, and equilibrium. The structural differences between **8** and **9** are that **8** has an acetylated N-terminus and that **9** has an additional amino acid at the C-terminus. Analysis of the reactions of peptides **7**−**9** would show how the different peptide structures might affect the SrtA-catalyzed coupling reactions between peptides and GPIs.

The peptide substrates **7**, **8**, and **9**, which contain both the CD52 peptide sequence and the sorting signal LPKTG, were prepared with an automatic peptide synthesizer employing the conventional Fmoc−amino acid chemistry. GPI analogue **4** was previously prepared in our laboratory,[39] while GPI analogues **5** and **6** were synthesized according to the procedures shown in Scheme 3. First, trisaccharide **16**[18] was converted into Schmidt glycosyl donor **18** in two steps, including oxidative hydrolysis of the anomeric center at the reducing end of **16** and trichloroacetimidation of hemiacetal **17** by trichloroacetonitrile in the presence of DBU. The reaction of **18** with 2-phenylethanol promoted by TMSOTf afforded an inseparable mixture of α and β anomers (3:1) that were separable by column chromatography after deacetylation with NaOMe/MeOH, eventually yielding **20**. The stereochemistry of **20** was confirmed by the $^1J_{C,H}$ coupling constants of the anomeric carbons. Phosphorylation of **20** was achieved by the *H*-phosphonate method using **21** and **22** as the phosphorylating reagents. The condensation reactions of **20** with **21** and **22** went smoothly in the presence of pivaloyl chloride and were followed by in situ oxidation of the resultant *H*-phosphonates using iodine. Finally, the benzyl and Cbz groups were simultaneously removed with 10% Pd/C

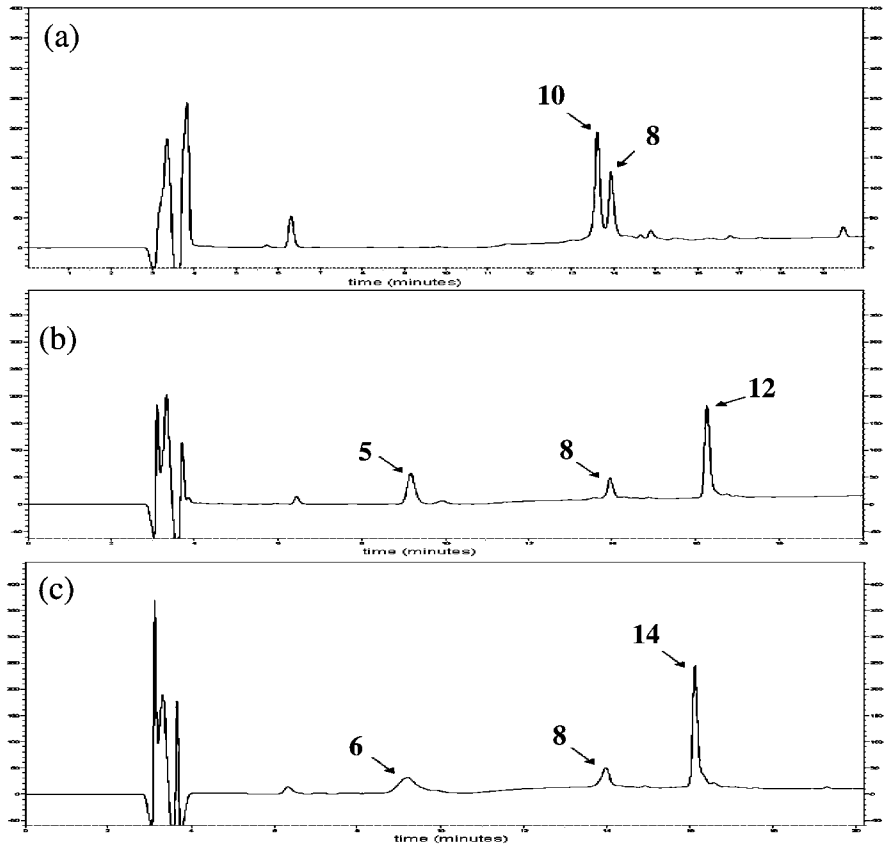


Figure 3. HPLC diagrams of SrtA-mediated coupling reactions of **8** with (a) **4**, (b) **5**, and (c) **6**. HPLC conditions: C_{18} column; gradient eluent, 10 to 60% CH_3CN/H_2O; monitoring at 220 nm.

under a hydrogen atmosphere to give **5** and **6**, respectively, which were ready for enzymatic ligation with peptides.

The enzymatic reactions outlined in Scheme 2 were performed in 0.3 M Tris-HCl buffer (pH ~7.5) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.2 mM mercaptoethanol. The peptide, GPI, and SrtA concentrations were set at 0.5 mM, 2.5 mM, and 12.6 μM, respectively. The reaction mixtures were incubated at 37 °C, and the reaction progress was monitored by HPLC and MALDI−TOF mass spectrometry (MS). After 24 h of incubation, the reactions were quenched with 0.1% trifluoroacetic acid (TFA), and the reaction yields were eventually determined using HPLC. It was observed that the reactions of **7** with **4** and **5** were very slow, and after 24 h of incubation, the corresponding desired products **10** and **12** were formed in only ~10% yield while the majority of **7** was unaffected. These results suggested that **7** was probably not a good SrtA substrate. On the contrary, the reactions of **8** with **4** and **5** were very fast, offering **10** and **12** in 61 and 78% yield, respectively (Figure 3). Under the same conditions, the reaction of **8** and **6**, which contained two Gly residues, was also fast and gave conjugate product **14** in an 82% yield. Furthermore, the reactions of **9** with **4**, **5**, and **6** were similar to the reactions of **8**, affording **11**, **13**, and **15** in ~65, 81, and 78% yield, respectively. Large-scale reactions of **8** with **4** and **5** were also performed. In this case, the ligation products **10** and **12** were purified by HPLC to obtain the samples in milligram quantities that were sufficient to perform NMR characterization in addition to MS analysis.

These studies revealed that SrtA accepts both monosaccharide and trisaccharide GPI analogues as the peptide acceptor in transpeptidation reactions, and more interestingly, trisaccharide analogues **5** and **6** seemed to be better substrates than the monosaccharide GPI analogue **4**. The results suggest that SrtA may accept more complex GPI analogues. In addition, **5** and **6**, which contain one and two Gly residues, respectively, were similar as peptide acceptors of the SrtA-catalyzed transpeptidation reactions. These studies further demonstrated that attaching the sorting signal directly to the peptidic C-terminus, such as in the case of **7**, does not give the best SrtA substrate. For efficient recognition by and reaction with SrtA, the sorting signal should be close to the C-terminus but not exposed. The observation that peptide substrates with either a dipeptide (such as KS[39] or GS) or a tripeptide (GRS) at the C-terminus gave almost identical results suggests that beyond the sorting signal, SrtA may require only a dipeptide to be present at the C-terminus and that the structure of this dipeptide can be variable.

The reactions of **8** with **4** and **5** were analyzed in detail to further understand the reactivity of SrtA. In these studies, the two reactions were carried out under the same conditions, and reaction aliquots were obtained at various time points, quenched with 0.1% TFA, and then analyzed by HPLC. As shown in Figure 4, both reactions were fast and reached equilibrium within 6 h. Evidently, the reaction of **5** was faster than that of **4**. After 30 min of incubation, **5** gave 60% conversion, as opposed to only 30% for **4**. To further examine the kinetics of these reactions, we derived the K_m and V_{max} values of **4** and **5** by fitting their initial reaction rates obtained at various concentrations (0.625, 1.25, 2.5, 5.0, 10.0 mM) [with fixed concentrations of **8** (0.5 mM) and SrtA (12.6 μM)] to the Michaelis−Menten equation using Lineweaver−Burk plots. The V_{max} values of **4** and **5** were 8.4612 and 12.9761 μM/sec, respectively, and the corresponding K_m values were 2.0928 and 0.8784 μM. These results clearly indicate that the reaction of **5** is faster and more

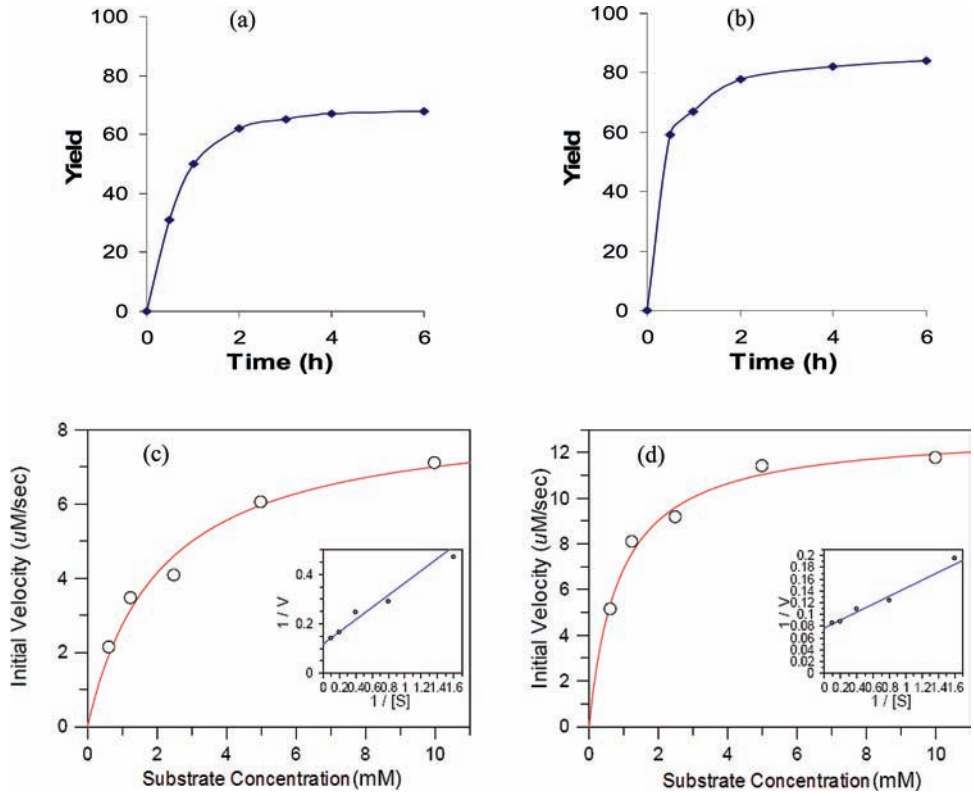
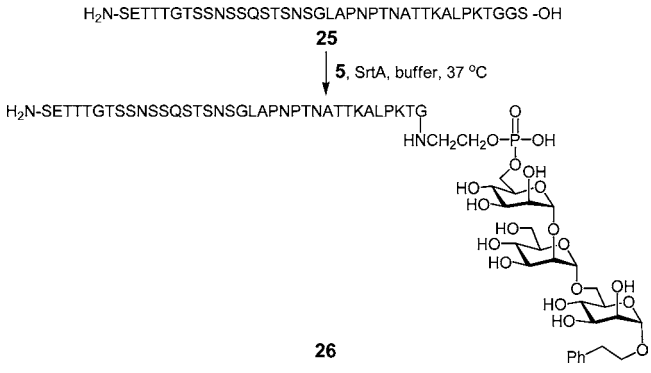


Figure 4. Kinetics analysis of SrtA-catalyzed reactions of **8** with **4** and **5**. (a) Results of the reaction between **4** (2.5 mM) and **8** (0.5 mM). (b) Results of reaction between **5** (2.5 mM) and **8** (0.5 mM). (c) Experiments to determine K_m and V_{max} for **4** at a fixed concentration of **8** (0.5 mM). (d) Experiments to determine K_m and V_{max} for **5** at a fixed concentration of **8** (0.5 mM).

Scheme 4. SrtA-Mediated Chemoenzymatic Synthesis of CD24 Analogue **26**



efficient than that of **4**. Therefore, the increased steric hindrance of trisaccharide **5** in comparison to **4** did not seem to have any impact on its reactivity with the acyl−enzyme complex. On the contrary, **5** and **6** may fit the catalytic site of SrtA better than **4**. To prove this postulation and address its rationale, additional structure−reactivity relationship studies are necessary. Nevertheless, these observations highlight the potential of accomplishing site-specific ligation between peptides and more complex GPI analogues via SrtA-catalyzed transpeptidation.

To further investigate whether the chemoenzymatic synthesis strategy is applicable to more complex peptides and proteins, we then used solid-phase peptide synthesis to prepare a small protein **25** containing the full sequence of the CD24 antigen and the sorting signal and evaluated its conjugation with GPI analogue **5** in the presence of SrtA (Scheme 4). The coupling reaction was carried out under the same conditions as described above, while the reaction progress was monitored by MALDI−TOF MS and HPLC. We found that the reaction afforded the desired GPI−protein conjugate **26** [MALDI−TOF MS (*m*/*z*): calcd for $[M + H]^+$, 4210.2; found, 4210.6] in 72.9% yield according to HPLC assays. This result demonstrates the potential of SrtA to be used for the synthesis of more complex GPI-anchored peptides/proteins.

Conclusion

In summary, the synthesis of several analogues of the human CD52 and CD24 antigens through SrtA-catalyzed ligation between peptides and GPI analogues has been described. It was observed that peptides with different lengths and sequences had very similar reactivities. On the other hand, the more sterically demanding trisaccharide GPI analogues **5** and **6** were better SrtA substrates than the monosaccharide GPI analogue **4**. On the basis of the present results, we are optimistic that the strategy may be applicable to more complex peptides and GPIs and that it may be developed into a general and practical method for the synthesis of GPI-anchored peptides, glycopeptides, proteins, and glycoproteins. The ligation of intact GPIs and full-size proteins/glycoproteins is now under investigation.

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Supporting Information Available: Experimental details for the preparation of SrtA and compounds **5**−**9** and **25**; conditions and protocols for the enzymatic reactions of **4**−**6** with **7**−**9** and of **5** with **25** to prepare **10**−**15** and **26**, respectively; HPLC results for the enzymatic reactions; and MS and NMR spectra of all intermediates and products. This material is available free of charge via the Internet at http://pubs.acs.org.

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