

Peptide–Sugar Ligation Catalyzed by Transpeptidase Sortase: A Facile Approach to Neoglycoconjugate Synthesis

Sharmishtha Samantaray, Uttara Marathe, Sayani Dasgupta, Vinay K. Nandicoori, and Rajendra P. Roy*

National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110 067, India

Received September 23, 2007; E-mail: rproy@nii.res.in

Development of new methods for linking sugars to peptides or proteins is an active area of research because natural glycopeptides or neoglycoconjugates play important roles in biology and medicine and are indispensable tools for probing several biological processes.^{1–4} However, despite dramatic progress in synthetic carbohydrate and protein chemistry in recent years, glycoconjugate synthesis involving sugar and polypeptide remains a formidable task. This is principally because synthetic protocols are quite demanding and involve multiple reaction steps with requirements of rather extensive protection of reactive functionalities. The problem may be in part or completely obviated through the intermediary of enzymes. Indeed, glycosidases and glycosyl transferases, in appropriate situations, have made the synthesis of oligosaccharides much simpler.^{5–7} Given the current ease with which peptides are assembled by solid phase methodology and proteins obtained from expression systems, the availability of enzymes capable of covalently linking a presynthesized sugar and a polypeptide would greatly facilitate the convergent semisynthesis of glycoconjugates with exquisite biological properties. We report here a novel enzymatic approach, using an unprecedented sortase-catalyzed transamidation reaction, for the facile one-pot synthesis of glycoconjugates comprising amino sugars and native polypeptides.

The transpeptidase sortase, present in the cell envelope of most gram-positive bacteria, catalyzes the covalent anchoring of several bacterial surface proteins to the peptidoglycan cross-bridges of the cell wall.^{8,9} Sortase A of *Staphylococcus aureus* recognizes a LPXTG pentapeptide sequence motif located near the C-terminus of the target proteins, cleaves at Thr–Gly peptide bond, and catalyzes the formation of a new peptide bond between the threonyl carboxyl and amino group of the peptidoglycan pentaglycine cross-bridges.¹⁰ The transpeptidation reaction proceeds in two steps without the aid of any extraneous molecule; the active site cysteine residue first attacks the target LPXTG substrate forming an acyl–enzyme intermediate which in the second step is resolved by the nucleophilic attack of the amino group of the terminal Gly residue of the peptidoglycan. In the absence of a suitable amino nucleophile, the LPXTG peptide substrate is slowly hydrolyzed. Sortase-mediated transpeptidation reaction involving LPXTG and aminoglycine containing polypeptides proceeds smoothly in vitro and has been applied to the synthesis of protein–peptide and peptide–nucleic acid conjugates that would have been difficult to obtain by purely chemical or genetic means.^{11–13} Interestingly, ligation of LPXTG substrates can occur even with polypeptides containing a single Gly residue at the amino terminus. This observation of relaxed specificity for the amine nucleophile and the fact that the transpeptidation reaction does not require intermediary of high-energy phosphates prompted us to explore sortase-mediated ligation of polypeptides to amino sugars with a view to develop an enzymatic approach to glycoconjugate synthesis.

We considered 6-aminoheptoses as potential sugar substrates with the idea that the $-\text{CH}_2-\text{NH}_2$ moiety present in these sugars might mimic some elements of the glycine structure. Accordingly, we tested the potential of sortase to ligate 6-deoxy-6-aminoglucose and 6-deoxy-6-aminomannose to a model YALPETGK peptide substrate. HPLC assays followed by MALDI-TOF or ESMS analyses revealed the formation of respective YALPET–sugar adducts, suggesting that the above amino sugars indeed acted as nucleophiles in the transamidation reaction (Supporting Information Figure 1). In contrast, the substrate peptide was hydrolyzed to YALPET without the formation of the YALPET–sugar adduct when glucosamine was used as a substrate. Consistent with the known tolerance of sortase for the LPXTG recognition motif,¹⁴ the YALPMTGK peptide sequence also reacted with 6-deoxy-6-aminoglucose or 6-deoxy-6-aminomannose but not with glucosamine.

To further probe the specificity requirements as well as to see if 6-aminoheptoses can serve as recognition tags for peptide–sugar ligations, we investigated the ability of sortase to ligate peptides to an aminoglycoside class of therapeutically important antibiotics. These antibiotics are built up by a variety of amino sugars of the 6-amino or the 2,6-diamino type besides containing several other amino functionalities¹⁵ (Figure 1). The central scaffold of aminoglycoside antibiotics is the 2-deoxystreptamine ring to which amino sugars are substituted at positions 4 and 6 (as in tobramycin and kanamycins) or 4 and 5 (as in ribostamycin, neomycin, and paromomycin). Sortase-mediated ligation of model peptide substrates to aminoglycoside antibiotics proceeded smoothly. Analyses of the reaction products by reversed phase HPLC (Figure 2) followed by MALDI (Supporting Information Table 2) revealed the formation of specific conjugates between antibiotics and peptides in the yields varying from 35 to 70% for the kanamycin class, and about 18–30% for the ribostamycin class of antibiotics. Electrospray mass spectrometry (Supporting Information Figures 2–5) of the respective conjugates produced fragmentations that unambiguously showed occurrence of peptide ligation exclusively at a single 6-amino site in ring A of kanamycins, tobramycin, and ribostamycin or ring D of paromomycin and neomycin. Thus, conjugation of peptide substrates was limited to the 6-amino site in the antibiotics despite the presence of a plethora of amino groups, indicating rather strict specificity and selectivity for the sugar amino groups by sortase.

Next, we explored conjugation of biologically relevant peptides to aminoglycoside antibiotics. Toward this, we considered peptide sequences derived from or based on Tat and Rev proteins of HIV because these proteins play important roles in virus replication through their interactions with structured viral RNA target sites, TAR in the case of Tat and RRE in the case of Rev.¹⁶ Interestingly, aminoglycoside antibiotics and analogues,¹⁷ as well as short

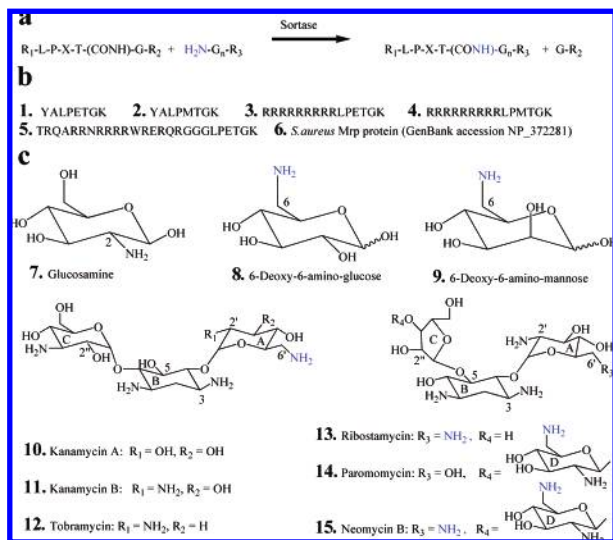


Figure 1. (a) General peptide ligation reaction catalyzed by sortase. (b) LPXTG peptide substrates used in the study. (c) Amino sugars used in the study. The 6-amino site is shown in blue.

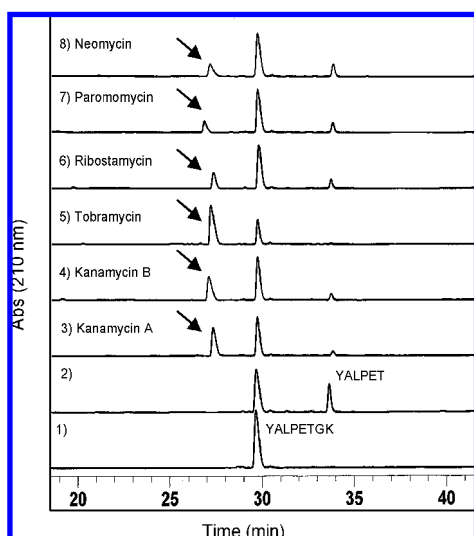


Figure 2. Sortase-catalyzed conjugation of YALPETGK model peptide to aminoglycosides. Reversed phase HPLC profiles 1 and 2 are peptide samples in the absence and presence of sortase, respectively. Chromatograms numbered 3 to 8 represent reactions of peptide with aminoglycoside antibiotics. The peptide–antibiotics conjugate is indicated by an arrow.

arginine-rich sequences derived from Tat and Rev^{17,18} or even a nonaarginine peptide mimic, have been shown to interfere with Tat–TAR or Rev–RRE interactions, leading to inhibition of virus replication.¹⁹ We prepared conjugates of nonaarginine (4) or a Rev sequence (5) with several antibiotics (Supporting Information Table 2 and Figure 6). We used the neomycin–Rev conjugate, as a test case, for evaluating the extent of RRE RNA binding. The gel retardation assays (Supporting Information Figure 7) yielded a RRE binding affinity (K_d) of 9.3 nM for the conjugate as compared to 114.3 nM for the Rev peptide, suggesting about 10-fold or more improvement of RNA binding in the conjugate. Together, these results demonstrate the utility of sortase for generating useful conjugates.

Finally, we tested the feasibility of using sortase for site-specific conjugation of sugars to proteins. For this, we expressed a protein

(Mrp protein, NP_372281) from *Staphylococcus aureus* nested with a LPNTG sequence motif in its carboxy terminal region. We employed HPLC assays and MALDI-TOF to investigate the ligation of tobramycin to Mrp (Supporting Information Figures 8 and 9). Incubation of Mrp alone with sortase led to generation of two fragments expected from hydrolysis at the T–G peptide bond. However, incubation of the protein with sortase in the presence of tobramycin produced specific conjugate (Mrp–LPNT–tobramycin) with a yield of more than 45% in 6 h.

In summary, our work demonstrates that sortase can transfer peptide substrates to oligosaccharides appended with a 6-deoxy-6-aminohexose moiety in a selective manner as that of an oligoglycine sequence. Such an enzymatic activity of peptide–sugar ligation, presumably promiscuous in origin for sortase, is hitherto unknown. This robust reaction provides a simple and straightforward method for covalent ligation of a prefabricated sugar containing a 6-aminohexose tag to synthetic peptides and expressed proteins encoded with a C-terminal LPXTG sortase recognition sequence. We envision several biotechnological applications of this methodology, including generation of novel glycopeptide-based immunovaccines comprising oligosaccharides substituted with multiple peptides and glycolabeling of proteins. Besides, the facile assembly of aminoglycoside antibiotic conjugates offers tremendous possibilities of generating new RNA ligands and therapeutics. The work on anti-HIV activity of antibiotics–Tat/Rev conjugates is in progress.

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Supporting Information Available: Peptide–sugar ligations, product characterization, and RNA binding data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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