Chemical Protein Synthesis Hot Paper

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Total Chemical Synthesis of the Enzyme Sortase $A_{\Delta N59}$ with Full Catalytic Activity**

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Abstract: *The enzyme sortase A is a ligase which catalyzes transpeptidation reactions.[1,2] Surface proteins, including virulence factors, that have a C terminal recognition sequence are attached to Gly_5 on the peptidoglycan of bacterial cell walls by sortase A.[1] The enzyme is an important anti-virulence and anti-infective drug target for resistant strains of Gram-positive bacteria.[2] In addition, because sortase A enables the splicing of polypeptide chains, the transpeptidation reaction catalyzed by sortase A is a potentially valuable tool for protein science.[3] Here we describe the total chemical synthesis of enzymatically active sortase A. The target 148 residue polypeptide chain of sortase $A_{\Delta N59}$ was synthesized by the convergent chemical ligation of four unprotected synthetic peptide segments. The folded protein molecule was isolated by size-exclusion chromatography and had full enzymatic activity in a transpeptidation assay. Total synthesis of sortase A will enable more sophisticated engineering of this important enzyme molecule.*

The sortase A mature polypeptide chain contains 206 amino acids.[4] The N-terminal sequence from amino acids 1 to 59 contains a transmembrane domain and is not required for enzyme catalytic activity: sortase $A_{\Delta N59}$ that was truncated by removal of amino acid residues 1 to 59 and that was prepared by recombinant expression showed full enzymatic activities.[5] The recombinant enzyme protein sortase $A_{\Delta N59}$ has been crystallized and its structure has been determined by X-ray diffraction.[6] In the course of enzyme-catalyzed transpeptidation, the side-chain thiol functionality of the single Cys (residue 184) forms a thioester-linked enzyme–peptide acyl-enzyme intermediate. This active-site Cys has been mutated to Ala and the co-crystal structure of the resulting inactive enzyme with a peptide substrate has been determined.[6] Based on that structure and on the results of site-directed mutagenesis studies, a chemical mechanism has been proposed for the sortase A catalyzed transpeptidation reaction.[1,2] Because chemical synthesis can in principle provide complete control over the covalent structure of a protein molecule, an effective total synthesis of sortase A would be a uniquely useful tool to test and refine the proposed transpeptidation mechanism.[7]

The amino acid sequence of the sortase $A_{\Delta N59}$ catalytic domain, from residue 60 to 206, is shown in Figure 1. For convenience of comparison with the literature, we retain the original numbering in describing the polypeptide chain of sortase $A_{\Delta N59}$. The N-terminal residue 60 is a glutamine and is not stable; when exposed to even mildly acid pH, the $^{\alpha}NH_2$ will react with the side chain carboxamide to form a pyrrolidonecarboxylic acid residue.[8] For that reason, a norleucine residue was added to the N-terminal of the target polypeptide as an isosteric replacement for the N-terminal methionine used in the recombinantly expressed enzyme.[9]

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          UQ     61 AKPQIPKDKS   71 KVAGYIEIPD   81 ADIKEPVYPG
 91 PATPEQLNRG   101 VSFAEENESL  111 DDQNISIAGH  121 TFIDRPNYQF
131 TNLKAAKKGS   141 UVYFKVGNET  151 RKYKUTSIRD  161 VKPTDVGVLA
171 EQKGKDKQLT   181 LITCDDYNEK  191 TGVWEKRKIF  201 VATEVK
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Figure 1. Amino acid sequence of synthetic Nle^{59}-[D170A] sortase $A_{\Delta N59}$. Ligation sites are underlined. U represents norleucine. Adapted from Uniprot ID Q9S446.

We set out to make the target 148 residue polypeptide chain of sortase $A_{\Delta N59}$ in a fully convergent fashion by the chemical ligation of four unprotected synthetic peptide segments (Scheme 1). Thioester-mediated, amide-forming chemical ligation takes place at Xaa-Cys sites by reaction of a peptide-thioester with a Cys peptide.[10] The lone cysteine residue in the sortase $A_{\Delta N59}$ polypeptide chain is not at a suitable position for use as a ligation site. Instead, we made use of Xaa-Ala ligation sites at Ala^{104}, Ala^{135}, and Ala^{170}; after ligation, the Xaa-Cys sequences are desulfurized to give the native Ala residues.[11] Replacement of Asp^{170} by alanine does not affect the catalytic activity of sortase A.[12] All methionines in the native sequence of sortase $A_{\Delta N59}$ were replaced by norleucines in order to avoid potential complications in post-ligation desulfurization.[9,11] Finally, the side chain thiol moiety of the catalytically essential Cys^{184} must be protected during the desulfurization of the full-length synthetic polypeptide chain.

Our synthetic strategy was based on the convergent chemical ligation of four peptide segments of similar size: Nle^{59}-103-thioester **1** (45 aa), Cys^{104}-134-thioester **2** (31 aa), Thz^{135}-169-thioester **3** (35 aa), and Cys^{170}-206-OH **4** (37 aa). The peptide segments were prepared by manual "in situ neutralization" Boc (*tert*-butoxycarbonyl) chemistry solid phase peptide synthesis (SPPS),[13] and were purified by reverse-phase HPLC and characterized by analytical HPLC–electrospray mass spectrometry (LC-MS). The left half of the

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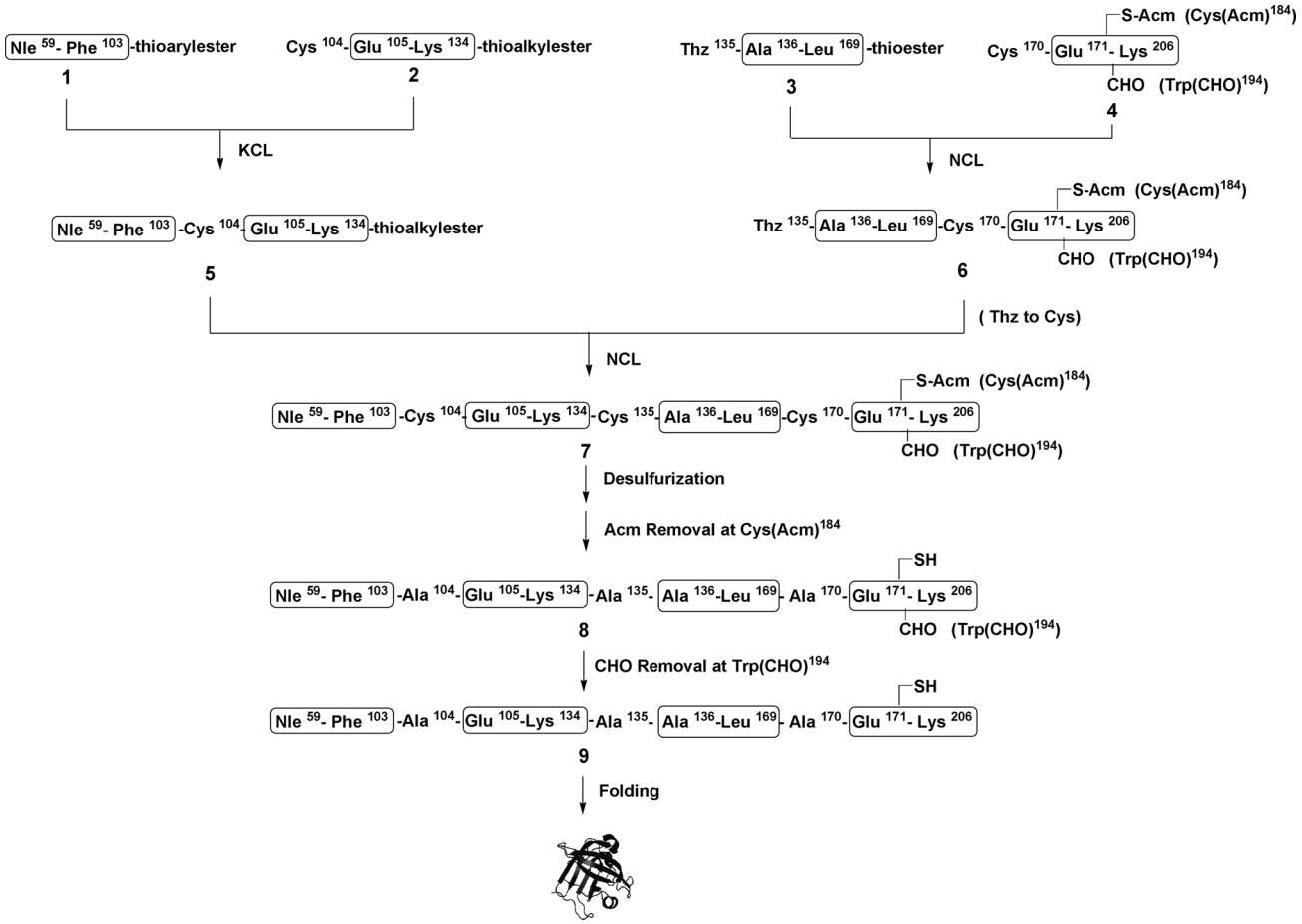

Scheme 1. Convergent synthesis of Nle[59]-[D170A] sortase $A_{\Delta N59}$. Designations: *S*-aryl, 4-mercaptophenylacetic acid thioester; Nle, norleucine; *S*-alkyl, a) 2-mercaptoethanesulfonate thioester for Cys[104]-134-*S*-alkyl **2**, b) 3-mercaptopropionic acid leucine amide for thioester for Thz[135]-169-*S*-alkyl **3**; KCL: kinetically controlled ligation; NCL, native chemical ligation; Thz, L-thiazolidine-4-carboxylic acid.

target polypeptide was prepared by the kinetically controlled ligation of a peptide-thioaryl ester with a Cys-peptide-thioalkyl ester, to give a product Nle[59]-134 peptide-thioalkyl ester **5**.[14] The right half of the target sequence was prepared by native chemical ligation (NCL) of a Thz-peptide-thioester with a Cys-peptide; after ligation, the product Thz[135]-206 peptide was converted to the desired Cys[135]-206 peptide. A final NCL step gave the full-length target 148 residue sortase $A_{\Delta N59}$ polypeptide chain (Scheme 1).

Peptide thioaryl ester Nle[59]-103-MPAA (**1**) and Cys[104]-134-thioalkyl ester **2** underwent kinetically controlled ligation at pH 6.5 in the presence of TCEP (TCEP = tris(2-carboxyethyl)phosphine.HCl)) and in the absence of added thiol catalyst (Figure S.1 in the Supporting Information).[14,15] One complication was as follows: the C-terminal of peptide Cys[104]-134-thioalkyl ester **2** is a lysine residue. Unwanted lactam formation was observed, presumably from reaction of the side-chain $^{\varepsilon}NH_2$ of Lys[134] with the C-terminal thioester moiety when the pH of the ligation reaction was above 6.5. When the reaction pH was kept near 6.5, this lactam formation side reaction was suppressed. Lactam formation at a peptide-Lys-thioester has also been reported by Brik et al.[16] Suppression of lactam formation at pH 6.5 in our hands may be due to the high (7 mM) concentrations of reacting peptide segments used, which would favor the intermolecular ligation reaction over the intramolecular lactam side reaction. The reaction was worked up by adding excess sodium 2-mercaptoethanesulfonate (MESNA), and the desired ligation product Nle[59]-134-thioalkylester **5** was isloated in 53% yield.

Peptide Thz[135]-169-thioalkylester **3** and peptide [Cys(Acm)[184],Trp(CHO)[194]]Cys[170]-206-OH (**4**; here CHO means formyl group on Trp[194]) were reacted overnight in 6 M GuHCl, 0.2 M Na_2HPO_4 at pH 7.0 in the presence of TCEP and with the aryl thiol (4-carboxymethyl)thiophenol mercaptoacetic acid (MPAA) as catalyst.[15] The ligation reaction (Figure S.2) was worked up by adding excess MESNA, and the product peptide [Cys(Acm)[184],Trp(CHO)[194]]Thz[135]-206-OH (**6**) was purified by preparative HPLC. The isolated yield was 56%. Product peptide Thz[135]-206-OH **6** was treated with $MeONH_2{\cdot}HCl$ at pH 3 for 5 h to give [Cys(Acm)[184],Trp(CHO)[194]]Cys[135]-206-OH in 70% isolated yield. Thz-to-Cys conversion was carried out on purified peptide [Cys(Acm)[184],Trp(CHO)[194]]Thz[135]-206-OH (**6**), because partial deprotection of side chain formyl group on tryptophan was observed when one-pot deprotection of Thz directly after ligation was attempted.

With the two halves of the target polypeptide chain in hand, the final native chemical ligation reaction was carried out between peptide Nle[59]-134-thioester **5** and peptide Cys[135]-206-OH. We employed careful control of the reaction at pH 6.5 in order to suppress lactam formation at the C-terminal Lys[134]-thioester and we used high concentrations of the reactant peptides and 200 mM MPAA as catalyst to facilitate the ligation reaction. The analytical data for this native chemical ligation are shown in Figure 2. Reaction

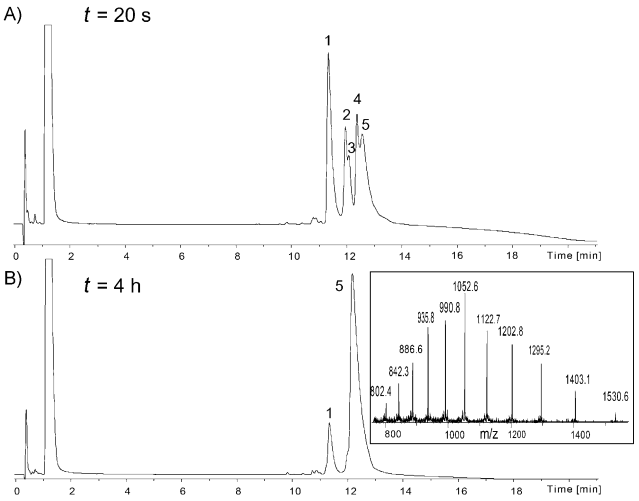

Figure 2. Final native chemical ligation of Nle[59]-134-thioester **5** and Cys[135]-206-OH. A) Analytical HPLC of ligation reaction of Nle[59]-134-*S*-alkyl thioester **5** and Cys[135]-206-OH at time 20 s. Peak 1: Cys[135]-206-OH; peak 2: Nle[59]-134-*S*-alkyl (MESNA) thioester **5**; peak 3: Nle[59]-134-thiolactone; peak 4: Nle[59]-134-MPAA thioester generated by MPAA thioester exchange; peak 5: product Nle[59]-206-OH **7**. B) HPLC trace of ligation reaction of Nle[59]-134-*S*-alkyl thioester **5** and Cys[135]-206-OH at time 4 h. The reaction was complete. Peak 1: remaining Cys[135]-206-OH; peak 5: product Nle[59]-206-OH **7**. ESI-MS of peak 5 is shown in the inset. Observed mass of Nle[59]-206-OH **7** was 16826.0 ± 1.2 Da (1.2 Da is calculated from standard deviation of multiple charge stage of mass spectra), calculated mass of Nle[59]-206-OH **7** was 16825.6 Da (average mass). Masses were obtained across the whole peak of UV signal during LC-MS analysis.

intermediates formed during the ligation reaction included Nle[59]-134-thiolactone and Nle[59]-134-MPAA thioester; because the peptide segment Cys[136]-206-OH was added in excess, those reaction intermediates were consumed and reaction was complete. The ligation reaction was worked up by adding excess MESNA and purified by preparative HPLC to give the desired full-length product polypeptide chain [Cys[104,135,170],Cys(Acm)[184],Trp(CHO)[194]]Nle[59]-206-OH (**7**) in 55% isolated yield.

To convert the three ligation-site Cys residues to native Ala residues we used TCEP-mediated radical desulfurization.[11c] First we screened thiols such as glutathione, cysteine, 2-mercaptoethanol, and *t*BuSH as hydrogen sources. Among those, *t*BuSH had an unpleasant smell, while 2-mercaptoethanol gave better results than cysteine or glutathione. Suppression of the desulfurization reaction by remnants of MPAA was observed, so residual MPAA was removed by preparative HPLC prior to the desulfurization reaction, and the reaction solution was sparged with helium to remove oxygen. The yield of [Cys(Acm)[184],Ala[104,135,170],Trp(CHO)[194]]Nle[59]-206-OH was 90% (see Figure S.3).

The side-chain thiol of Cys184 remained protected by an Acm group. We explored different conditions for the removal of the S-Acm group by silver acetate (AgOAc) in organic/water solution.[17] Our preferred conditions used 100 equivalents of AgOAc relative to peptide in AcOH/H_2O (v/v 1:1) and gave the desired [Trp(CHO)[194]]Nle[59]-206-OH **8** in 80% yield. The formyl group on Trp194 was removed by treatment with piperidine/2-mercaptoethanol in 6 M GuHCl, at 0 °C for 1 h to give the full-length target polypeptide Nle[59]-206-OH **9**.[18]

We hypothesized that both [Trp(CHO)194] sortase A and sortase A would catalyze typical transpeptidation reactions, because it had been shown that mutation of Trp194 to alanine only moderately decreased sortase A catalytic activity even though Trp194 is close to the catalytic cysteine 184.[5] For that reason, both the full-length polypeptide products [Trp(CHO)[194]]Nle[59]-206-OH (**8**) and Nle[59]-206-OH (**9**) were separately folded by dialysis against 50 mM Tris buffer, 150 mM NaCl, pH 7.5.

Size exclusion chromatography of the folded product [Trp(CHO[194]]Nle[59]-206-OH is shown in Figure 3 and analytical LCMS of both purified folded products is shown in Figure 4. After Superdex purification, the synthetic protein products sortase A and [Trp(CHO)[194]]sortase A had satisfactory purities and correct masses within experimental error.

Sortase A catalyzes transpeptidation reactions at the Thr–Gly bond in the recognition sequence Leu-Pro-Xaa-Thr-Gly (LPXTG in single letter code). Enzymatic activities of synthetic sortase A and [Trp(CHO)[194]]sortase A were evaluated by a ligation reaction between the peptide substrates AQALPETGEE.amide and GGGGGL.amide which were synthesized on our recently reported MBHA (4-methyl)-benzhydrylamine) linker on aminomethyl-resin.[19] The enzyme-catalyzed ligation reaction was carried out in 50 mM Tris buffer, 150 mM NaCl, pH 7.5, in the presence and absence

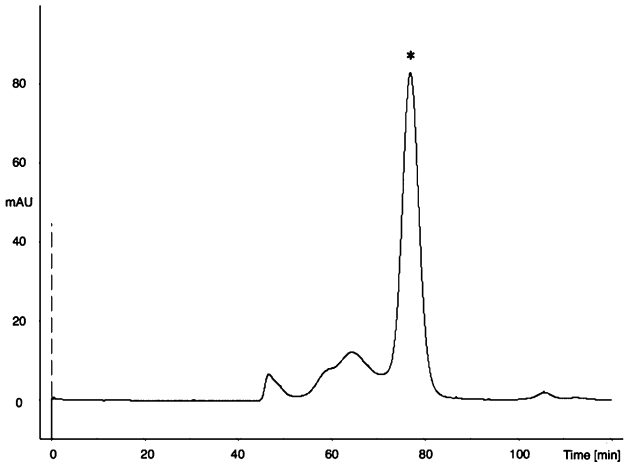

Figure 3. Size-exclusion chromatography of folded synthetic polypeptide [Trp(CHO)[194]]Nle59-206-OH (**8**) (*) on a Superdex 75-column in buffer 10 mM Tris, 100 mM NaCl, pH 7.5.

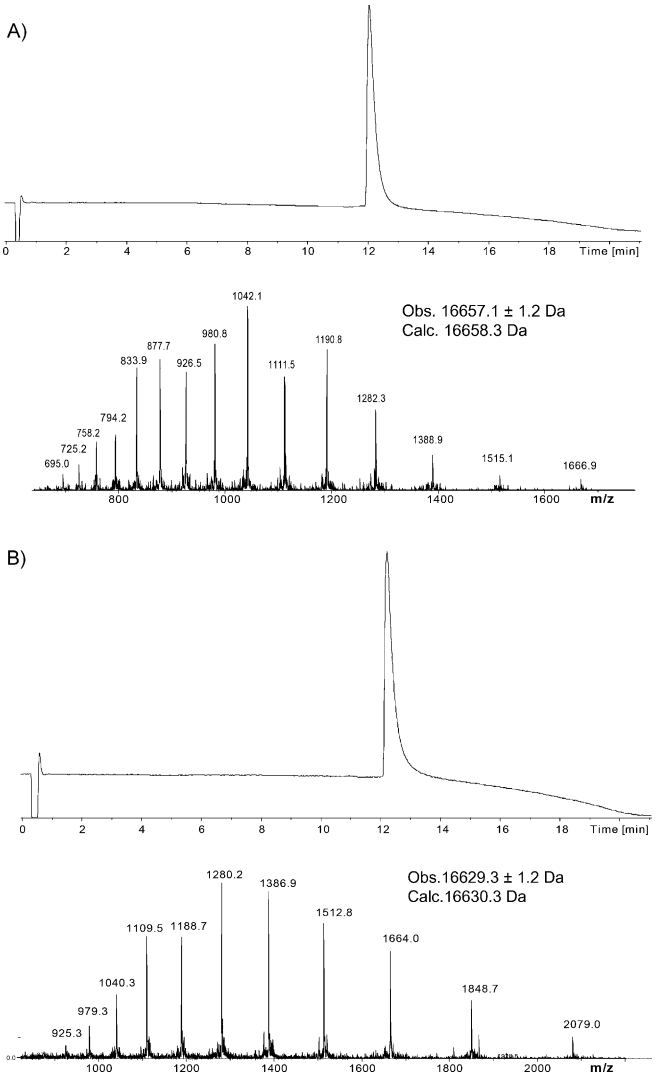

Figure 4. Characterization of synthetic [Trp(CHO)^{194}] sortase $A_{\Delta N59}$ and synthetic sortase $A_{\Delta N59}$ after folding and Superdex purification. A) LC-MS data for synthetic [Trp(CHO)^{194}] sortase A. The calculated mass was 16658.3 Da (average isotope composition) and the observed mass was 16657 ± 1.2 Da. B) LC-MS data for synthetic sortase A. The calculated mass was 16630.3 Da (average mass) and the observed mass was 16629 ± 1.2 Da. MS data were obtained across the whole peak of UV signal during LC-MS analysis and the uncertainties in masses were calculated based on standard deviation of masses of multiple charge states.

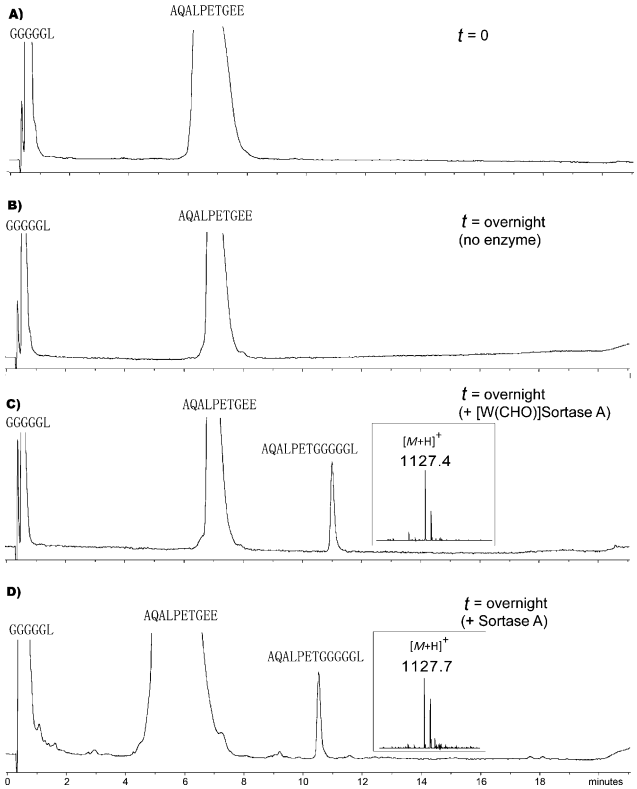

Figure 5. Transpeptidation of AQALPETGEE.amide and GGGGGL.amide catalyzed by synthetic sortase $A_{\Delta N59}$ and synthetic [Trp(CHO)^{194}] sortase $A_{\Delta N59}$ monitored by LC-MS. A) Ligation reaction at time zero. B) Control reaction: without adding enzyme, no AQALPETGGGGGL.amide product was formed. C) After adding synthetic [Trp(CHO)^{194}] sortase $A_{\Delta N59}$, the expected ligation product AQALPETGGGGGL.amide was formed after overnight reaction. D) After adding synthetic sortase $A_{\Delta N59}$, the expected ligation product AQALPETGGGGGL.amide was formed after overnight reaction. The smaller product AQALPETGGGGL.amide peak in panel (D) is because 5-times less enzyme was used than in panel (C).

of Ca^{2+}. As shown in Figure 5, the expected ligation product, peptide AQALPETGGGGGL amide was observed in both cases, with catalytic activity dependent on Ca^{2+}. Based on those observations, our synthetic sortase A and [Trp(CHO)^{194}] sortase A were both enzymatically active.

For quantitative assessment of the enzymatic activities of synthetic sortase $A_{\Delta N59}$ and the analogue enzyme [Trp(CHO)^{194}] sortase $A_{\Delta N59}$ the two synthetic enzymes were compared with recombinant sortase A using the fluorogenic substrate Abz-LPATG-Dap(DNP) (Abz: *ortho*-aminobenzoic acid; Dnp: 2,4-dinitrophenyl). Recombinant sortase A was obtained by expression of the sortase A gene in *E. coli* cells. Substrate was incubated with enzyme in 50 mM Tris buffer, 150 mM NaCl, pH 7.5 in the presence of 5 mM Ca^{2+} and 5 mM triglycine as nucleophile, at 24 °C. The resulting data are shown in Table 1. In this assay, synthetic sortase A and [Trp(CHO)^{194}] sortase A had closely similar enzymatic activities. Clearly, the presence of the formyl group on the indole nitrogen of Trp^{194} does not affect the activity of sortase A. Furthermore, the activities observed for the synthetic enzymes were comparable to recombinant sortase A, with K_m values identical within experimental uncertainty. Interestingly, both the synthetic sortase A and [Trp(CHO)^{194}] sortase A had turnover numbers (k_{cat}) somewhat higher than recombinant sortase A; this might be attributed to the

Table 1: Comparison of enzymatic activities of recombinant sortase A and synthetic sortase A.

	k_{cat} [s^{-1}] ($\times 10^4$)	K_m [μM]
recombinant sortase A	7.9 ± 0.3	49 ± 4
synthetic [Trp(CHO)^{194}] sortase A	22 ± 2	45 ± 8
synthetic sortase A	16 ± 1	41 ± 6

number of purification steps before and after folding the synthetic enzyme molecules. The K_m and k_{cat} data for the synthetic sortase A enzymes were also comparable with literature data reported for recombinant sortase A with the substrate Abz-LPETG-Dnp.[20]

In conclusion, we have developed an effective total chemical synthesis of the enzyme sortase $A_{\Delta N59}$ and showed that this synthetic enzyme and its analogue [Trp(CHO)^{194}] sortase $A_{\Delta N59}$ both catalyze a transpepetidation reaction between LPXTG and pentaglycine with enzymatic activities comparable to recombinant *Staphylococcus aureus* sortase $A_{\Delta N59}$ expressed in *E. coli*. The formyl group on the indole nitrogen of tryptophan 194 does not affect the enzymatic activity of sortase A. Furthermore, the aspartic acid 170 to alanine mutation and the replacement of the methionines with norleucine residues also do not change the observed enzymatic activities which is consistent with previous mutation studies on sortase A.[12] The total synthesis of sortase $A_{\Delta N59}$ described in this work could be further exploited for detailed studies of the catalytic mechanism of sortase A. The consequent enhanced understanding of the sortase A transpeptidation reaction will be useful as a basis for further engineering of this important enzyme molecule.

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