JOC The Journal of Organic Chemistry

Featured Article

pubs.acs.org/joc

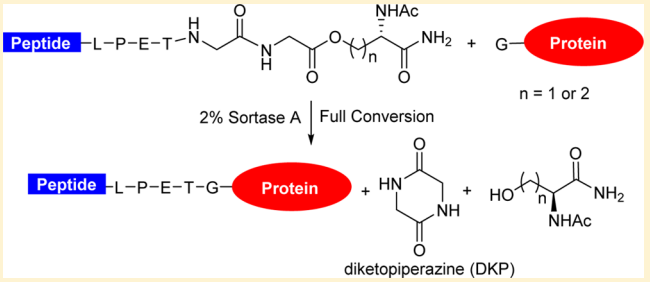
Irreversible Sortase A-Mediated Ligation Driven by Diketopiperazine Formation

Fa Liu,* Ethan Y. Luo, David B. Flora, and Adam R. Mezo

Lilly Research Laboratories, Indianapolis, Indiana 46285, United States

Supporting Information

ABSTRACT: Sortase A (SrtA)-mediated ligation has emerged as an attractive tool in bioorganic chemistry attributing to the remarkable specificity of the ligation reaction and the physiological reaction conditions. However, the reversible nature of this reaction limits the efficiency of the ligation reaction and has become a significant constraint to its more widespread use. We report herein a novel set of SrtA substrates (LPETGG-isoacyl-Ser and LPETGG-isoacyl-Hse) that can be irreversibly ligated to N-terminal Gly-containing moieties via the deactivation of the SrtA-excised peptide fragment through diketopiperazine (DKP) formation. The convenience of the synthetic procedure and the stability of the substrates in the ligation buffer suggest that both LPETGG-isoacyl-Ser and LPETGG-isoacyl-Hse are valuable alternatives to existing irreversible SrtA substrate sequences.



INTRODUCTION

Staphylococcal sortase A (SrtA) is a transpeptidase that exists in almost all Gram-positive bacteria and attaches surface proteins to the bacterial cell wall through newly formed peptide amide bonds.[1] The SrtA mechanism is as follows: SrtA first cleaves the Thr-Gly amide bond within the LPXTG (X = D, E, A, N, Q, or K) recognition motif (substrate A) to form a Thr thioester intermediate that can then react with a N-terminal Gly-containing peptide (nucleophile B) to reform a Thr-Gly peptide bond linking the two substrates (A–B, Scheme 1A).[2,3] The remarkable specificity of the SrtA-mediated ligation and the physiological reaction conditions have resulted in numerous successful applications of this reaction, including (1) the preparation of fusion constructs between small molecules, peptides, proteins and antibodies,[4−8] (2) the generation of circular proteins,[9,10] and (3) its application in protein purification.[11] When using synthetically generated peptides or small molecules, this method can effectively circumvent the challenge of introducing non-native amino acids or other nonstandard features such as C-terminal amides into recombinantly derived proteins. It can also efficiently combine two recombinant proteins which otherwise could be difficult to produce as a single fusion protein due to low expression yields or improper folding.[6,8]

However, despite the attractive features of SrtA-mediated ligation, its broader application has been largely hampered by the reversibility of the process which leads to less than quantitative conversion yields. A few approaches have been reported to improve conversion yields. For example, Nagamune and co-workers incorporated a rigid β-hairpin secondary structure flanking the LPETG motif in the ligated product. The ligated product is a poor substrate for the reverse SrtA reaction, thereby driving the forward ligation reaction toward completion.[12] As another example, both the Ploegh and Turnbull groups replaced the Thr-Gly amide bond in the LPETG recognition sequence of the substrate with an ester bond. Upon ligation, the excised fragment contained a terminal hydroxyl group, instead of a terminal amine, which dramatically reduced its reactivity and prevented the reverse ligation.[13,14] Among these methods, the depsipeptide approach from Turnbull and co-workers[14] appears particularly attractive; however, the preparation of this depsipeptide requires a nonstandard dipeptide which is not convenient to obtain using standard solid-phase peptide synthesis. In addition, the stability of this depsipeptide under standard SrtA ligation conditions is limited because it is susceptible to hydrolysis via its ester bond. This may become problematic should the ligation proceed slowly. We report herein novel alternative modifications of the SrtA recognition sequence designed to generate an irreversible ligation reaction by deactivating the N-terminal glycine of the excised peptide through diketopiperazine (DKP) formation (Scheme 1B).

RESULTS AND DISCUSSION

Recombinant SrtA, containing a N-terminal deletion of 59 amino acids and a C-terminal hexa-His tag, was produced as described[15,16] and used throughout the study. The peptide GGGGAEW-NH_2 (**1**, Table 1) was used as the nucleophile in all ligation experiments described below. In the search for suitable R_3 and R_4 groups to enable the formation of DKP (Scheme 1B), the first sequence evaluated was FLQLYGL-

Received: November 13, 2013
Published: December 30, 2013

   dx.doi.org/10.1021/jo4024914 | J. Org. Chem. 2014, 79, 487−492

Scheme 1. (A) Reversible SrtA-Mediated Ligation. (B) Irreversible SrtA Ligation Mediated by DKP Formation (R_1, R_2: Any Moiety of Interest; R_3: Any Suitable Group; R_4: Any Leaving Group except Glycine)

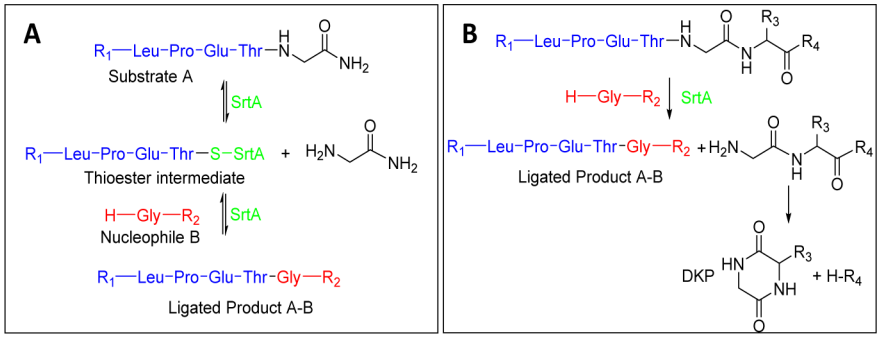
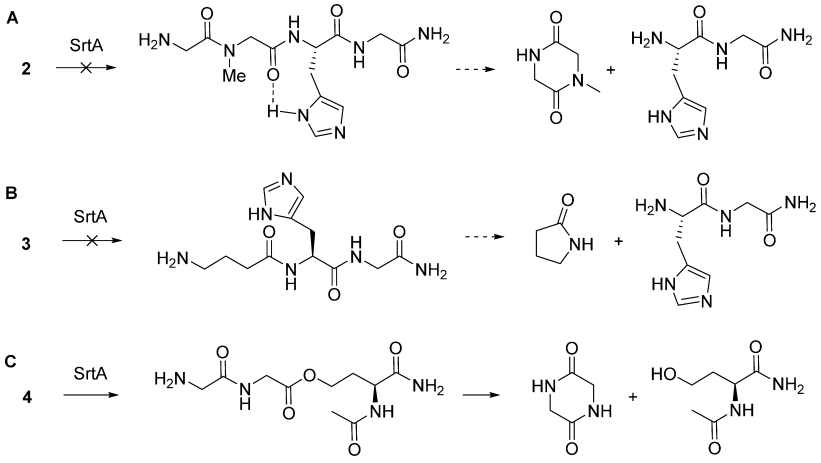


Table 1. Sequence and Characterization of the Synthetic Model Peptides

peptide no.	sequence[a,b]	MW (M + H)$^+$ calcd	MW (M + H)$^+$ obsd	amt (mg)[c]	yield (%)	purity[d] (%)
1	GGGGAEW-NH_2	632.3	632.0	50[e]	79	96
2	FLQLYGLPETGSarHG-NH_2	1501.8	1502.0	110[e]	73	80
3	FLQLYGLPETGabaHG-NH_2	1458.8	1458.1	105[e]	72	90
4	FLQLYGLPETGG-isoacyl-Hse(Ac)-NH_2	1436.7	1436.0	95	66	98
9	FLQLYGLPETGGG-NH_2	1350.7	1350.9	115	85	98
10	FLQLYGLPETGP-isoacyl-Hse(Ac)-NH_2	1476.8	1476.9	10	7	98
11	FLQLYGLPETGAib-isoacyl-Hse(Ac)-NH_2	1464.8	1464.9	10	7	98
12	FLQLYGLPETGG-isoacyl-S(Ac)-NH_2	1422.7	1422.9	50	35	98
13	FLQLYGLPET-OCH_2CO-NH_2	1237.6	1238.0	110[e]	89	95
14	FLQLYGLPETGS-isoacyl-S(Ac)-NH_2	1452.7	1452.9	20	14	98
15	FLQLYGLPETGY-isoacyl-Hse(Bz)-NH_2	1604.8	1604.9	12	8	93

[a]The nonunderlined residues were selected randomly. [b]Unusual amino acid abbreviations: Sar, *N*-methylglycine; Gaba, γ-aminobutyric acid; Hse, homoserine; Aib: 2-aminoisobutyric acid. [c]The amount of product obtained from 0.10 mmol scale synthesis. [d]Based on the RP-HPLC at 210 nm. [e]The weight of the crude peptide, which was used without further purification.

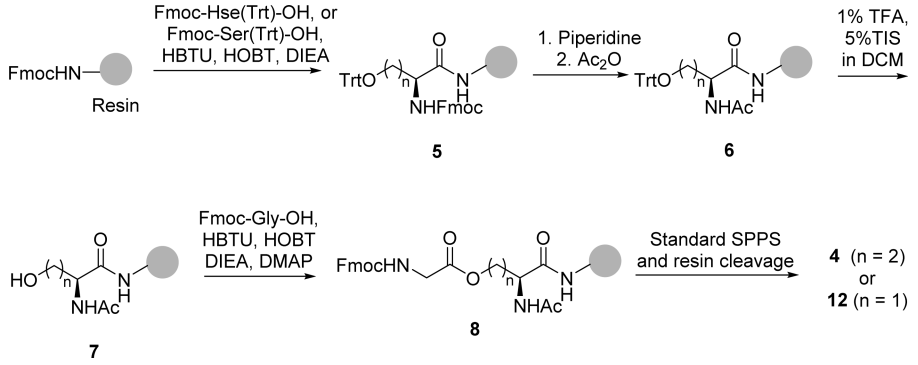
Scheme 2. Proposed Pathways for Deactivating the N-Terminus of Excised Peptide during SrtA-Mediated Ligation



PET^{0}G^{1}Sar^{2}H^{3}G-NH_2 (**2**, the positions of selected residues are labeled as the superscripted numbers, Table 1). To achieve accelerated DKP formation, two hypotheses were pursued: (1) Sar^2 was incorporated into substrate peptide **2** to potentially increase the *cis*-amide bond population, and (2) His^3 was incorporated to potentially activate the Sar-His amide bond (Scheme 2A).[17−20] However, it was found that the ligation did not proceed, as no conversion was detected by HPLC (high-performance liquid chromatography) and MS (mass spectrometry) after 24 h incubation at 37 °C with 5% (mol/mol) SrtA. It is likely that SrtA did not recognize substrate peptide **2**, possibly a result of the *N*-methylamino acid at the 2 position. Under a similar hypothesis, the N-terminal Gaba (γ-aminobutyric acid) moiety of the excised fragment from peptide FLQLYGLPET^{0}Gaba^{1,2}H^{3}G-NH_2 (**3**, Table 1) could undergo five-membered ring γ-lactam formation to form 2-pyrrolidone,[19] thereby exposing the SrtA-unreactive histidine at the N-terminus (Scheme 2B). However, similar to peptide **2**, SrtA-mediated ligation of peptides **3** and **1** did not provide any ligated product after 24 h at 37 °C with 5% SrtA, reinforcing the need for natural amino acids at the 1 and 2 position in the consensus recognition sequence.

dx.doi.org/10.1021/jo4024914 | J. Org. Chem. 2014, 79, 487−492

Scheme 3. Synthesis of Isoacyl Peptides 4 and 12



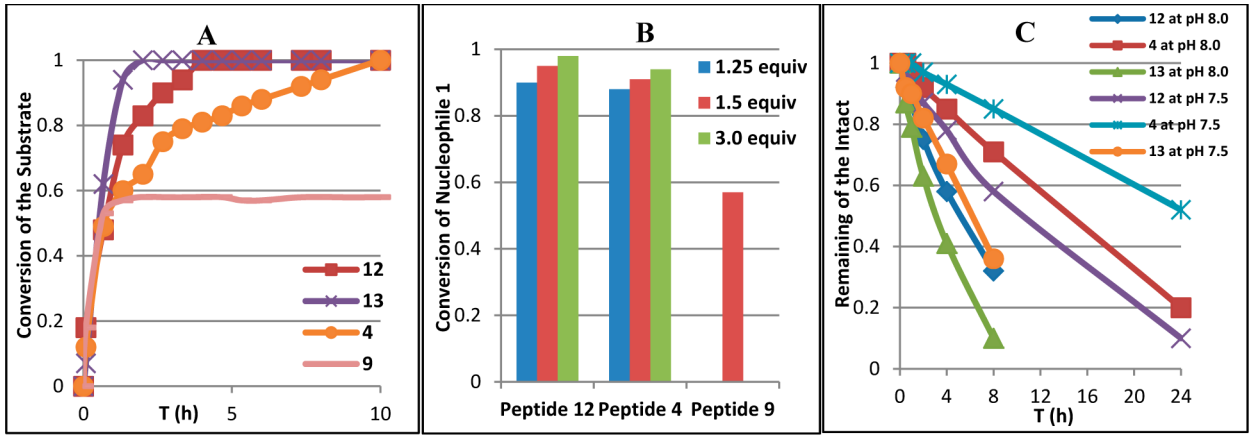


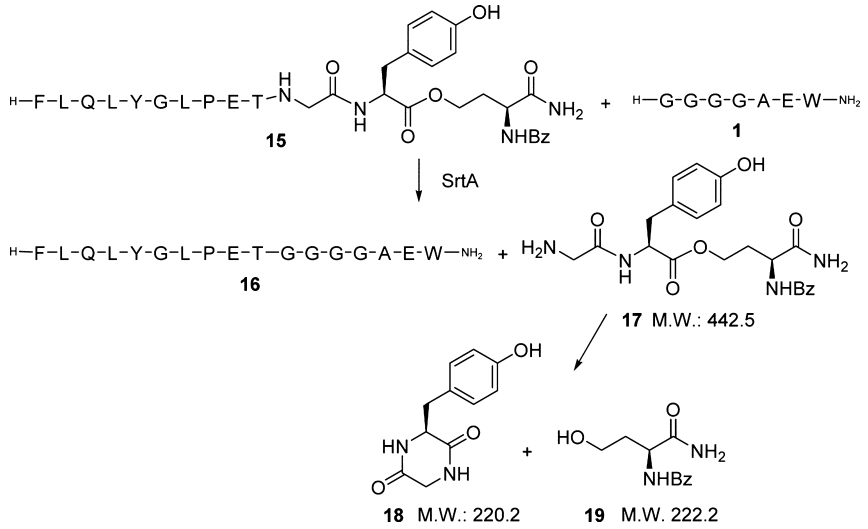
Figure 1. (A) Time-course of SrtA-mediated ligation of peptides **4**, **9**, **12**, or **13** with **1** (1.20 equiv) and 2% SrtA (mol/mol). (B) Efficiency of peptides **4**, **12** (1.25−3.0 equiv), and **9** (1.5 equiv) for converting nucleophile **1** to ligation products. (C) Stability of substrate peptides **4**, **12**, and **13**. All experiments were conducted in 50 mM HEPES, 150 mM NaCl and 10 mM $CaCl_2$ at 37 °C; pH 8.0 for panels A and B, pH 7.5 and 8.0 for panel C.

Learning from these two unsuccessful attempts with peptides **2** and **3**, subsequent model substrate peptides utilized glycine at both positions 1 and 2 to ensure efficient recognition by SrtA. In order to promote DKP formation, an ester bond between Gly^2 and isoacyl-Hse^3 (FLQLYGLPETG^{1}G^2-isoacyl-Hse^3(Ac)-NH_2, peptide **4**) was employed in place of the regular amide bond (Table 1 and Scheme 2C). The synthesis of peptide **4** began with loading Fmoc-Hse(Trt)-OH onto Rink amide resin, followed by treatment with piperidine and acetic anhydride to provide resin **6** (Scheme 3). The trityl group of resin **6** was removed with 1% TFA (trifluoroacetic acid) and 5% TIS (triisopropylsilane) in DCM (dichloromethane), and the Fmoc-Gly-OH was coupled to resin **7** with HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate)/HOBt (hydroxybenzotriazole)/DIEA (*N*,*N*-diisopropylethylamine) and a catalytic amount of DMAP (4-(dimethylamino)pyridine). The remaining residues were then assembled on resin **8** by means of an automated peptide synthesizer using standard Fmoc/t-Bu protocols. In order to effectively compare the ligation efficiency of peptide **4**, a comparator peptide was also synthesized which contained all natural amino acids (peptide **9**, Table 1). The ligation of peptides **4** or **9** was performed with 1.2 equiv of nucleophile **1** and 2% SrtA (mol/mol, based on substrate peptides **4** or **9**) at 37 °C, and the ligation reaction was monitored by analytical HPLC. It was found that peptide **4** was progressively consumed and the conversion of **4** to its ligated product was complete by 10 h. In contrast, the ligation of peptides **9** and **1** reached equilibrium at 2 h with only 58% conversion of peptide **9** to its ligated product (Figure 1A; Figures S1A and S1B and Table S1, Supporting Information).

With the encouraging data from peptide **4**, we again hypothesized that the rates of DKP formation could be increased by inducing a greater proportion of the *cis*-conformation in the C-terminal amide of Gly^1 via the replacement of Gly^2 with proline or 2-aminoisobutyric acid (Aib). However, the SrtA-mediated ligation of peptide **1** with both substrate peptides **10** (FLQLYGLPET^{0}G^{1}P^2-isoacyl-Hse-(Ac)-NH_2) and **11** (FLQLYGLPET^{0}G^{1}Aib^2-isoacyl-Hse(Ac)-NH_2) proceeded much more slowly than the ligation of peptides **4** and **1**, possibly a result of poor recognition of sequences LPETG^{1}P^2 and LPETG^{1}Aib^2 by SrtA as compared to LPETG^{1}G^2. Our focus then shifted to the possibility of increasing the leaving ability of the alcohol to also accelerate DKP formation. Serine was tested instead of Hse at position 3 of peptide **4** since the hydroxyl of the serine side-chain possesses a lower pK_a than the hydroxyl of Hse, and therefore serine was expected to be more reactive. The resulting peptide **12** (FLQLYGLPETG^{1}G^2-isoacyl-S^3(Ac)-NH_2) was evaluated under the identical ligation conditions as for **4** and **9**, and it was found that full conversion of **12** was achieved at 4 h (vs 10 h for **4**) (Figure 1A; Figure S1C and Table S1, Supporting Information). To compare with the previously reported depsipeptide approach,[14] substrate peptide **13** (FLQLYGL-PET-OCH_2CO-NH_2) was prepared and tested, and it was found to be fully converted to ligation product after 2 h (Figure

dx.doi.org/10.1021/jo4024914 | *J. Org. Chem.* 2014, 79, 487−492

Scheme 4. Determination of the DKP Formation during the Ligation between Peptides 15 and 1



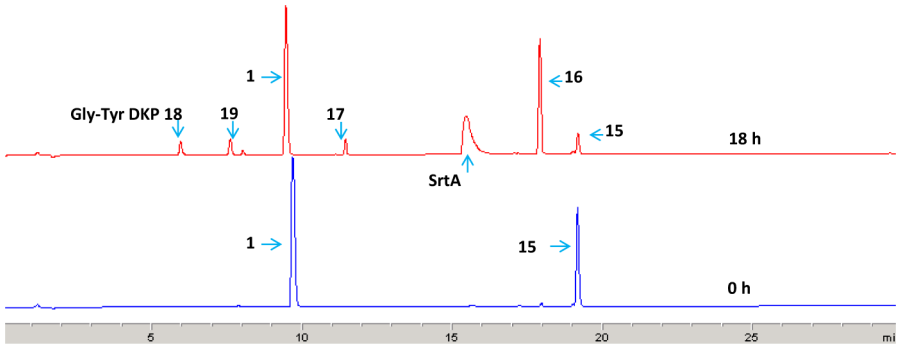


Figure 2. Analytical RP-HPLC traces of the ligation between peptides **15** and **1** (at 210 nm).

1A; Figure S1D and Table S1, Supporting Information). It should be noted that although the ligations proceeded to completion, hydrolysis of the substrate peptides **4**, **12**, and **13** was observed to a similar extent of approximately 15% for each (Table S2, Supporting Information). To test if other residues at position 2 could reduce the extent of hydrolysis, substrate peptide **14** (FLQLYGLPETG^{1}S^{2}-isoacyl-S^{3}(Ac)-NH_2), with serine at position 2, was synthesized and tested in the ligation reaction. It was found that the extent of hydrolysis of **14** was approximately 20% under the same ligation conditions as for peptide **12** (Figure S3, Supporting Information). Considering the low efficiency of coupling residues other than glycine to the side-chain hydroxyl of serine, additional amino acids at position 2 were not pursued.

To examine the efficiency of the substrate peptides **4** and **12** on converting a fixed amount of nucleophile **1** into the SrtA ligation product, a range of molar ratios of **4** and **12** to **1** (1.25−3.0) were evaluated. It was found that 1.5 equiv of substrate peptide **4** converted 91% nucleophile **1** within 20 h, while the same amount of peptide **12** achieved 96% conversion of **1** within 6 h (Figure 1B; Figures S2A−F, Supporting Information). As described earlier,[14] SrtA (2 mol % of the substrate peptides) maintains an equilibrium between the product and the thioester intermediate making it difficult to achieve 100% conversion of nucleophile **1**. As a comparison, 1.5 equiv of the natural amino acid-containing substrate peptide **9** only converted 57% of peptide **1** under identical ligation conditions (Figure 1B; Figure S2G, Supporting Information)

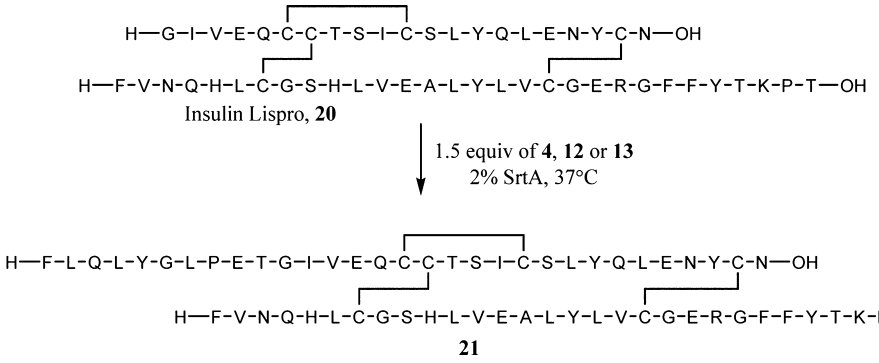
In addition to overcoming the reversibility of the reaction, the stability of the substrate peptides in the ligation buffer is also important for achieving high conversion yields. The $t_{1/2}$ (half-life time) of peptides **4**, **12**, and **13** was measured in the ligation buffer of 50 mM HEPES, 150 mM NaCl, and 10 mM $CaCl_2$ at pH 7.5 or 8.0 at 37 °C. It was determined that at pH 8.0 the $t_{1/2}$ was ∼15 h for peptide **4**, ∼5 h for peptide **12**, and ∼3 h for peptide **13** (Figure 1C; Table S3, Supporting Information). The trends at pH 7.5 were similar with $t_{1/2}$'s approximately 2-fold higher for each peptide. The longer $t_{1/2}$'s of peptides **4** and **12** could be beneficial in cases where the ligation proceeds slowly (e.g., due to steric hindrance of the substrates) and the reaction needs more time for completion.

To confirm that the SrtA-mediated ligation of substrate peptides **4** or **12** was indeed driven by DKP formation as originally hypothesized, peptide **15** (FLQLYGLPETGY-isoacyl-Hse(Bz)-NH_2) was designed with tyrosine and benzamide incorporated into the SrtA-excised fragment to facilitate monitoring of the byproduct by HPLC/UV and MS. Upon analysis of the ligation mixture of peptides **15** and **1** using LC−MS, a side product with the molecular weight of 220 Da was identified which unambiguously confirmed the formation of Gly-Tyr DKP **18** (Scheme 4 and Figure 2; Figure S4, Supporting Information).

The practical utility of substrate peptides **4** and **12**, in comparison to peptide **13**, was evaluated by performing a ligation with insulin lispro **20**. Conveniently, insulin lispro contains a glycine at the N-terminus of its A-chain. Each of the

dx.doi.org/10.1021/jo4024914 | J. Org. Chem. 2014, 79, 487−492

Scheme 5. SrtA-Mediated Ligation between Insulin Lispro 20 and Substrate Peptides 4, 12, and 13



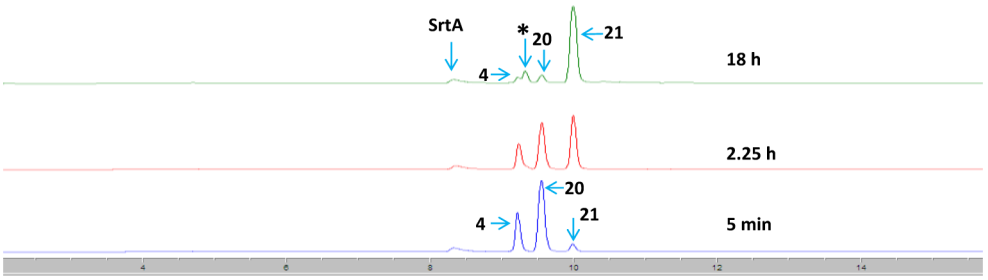


Figure 3. Analytical RP-HPLC traces of the SrtA-mediated ligation between insulin lispro **20** and peptide **4** (at 210 nm). 93% of insulin lispro **20** was converted to **21** at 18 h. Asterisk indicates the hydrolyzed product of **4** as confirmed by mass spectrometry.

substrate peptides (1.5 equiv each; **4**, **12**, or **13**) was incubated with lispro **20** in the presence of 2% SrtA at 37 °C (Scheme 5). After 18 h, the conversion of lispro **20** to the ligation product **21** was nearly identical for all three substrate peptides: 92% for peptide **4**, 93% for peptide **12**, and 92% for peptide **13** (Figures 3; Figures S5A–C, Supporting Information).

■ CONCLUSION

In conclusion, we have developed a novel set of SrtA substrates that, through DKP formation of the N-terminal glycine of the SrtA-excised peptide fragment, can be irreversibly ligated to the N-terminus of Gly-containing nucleophiles. Peptides comprising both LPETGG-isoacyl-Ser (**4**) and LPETGG-isoacyl-Hse (**12**) were demonstrated to be effective, irreversible SrtA substrates, and each peptide can be readily prepared through standard solid-phase procedures using commercially available amino acid building blocks. Additionally, the convenience of the synthetic procedures and the higher stability (vs substrate peptide **13**) in the ligation buffer suggest that both LPETGG-isoacyl-Ser and LPETGG-isoacyl-Hse are valuable alternatives to existing irreversible SrtA substrate sequences.

■ EXPERIMENTAL SECTION

General Information. All solvents [*N*,*N*′-dimethylforamide (DMF), methanol (MeOH), dichloromethane (DCM), acetonitrile (ACN), diethyl ether (Et_2O)] and reagents [hydroxybenzotriazole (HOBT), *N*,*N*′-diisopropylcarbodiimide (DIC), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), tris-(hydroxymethyl)aminomethane (Tris), collidine, acetic anhydride, triphosgene] were purchased and used directly. The dipeptide Fmoc-Thr(t-Bu)-OCH_2CO_2H was purchased from the Chinese Peptide Company. Insulin lispro was obtained from Eli Lilly (Indianapolis, IN). Water (H_2O) was obtained from a Milli-Q water purification system (Millipore). Polystyrene Rink amide (RAM) resin was purchased from Rapp Polymere GMBH. Fmoc-chemistry based amino acid cartridges were purchased from several vendors and used directly. LC-MS system: Agilent 1100 series liquid chromatography mass spectrometer, model G1956A/B. General analytical RP-HPLC condition: All samples were analyzed using a Waters SymmetryShield RP18 Column (cat. no. 186000179, 3.5 μm, 4.6 × 100 mm.): a linear gradient from 6% aqueous ACN (0.1% trifluoroacetic acid) to 60% aqueous ACN (0.1% trifluoroacetic acid) over 14 min at 60 °C and a flow rate of 1.0 mL/minute.

General Procedure for Solid-Phase Synthesis of Peptides 1–3, 9, and 13. Peptide synthesis was conducted using 0.10 mmol of Rink Amide resin (Rapp Polymere GMBH) and a ABI433 peptide synthesizer, using a standard Fmoc_HOBT_DCC_0.10 mmol method (Table S4, Supporting Information). Cleavage was conducted by treatment of this resin with 10 mL of a TFA solution containing 2.5% TIS, 2.5% H_2O at rt, with gentle shaking for 1.5 h. The resin was filtered and washed with TFA (2 mL × 2). The combined filtrate was precipitated with cold ether (80 mL). The precipitate was collected by centrifugation, then washed with cold ether (80 mL × 2), and purified by preparative RP-HPLC using a Waters SymmetryPrep C18 Column (cat. no. WAT066245, 7 μm, 19 × 300 mm). Purification was achieved using a linear gradient from 5% aqueous ACN (0.1% trifluoroacetic acid) to 50% aqueous ACN (0.1% trifluoroacetic acid) over 80 min at a flow rate of 15 mL/minute. Selected fractions were pooled and lyophilized to provide the target peptides as lyophilized powders. Identity was confirmed using mass spectrometry (Table 1; Figure S6, Supporting Information).

General Procedure for Solid-Phase Synthesis of Peptides 4 and 10–12, 14, and 15. Rapp Polymere Rink amide resin (Rapp Ploymere, cat no. H 400 23) (0.10 mmol) was treated with 20% piperidine in DMF (10 min × 2) and washed with DMF (3 mL × 6). In a separate vial, Fmoc-Hse(Trt)-OH (1.0 mmol) or Fmoc-Ser(Trt)-OH (1.0 mmol), HBTU (1.0 mmol), HOBt (1.0 mmol), and DIEA (1.0 mmol) were mixed in DMF (3 mL) for 5 min before being transferred to the above resin. The resulting slurry was gently mixed for 2 h at rt, washed with DMF (3 mL × 3), treated with 20% piperidine in DMF (10 min × 2), and washed with DMF (3 mL × 3) before being mixed with acetic anhydride (2 mmol) and DIEA (2 mmol) in DCM (5 mL) at rt for 1 h. The resulting resin was washed

dx.doi.org/10.1021/jo4024914 | J. Org. Chem. 2014, 79, 487–492

with DCM (3 mL × 6), treated with 5 mL of 1% TFA and 5% TIS in DCM for 2 min, drained, and retreated 5 more times. Formation of the ester bond in peptides **4**, **10**, **12**, **14**, and **15** was achieved by mixing Fmoc-Gly-OH (1.0 mmol), HBTU (1.0 mmol), HOBt (1.0 mmol), DIEA (1.0 mmol), and DMAP (0.6 mg, 5% of the resin substitution) in 3 mL of DMF and then adding this mixture to the resin for 2 h at rt. This coupling was repeated once. Formation of the ester bond in peptide **11** was achieved by in situ acyl chloride formation using triphosgene:[21] the resin was pretreated with DIEA/THF (0.60 mL, 1:1 by volume) for 10 min. In a separate vial, Fmoc-Aib−OH (1.0 mmol) and BTC (triphosgene, 100 mg, 0.33 mmol) were dissolved in anhydrous THF (5 mL). To this mixture was added collidine (0.40 mL) and the resulting slurry was mixed for 1 min, and transferred to the above DIEA/THF pretreated resin followed by the addition of DMAP (1.2 mg). The resin was gently agitated at rt for 4 h. For both coupling methods, the resulting resin-bound isoacyl peptides were transferred to the ABI433 peptide synthesizer to assemble the remainder of the peptide sequence using the standard Fmoc_HOBT_DCC_0.10 mmol method (Table S4, Supporting Information). Resin cleavage and peptide purification was conducted as described above for the synthesis of peptides **1**−**3**, **9**, and **13**.

General Procedure for SrtA Ligation. The substrate peptides (**2**−**4** and **9**−**15**) and the nucleophile peptides (**1** or insulin lispro **20**) were mixed in a HEPES ligation buffer (50 mM HEPES, 5 mM $CaCl_2$, 150 mM NaCl, pH 8.0) at the desired concentration and molar ratio with the presence of SrtA (2% mol/mol based on the substrate peptides) at 37 °C or room temperature (21 °C). The reaction was monitored by analytical HPLC and/or LC−MS.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

RP-HPLC traces and mass spectrometry data for the SrtA ligations and the synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: liufx@lilly.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Yuewei Qian and Ms. Xiaohua He (Eli Lilly) for the expression and purification of SrtA.

■ REFERENCES

(1) Hendrickx, A. P.; Budzik, J. M.; Oh, S. Y.; Schneewind, O. *Nat. Rev. Microbiol.* **2011**, *9*, 166.
(2) Ton-That, H.; Mazmanian, S. K.; Faull, K. F.; Schneewind, O. *J. Biol. Chem.* **2000**, *275*, 9876.
(3) Aulabaugh, A.; Ding, W.; Kapoor, B.; Tabei, K.; Alksne, L.; Dushin, R.; Zatz, T.; Ellestad, G.; Huang, X. *Anal. Biochem.* **2007**, *360*, 14.
(4) Mao, H.; Hart, S. A.; Schink, A.; Pollok, B. A. *J. Am. Chem. Soc.* **2004**, *126*, 2670.
(5) Proft, T. *Biotechnol. Lett.* **2010**, *32*, 1.
(6) Tsukiji, S.; Nagamune, T. *ChemBioChem* **2009**, *10*, 787.
(7) Wu, Z.; Guo, Z. *J. Carbohydr. Chem.* **2012**, *31*, 48.
(8) Popp, M. W.; Ploegh, H. L. *Angew. Chem., Int. Ed.* **2011**, *50*, 5024.
(9) Antos, J. M.; Popp, M. W.; Ernst, R.; Chew, G. L.; Spooner, E.; Ploegh, H. L. *J. Biol. Chem.* **2009**, *284*, 16028.
(10) Parthasarathy, R.; Subramanian, S.; Boder, E. T. *Bioconjugate Chem.* **2007**, *18*, 469.
(11) Mao, H. *Protein Expr. Purif.* **2004**, *37*, 253.
(12) Yamamura, Y.; Hirakawa, H.; Yamaguchi, S.; Nagamune, T. *Chem. Commun. (Cambridge)* **2011**, *47*, 4742.
(13) Antos, J. M.; Chew, G. L.; Guimaraes, C. P.; Yoder, N. C.; Grotenbreg, G. M.; Popp, M. W.; Ploegh, H. L. *J. Am. Chem. Soc.* **2009**, *131*, 10800.
(14) Williamson, D. J.; Fascione, M. A.; Webb, M. E.; Turnbull, W. B. *Angew. Chem., Int. Ed.* **2012**, *51*, 9377.
(15) Ilangovan, U.; Ton-That, H.; Iwahara, J.; Schneewind, O.; Clubb, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6056.
(16) Ton-That, H.; Liu, G.; Mazmanian, S. K.; Faull, K. F.; Schneewind, O. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12424.
(17) Suaifan, G. A. R. Y.; Mahon, M. F.; Arafat, T.; Threadgill, M. D. *Tetrahedron* **2006**, *62*, 11245.
(18) Santos, C.; Mateus, M. L.; dos Santos, A. P.; Moreira, R.; de Oliveira, E.; Gomes, P. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1595.
(19) Gomes, P.; Vale, N.; Moreira, R. *Molecules* **2007**, *12*, 2484.
(20) Goolcharran, C.; Borchardt, R. T. *J. Pharm. Sci.* **1998**, *87*, 283.
(21) Thern, B.; Rudolph, J.; Jung, G. *Tetrahedron Lett.* **2002**, *43*, 5013.