Macrolactamization of Glycosylated Peptide Thioesters by the Thioesterase Domain of Tyrocidine Synthetase

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Summary
The 35 kDa thioesterase (TE) domain excised from the megaladton tyrocidine synthetase (Tyc Syn) retains autonomous capacity to macrocyclize peptidyl thioesters to D-Phe1-L-Leu10-macrolactams. Since a number of nonribosomal peptides undergo O-glycosylation events during tailoring to gain biological activity, the Tyc Syn TE domain was evaluated for cyclization capacity with glycosylated peptidyl-S-NAC substrates. First, Tyr7 was replaced with Tyr(β-D-Gal) and Tyr(β-D-Glc) as well as with Ser-containing β-linked D-Gal, D-Glc, D-GlcNAc, and D-GlcNH2, and these new analogs were shown to be cyclized with comparable kcat/Km catalytic efficiency. Similarly, Gal- or tetra-O-acetyl-Gal-Ser could also be substituted at residues 5, 6, and 8 in the linear decapetidyl-S-NAC sequences and cyclized without substantial loss in catalytic efficiency by Tyc Syn TE. The cyclic glycopeptides retained antibiotic activity as membrane perturbants in MIC assays, opening the possibility for library construction of cyclic glycopeptides by enzymatic macrocyclization.

Introduction
A variety of peptidyl, lipopeptidyl, and hybrid polyketidyl-peptidyl natural products are generated as macrocyclic lactams and lactones in which the conformational constraints introduced by cyclizations restrict architecture to biologically active conformations [1, 2]. These classes of natural products are elongated from simple amino acid and acyl CoA monomer units by multimodular enzymatic assembly lines as a series of acyl-S-enzyme covalent intermediates [1, 2]. Full-length acyl-S-enzymes undergo self-catalyzed chain termination by 30–40 kDa thioesterase domains, situated at the C-terminal end of the assembly lines [1, 2]. A variety of TE domains, when excited and expressed as a single domain, retain autonomous activity to act as regioselective and stereoselective macrocyclization catalysts [3–6], both on soluble thioester substrates and for substrates tethered on solid support beads [3, 7].

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The nascent cyclic products released by TE domain catalysis often undergo a series of subsequent tailoring reactions, most commonly oxidations and glycosylations, carried out by dedicated tailoring enzymes encoded with the assembly line genes in biosynthetic gene clusters [1, 8]. The glycosylations may be required for gain of biological activity, as in the erythromycin family [9], or as scaffolds for further enzymatic decoration to create the pharmacophores [10]. We have focused on characterization of such glycosyltransferases as an approach to diversify natural product structure and activity [11–15].

In parallel, we have begun to explore chemical incorporation of glycosylated amino acids into linear peptide precursors to act as substrates for the macrocyclizing thioesterases. As an initial test, we turned to the tyrocidine synthetase TE domain (Tyc Syn TE), which we have previously determined to be an efficient head-to-tail cyclization catalyst with decapetidyl thioesters, generating the natural cyclic decapetidyl tyrocidine and a variety of backbone analogs [3, 16–19]. In particular, we have previously shown that Tyc Syn TE will tolerate replacement of residues 5–8 of peptidyl thioesters. Therefore, we started with Tyr7 and replaced it with protected glycosyl-[β]-Tyr and glycosyl-[β]-Ser residues via solid-phase synthesis of monoglycosyl decapetidyl thioesters. We also substituted residues 5–8 with galactosyl-[β]-Ser to evaluate the permissiveness of Tyc Syn TE to macrocyclize the regiosomeric monoglycosyl peptidyl thioesters. All cyclic products were tested for minimal inhibitory concentrations as antibiotics and for minimal hemolysis concentrations.

Results
Synthesis of Glycopeptide Thioesters
The decapetidyl thioesters were synthesized essentially as previously reported [3], by solid-phase peptide synthesis with Fmoc-protected amino acids, including Tyr(AC4-β-D-Gal), Tyr(AC4-β-D-Glc), Ser(AC4-β-D-Gal), Ser(AC4-β-D-Glc), Ser(AC2-β-D-Glcnac), and Ser(AC2-β-D-GlcNBoc) in place of Tyr7, as depicted in Figure 1. The hydroxyl groups of the sugar were protected with acetyl groups, which were removed before conversion to the N-acetylcysteamine (NAC) thioester, and enzymatic cyclization assays were performed as noted. Analogously Fmoc-Ser(AC4-β-D-Gal) was used in place of Asn6, Gin6, and Val9 during solid-phase synthesis of the linear peptides (Figure 1).

Substrate Behavior of Glycosylated Peptidyl-S-NACs
The excised TE domain from tyrocidine synthetase will accept soluble decapetidyl-S-NAC thioesters in place of the decapetidyl-S-panthethiynyl-phospho-carrier protein domain that is the natural acyl-S-enzyme upstream substrate in tyrocidine synthetase [3]. The head-to-tail cyclization is shown in Figure 2 for both the wild-type
decapeptidyl-S-NAC and the monoglucosyl-serine versions at residue 7 (Figure 2).

Table 1 shows, as expected from earlier studies [17, 19], that Tyc Syn TE tolerates replacement of Tyr7 by Ser, albeit with cyclization kinetics of a 10-fold increase in $K_M$ and a 2-fold drop in $k_{cat}$, yielding a 20-fold
Table 1. Kinetic Parameters of Tyc Syn TE-Catalyzed Cyclization of Peptide Thioesters Substituted with Different Glycosylic Tyr and -Ser at Position 7

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (min$^{-1}$)$^a$</th>
<th>$K_m$ (μM)$^a$</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$)</th>
<th>Cyclization to Hydrolysis Ratio at 5 μM$^b$</th>
<th>Cyclization to Hydrolysis Ratio at 50 μM$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrocidine</td>
<td>110</td>
<td>1.4</td>
<td>79</td>
<td>17.2 ± 0.4</td>
<td>12.3 ± 0.3</td>
</tr>
<tr>
<td>Tyr(β-D-Gal)</td>
<td>59</td>
<td>8.5</td>
<td>6.9</td>
<td>10.1 ± 0.4</td>
<td>8.6 ± 0.1</td>
</tr>
<tr>
<td>Tyr(β-D-Glc)</td>
<td>82</td>
<td>8.3</td>
<td>3.9</td>
<td>10.8 ± 0.2</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>Ser7</td>
<td>69</td>
<td>15.0</td>
<td>26.5</td>
<td>6.7 ± 0.5</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Ser7(β-D-Gal)</td>
<td>55</td>
<td>12.2</td>
<td>11.4</td>
<td>6.2 ± 0.1</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>Ser7(β-D-Glc)</td>
<td>55</td>
<td>15.4</td>
<td>3.8</td>
<td>10.8 ± 0.1</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Ser7(β-D-GlcNH$_2$)</td>
<td>101</td>
<td>11.4</td>
<td>9.9</td>
<td>10.6 ± 0.2</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Ser7(β-D-GlcNH$_2$)</td>
<td>140</td>
<td>4.3</td>
<td>32.6</td>
<td>11.1 ± 0.1</td>
<td>8.0 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$For the $k_{cat}$ and $K_m$ measurements, reactions were carried out with 5 nM TE, and substrate concentrations used were 0.5, 1, 2, 5, 10, 20, and 40 μM.

$^b$For the cyclization to hydrolysis ratio measurement, reactions were carried out with 50 nM TE at two different substrate concentrations, 5 and 50 μM. The cyclization to hydrolysis ratios were measured by LCMS after the reactions were complete in 1 hr.

decrease in catalytic efficiency. Tyr$_7$(β-D-Gal) and Tyr$_7$(β-D-Glc) are slightly poorer substrates than the wild-type Tyr$_7$, peptidyl-S-NAC by only about 8-fold in $K_m$ and are almost equivalent in $k_{cat}$, confirming tolerance by the TE to the addition of a hydrophilic sugar. Substitution of Tyr$_7$ by Ser or any of four glycosyl-serines, including N-acetyl-glucosaminyl- and 2-amino-2-deoxy-glucosyl-O-Ser$_7$, could be done without substantial penalty for subsequent head-to-tail macrocyclization by the enzyme. This suggests a prospect of subsequent chemical and/or enzymatic acylation strategies to make cyclic lipoglycopeptides by these approaches. In terms of the cyclization to hydrolysis ratio mediated by the TE domain, the Tyr$_7$(β-D-Gal) and Tyr$_7$(β-D-Glc) substrates gave ratios of 8:1–10:1 under conditions in which the Tyr$_7$ wild-type decapetidyl-S-NAC sequence was cyclized at ratios of 12:1–17:1. These ratios provide synthetically useful flux to regiospecific macrocyclization of the monoglycosylated peptides. Table 1 also displays data for the corresponding TE domain-mediated cyclization of Ser$_7$(β-D-Gal)$_7$-, Ser$_7$(β-D-Glc)$_7$-, Ser$_7$(β-D-GlcNAc)$_7$-, and Ser$_7$(β-D-GlcNH$_2$)$_7$-decapeptidyl thioesters. All four glycosylated Ser$_7$ peptides are macrocyclized with 85%–90% of the flux to the macro-lactam (Table 1).

To evaluate the tolerance of the Tyc Syn TE domain for glycosylated residues at other sites, Ser(β-D-Gal)- or Ser(β-D-Glc)-decapeptidyl thioesters were synthesized with the sugar substituent displayed at residue 5, 6, or 8 on the decapetidyl thioester backbone, replacing Asn$_5$, Glu$_6$, or Val$_8$, respectively. In all cases, the unglycosylated serine-containing peptides (Ser$_5$, Ser$_6$, Ser$_7$) were prepared and assayed with the TE domain for comparison to the substrates containing glycosylserine. At positions 5–7, the galactosyl and tetra-O-acetylgalactosyl substituents were accommodated with remarkably little perturbation (Table 2), consistent with the view [16] that this portion of the linear substrate does not make significant contact with the enzyme active site. Cyclization to hydrolysis ratios ranged from 7.1 to 13:1, indicating that 85%–95% of the flux is directed by the enzyme to intramolecular capture by the amine nucleophile of D-Phe$_1$. At residue 8, while the replacement of Val$_8$ for Ser is well tolerated, the galactosyl-O-serine substitution, with and without the four acetyl groups, has a substantial effect on the product-determining step. While $k_{cat}$ is not substantially altered, now 50% of the flux in the Ser$_6$(β-D-Gal) and 80% of the flux in the Ser$_7$(β-D-Gal) is to hydrolysis, suggesting that a cyclization conformer in the acyl-O-TE active site is disfavored by the sugar substituent at residue 8 (Table 2).

Biological Activity of Glycosylated Tyrocidine Derivatives

The antibiotic activity of tyrocidine arises from its penetration into bacterial membranes and subsequent pore formation. The selectivity of bacterial membranes versus eukaryotic membranes is not particularly high, limiting the systemic use of this class of cyclic peptide antibiotics [20]. We evaluated the minimal inhibitory concentration (MIC) against Bacillus subtilis and the minimal hemolytic concentration (MHC) against human erythrocytes as a typical eukaryotic cell membrane toxicity [21, 22]. As shown in Table 3, the MIC for the parent tyrocidine was 1.5 μM, while the MHC was 25 μM, yielding an MHC/MIC ratio of 16. The Ser$_5$, Ser$_6$, Ser$_7$, and Ser$_8$ variants had MHC/MIC ratios of 4–8, as did the Tyr$_7$(β-D-Glc) and Tyr$_7$(β-D-Gal) cyclic macrolactams. The presence of galactosyl, 2-amino-glucosyl, and GlcNAC on Ser$_7$ or galactosyl and tetra-O-acetyl galactosyl residues on Ser$_5$–Tyr$_7$ had only minimal effects on MIC and MHC values. However, the galactosylation at Ser$_8$ in both acylated and unprotected form raised the MIC 15- and 2-fold, respectively, while the MHC values increased 8- and 4-fold, respectively. Thus, glycosyl substitution at residue 8 appears to impair the membrane activity of the cyclic peptide (Table 3).

Discussion

The tyrocidine synthetase TE domain acts as the last domain in a 28-domain, 3-protein, megadalton nonribosomal peptide synthetase assembly line [23]. As the most downstream domain in the assembly line, the 30 kDa TE acts as the chain termination catalyst, causing head-to-tail macrocyclization of the amino group of D-Phe$_1$ on the activated carbonyl of L-Leu$_{10}$. The excised TE domain is soluble, well folded, and autonomously active as a regiospecific macroactamization catalyst. Prior work has indicated a broad tolerance of the excised TE for replacement of the side chains of the four residues, D-Phe$_{4i}$, Asn$_{5r}$, Glu$_{6r}$, and Tyr$_7$, with re-
Table 2. Kinetic Parameters of Tyc Syn TE-Catalyzed Cyclization of Peptide Thioesters Substituted with Ser, Ser(B-D-Gal), and Ser(Ac4-B-D-Gal) at Positions 5–8

<table>
<thead>
<tr>
<th></th>
<th>k_{cat} (min^{-1})</th>
<th>K_m (μM)</th>
<th>k_{cat}/K_m (min^{-1} μM^{-1})</th>
<th>Cyclization to Hydrolysis Ratio at 5 μM</th>
<th>Cyclization to Hydrolysis Ratio at 50 μM</th>
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<tbody>
<tr>
<td>Ser_5</td>
<td>46</td>
<td>0.8</td>
<td>58.5</td>
<td>7.3 ± 0.4</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>68</td>
<td>2.0</td>
<td>34</td>
<td>13.0 ± 1.6</td>
<td>8.9 ± 0.1</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>111</td>
<td>4.4</td>
<td>25.2</td>
<td>6.9 ± 0.6</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>42</td>
<td>0.5</td>
<td>84</td>
<td>13.6 ± 0.2</td>
<td>11.8 ± 0.7</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>22</td>
<td>2.5</td>
<td>8.8</td>
<td>8.1 ± 0.1</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>69</td>
<td>15.0</td>
<td>4.6</td>
<td>6.7 ± 0.5</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>55</td>
<td>12.2</td>
<td>4.5</td>
<td>6.2 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>40</td>
<td>13.8</td>
<td>2.9</td>
<td>3.9 ± 0.4</td>
<td>7.7 ± 0.1</td>
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<tr>
<td>Ser_6</td>
<td>112</td>
<td>6.2</td>
<td>18.1</td>
<td>9.8 ± 0.1</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Ser_6(B-D-Gal)</td>
<td>39</td>
<td>9.6</td>
<td>4.1</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Ser_6(Ac4-B-D-Gal)</td>
<td>94</td>
<td>14.3</td>
<td>6.6</td>
<td>0.21 ± 0.02</td>
<td>0.27 ± 0.01</td>
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Assay conditions were the same as in Table 1.

Table 3. MIC, MHC, and Therapeutic Index of Glycopeptides Made

<table>
<thead>
<tr>
<th></th>
<th>MIC (μM)</th>
<th>MHC (μM)</th>
<th>Therapeutic Index (MHC/MIC)</th>
</tr>
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<tbody>
<tr>
<td>Tyrocidine</td>
<td>1.5</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Ser_5</td>
<td>3</td>
<td>12.5</td>
<td>4</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>6</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>6</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Ser_5</td>
<td>1.5</td>
<td>12.5</td>
<td>8</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>3</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>6</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>3</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Ser_5(Ac4-B-D-Gal)</td>
<td>3</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>6</td>
<td>50</td>
<td>8</td>
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<td>Ser_5(B-D-Gal)</td>
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<td>8</td>
</tr>
<tr>
<td>Ser_5(B-D-Gal)</td>
<td>6</td>
<td>50</td>
<td>8</td>
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</tbody>
</table>

Significance

A variety of nonribosomal peptide and polyketide natural products undergo regiospecific macrocyclization as the chain release step from their enzymatic assem-
ably lines. Many of these cyclic products then undergo glycosylation by dedicated tailoring glycosyltransferases. Glycosylation increases aqueous solubility, can protect the producer microorganism from the actions of the antibiotic, and/or can be required for biological activity. An alternative to nature’s use of tandem enzymatic steps of macrolactamization and glycosylation is a chemoenzymatic approach of glycopeptide thioester synthesis followed by enzymatic macrolactamization. The excised thioesterase domain of tyrocidine synthetase was used as a prototype to validate that TyrF could be replaced by glycosyl-O-Tyr and glycosyl-O-Ser residues via solid-phase synthesis, and the modified peptidyl thioesters then undergo TE-mediated D-Phe-L-Leu-10 head-to-tail macrolactamization. Enzymatic macrolactamization proceeded with equal efficiency (80%–95%) for both glycosylated and non-glycosylated peptide thioesters. Three other residues, Asn, Gln, and Val, could also be replaced with galactosyl-O-Ser residues and could be cyclized by the TE domain. Only the galactosyl-O-Ser6 substrate showed slower flux to cyclization. The glycosylated-O-tyrocidine peptides retained antimicrobial activity but tended to be slightly less hemolytic. This chemoenzymatic approach to glycopeptides should be generalizable to TE domains from NRPS, PKS, and hybrid NRPS/PKS assembly lines, with distinct regiochemical assembly. Many of these cyclic products then undergo enzymatic steps of macrolactamization and glycosylation by dedicated tailoring glycosyltransferases. Glycosylation increases aqueous solubility, can protect the producer microorganism from the actions of the antibiotic, and/or can be required for biological activity. An alternative to nature’s use of tandem enzymatic steps of macrolactamization and glycosylation is a chemoenzymatic approach of glycopeptide thioester synthesis followed by enzymatic macrolactamization. The excised thioesterase domain of tyrocidine synthetase was used as a prototype to validate that TyrF could be replaced by glycosyl-O-Tyr and glycosyl-O-Ser residues via solid-phase synthesis, and the modified peptidyl thioesters then undergo TE-mediated D-Phe-L-Leu-10 head-to-tail macrolactamization. Enzymatic macrolactamization proceeded with equal efficiency (80%–95%) for both glycosylated and non-glycosylated peptide thioesters. Three other residues, Asn, Gln, and Val, could also be replaced with galactosyl-O-Ser residues and could be cyclized by the TE domain. Only the galactosyl-O-Ser6 substrate showed slower flux to cyclization. The glycosylated-O-tyrocidine peptides retained antimicrobial activity but tended to be slightly less hemolytic. This chemoenzymatic approach to glycopeptides should be generalizable to TE domains from NRPS, PKS, and hybrid NRPS/PKS assembly lines, with distinct regiochemical assembly of macrolactamization and macrolactonization to create libraries of neoglycosylated natural products. Experimental Procedures General Procedures for Chemical Synthesis All chemicals were purchased as reagent grade and were used without further purification. Dichloromethane (CH2Cl2) was distilled over calcium hydride, and tetrahydrofuran (THF) and diethyl ether were distilled over sodium/benzophenone ketyl. Anhydrous DMF was purchased from a commercial source. Reactions were monitored with analytical thin-layer chromatography (TLC) on silica gel (25%–50%) run three times, Rf = 0.08; (1.12 g, 0.87 mmol, 1 eq) and 1,3,4,6-tetra-O-acetyl-2,2'-dichloroethylenedioxybenzylamine) and 3,4,6-tri-O-acetyl-D-glucopyranosyl)-L-serine (2b) was prepared by reaction of Fmoc-Ser-OAll (0.8 mg, 0.14 mmol) and morpholine (24 μmol, 0.27 mmol) in CH2Cl2 (650 μL) according to the general procedure; spectral data are in agreement with those reported [26, 27].
aturation according to the general procedure, except the reaction was stirred with 1 eq glycosyl donor at room temperature for 12 hr before addition of the remaining 0.7 eq. The reaction was monitored by TLC with 25% ethyl acetate/hexanes run four times, Rf = 0.2: (1.8 g, 88%); Rf = 0.2 (40% ethyl acetate/hexanes); spectral data are in agreement with those reported [28].

N-(9-Fluorenylethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine Allyl Ester (3a)
Glycosyl amino acid 3 (150 mg, 0.18 mmol) and activated Zn dust (180 mg, 2.75 mmol) were dissolved in glacial acetic acid (2 ml) and stirred vigorously at room temperature for 4 hr. The reaction was filtered through Celite and dried under vacuum. Pyridine (2.25 ml) and acetic anhydride (1.13 ml) were added to this crude intermediate. The reaction was stirred overnight at room temperature and then concentrated. The residue was dissolved in ethyl acetate (20 ml) and then washed with 0.1 N HCl twice, saturated NaHCO3, and brine. The organic layer was dried over Na2SO4 and concentrated. The residue was purified by chromatography on a silica gel column; elution with a gradient of ethyl acetate in hexanes (60%-90%) afforded glycaside 3a as a white solid (108 mg, 86%); spectral data are in agreement with those reported [28].

N-(9-Fluorenylethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine Allyl Ester (3a)
MALDI-MS of C35H42N2O14 (M, 715) m/z 738 resin, filtered, and concentrated. Alternatively, the acetyl protecting groups on sugars, the peptides were treated with 0.2 M sodium methoxide in methanol for 15 min, then the reaction was quenched with Dowex 50WX8-200 acidic resin, filtered, and concentrated. Alternatively, the acetyl protecting groups on sugars, the peptides were treated with 0.2 M sodium methoxide in methanol for 15 min, then the reaction was quenched with Dowex 50WX8-200 acidic resin, filtered, and concentrated. Alternatively, the acetyl protecting

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N-(9-Fluorenylethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine Allyl Ester (3a)
MALDI-MS of C35H42N2O14 (M, 715) m/z 738 resin, filtered, and concentrated. Alternatively, the acetyl protecting
groups can be removed with 1:7 hydrazine hydrate (55%) methanol for 5 hr before cleavage of the peptide from the solid support.

To make the N-acetylcysteamine thioester, the peptides (25 μmol) were mixed with NAC (53 μL, 500 μmol), dicyclohexylcarbodi-imide (16 mg, 75 μmol), HOBT (12 mg, 75 μmol), and diisopropylthylamine (17 μL, 100 μmol) in THF (0.5 mL). The reaction was agitated at room temperature for 3 hr to overnight. After removal of the THF, the peptide thioesters were deprotected with 95:5 TFA/水/isopropanolamine (2 ml) or 16:3:1 TFA:CH2Cl2:NAC at ambient temperature for 4 hr. Then the peptide thioesters were precipitated with cold ether (40 mL), and the precipitates were collected by centrifugation. The precipitates were then dissolved in 50% acetonitrile/water and purified by reverse-phase (C18) HPLC with a gradient of 0–60% acetonitrile in a gradient of 0–100% acetonitrile over 30 min. The lyophilized products were dissolved in water for TE-catalyzed cyclization reactions. The reactions were carried out in 25 mM MOPS buffer (pH 7) with 0.1% Brij58. The substrate concentrations used were 0.5, 1, 2, 5, 10, 20, and 40 μM, and the enzyme concentration used was 5 nM. Otherwise, the procedure was the same as reported earlier [3]. The products were characterized by MALDI-MS: Tyc-S-NAC m/z 1390 ([M+H]+), calcd 1388.7; Ser, S-NAC m/z 1363 ([M+H]+), calcd 1361.7; Ser(ε-ε-D-Gal)-S-NAC m/z 1691 (M+), calcd 1691.8; Ser, S-D-Gal)-S-NAC m/z 1476 (M+), calcd 1474.7; Ser, S-D-Glcnac)-S-NAC m/z 1707 (M+), calcd 1706.8; Ser, S-D-Gal)-S-NAC m/z 1539 (M+), calcd 1538.7.

Determination of K 장, K substrate, and Cyclization to Hydrolysis Ratio
The lyophilized products were dissolved in water for TE-catalyzed cyclization reactions. The reactions were carried out in 25 mM MOPS buffer (pH 7) with 0.1% Brij58. The substrate concentrations used were 0.5, 1, 2, 5, 10, 20, and 40 μM, and the enzyme concentration used was 5 nM. Otherwise, the procedure was the same as reported earlier [3]. To get K 장, K substrate, and the concentration of absorption at 220 nm.

Preparation of Cyclized Glycopeptides
100 μM peptide-S-NAC thioesters were incubated for 3 hr with 1 μM Tyc TE in 25 mM MOPS buffer (pH 7) with 0.1% Brij58. The cyclic peptides were purified by preparative HPLC. Lyophilized cyclic peptides were dissolved in methanol. The purities were checked by analytical RP-HPLC (20–100% acetonitrile/0.1% TFA), and the products were characterized by preparative HPLC. Lyophilized cyclic peptides were dissolved in methanol. The purities were checked by analytical RP-HPLC (20–100% acetonitrile/0.1% TFA), and the areas of absorption at 220 nm with a known concentration of tyrosyl cyclase in 25 mM MOPS buffer (pH 7) with 0.1% Brij58. The substrate concentrations used were 5 μM and 50 μM, and the TE concentration was 50 mM. After 1 hr, the reaction was complete, and the cyclization to hydrolysis ratio was determined by LCMS by using the area of absorption at 220 nm.

MIC and MHC Determination for Variant Cyclic Peptides
In a 96-well plate, cyclic peptides were serially diluted in methanol and dried in a speedvac. For minimal inhibitory concentration (MIC) testing, an overnight B. subtilis PY79 culture was diluted (1/10,000) with LB media, and 80 µl of the diluted culture was added to each well. After overnight incubation at 30°C, the concentrations required for complete inhibition of bacterial cell growth were determined by visual inspection. Minimal hemolytic concentration (MHC) values were determined by the addition of 80 μl of human red blood cells (Research Blood Components) diluted (1:100) with PBS buffer (pH 7.4) to dried peptides. RBCs were incubated at room temperature overnight, and concentrations required for complete lysis were determined visually.

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