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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 megadalton tyrocidine synthetase (Tyc Syn) retains autonomous capacity to macrocyclize peptidyl thioesters to D-Phe$_1$-L-Leu$_{10}$-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, Tyr$_7$ 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-GlcNH$_2$, and these new analogs were shown to be cyclized with comparable k_{cat}/K_m catalytic efficiency. Similarly, Gal- or tetra-*O*-acetyl-Gal-Ser could also be substituted at residues 5, 6, and 8 in the linear decapeptidyl-*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 excised 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 decapeptidyl thioesters, generating the natural cyclic decapeptide 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 Tyr$_7$ and replaced it with protected glycosyl-β-Tyr and glycosyl-β-Ser residues via solid-phase synthesis of monoglycosyl decapeptidyl thioesters. We also substituted residues 5–8 with galactosyl-β-Ser to evaluate the permissiveness of Tyc Syn TE to macrocyclize the regioisomeric 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 decapeptidyl thioesters were synthesized essentially as previously reported [3], by solid-phase peptide synthesis with Fmoc-protected amino acids, including Tyr(Ac$_4$-β-D-Gal), Tyr(Ac$_4$-β-D-Glc), Ser(Ac$_4$-β-D-Gal), Ser(Ac$_4$-β-D-Glc), Ser(Ac$_3$-β-D-GlcNAc), and Ser(Ac$_3$-β-D-GlcNBoc) in place of Tyr$_7$, 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(Ac$_4$-β-D-Gal) was used in place of Asn$_5$, Gln$_6$, and Val$_8$ 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 decapeptidyl-*S*-NAC thioesters in place of the decapeptidyl-*S*-pantotheinyl-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

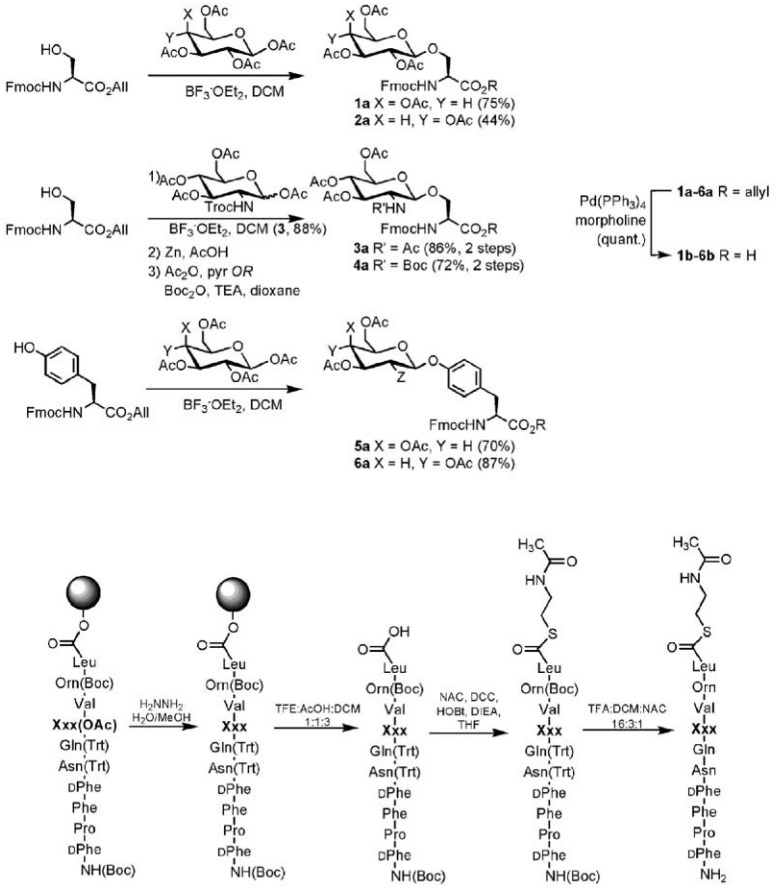

Figure 1. Synthesis of Glycosylated Amino Acids and Incorporation into Linear Peptidyl-*S*-NAC Thioesters via Solid-Phase Peptide Synthesis

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 Tyr_7 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

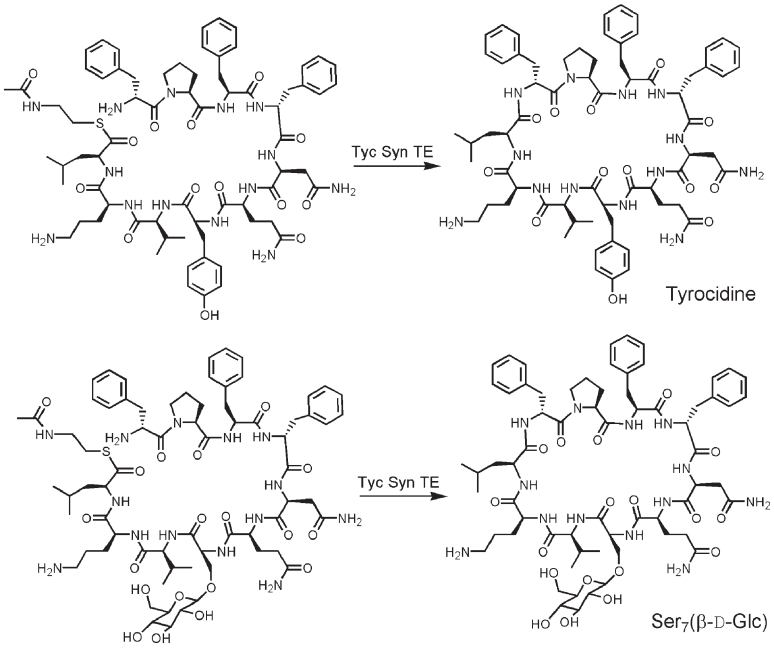

Figure 2. Tyc Syn TE-Catalyzed Head-to-Tail Cyclization to Produce Tyrocidine and Glycosyl-Tyrocidine Variants, with Ser_7(β-D-Glc) Shown as an Example

Table 1. Kinetic Parameters of Tyc Syn TE-Catalyzed Cyclization of Peptide Thioesters Substituted with Different Glycosyl-Tyr and -Ser at Position 7

	k_{cat} (min^{-1})[a]	K_m (μM)[a]	k_{cat}/K_m (min^{-1} μM^{-1})	Cyclization to Hydrolysis Ratio at 5 μM[b]	Cyclization to Hydrolysis Ratio at 50 μM[b]
Tyrocidine	110	1.4	79	17.2 ± 0.4	12.3 ± 0.3
Tyr_7(β-D-Gal)	59	8.5	6.9	10.1 ± 0.4	8.6 ± 0.1
Tyr_7(β-D-Glc)	82	8.3	9.9	10.8 ± 0.2	8.2 ± 0.1
Ser_7	69	15.0	4.6	6.7 ± 0.5	5.3 ± 0.5
Ser_7(β-D-Gal)	55	12.2	4.5	6.2 ± 0.1	5.6 ± 0.1
Ser_7(β-D-Glc)	55	15.4	3.6	10.8 ± 0.1	7.7 ± 0.1
Ser_7(β-D-GlcNAc)	101	11.4	8.9	10.6 ± 0.2	6.1 ± 0.2
Ser_7(β-D-$GlcNH_2$)	140	4.3	32.6	11.1 ± 0.1	8.0 ± 0.1

[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 decapeptidyl-*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)-, Ser_7(β-D-Glc)-, Ser_7(β-D-GlcNAc)-, and Ser_7(β-D-$GlcNH_2$)-decapeptidyl thioesters. All four glycosylated Ser_7 peptides are macrocyclized with 85%–90% of the flux to the macrolactam (Table 1).

To evaluate the tolerance of the Tyc Syn TE domain for glycosylated residues at other sites, Ser(β-D-Gal)- or Ser(Ac_4-β-D-Gal)-peptidyl thioesters were synthesized with the sugar substituent displayed at residue 5, 6, or 8 on the decapeptidyl thioester backbone, replacing Asn_5, Gln_6, or Val_8, respectively. In all cases, the unglycosylated serine-containing peptides (Ser_5, Ser_6, Ser_8) were prepared and assayed with the TE domain for comparison to the substrates containing glycosyl-serine. At positions 5–7, the galactosyl and tetra-*O*-acetyl-galactosyl 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_8(β-D-Gal) and 80% of the flux in the Ser_8(Ac_4-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-aminoglucosyl, and GlcNAc on Ser_7 or galactosyl and tetra-*O*-acetyl galactosyl residues on $Ser_{5–7}$ had only minimal effects on MIC and MHC values. However, the galactosylation at Ser_8 in both acetylated and nonprotected 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 macrolactamization catalyst. Prior work has indicated a broad tolerance of the excised TE for replacement of the side chains of the four residues, D-Phe_4, Asn_5, Gln_6, and Tyr_7, with re-

Table 2. Kinetic Parameters of Tyc Syn TE-Catalyzed Cyclization of Peptide Thioesters Substituted with Ser, Ser(β-D-Gal), and Ser(Ac_4-β-D-Gal) at Positions 5–8

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	Cyclization to Hydrolysis Ratio at 5 μM	Cyclization to Hydrolysis Ratio at 50 μM
Ser_5	46	0.8	58.5	7.3 ± 0.4	5.3 ± 0.1
Ser_5(β-D-Gal)	68	2.0	34	13.0 ± 1.6	8.9 ± 0.1
Ser_5(Ac_4-β-D-Gal)	111	4.4	25.2	6.9 ± 0.6	4.7 ± 0.1
Ser_6	42	0.5	84	13.6 ± 0.2	11.8 ± 0.7
Ser_6(β-D-Gal)	22	2.5	8.8	8.1 ± 0.1	7.0 ± 0.1
Ser_6(Ac_4-β-D-Gal)	24	2.5	9.6	10.0 ± 0.3	7.7 ± 0.1
Ser_7	69	15.0	4.6	6.7 ± 0.5	5.3 ± 0.5
Ser_7(β-D-Gal)	55	12.2	4.5	6.2 ± 0.1	5.6 ± 0.1
Ser_7(Ac_4-β-D-Gal)	40	13.8	2.9	3.9 ± 0.4	7.7 ± 0.1
Ser_8	112	6.2	18.1	9.8 ± 0.1	5.8 ± 0.3
Ser_8(β-D-Gal)	39	9.6	4.1	1.1 ± 0.2	1.3 ± 0.1
Ser_8(Ac_4-β-D-Gal)	94	14.3	6.6	0.21 ± 0.02	0.27 ± 0.01

Assay conditions were the same as in Table 1.

tention of regiospecific macrocyclization capacity [3, 7, 16–19].

Since many nonribosomal peptides undergo post-assembly line tailoring by dedicated glycosyltransferases [1, 8, 24] to create glycopeptides in which solubility and/or biological activity is altered by the tailoring process, we have begun to explore in this work whether one could provide glycosylated substrates to this TE domain and still see effective head-to-tail cyclization. To that end, we have replaced each of residues 5–8 with a serine side chain and observed maintenance of good ratios of enzymatic cyclization to hydrolysis of the serine-containing peptidyl thioesters. Our initial focus on Tyr_7 shows that incorporation of Tyr(β-D-Gal) or Tyr (β-D-Glc) during solid-phase synthesis of the decapeptidyl-*S*-NACs caused no problem for enzyme-directed flux of head-to-tail cyclization. Furthermore, four different β-glycosyl-Ser_7 substitutions for Tyr_7 were also not deleterious to TE-mediated flux to macrolactamization. Subsequently, we substituted Ser(β-D-Gal) in deprotected or tetra-*O*-acetyl-protected forms at each of residues 5–8. Only at residue 8 did the substitution interfere noticeably with the D-Phe_1-L-Leu_{10} enzymatic macrocyclization.

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

	MIC (μM)	MHC (μM)	Therapeutic Index (MHC/MIC)
Tyrocidine	1.5	25	16
Ser_5	3	12.5	4
Ser_5(β-D-Gal)	6	50	8
Ser_5(Ac_4-β-D-Gal)	6	25	4
Ser_6	1.5	12.5	8
Ser_6(β-D-Gal)	3	50	16
Ser_6(Ac_4-β-D-Gal)	6	25	4
Ser_7	1.5	12.5	8
Ser_7(β-D-Gal)	3	50	16
Ser_7(Ac_4-β-D-Gal)	3	25	8
Ser_7(β-D-Glc)	6	50	8
Ser_7(β-D-GlcNAc)	6	50	8
Ser_7(β-D-$GlcNH_2$)	6	50	8
Tyr_7(β-D-Gal)	6	50	8
Tyr_7(β-D-Glc)	6	50	8
Ser_8	6	25	4
Ser_8(β-D-Gal)	100	200	2
Ser_8(Ac_4-β-D-Gal)	12	100	8

We do not expect a priori for glycosylation of the membrane-seeking cyclic tyrocidine peptides to improve either antibiotic efficacy or necessarily reduce the liability of nonspecific pore formation in eukaryotic membranes (measured as hemolysis of human red cells). In fact, most of the glycosylated tyrocidines retained their antibiotic activity with equal or marginally decreased tendency to hemolysis. These initial data suggest that one could create libraries of glycosylated peptides to evaluate these and other biological activities.

Using the combination of chemoenzymatic steps noted here, it should be feasible to make libraries of cyclic tyrocidine peptides with monosaccharides or oligosaccharides at multiple residues. However, there is no reason to believe this approach is limited to the tyrocidine synthetase TE. We and others have noted that TE domains excised from additional NRPS assembly lines [4–6] retain stereo- and regiospecificity as catalysts to make branched macrocycles, both macrolactams and macrolactones. Therefore, libraries of glycopeptides on different peptide scaffolds should be accessible by use of glycosyl amino acids in solid-phase synthesis followed by macrocyclization by TE domains. This approach should be equally valid for converting lipopeptide antibiotics (e.g., daptomycin) into lipoglycopeptides. Further, converting the backbones of hybrid polyketide/nonribosomal peptides to regiospecific glycosylated versions may also be possible, provided the macrocyclizing TE domains show appropriate tolerance to glycosylation away from the site of macrocyclization. It should also be possible to chemically incorporate oligosaccharyl as well as monosaccharyl chains before or after TE-mediated scaffold peptide cyclizations, thereby providing a useful way to make glycopeptide immunogens.

Significance

A variety of nonribosomal peptide and polyketide natural products undergo regiospecific macrocyclization as the chain release step from their enzymatic assem-

bly 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 macrocyclization and glycosylation is a chemoenzymatic approach of glycopeptide thioester synthesis followed by enzymatic macrocyclization. The excised thioesterase domain of tyrocidine synthetase was used as a prototype to validate that Tyr_7 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_1-L-Leu_{10} head-to-tail macrocyclization. Enzymatic macrolactamization proceeded with equal efficiency (80%–95%) for both glycosylated and nonglycosylated peptide thioesters. Three other residues, Asn_5, Gln_6, and Val_8, could also be replaced with galactosyl-*O*-Ser residues and could be cyclized by the TE domain. Only the galactosyl-*O*-Ser_8 substrate showed lower 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 regio- and stereochemistry 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 (CH_2Cl_2) 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 60 F254 plates and visualized under UV (254 nm) and/or by staining with acidic cerium ammonium molybdate. ^{1}H NMR spectra were recorded on a 500 MHz spectrometer. Fmoc-Ser-OAll and Fmoc-Tyr-OAll were prepared according to the published procedure for allyl ester protection of amino acids [25].

General Procedure for Glycosylation of Amino Acid Allyl Esters

The Fmoc amino acid allyl ester (1 eq) and the peracetylated glycosyl donor (1 eq) were dissolved in dry CH_2Cl_2 under argon. The solution was cooled to 0°C, and $BF_3{\cdot}OEt_2$ (3 eq) was added dropwise. The reaction was monitored by TLC, and after the glycosyl donor was nearly consumed (~2 hr, unless otherwise noted), additional glycosyl donor (0.7–1 eq) and $BF_3{\cdot}OEt_2$ (0.7–1 eq) were added. If the glycosyl donor was consumed again and amino acid acceptor remained, additional glycosyl donor (1 eq) and $BF_3{\cdot}OEt_2$ (1 eq) were added, and the reaction was stirred overnight. The reaction was diluted with CH_2Cl_2, washed with 0.5 M aqueous $NaHSO_4$, and then washed with H_2O. The organic phase was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography on a silica gel column; elution with a gradient of ethyl acetate in hexanes (25%–50%) afforded the Fmoc glycosyl amino acid allyl ester as a white foam.

General Procedure for Allyl Ester Removal from Glycosyl Amino Acids

The Fmoc glycosyl amino acid (1 eq) and tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$, 0.01 eq) were dissolved in dry CH_2Cl_2 under argon. Morpholine (1.9 eq) was added dropwise. The reaction was stirred for 20 min, at which point the reaction was complete by TLC. The reaction was diluted with CH_2Cl_2, washed with 10% citric acid twice, and washed with brine once. The organic layer was dried over $MgSO_4$ and concentrated. The product was determined to be pure by NMR and was either used as is or after removal of the palladium with MP-TMT resin (Argonaut).

N-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine Allyl Ester (1a)

Compound **1a** was prepared by reaction of Fmoc-Ser-OAll (0.8 g, 2.18 mmol, 1 eq) and β-D-galactose pentaacetate (2.55 g, 6.53 mmol, 3 eq) in dry CH_2Cl_2 under $BF_3{\cdot}OEt_2$ promotion at 4°C by adding the glycosyl donor in three separate 1 eq additions according to the general procedure. The reaction was monitored by TLC with 25% ethyl acetate/hexanes run three times, R_f = 0.08: (1.12 g, 74%); R_f = 0.3 (40% ethyl acetate/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.99, 2.01, 2.03, 2.15 (4s, 3H each, Ac), 3.85 (t, 1H, *J* = 6.5 Hz, H-5), 3.89 (dd, 1H, *J* = 3.5 and 11 Hz, H-β), 4.11 (d, 2H, *J* = 7.0 Hz, H-6, H-6′), 4.24 (t, 1H, *J* = 7.0 Hz, Fmoc CH), 4.29 (dd, 1H, *J* = 3.0 and 10.5 Hz, H-β′), 4.4–4.45 (m, 1H, Fmoc OCH_2), 4.44 (d, 1H, *J* = 8.0 Hz, H-1), 4.45–4.55 (m, 2H, H-α, Fmoc OCH_2), 4.68 (d, 2H, *J* = 5.5 Hz, allyl OCH_2), 5.00 (dd, 1H, *J* = 3.5 and 10.5 Hz, H-3), 5.16 (dd, 1H, *J* = 8.0 and 10 Hz, H-2), 5.25 (dd, 1H, *J* = 1.0 and 10.5 Hz, allyl CH=CH_2), 5.34 (d, 1H, *J* = 17 Hz, allyl CH=CH_2), 5.38 (d, 1H, *J* = 3.0 Hz, H-4), 5.59 (d, 1H, *J* = 8.0 Hz, NH), 5.90 (m, 1H, allyl C*H*=CH_2), 7.3–7.8 (dd, dd, t, m, 8H, Fmoc Ar); MALDI-MS of $C_{35}H_{39}NO_{14}$ (M, 698) m/z 721 ($[M+Na]^+$); high-resolution MS (HRMS, ESI-TOF high-acc) *m/z* calcd for $C_{35}H_{39}NO_{14}$ ($[M+Na]^+$) 720.2263, found 720.2259.

N-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine (1b)

Compound **1b** was prepared by reaction of **1a** (600 mg, 0.86 mmol) with $Pd(PPh_3)_4$ (9.9 mg, 8.6 μmol) and morpholine (143 μl, 1.63 mmol) in CH_2Cl_2 (4 ml) according to the general procedure; spectral data are in agreement with those reported [26, 27].

N-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine Allyl Ester (2a)

Compound **2a** was prepared by reaction of Fmoc-Ser-OAll (414 mg, 1.13 mmol, 1 eq) and β-D-glucose pentaacetate (1.3 g, 3.33 mmol, 3 eq) in dry CH_2Cl_2 under $BF_3{\cdot}OEt_2$ promotion at 4°C by adding the glycosyl donor in three separate 1 eq additions according to the general procedure. The reaction was monitored by TLC with 25% ethyl acetate/hexanes run three times, R_f = 0.08: (346 mg, 44%); R_f = 0.3 (40% ethyl acetate/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.00, 2.01, 2.03, 2.07 (4s, 3H each, Ac), 3.65 (m, 1H, H-5), 3.86 (dd, 1H, *J* = 3 and 10.5, H-β), 4.10 (dd, 1H, *J* = 12 Hz), 4.23 (m, 2H, H-6′, Fmoc CH), 4.29 (dd, 1H, *J* = 2.0 and 10.5 Hz, H-β′), 4.42 (dd, 1H, *J* = 7.0 and 10.5, Fmoc OCH_2), 4.46–4.50 (m, 3H, Fmoc OCH_2, H-1 (*J* = 7.5 Hz), H-α), 4.66 (d, 2H, *J* = 4.0 Hz, allyl OCH_2), 4.95 (dd, 1H, *J* = 8.5 and 9.0 Hz, H-2), 5.06 (t, 1H, *J* = 9.5 Hz, H-4), 5.19 (t, 1H, *J* = 10.5 Hz, H-3), 5.24 (d, 1H, *J* = 10.5 Hz, allyl CH=CH_2), 5.32 (d, 1H, *J* = 17.5 Hz, allyl CH=CH_2), 5.62 (d, 1H *J* = 13.0 Hz), 5.89 (m, 1H, allyl CH), 7.3–7.8 (t, t, d, d, 8H, Fmoc Ar); MALDI-MS of $C_{35}H_{39}NO_{14}$ (M, 698) m/z 721 ($[M+Na]^+$); HRMS (ESI-TOF high-acc) calcd for $C_{35}H_{39}NO_{14}$ ($[M+H]^+$) 698.2443, found 698.2438.

N-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine (2b)

Compound **2b** was prepared by reaction of **2a** (100 mg, 0.14 mmol) with $Pd(PPh_3)_4$ (1.66 mg, 1.4 μmol) and morpholine (24 μl, 0.27 mmol) in CH_2Cl_2 (650 μl) according to the general procedure; spectral data are in agreement with those reported [26, 27].

N-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2-deoxy-2-(2′,2′,2′-trichloroethoxycarbonylamino)-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-L-serine Allyl Ester (3)

Compound **3** was prepared by reaction of Fmoc-Ser-OAll (1.0 g, 2.72 mmol, 1 eq) and 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-(2′,2′,2′-trichloroethoxycarbonylamino)-D-glucopyranoside (2.42 g, 4.62 mmol, 1.7 eq) in dry CH_2Cl_2 under $BF_3{\cdot}OEt_2$ promotion at room temper-

ature 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, R_f = 0.2: (1.8 g, 88%); R_f = 0.2 (40% ethyl acetate/hexanes); spectral data are in agreement with those reported [28].

***N*-(9-Fluorenylmethoxycarbonyl)-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 $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 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 glycoside **3a** as a white solid (108 mg, 86%); spectral data are in agreement with those reported [28].

***N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine (3b)**

Compound **3b** was prepared by reaction of **3a** (63 mg, 0.09 mmol) with $Pd(PPh_3)_4$ (1.05 mg, 0.9 μmol) and morpholine (15 μl, 0.17 mmol) in CH_2Cl_2 (500 μl) according to the general procedure, except the workup was done with only one wash with 10% citric acid; spectral data are in agreement with those reported [28].

***N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-*tert*-butoxycarbonylamino-2-deoxy-β-D-glucopyranosyl)-L-serine Allyl Ester (4a)**

Glycosyl amino acid **3** (1 g, 1.2 mmol) and activated Zn dust (1.3 g, 20 mmol) were dissolved in glacial acetic acid (10 ml) and stirred vigorously at room temperature for 5 hr. The reaction was filtered through Celite and dried under vacuum. To this crude intermediate, under argon, was added di-*tert*-butyl-dicarbonate (369 mg, 1.69 mmol) and dry 1,4-dioxane (10 ml). Then, triethylamine (505 μl, 3.62 mmol) was added dropwise. The reaction was stirred overnight at room temperature and then concentrated. The residue was dissolved in ethyl acetate (35 ml) and then washed with saturated $NaHCO_3$ and then H_2O. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on a silica gel column; elution with a gradient of ethyl acetate in hexanes (40%–60%) afforded glycoside **4a** as a white solid (659 mg, 72%); R_f = 0.4 (50% ethyl acetate/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$)· δ 1.41 (s, 9H, *t*-Bu), 2.02, 2.04, 2.06 (3s, 3H each, Ac), 3.50 (m, 1H, H-2), 3.6–3.7 (m, 1H, H-5), 3.90 (dd, 1H, *J* = 2.5 and 10.5, H-β), 4.10 (dd, 1H, *J* = 2.0 and 12.5, H-6), 4.2–4.3 (m, 3H, H-6′, H-β′, Fmoc CH), 4.33 (dd, 1H, *J* = 7.5 and 10.5, Fmoc OCH_2), 4.48 (dd, 1H, *J* = 7.0 and 10.5, Fmoc OCH_2), 4.56 (m, 1H, H-α), 4.6–4.7 (m, 2H, allyl OCH_2), 4.74 (br d, 1H, H-1), 5.02 (dd, 1H, *J* = 9.5 and 10, H-4), 5.2–5.4 (m, 3H, H-3, allyl CH=CH_2), 5.87 (m, 2H, NH, allyl C*H*=CH_2), 7.3–7.8 (m, t, t, d, 8H, Fmoc Ar); MALDI-MS of $C_{38}H_{46}N_2O_{14}$ (M, 755) m/z 778 ([M+Na]$^+$); HRMS (ESI-TOF high-acc) *m/z* calcd for $C_{38}H_{46}N_2O_{14}$ ([M+Na]$^+$) 777.2847, found 777.2845.

***N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(3,4,6-tri-*O*-acetyl-2-*tert*-butoxycarbonylamino-2-deoxy-β-D-glucopyranosyl)-L-serine (4b)**

Compound **4b** was prepared by reaction of **4a** (68.3 mg, 0.09 mmol) with $Pd(PPh_3)_4$ (1.05 mg, 0.9 μmol) and morpholine (15 μl, 0.17 mmol) in CH_2Cl_2 (500 μl) according to the general procedure; R_f = 0.4 (10% methanol/CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$)·δ 1.40 (s, 9H, *t*-Bu), 2.01, 2.03, 2.06 (3s, 3H each, Ac), 3.55 (m, 1H, H-2), 3.65 (m, 1H, H-5), 3.96 (dd, 1H, *J* = 7.0 Hz), 4.1–4.2 (m, 1H, H-6), 4.2–4.3 (m, 3H, H-6′, H-β, Fmoc CH), 4.34 (dd, 1H, *J* = 7.5 and 10.5 Hz, Fmoc OCH_2), 4.45 (dd, 1H, *J* = 7.0 and 8.0 Hz, Fmoc OCH_2), 4.56 (m, 1H, H-α), 4.69 (br d, 1H, H-1), 5.02 (dd, 1H, *J* = 9.0 and 10.0 Hz, H-4), 5.24 (m, 1H, H-3), 6.00 (d, 1H, *J* = 8.0 Hz, NH), 7.3–7.8 (m, t, dd, d, 8H, Fmoc Ar); MALDI-MS of $C_{35}H_{42}N_2O_{14}$ (M, 715) m/z 738 ([M+Na]$^+$); HRMS (ESI-TOF high-acc) *m/z* calcd for $C_{35}H_{42}N_2O_{14}$ ([M+Na]$^+$) 737.2528, found 737.2530.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-tyrosine Allyl Ester (5a)**

Compound **5a** was prepared by reaction of Fmoc-Tyr-OAll (113.4 mg, 0.26 mmol, 1 eq) and β-D-galactose pentaacetate (300.5 mg, 0.77 mmol, 3 eq) in dry CH_2Cl_2 under BF_3·OEt_2 promotion at 4°C by adding the glycosyl donor in three separate 1 eq additions according to the general procedure (138 mg, 70%). The reaction was monitored by TLC with 40% ethyl acetate/hexanes run twice, R_f = 0.3: (138 mg, 70%); R_f = 0.16 (40% ethyl acetate/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.02, 2.03, 2.04, 2.16 (4s, 3H each, Ac), 3.0–3.2 (m, 2H, H-β, and -β′), 3.91 (t, 1H, *J* = 6.5 Hz, Fmoc CH), 4.05–4.2 (m, 3H, Fmoc OCH_2 and H-5), 4.28 (dd, 1H, *J* = 7.0 and 10.5 Hz, H-6), 4.43 (dd, 1H, *J* = 7.0 and 10.5 Hz, H-6′), 4.62 (d, 2H, *J* = 5.5 Hz, allyl OCH_2), 4.67 (m, 1H, H-α), 4.91 (d, 1H, *J* = 8.0 Hz, H-1), 5.08 (dd, 1H, *J* = 3.0 and 4.0 Hz, H-3), 5.25 (d, 1H, *J* = 10.5, allyl CH=CH_2), 5.30 (d, 1H, *J* = 17.5, allyl CH=CH_2), 5.35–5.5 (m, 3H, NH, H-2, H-4), 5.8–5.95 (m, 1H, C*H*=CH_2), 6.90 (d, 2H, *J* = 8.5 Hz, Tyr Ar), 7.04 (d, 2H, *J* = 8.5 Hz, Tyr Ar), 7.2–7.8 (m, t, dd, d, 8H, Fmoc Ar); MALDI-MS of $C_{41}H_{43}NO_{14}$ (M, 774) m/z 797 ([M+Na]$^+$); HRMS (ESI-TOF high-acc) *m/z* calcd for $C_{41}H_{43}NO_{14}$ ([M+H]$^+$) 774.2756, found 774.2750.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-tyrosine (5b)**

Compound **5b** was prepared by reaction of **5a** (70 mg, 0.09 mmol) with $Pd(PPh_3)_4$ (1.05 mg, 0.9 μmol) and morpholine (15 μl, 0.17 mmol) in CH_2Cl_2 (500 μl) according to the general procedure; spectral data are in agreement with those reported [26].

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-tyrosine Allyl Ester (6a)**

Compound **6a** was prepared by reaction of Fmoc-Tyr-OAll (414.3 mg, 1.13 mmol, 1 eq) and β-D-glucose pentaacetate (1.3 g, 3.33 mmol, 3 eq) in dry CH_2Cl_2 under BF_3·OEt_2 promotion at 4°C by adding the glycosyl donor in three separate 1 eq additions according to the general procedure (346 mg, 44%). The reaction was monitored by TLC with 40% ethyl acetate/hexanes run twice, R_f = 0.3: (346 mg, 44%); R_f = 0.16 (40% ethyl acetate/hexanes); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.02–2.04 (4s, 3H each, Ac), 3.0–3.2 (m, 2H, H-β, and -β′), 3.71 (m, 1H, H-5), 4.09 (m, 1H, H-6′), 4.17 (t, 1H, *J* = 7.0 Hz, Fmoc CH), 4.26 (m, 2H, H-6 and Fmoc OCH_2), 4.44 (dd, 1H, *J* = 7.0 and 10.5 Hz, Fmoc OCH_2), 4.62 (d, 2H, *J* = 5.5 Hz, allyl OCH_2), 4.68 (q, 1H, *J* = 6.0 Hz, H-α), 4.94 (d, 1H, *J* = 7.0 Hz, H-1), 5.14 (t, 1H, *J* = 9 Hz, H-4), 5.2–5.35 (m, 4H, H-2, H-3, Fmoc CH=CH_2), 4.1 (d, 1H, *J* = 7.5 Hz, NH), 5.87 (m, 1H, allyl C*H*=CH_2), 6.89 (d, 2H, *J* = 8 Hz, Tyr Ar), 7.05 (d, 2H, *J* = 8 Hz, Tyr Ar), 7.2–7.8 (m, t, dd, d, 8H, Fmoc Ar); MALDI-MS of $C_{41}H_{43}NO_{14}$ (M, 774) m/z 797 ([M+Na]$^+$); HRMS (ESI-TOF high-acc) *m/z* calcd for $C_{41}H_{43}NO_{14}$ ([M+H]$^+$) 774.2756, found 774.2742.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-tyrosine (6b)**

Compound **6b** was prepared by reaction of **6a** (100 mg, 0.13 mmol) with $Pd(PPh_3)_4$ (1.5 mg, 1.3 μmol) and morpholine (21.4 μl, 0.25 mmol) in CH_2Cl_2 (600 μL) according to the general procedure; spectral data are in agreement with those reported [26].

Synthesis of Linear Peptide *N*-acetylcysteamine Thioesters

Linear peptides were synthesized by hand or on a Symphony peptide synthesizer by using leucine-derivatized 2-chlorotrityl resin with five equivalents of 0.2 M Fmoc-protected amino acids in DMF and diisopropylcarbodiimide/hydroxybenzotriazole (DIPCDI/HOBt) activation, except that the last amino acid, D-Phe_1, was Boc protected. The peptides were cleaved from the solid support with 1:1:3 trifluoroethanol:acetic acid:CH_2Cl_2, and the protected linear peptides were obtained by azeotrope with hexanes to remove the cleavage reagents.

To remove 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 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), dicyclohexylcarbodiimide (16 mg, 75 μmol), HOBt (12 mg, 75 μmol), and diisopropylethylamine (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 95:5:5 TFA:water:triisopropylsilane (2 ml) or 16:3:1 TFA:CH_2Cl_2: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 (C_{18}) HPLC with a gradient of 20%–100% acetonitrile/0.1% TFA over 60 min. The peptidyl-*S*-NACs were characterized by MALDI: Tyc-*S*-NAC *m/z* 1390 ([M+H]$^+$), calcd 1388.7; Ser_5-*S*-NAC *m/z* 1363 ([M+H]$^+$), calcd 1361.7; Ser_5(Ac_4-β-D-Gal)-*S*-NAC *m/z* 1691(M$^+$), calcd 1691.8; Ser_5(β-D-Gal)-*S*-NAC *m/z* 1524 (M$^+$), calcd 1523.7; Ser_6-*S*-NAC *m/z* 1348 (M$^+$), calcd 1347.7; Ser_6(Ac_4-β-D-Gal)-*S*-NAC *m/z* 1677 (M$^+$), calcd 1677.8; Ser_6(β-D-Gal)-*S*-NAC *m/z* 1510 (M$^+$), calcd 1509.7; Ser_7-*S*-NAC *m/z* 1313 (M$^+$), calcd 1312.7; Ser_7(Ac_4-β-D-Gal)-*S*-NAC *m/z* 1643 (M$^+$), calcd 1642.8; Ser_7(β-D-Gal)-*S*-NAC *m/z* 1476 ([M+H]$^+$), calcd 1474.7; Ser_7(β-D-Glc)-*S*-NAC *m/z* 1476 ([M+H]$^+$), calcd 1474.7; Ser_7(β-D-GlcNAc)-*S*-NAC *m/z* 1517 ([M+H]$^+$), calcd 1515.7; Ser_7(β-D-GlcNH_2)-*S*-NAC *m/z* 1475 ([M+H]$^+$), calcd 1473.7; Tyr_7(β-D-Gal)-*S*-NAC *m/z* 1552 ([M+H]$^+$), calcd 1550.8; Tyr_7(β-D-Glc)-*S*-NAC *m/z* 1552 ([M+H]$^+$), calcd 1550.8; Ser_8-*S*-NAC *m/z* 1377 (M$^+$), calcd 1376.7; Ser_8(Ac_4-β-D-Gal)-*S*-NAC *m/z* 1707 (M$^+$), calcd 1706.8; Ser_8(β-D-Gal)-*S*-NAC *m/z* 1539 (M$^+$), calcd 1538.7.

Determination of k_{cat}, K_m, 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_{cat}, K_m values, the initial reaction rates obtained were fit to the Michaelis-Menten equation, with R values greater than 0.96.

To determine the cyclization to hydrolysis ratio, reactions were carried out in triplicate 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 nM. 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.

Preparation of Cyclized Glycopeptides

100 μM peptidyl-*S*-NAC thioesters were incubated for 3 hr with 1 μM Tyc TE in 25 mM MOPS (pH 7) and 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 concentrations were determined by comparing the area of absorption at 220 nm with that of a known concentration of tyrocidine linear peptide thioester. The cyclic peptides were characterized by LCMS or MALDI: Tyc *m/z* 1271 ([M+H]$^+$), calcd 1269.7; Ser_5 *m/z* 1243 (M$^+$), calcd 1242.6; Ser_5(Ac_4-β-D-Gal) *m/z* 1573 (M$^+$), calcd 1572.7; Ser_5(β-D-Gal) *m/z* 1405 (M$^+$), calcd 1404.7; Ser_6 *m/z* 1229 (M$^+$), calcd 1228.6; Ser_6(Ac_4-β-D-Gal) *m/z* 1559 (M$^+$), calcd 1558.7; Ser_6(β-D-Gal) *m/z* 1391 (M$^+$), calcd 1390.7; Ser_7 *m/z* 1194 (M$^+$), calcd 1193.6; Ser_7(Ac_4-β-D-Gal) *m/z* 1524 (M$^+$), calcd 1523.7; Ser_7(β-D-Gal) *m/z* 1356 (M$^+$), calcd 1355.7; Ser_7(β-D-Glc) *m/z* 1356 (M$^+$), calcd 1355.7; Ser_7(β-D-GlcNAc) *m/z* 1397 (M$^+$), calcd 1396.7; Ser_7(β-D-GlcNH_2) *m/z* 1355 (M$^+$), calcd 1354.7; Tyr_7(β-D-Gal) *m/z* 1432 (M$^+$), calcd 1431.7; Tyr_7(β-D-Glc) *m/z* 1432 (M$^+$), calcd 1431.7; Ser_8 *m/z* 1258 (M$^+$), calcd 1257.6; Ser_8(Ac_4-β-D-Gal) *m/z* 1588 (M$^+$), calcd 1587.7; Ser_8(β-D-Gal) *m/z* 1420 (M$^+$), calcd 1419.7.

MIC and MHC Determination for Variant Cyclic Peptides

In a 96-well plate, cylic 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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