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A Chemoenzymatic Approach to Glycopeptide Antibiotics

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Abstract: Many biologically active natural products are constrained by macrocyclization and modified with carbohydrates. These two types of modifications are essential for their biological activities. Here we report a chemoenzymatic approach to make carbohydrate-modified cyclic peptide antibiotics. Using a thioesterase domain from the decapeptide tyrocidine synthetase, 13 head-to-tail cyclized tyrocidine derivatives were obtained with one to three propargylglycines incorporated at positions 3−8. These cyclic peptides were then conjugated to 21 azido sugars via copper(I)-catalyzed cycloaddition. Antibacterial and hemolytic assays showed that the two best glycopeptides, Tyc4PG-**14** and Tyc4PG-**15**, have a 6-fold better therapeutic index than the natural tyrocidine. We believe this method will also be useful for modifying other natural products to search for new therapeutics.

Introduction

A variety of natural products with antibiotic activities are of polyketide (PK) origin, such as erythromycin and daunomycin, or of nonribosomal peptide (NRP) origin, such as novobiocin and vancomycin, or are PK/NRP hybrids, such as bleomycin (Figure 1). Macrocyclization and glycosylation are the two late-stage modifications in the biosynthesis of many PK or NRP natural products, the stage that usually confers the biological activity *per se*, or an improved property such as solubility or target affinity.[1] The widespread occurrence of such tailoring macrocyclization and glycosylation encourages us to make novel glycosylated cyclic peptides and to study the effect of different carbohydrates on their activity.

We have designed a chemoenzymatic approach to make carbohydrate-modified cyclic peptides, which begins, as the first example, with the cyclic decapeptide tyrocidine (Tyc) as the peptide scaffold onto which various carbohydrates will be attached. The head-to-tail cyclic peptide scaffold of Tyc can be generated enzymatically by the excised thioesterase domain (TE) from the tyrocidine synthetase using the corresponding linear peptide *N*-acetyl cysteamine (SNAC) thioester as the substrate (Figure 2A).[2] This 35 kDa TE has relaxed substrate specificity and can tolerate substitutions at most of the substrate's 10 residues.[2,3] Cyclization is accompanied by variable amounts of enzyme-mediated, competing thioester hydrolysis. Utilizing Tyc TE's relaxed substrate specificity, we planned to make alkyne-containing cyclic peptides by enzymatic macrocyclization and then conjugated them to azido sugars to produce glycosylated cyclic peptides using the copper(I)-catalyzed [3 + 2] cycloaddition, a reaction condition that was recently developed by Sharpless and co-workers (Figure 2B).[4] This cycloaddition reaction has been used by several groups in various bioconjugation experiments and has proved to be robust.[5−9] In the past two decades, the ability to make complex glycoconjugates, including glycopeptides and glycoproteins, has increased dramatically.[10,11] In the field of natural product modification, both chemical and enzymatic methods and natural and unnatural linkages have been used to conjugate carbohydrates to peptides.[12−19] However, chemical methods to form glycoconjugates normally require several transformations to install appropriate functional groups for the conjugation reaction, and the yield could be low. On the other hand, enzymatic methods suffer from the availability of glycosyl donor substrates (normally TDP-sugars, which are difficult to make even via

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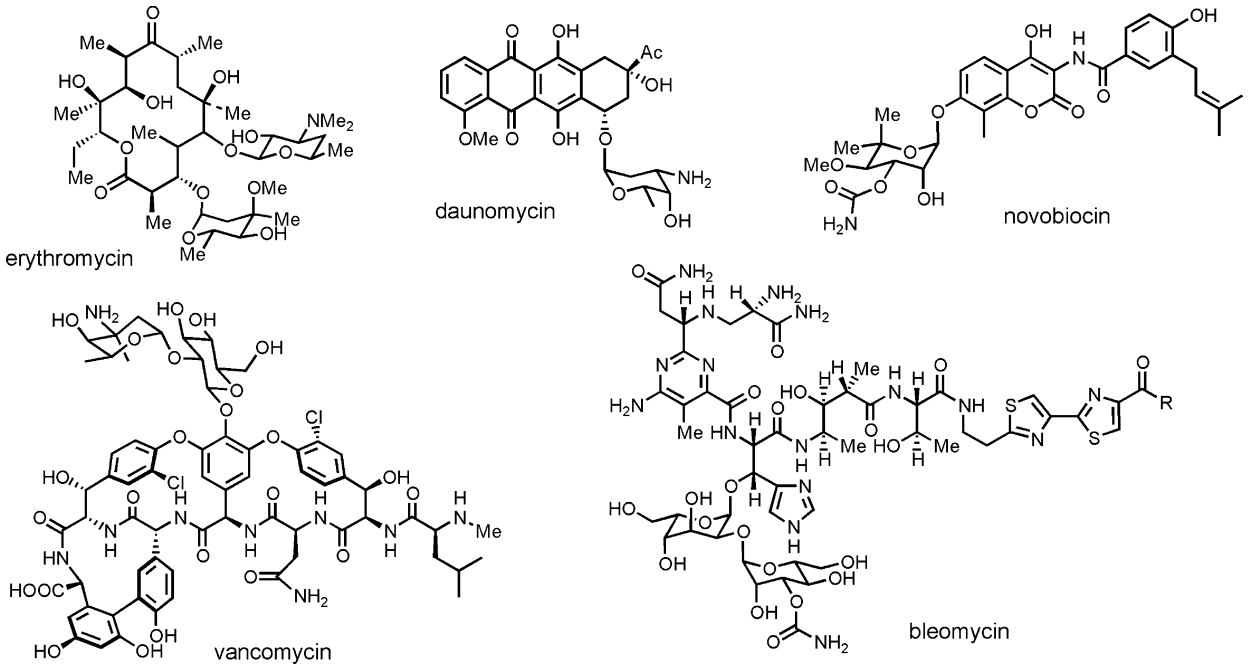

Figure 1. Structures of representative natural antibiotics with carbohydrate moieties.

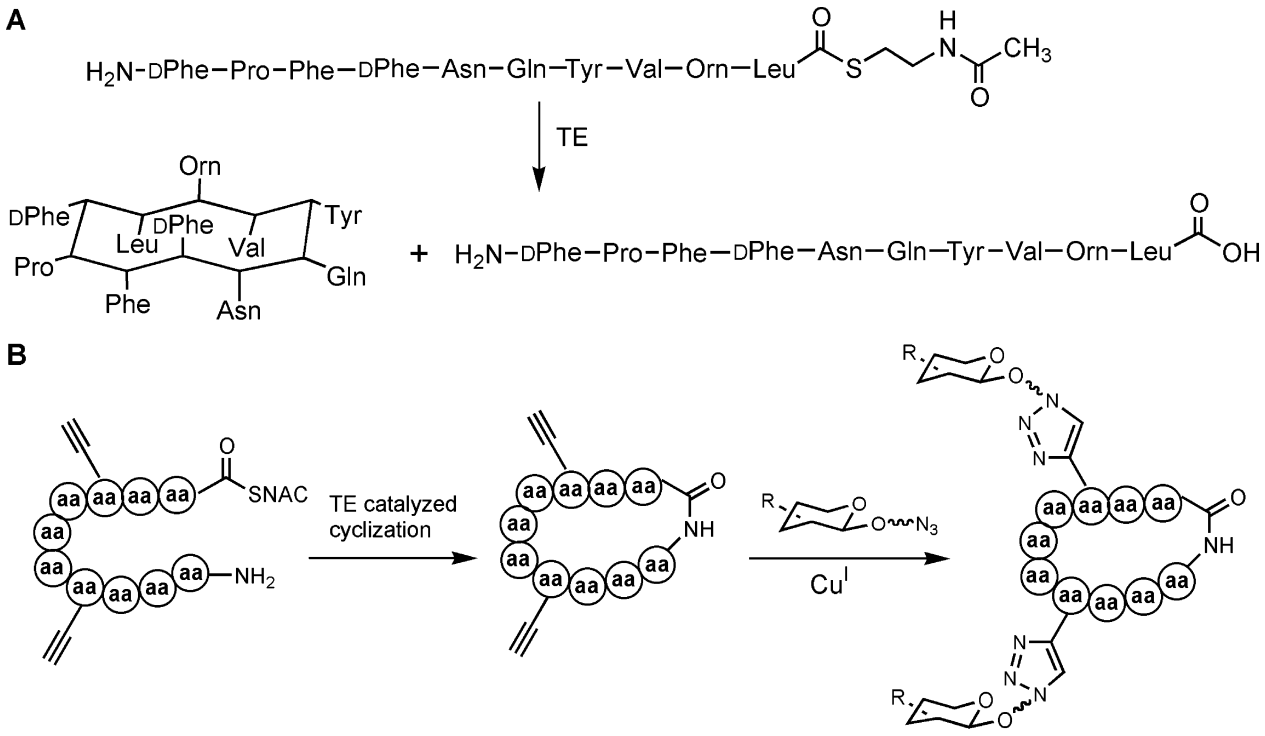

Figure 2. Chemoenzymatic approach (B) to make glycopeptidevariants of the antibiotic tyrocidine using Tyc TE catalyzed macrocyclization (A) and copper(I) catalyzed [3 + 2] cylcoaddition.

enzymatic methods[19]) and substrate specificity of the glycosyltransferases. Therefore, new chemoselective ligation reactions could potentially offer advantages.

Tyc is a NRP antibiotic, and it is believed that it targets the lipid bilayer of bacteria to make pores.[20] Because there is no specific bacterial protein target for the cyclic peptides of the tyrocidine class, drug resistance is rare, which is particularly beneficial considering the increasing antibiotic resistance in bacteria. However, Tyc has the liability that it can also insert into eukaryotic membranes and cause lysis of human red blood cells, which limits its systemic application.[21] Here, by introducing carbohydrates into Tyc, we seek to improve its therapeutic index which is defined by the ratio of the minimal hemolytic concentration (MHC) to the minimal inhibitory concentration (MIC) of bacteria growth. It should be noted that even though carbohydrates are important for the functions of many natural

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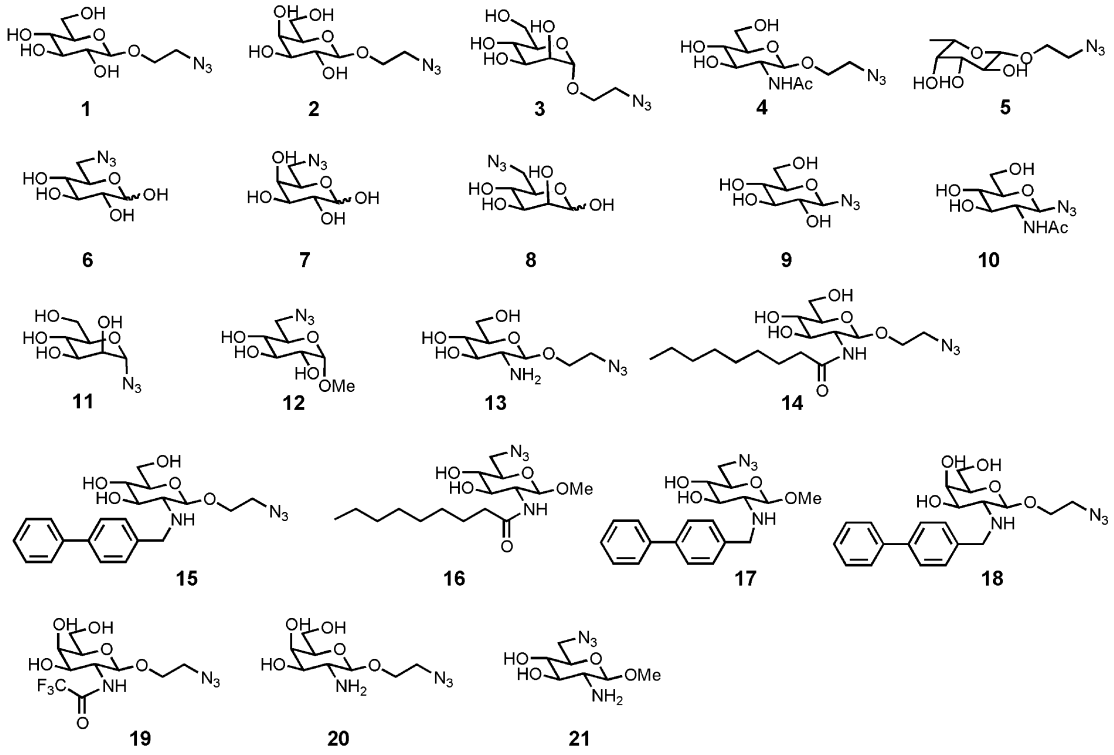

Figure 3. 21 azido sugars used in this study.

products, modification of a natural product's sugar portion may not always confer beneficial effects, as indicated by related work on vancomycin.[14,18,19] Therefore, to maximize the chance of our success with the modification of tyrocidine, we decided to take a combinatorial approach, and as will be shown, the choice of Sharpless's click chemistry greatly facilitates its implementation.

Results

Macrocyclization of Propargylglycine Substituted Tyc Linear Peptide−Thioesters by TE. Tyc linear peptides containing propargylglycine (PG) at different positions were synthesized on solid support. For monosubstituted peptides, position 2−8 were used. For di- and tri-substituted peptides, only positions 5−8 were used because these positions are more tolerant to substitutions than others for subsequent TE-mediated head-to-tail macrolactamization. After cleavage from solid support, these peptides were then coupled to *N*-acetylcysteamine and deprotected using published procedures.[2] Seven mono-substituted, six di-substituted, and four trisubstituted propargylglycine variant peptide-SNACs were synthesized. These 17 peptide-SNACs were then subjected to TE-catalyzed cyclization. Most of them (13 out of 17) can be cyclized with reasonable efficiency (Table 1). The competing reaction is TE-mediated hydrolysis of the peptidyl-SNAC as noted by cyclization/hydrolysis ratio. For these 13 peptide−SNACs, preparative scale reactions were carried out to give enough quantities of the cyclic peptides to be used in the subsequent cycloaddition reaction.

Choice and Synthesis of Azido Monosacchrides. For azido sugars, we have focused initially on monohexoses (Figure 3), most of which (**1**−**12**) can be easily accessed following published procedures.[22−28] The acyl chain and the biphenyl group in azido sugars **14**−**18** were incorporated to increase membrane affinity as inspired by teicoplanin and other vancomycin-type lipoglycopeptide antibiotics.[29] The detailed synthesis of **13**−**21** can be found in the Supporting Information.

Table 1. Cyclization Efficiency of PG-Substituted Peptide−SNACs

peptide−SNAC substrate	complete consumption of substrate?[a]	cyclization-to-hydrolysis ratio[a]	cyclic peptide prepared?
2PG	−	ND[b]	−
3PG	+	3.5	+
4PG	+	6.2	+
5PG	+	3.7	+
6PG	+	11.1	+
7PG	+	3.2	+
8PG	+	3.3	+
56PG	−	0.04	−
57PG	+	1.2	+
58PG	+	2.3	+
67PG	−	0.1	−
68PG	+	3.8	+
78PG	+	2.4	+
567PG	−	0.9	+
568PG	−	ND[b]	−
578PG	+	0.5	+
678PG	+	3.4	+

[a] Determined with 100 μM substrate and 100 nM TE, room temperature, 1 h. [b] ND: not determined because almost no reaction occurred.

Preparation of the Glycopeptide Library by Click Chemistry. We carried out the cycloaddition reaction in 96-well plates to facilitate subsequent MIC and MHC assays. Sharpless's

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Table 2. MIC and MHC Values for Reagents Used in the [3 + 2] Cycloaddition Reaction

reagent	MIC/μM	MHC/μM
Cu^{2+}	1250	160
ascorbate	>10000	>10000
TCEP	5000	1250
tris(triazolyl)amine ligand	5000	>10000
tyrocidine	1.5	25
tyrocidine with 2.5 equiv of Cu^{2+}, 5 equiv of ascorbate, and 5 equiv of ligand	1.5	12.5

original condition for azido−alkyne [3 + 2] cycloaddition is to use sodium ascorbate to reduce Cu^{II} to Cu^{I}.[4] However, in later publications, slightly different conditions have been used; for example, the use of Cu wire or tris(carboxyethyl)phosphine (TCEP) as reducing reagent and the use of a tris(triazolyl)amine ligand.[5−8] For our experiments, we chose to run the reaction at about millimolar concentrations and then carry out MIC and MHC assays initially without any purification process. Therefore, we utilized reagents that give fast reaction rates and do not interfere with the MIC and MHC assays. Sodium ascorbate was the reducing reagent of choice since it is less toxic than TCEP to both bacteria and human red blood cells (Table 2). Using cyclic peptide Tyc3PG and azido sugar **1**, we found conditions (0.7 mM peptide, 1.4 mM azido sugar, 1.7 mM $CuSO_4$, 3.4 mM ascorbate in a 60 μL reaction) that give complete conversion of starting material for mono-PG-substituted peptides in 2 h. The reaction products, however, contain both the desired triazole product and a minor product whose mass is the desired triazole product plus 16. We assigned this M + 16 product as the 5-hydroxytriazole product, which has been observed before.[4] Under similar conditions, the reactions involving di- and trisubstituted peptides could not go to completion. However, this problem was solved by the addition of tris(triazolyl)amine ligand (0.7 mM peptide, 2.7 or 3.6 mM azido sugar, 1.8 mM $CuSO_4$, 3.6 mM ligand, and 22 mM ascorbate in a 55 μL reaction). Under this condition, the formation of 5-hydroxytriazole is almost completely suppressed.

Using these two sets of conditions, a library of 13 × 21 cycloaddtion reactions were conducted. The quality of the product library was determined by LCMS. For most monosubstituted peptides the quality is good, with >90% conversion to products and minimal 5-hydroxytriazole products. But for disubstituted peptides, incomplete reactions were observed when using azido sugars **17** and **18**. For trisubstituted peptides, incomplete reactions were observed when using azido sugars **13**, **15**, **17**, **18**, **20**, and **21**. Therefore, the total number of glycopeptides made in this two-step enzymatic macrocyclization, triazole-forming glycosylation is 247.

Biological Activity Assays of the Glycopeptide Library. We then carried out biological activity assays of these glycopeptides. After the cycloaddition reaction, the reaction mixtures were used directly for MIC and MHC assays.[3] As a control it was important to make sure that the reagents (Cu^{2+}, ascorbate, and the ligand) used in the cycloaddition do not interfere with the assays at the concentrations used. Data in Table 2 showed that all the reagents used have MIC and MHC values greater than 1 mM, except that copper(II) has a MHC of 160 μM. Since tyrocidine's MIC is only 1.5 μM and MHC is 25 μM, these reagents, when used in severalfold excess, should not interfere with the MIC and MHC assays. This is further supported by tyrocidine's MIC and MHC values measured in the presence of all three reagents in 2.5- or 5-fold excess. The MIC and MHC values of the 21 azido sugars were also tested and are all greater than 200 μM. Therefore the use of more than 1 equiv of azido sugars should cause no problems for the assays.

The tested concentration range of glycopeptides is from 200 to 1.5 μM for MHC and 25 to 0.1 μM for MIC. The MIC and MHC data for the mono-glycosylated peptides are shown in Table S2 in the Supporting Information. These data reveal two trends: first, most simple sugars cannot increase the therapeutic index (MHC/MIC) of the peptides, and the best sugars (**14** and **15**) are those that have either a nonoyl group or a biphenyl group; second, the best position to put the sugars is position 4, while positions 5 and 6 are also acceptable.

Among the di- and tri-glycosylated peptides, none has better therapeutic index (data not shown) than tyrocidine. In fact, most of them have MIC's higher than 25 μM. It seems that modifying more than one position with the 20 or so sugars is detrimental to the antibiotic activity, at least for positions 5−8.

Biological Activity Assays of Selected Glycopeptides Using Purifed Products. To verify the library MIC and MHC assay results, the cycloaddition was repeated to synthesize the two glycopeptides with the best therapeutic index, Tyc4PG-**14** and Tyc4PG-**15** (Figure 4). MIC and MHC assays were then carried out using HPLC purified compounds. The result indicates that Tyc4PG-**14** and Tyc4PG-**15** have at least a 6-fold better therapeutic index compared with wild-type tyrocidine (entries 1−3 in Table 3). Two other glycopeptides Tyc4PG-**18** and Tyc7PG-**1** were also purified and assayed (entries 4 and 5 in Table 3) to further examine the results from library screening. The assay results using the cycloaddition mixtures are similar to those obtained using purified compounds.

Divalent Display of Azido Sugars at Postion 4. Since the best position to bear the sugar is position 4, it was of interest to see whether more than one sugar at position 4 would further affect MHC and MIC values. To this end, two branched glycan chain peptides, Tyc4PG-$\mathbf{14_2}$ and Tyc4PG-$\mathbf{15_2}$ (Figure 4), were synthesized. However, the MIC and MHC assays showed these two glycopeptides have no advantage compared with Tyc4PG-**14** and Tyc4PG-**15**, even though Tyc4PG-$\mathbf{15_2}$ is still slightly better than tyrocidine itself (Table 3, entries 6 and 7).

Discussion

We have generated a library of 247 glycopeptides by combining enzymatic macrocyclization by TE and the ligation reaction via click chemistry. The relaxed substrate specificity of TE from the tyrocidine synthetase enabled the preparation of unprotected Tyc cyclic peptide derivatives with propargylglycine substituted at various positions, further demonstrating the utility of this enzyme. The unique features of the copper(I)-catalyzed [3 + 2] cycloaddition, such as the mild reaction condition, the orthogonality of azide and alykne with other functional groups, the ease of preparing azido glycosides, and the ability of the reaction to proceed at less than 1 mM concentration on a 40 nmol scale, greatly facilitated the construction of the library in a relatively short period of time. It also facilitated the subsequent MIC and MHC assays because the reaction is clean and the reagents used do not interfere with the assays at the concentrations used. For a combinatorial library approach, these features are essential and are advantageous

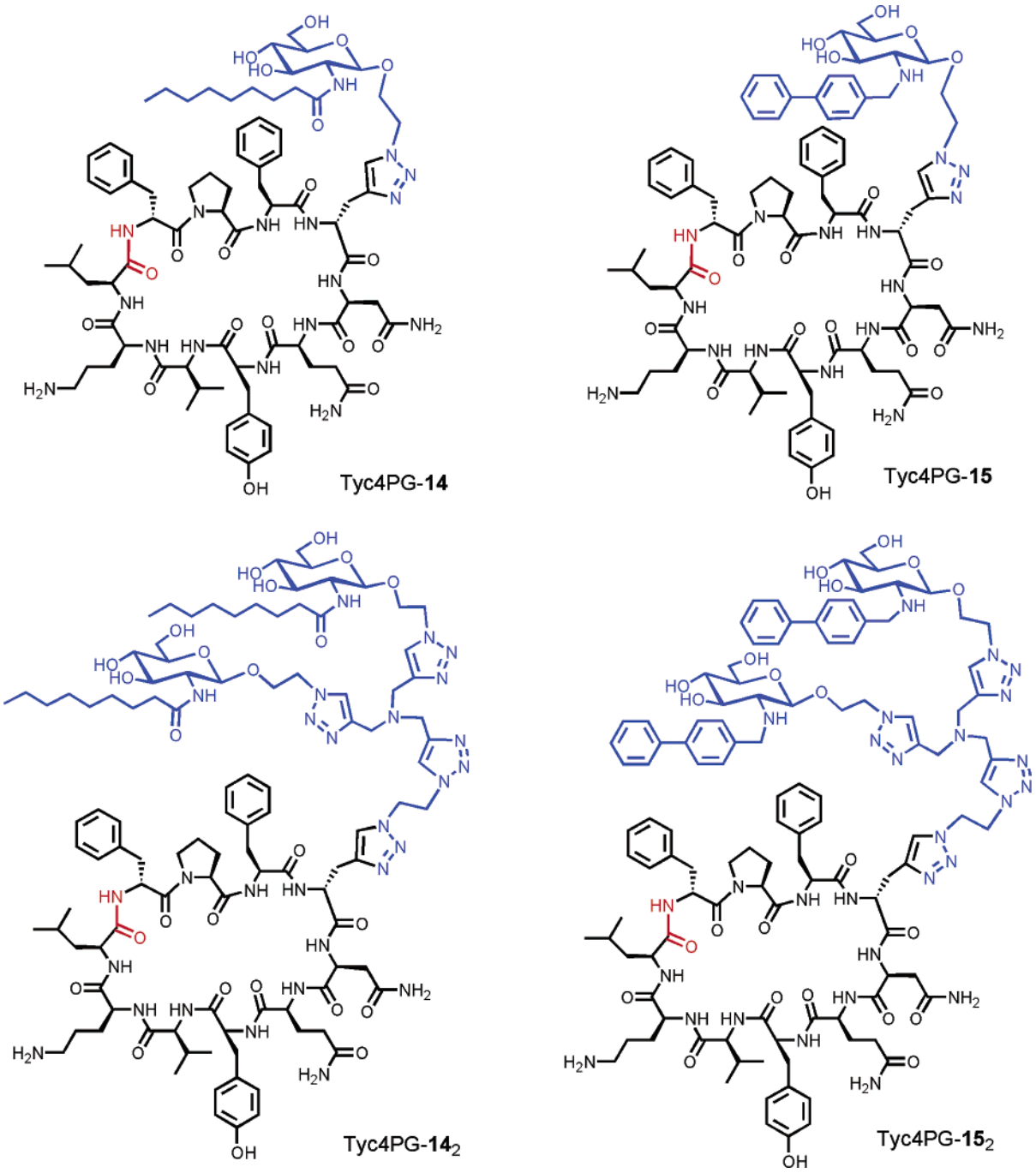

Figure 4. Structures of glycopeptide Tyc4PG-**14**, Tyc4PG-**15**, Tyc4PG-**14**$_2$, and Tyc4PG-**15**$_2$.

Table 3. MIC and MHC Values Obtained Using Purified Glycopeptides

	peptides	MIC/μM	MHC/μM	MHC/MIC
1	tyrocidine	1.5	25	16
2	Tyc4PG-**14**	3	>300	>100
3	Tyc4PG-**15**	3	>300	>100
4	Tyc4PG-**18**	6	100	16
5	Tyc7PG-**1**	6	25	4
6	Tyc4PG-**14**$_2$	12	25	2
7	Tyc4PG-**15**$_2$	1.5	50	32

compared with the *in vitro* glycorandomization method reported in a recent publication,[19] in which only a portion of the library generated was assayed for biological activity. Of course, as with any other method, limitations do exist for the click chemistry approach. For example, it is not easy to simultaneously ligate different azido sugars to the Tyc scaffold with multiple propargylglycine residues.

From this 247-member library, glycopeptides with more than 6-fold improved therapeutic indexes over the native Tyc were obtained while maintaining Tyc's antibacterial potency. It is worth noting that carbohydrate randomization of natural products does not always improve activity.[14,18,19] This is probably because the functional mechanisms of the carbohydrate portion and/or the rest of the natural product in each case could be different. Such complication is also clearly evident in our study. For example, we found that position 4 is the best position to attach the sugars, and the best sugars are those with lipophilic chains (**14** and **15**). While it is easy to explain the function of the

lipophilic chains' since they are known to increase membrane affinity, it is difficult to explain why similar azido sugars with only slight changes in the sugar portion, such as **16**−**18**, gave different results from those of **14** and **15**. Similarly, without molecular details of the interactions of these glycopeptides with bacterial and human membranes, it is difficult to explain the selectivity observed. This emphasizes the importance of a combinatorial library approach for this kind of study.

It is also interesting that displaying multiple sugars on the Tyc scaffold did not further improve the therapeutic index of tyrocidine, even with azido sugars **14** and **15**. For Tyc cyclic peptides with multiple propargylglycine residues at positions 5−8, one could argue that the sugars are not displayed at the right positions. However, for Tyc4PG-$\mathbf{14_2}$ and Tyc4PG-$\mathbf{15_2}$, the result is still negative. For Tyc4PG-$\mathbf{15_2}$, both MIC and MHC are lower than those of Tyc4PG-**15**, indicating that displaying two copies of **15** does increase membrane affinity. However, the change in MHC is more significant than the change in MIC, resulting in a lower therapeutic index compared with Tyc4PG-**15**. Multivalent effect can exist when a targeted partner has multiple binding sites.[30] *A priori*, it is not clear that this condition would apply to tyrocidine, and it is possible that the conjugated sugars could somehow block the function of tyrocidine as implied by the MIC and MHC of Tyc4PG-$\mathbf{14_2}$. Therefore, it may not be surprising that displaying multiple sugars on the Tyc scaffold did not further improve the therapeutic indexes in this study.

There is no reason to think this approach will be limited to the tyrocidine skeleton. We believe this combination of enzyme-mediated linear precursor macrocyclization followed by azide glycoside cycloaddition will be generalizable to other TE-mediated macrocyclizations in both polyketide and hybrid polyketide/nonribosomal peptide scaffolds.[31−33] By combining chemical modifications to introduce alkyne groups, this strategy should also be useful for modification of other natural products that are not accessible through TE-mediated cyclization to search for new therapeutics.

Experimental Section

Preparation of PG-Substituted Tyc Cyclic Peptides. Tyc TE was expressed and purified as previously reported.[2] Linear peptides were synthesized on a Symphony peptide synthesizer using standard Fmoc solid-phase peptide synthesis (the last residue D-Phe_{10} was Boc protected). The peptides were cleaved from the 2-Cl Trt resin (3:1:1 dichloromethane:trifluoroethanol:acetic acid), and solvents were removed by azeotroping with hexanes. The peptides (∼50 μmol) were then coupled to *N*-acetylcysteamine (10 equiv) using dicyclohexylcarbodiimide (2 equiv)/1-hydroxybenzotriazole hydrate (2 equiv)/*N*,*N*-diisopropylethylamine (4 equiv) in tetrahydrofuran (THF; 1 mL). After removal of THF, the peptide−SNAC thioesters were deproteced using 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropylsilane and HPLC purified (20−100% acetonitrile/0.1% TFA over 50 min). Cyclic peptides were prepared using 100 μM peptide−SNAC thioesters and 100 nM TE in 25 mM MOPS buffer (pH 7) with 0.1% Brij58 at room temperature for 3 h. The cyclization-to-hydrolysis ratio was calculated on the basis of the areas of absorption at 220 nm from LCMS. The cyclic peptides were purified by HPLC (30−100% acetonitrile/0.1% TFA over 40 min). The lyophilized peptides were dissolved in methanol, and the concentrations were determined by analytical HPLC using a known concentration of Tyc peptide−SNAC thioester.

[3 + 2] Cycloaddition for Mono-PG-Substituted Tyc Peptides. The following solutions were made: 1.5 mM cyclic peptides in methanol (solution A), 50 mM azido sugars in methanol (solution B), 10 mM $CuSO_4$ in water (solution C), and 10 mM sodium ascorbate (solution D, make before use). The reactions were carried out in 96-well plates. Reagents were added in the following sequence: 27 μL of solution A, 1.6 μL of solution B, 10 μL of solution C, and 20 μL of solution D. The wells were then immediately sealed with Thermowell sealers (Corning Incorporated, Catalog No. 6570), and the plates were incubated with shaking at room temperature for 3 h. The reactions were checked by LCMS to ensure the quality of the glycopeptide library.

[3 + 2] Cycloaddition for Di- and Tri-PG-Substituted Tyc Peptides. The condition was similar to that for the mono-PG-substituted Tyc peptides unless otherwise stated. The following solutions were made in addition to solutions A and B: 20 mM $CuSO_4$ in water (solution E), 120 mM sodium ascorbate (solution F, make before use), and 20 mM tris(triazolyl)amine ligand (solution G). Reagents were added in the following sequence: 27 μL of solution A, 3 μL (for di-PG-substituted peptides) or 4 μL (for tri-PG-substituted peptides) of solution B, 5 μL of solution E, 10 μL of solution G, and 10 μL of solution F.

MHC Assay of the Glycopeptide Library. To the reaction mixtures were added appropriate amounts of methanol to make the final volume 100 μL. Then 50 μL was transferred to new 96-well plates to make serial 2× dilutions (200 μM to 1.6 μM) of the cyclic peptides with methanol. Methanol was then removed with a speedvac. Human red blood cells (Research Blood Components, Boston, MA) were diluted 100× with PBS buffer (pH 7.4), and 50 μL was added to each well. The plates were incubated at room temperature with rocking, and the concentrations required for complete lysis were determined visually after overnight incubation.

MIC Assay of the Glycopeptide Library. A 10 μL aliquot of the diluted reaction mixture was transferred to 96-well plates to make serial 2× dilutions (25 μM to 0.2 μM) with methanol. Methanol was then removed with a speedvac. An overnight *B. subtilis* PY79 culture was diluted 10000× with LB media, and 80 μL of the diluted culture was added to each well. After incubation in a shaker at 30 °C overnight, the concentrations required for complete inhibition of bacterial cell growth were determined visually.

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Supporting Information Available: The synthesis of azido sugars **13**−**21**, $\mathbf{14_2}$, and $\mathbf{15_2}$ and MS, MIC, and MHC data for the monosubstituted glycopeptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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