Tailoring of Glycopeptide Scaffolds by the Acyltransferases from the Teicoplanin and A-40,926 Biosynthetic Operons

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Summary

The teicoplanin acyltransferase (Atf) responsible for N-acylation of the glucosamine moiety to create the teicoplanin lipoglycopeptide scaffold has recently been identified. Here we use that enzyme (tAtf) and the cognate acyltransferase from the related A-40,926 biosynthetic cluster (aAtf) to evaluate specificity for glycopeptide scaffolds and for the acyl-CoA donor. In addition to acylation of 2-aminoethylglycosylpectide scaffolds with $k_{cat}$ values of $400–2000 \text{ min}^{-1}$, both Atfs transfer acyl groups to regioisomeric 6-amino-glucosyl scaffolds and to glucosyl scaffolds at rates of 0.2–0.5 $\text{min}^{-1}$ to create variant lipoglycopeptides. Using the teicoplanin glycosyltransferase tGtfA, tAtf, and GtFD, a glycosyltransferase from the vancomycin producer, it is possible to assemble a novel lipoglycopeptide with GlcNAc at $\beta$-OH-Tyr$_6$ and an $N_\beta$-acyl-glucosaminyln-vancosamine at Phegly$_4$. This study illustrates the utility of chemo- and regioselective acyltransferases and glycosyltransferases to create novel lipoglycopeptides.

Introduction

The family of glycopeptide antibiotics is composed of two subfamilies containing the clinically useful antibiotics vancomycin and teicoplanin, respectively (Figure 1A). Each has an oxidatively crosslinked heptapeptide scaffold constructed by nonribosomal peptide synthetase assembly lines [1, 2], with sugars added by dedicated glycosyltransferases (Gtfs) in post assembly line tailoring (Figure 1B). Vancomycin has the disaccharide L-vancomosaminyl-1,2-D-glucose attached to the phenolic hydroxyl of phenylglycine$_6$ (Phegly$_6$) of the scaffold by the tandem actions of GtfE and GtfD [3, 4]. Teicoplanin is glycosylated with glucosamine on Phegly$_4$ by tGtfB and is then N-acylated, capping the sugar against further elongation. A separate Gtf, tGtfA, adds $N_\beta$-acyetylglucosamine (GlcNAc) to the $\beta$-OH of $\beta$-OH-Tyr$_6$, and finally, mannosylation occurs on residue 7 of the crosslinked heptapeptide by a mannosyltransferase. The teicoplanin family member A-40,926, also shown in Figure 1A, differs from teicoplanin by positions of aromatic ring chlorination, an N-terminal methyl group, the identity of the acyl chain, the absence of a GlcNAc at the $\beta$-OH of scaffold residue 6, and a $\text{COOH}$ oxidation state on the N-decanoyl-sugar residue [5].

Perhaps the critical difference between teicoplanin, a lipoglycopeptide, and vancomycin, a glycopeptide, is the presence of the acyl chain which has been implicated in its mechanism of antimicrobial activity [6]. Teicoplanin retains efficacy against type B strains of vancomycin-resistant enterococci (VRE), while type A strains of VRE are resistant to both teicoplanin and vancomycin [7, 8]. The DNA sequences of biosynthetic gene clusters for the vancomycin analog chlormycamin [9], for teicoplanin [1, 2], and for the teicoplanin analogs A-49,734 [10] and A-40,926 [5] have been reported. This has enabled the identification and characterization of tailoring Gtfs [3, 4] and more recently of the tailoring acyltransferase (Atf) that adds the decanoyl group to the glucosamine moiety of teicoplanin [1].

The A-40,926 cluster, but not the A-49,734 cluster, has a homologous Atf [1, 5, 10], and we have now subcloned that enzyme and purified it from E. coli. The two enzymes share 67% sequence identity and 79% homology. We report the characterization and substrate specificities of both the teicoplanin Atf (tAtf) and the A-40,926 Atf (aAtf) using several glycopeptide intermediates from both the vancomycin and teicoplanin subfamilies as scaffold substrates and a variety of acyl-CoAs as acyl donor substrates. Remarkably, while the normal substrate for the Atfs is a 2-aminoethylglucosamine scaffold, we find that a regioisomeric 6-amino-glucosyl scaffold on both the teicoplanin scaffold and the vancomycin scaffold can be acylated at high pH. $k_{cat}$ values of 0.2 $\text{min}^{-1}$, down about three orders of magnitude from 400–2000 $\text{min}^{-1}$ determined here with natural 2-aminoethylglycosyl scaffolds, still suffice for preparative reactions. The $N_\beta$-acylglicosaminyl scaffolds can be further glycosylated, on the 2-OH, by the L-vancomosaminyl transferase in the vancomycin maturation pathway to create new lipoglycopeptide antibiotics. This approach offers a chemoenzymatic route to this class of compounds which shows good activity against vancomycin-resistant strains [11–13].

Results

Cloning, Expression, and Purification of Acyltransferases

Spencer and colleagues have recently identified the acyltransferase in the teicoplanin biosynthetic gene cluster, tei orf 11′ (tAtf), and validated its activity after purification from E. coli overproduction, although no kinetic or specificity characterization was reported [1]. They have also proposed that dbv orf 8 (aAtf) is the corresponding aminosugar $N$-acyltransferase from the A-40,926 producer [1]. In this paper we focus on the kinetic characterization and substrate specificities of these two enzymes. The two enzymes were PCR am-
Figure 1. Biosynthesis of Glycopeptide Antibiotics

(A) Chemical structures of three important glycopeptide and lipoglycopeptide antibiotics.
(B) The linear peptide backbone of the glycopeptide family of antibiotics is synthesized by NRPS machinery. The backbone is then acted on by a number of tailoring enzymes including crosslinking oxidases, glycosyltransferases, acyltransferases, and methylases.

Splified from cosmid DNA provided to us by the Donadio group at Vicuron Pharmaceuticals [2, 5], cloned into the pET22b expression vector, and expressed in E. coli as C-terminal His6-tagged proteins. The proteins were purified by nickel chromatography to greater than 95% purity based on SDS-PAGE analysis with a yield of 27 mg protein per liter of cell culture for each enzyme (data not shown).

Chemoenzymatic Synthesis of Glycopeptide Scaffolds

The Atf enzymes acylate the sugar amino group of their corresponding 2-aminoglucosyl crosslinked heptapeptide scaffolds [1]. While there are slight variations between the teicoplanin and A-40,926 backbones (i.e., N-terminal methylation, differential aromatic chlorination, the absence of a GlcNAc at the β-OH of scaffold residue 6, and a C6-COOH oxidation state on the N-decanoyl-sugar residue), we have chosen to use the teicoplanin scaffold due to its availability [5]. The teicoplanin aglycone (AGT) and vancomycin aglycone (AGV) can be obtained from teicoplanin or vancomycin through the acid hydrolysis of the sugar groups in trifluoroacetic acid [14, 15]. The aglycone can then be glycosylated with a variety of different sugar groups using the vancomycin or chloreremomycin glycosyltransferases as previously described [3, 4]. We enzymatically synthesized 10 mg of 2-aminoglucosyl AGT, 6-aminoglucosyl AGT, glucosyl AGT, and 2-aminoglucosyl AGV, using the GtfE enzyme, the corresponding UDP-sugar, and the heptapeptide aglycone scaffold as shown in Figure 2A.

Since GtfE has relaxed aglycone specificity [4], we were also able to glycosylate scaffolds where the amino terminus was protected with the Alloc protecting group [6]. This allowed the synthesis of the N-decanoyl-6-aminoglucosyl AGT standard chemoenzymatically by enzymatically glycosylating the protected aglycone, chemically acylating the free amine on the 6-aminoglucosyl residue, and finally deprotecting the N terminus of the heptapeptide.

Initial Kinetic Characterization of the A-40,926 Acyltransferase

A Tf was incubated with 2-aminoglucosyl AGT and octanoyl-CoA, and the ensuing reaction was monitored by HPLC. A representative A280 HPLC chromatogram is
Figure 2. Synthesis of Glycopeptides and UDP-6-Aminoglucose

(A) Chemoenzymatic synthesis of glycopeptides used in this study. (a) 500 μM aglycone scaffold, 500 μM UDP-sugar, 450 μM GtfE. (b) decanoyl succinimide (3 equiv.), Et3N, DMF, 16 hr, 57%. (c) Me2NH·BH3 (6 equiv), Pd(PPh3)4 (0.4 equiv), DMF, 30 min, 66%.

(B) Synthesis of UDP-6-aminoglucose used in the chemoenzymatic synthesis of 6-aminoglucosyl-containing scaffolds. (d) AcOH/H2O 4:1; (e) Ac2O, pyridine, DMAP, 81% (two steps); (f) hydrazine acetate, DMF, 73%; (g) 1. dicyanoethyl diisopropylphosphoramidite, tetrazole, CH2Cl2; 2. mCPBA, CH2Cl2; 3. isomerization with BF3·OEt2, 69%; (h) NaOMe, MeOH; (i) H2, Pd-C, MeOH; (j) Tce succinimide, 80% (three steps); (k) UMP morpholidate, tetrazole, pyridine, 2 d; (l) Bu4NF, DMF/THF, 45% (two steps).

shown in Figure 4E (bottom trace). The formation of a new product with a retention time of 12 min was observed, and the mass of the new peak determined by LCMS [M + H]+ = 1485.7 was consistent with the calculated mass of N-octanoyl-2-aminoglucosyl AGT [M + H]+ = 1485.4. To further verify the product of the acylation reaction, we compared a reaction mixture containing decanoyl-CoA to a previously reported authentic standard, N-decanoyl-2-aminoglucosyl AGT [6]. Coinjection of the reaction mixture and the standard showed that the enzymatic product coeluted with the chemically synthesized standard (data not shown). Steady-state kinetic analysis shows that the kcat of aAtf is 480 min−1, the Km of 2-aminoglucosyl AGT is 136 μM, and the Km of octanoyl-CoA is 22 μM at pH 7 (see Table 1). Parallel determination of the catalytic rate of tAtf re-
Table 1. aAtf Kinetic Parameters

<table>
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<tr>
<th>Scaffold</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ $\mu$M$^{-1}$)</th>
<th>pH</th>
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<tbody>
<tr>
<td>2-NH$_2$-glucosyl AGT</td>
<td>480</td>
<td>136</td>
<td>3.53</td>
<td>7</td>
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<tr>
<td>2-NH$_2$-glucosyl AGV</td>
<td>214</td>
<td>209</td>
<td>1.02</td>
<td>7</td>
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<tr>
<td>2-NH$_2$-glucosyl AGT</td>
<td>592</td>
<td>18</td>
<td>32.9</td>
<td>9</td>
</tr>
<tr>
<td>6-NH$_2$-glucosyl AGT</td>
<td>0.253</td>
<td>29</td>
<td>0.0087</td>
<td>9</td>
</tr>
<tr>
<td>Glucosyl AGT</td>
<td>0.574</td>
<td>42</td>
<td>0.0137</td>
<td>9</td>
</tr>
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Parameters reported are the average of triplicate experiments.

revealed a robust $k_{cat}$ of 1800 min$^{-1}$, approximately 3-fold faster than the A-40,926 enzyme.

**Acyl Chain Specificity**

Teicoplanin and A-40,926 are naturally found as a mixture of related compounds differing in the identities of their acyl chains. The predominant form of A-40,926 has an N-decanoyl modification, while there are five major teicoplanin counterparts, all having between 10 and 11 carbons on their acyl chains [1, 2, 5]. These natural substrate tolerances led us to further explore the capabilities of these Atfs to act as combinatorial chemoenzymatic tools to generate libraries of novel lipoglycopeptides. The specificities of both Atfs were tested using the series of acyl-CoA substrates shown in Figure 3A. With the exception of hexanoyl-CoA and biphenyl-CoA, which were synthesized using the general fatty acid/coenzyme A condensation strategy previously reported [16], all of these substrates were commercially available.

The results of the acyl chain specificity analysis are shown in Figures 3B and 3C. Both enzymes had a preference for long-chain acyl-CoAs. While trace amounts of product formation were observed using isobutyryl-, β-hydroxybutyryl-, crotonoyl-, and benzoyl-CoA by LCMS, the turnover was too slow to be quantified by HPLC. Biphenyl-CoA was not a substrate for either enzyme. The best substrate for aAtf was the C$_{12}$ acyl chain of lauroyl-CoA, while the best substrate for tAtf was decanoyl-CoA. In general the most robust substrates were acyl chains containing between 6 and 14 carbons. Across the acyl chain range, tAtf maintained a higher activity than aAtf.

**Sugar Specificity**

In addition to scaffold and acyl chain specificities, the ability of the acyltransferase enzymes to recognize alternate sugars was examined. In the physiologic setting, the Atf enzymes recognize the nucleophilic amino nitrogen at the 2 position of the glucosyl ring. To see if acylation would proceed at other positions on the ring, 6-aminoglucosyl AGT was synthesized and tested. When
Figure 4. pH Dependence on aAtf Activity with Alternate Substrates
(A) pH dependence on 2-aminoglucosyl AGT utilization. Data were fit to a single ionization resulting in an estimated pKa of 6.7.
(B) pH dependence on glucosyl AGT utilization.
(C) pH dependence on 6-aminoglucosyl AGT utilization.
(D) Plot depicting how the ratio of products changes with increasing pH for reactions containing glucosyl AGT or 6-aminoglucosyl AGT.
(E) Representative A280 HPLC traces showing the substrates and products from reactions where 2-aminoglucosyl-, 6-aminoglucosyl-, and glucosyl AGT served as substrates at pH 7.0. The y axis is linear and is in units of absorbance.

Table 2. LCMS Results for Heptapeptide Acylation with Octanoyl-CoA

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<tbody>
<tr>
<td>2-NH₂-glucosyl AGT</td>
<td>1359.3</td>
<td>1359.8</td>
<td>+2</td>
<td>1485.4</td>
<td>1485.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-NH₂-glucosyl AGV</td>
<td>1304.4</td>
<td>1305.6</td>
<td>+2</td>
<td>1430.5</td>
<td>1431.0</td>
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<tr>
<td>6-NH₂-glucosyl AGT</td>
<td>1359.3</td>
<td>1359.6</td>
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<td>1485.4</td>
<td>1486.6</td>
<td>1486.8</td>
<td>+1</td>
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<tr>
<td>Glucosyl AGT</td>
<td>1360.3</td>
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<td>+1</td>
<td>1486.3</td>
<td>1487.1</td>
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flies as a singly charged ion by LCMS analysis as expected, since there is only one amine available on the molecule for ionization. In order to provide NMR evidence for the position of acylation on glycosyl scaffolds, we choose to acylate the glycosyl vancomycin scaffold. We isolated 5 mg of the major acylated product by HPLC. The presence of a 2→O→acyl group was confirmed by its 1H NMR spectrum (500 MHz, d6 DMSO). The signal for the H-2 proton (δ = 4.85) of the glucose residue (assigned based on the COSY cross peaks) showed a distinct shift compared to the H-2 signal obtained for glycosyl vancomycin (δ = 3.55), characteristic for an acyl group attached to this position. No shift was observed at the other positions of the glucose ring.

To further explore this 2-OH and 6-NH2 activity, we determined the pH optimum of aAtf using 2-aminoglucosyl-, 6-aminoglucosyl-, and glucosyl AGT. With the natural substrate (2-aminoglucosyl AGT), the pH dependence leveled off at pH 8 (Figure 4A). Fitting the pH dependence to a single ionization resulted in a pKa of 6.7. The pH dependence of both the glucosyl- and 6-aminoglucosyl AGT scaffolds shows an increasing rate with pH up to the pH of enzyme stability, pH 10 (Figures 4B and 4C). Interestingly, at higher pH values, a second minor monacylated product could be seen forming in the glucosyl AGT reactions similar to the second minor product observed in the 6-aminoglucosyl AGT reactions. Due to the presence of two products, the velocity determined in these pH dependence experiments was taken as the sum of the rates of formation of each individual product. Figure 4D shows how the ratio between the two products changes as a function of pH for both substrates; at lower pH’s acylation at C2 predominates, while the amount of C6 acylation increases at higher pH’s.

While the activity was highest at pH 10, the formation of the minor product could be minimized by reducing the pH and kinetic parameters for these substrates were determined at pH 9 (summarized in Table 1). The Km for 2-aminoglucosyl AGT was reduced about one order of magnitude to 18 µM at pH 9 as compared to pH 7, resulting in a higher catalytic efficiency. The 6-aminoglucosyl- (29 µM) and glucosyl (42 µM) AGT scaffolds had similar Km values as the natural substrate, while their kat values were reduced about 1000-fold (0.253 min⁻¹ and 0.574 min⁻¹, respectively).

These results highlight two important findings: first, that tAtf and aAtf can catalyze the acylation of sugar hydroxyl groups, and second, that they can also acylate amines at position C6 of the glucosyl ring. Although both activities occur at significantly reduced rates, they can be preparatively useful in the chemoenzymatic synthesis of novel lipoglycopeptides.

Synthesis of Novel Lipoglycopeptides

To demonstrate the utility of the glycosyltransferase and acyltransferase enzymes from the glycopeptide biosynthetic machinery to generate novel lipoglycopeptides, two lipoglycopeptide derivatives were generated as summarized in Figure 5. The first, compound 16, is a teicoplanin analog that contains an acylated vancomycin disaccharide chain at Phegly4 and is missing its N-acetylglucosamine sugar at β-OH-Tyr6. This utilized the 6-aminoglucosyl AGT scaffold with aAtf-catalyzed enzymatic acylation of the 6-amino group on the sugar with decanoyl-CoA. Then, GtfD formed the N-acylated disaccharide by adding L-vancosamine (Figure 5A). In the second derivative, compound 19, the N-acetylglucosamine sugar was added to the β-OH-Tyr6 hydroxyl of 6-aminoglucosyl AGT, using GtfA (the GtfA analog from the teicoplanin cluster). Then aAtf and GtfD-mediated steps were employed sequentially to complete the three tailoring enzyme sequence (Figure 5A). All reactions were monitored by HPLC and by LCMS. Representative HPLC traces showing the formation of compound 16 are shown in Figure 5B.

Discussion

Natural products generated on nonribosomal peptide synthetase multimodular assembly lines commonly undergo a series of post assembly line tailoring reactions catalyzed by dedicated tailoring enzymes encoded in the biosynthetic genes clusters [17]. These enzymatic maturation steps may be crucial for the gain of biological activity, as typified by glycosylations of deoxerythronolide B on the way to the antibiotic erythromycin and by the tandem oxidative crosslinkings of the acyclic heptapeptide to generate the rigid aglycone scaffolds for vancomycin and teicoplanin family antibiotics.

In toto, vancomycin undergoes three types of post assembly line enzymatic tailoring: Leu1-N-methylation, the oxidative crosslinking of side chains 2–4, 4–6, and 5–7, and two glycosylations. Teicoplanin undergoes four oxidative crosslinkings, including side chains of residues 1–3, three glycosylations, and one acylation of the 2-aminoglucosyl moiety. The teicoplanin family member A-40,926 undergoes all of the teicoplanin modifications, except β-OH-Tyr6 glycosylation, plus two additional ones: Leu1-N-methylation and 2-aminoglucosyl double oxidation at C6 to the COOH oxidation state (Figure 1).

In this study we have focused on the acyltransferases from the teicoplanin and A-40,926 clusters, the enzymes responsible for conversion of glycopeptide to lipoglycopeptide scaffolds. Sequencing of the clusters identified likely candidates for the Atfs [1, 5], and the activity of tAtf was validated experimentally by the studies of Spencer and colleagues that showed that, when heterologously expressed in and purified from E. coli, tAtf was active for acylation of the 2-aminoglucosyl moiety both on the crosslinked heptapeptide scaffold and as UDP-2-aminoglucose [1]. Here we have performed initial kinetic characterization of both the teicoplanin tAtf and the homologous A-40,926 aAtf for both scaffold and acyl-CoA substrates.

Both Atfs are robust catalysts, with kat values of 400 min⁻¹ and 2000 min⁻¹ for aAtf and tAtf, respectively, using the substrate pair 2-aminoglucosyl AGT and octanoyl-CoA at pH 7. Both Atfs show reasonable utilization of C12-C14 acyl-CoA substrates but very low activities with short chain and aromatic acyl-CoAs.

Analysis of glycosyl-crosslinked heptapeptide scaffolds as substrates confirms that the 2-aminoglucosyl...
moiety is the preferred nucleophilic cosubstrate. As the pH is raised, detectable activity in the HPLC/MS assay is observed with glucosyl AGT for monoacylation with octanoyl-CoA as donor. Assuming the regiochemistry is maintained, this would represent a substitution of the 2-NH₂ group by the 2-OH group of the sugar. We isolated 5 mg of acylated glucosyl vancomycin scaffold, and NMR analysis shows a distinctive shift of the H-2 proton of the glucose ring confirming the site of acylation. The pKa of the -OH versus -NH₃⁺ is about 5 units higher for generation of the 2-O⁻, consistent with the monotonic rise of kcat for the glucosyl acceptor substrate, while the 2-NH₂-substrate shows a plateau above pH 8.

When 6-aminoglucosyl AGT was evaluated as a substrate, a pH profile and kcat comparable to the glucosyl-scaffold was observed. In this case two monoacylated products are detected (Figure 4E, middle trace). One of these is likely to be the 2-O-octanoyl-6-NH₂-glucosyl product, which appears to be the major kinetic product, while the other is likely to be the N6-octanoyl-glucosyl product. We have verified the location of this N6 acylation by comparing an authentic standard made chemically to the enzymatic product by HPLC and LCMS analysis. This apparent relaxation of the regioselectivity of the Atfs might be useful in the chemoenzymatic synthesis of glycopeptide derivatives where acyl chains can be appended to either the 2 or the 6 position of the glucosyl ring.

In particular, if the 2-OH of the 6-aminoglucosyl moiety is still free, one might expect this should enable further modification of the glucosyl moiety by vancomamine or epivancosamine by GtfD or GtfC, respectively. To that end the tandem incubations depicted in Figure 5 were conducted where the 6-aminoglucosyl AGT scaffold was subjected to Atf action, followed by GtfD and TDP-L-vancosamine. These were done in parallel following glycosylation of the β-OH of β-OH-Tyr₆ with GlcNAc by the teicoplanin tGtfA glycosyltransferase. In both cases decanoyl transfer was followed by vancosaminy transfer to generate compounds 16 and 19, containing the N₆-decanoyl-glucosyl-2-L-vancosaminyl substituent. These are lipoglycopeptides with the vancomycin-type disaccharide grafted on the teicoplanin scaffold, and the decanoyl side chain placed, not at N₂, but at N₆. Compound 16 is an analog of a vancomycin derivative previously synthesized chemically [13], while 19 has the three-sugar (disaccharide at residue four, monosaccharide at residue six) pattern characteristic of chloroeremomycin. The decanoyl-N₆ regiochemistry is intriguing and distinct from the N-chlorobiphenyl substitution on the amino group of the vancosaminyl sugar found in the clinical candidate oritavancin that is highly active against vancomycin-resistant enterococci.

Lipoglycopeptides are of interest, given that many of these compounds show improved activity against vancomycin-resistant bacteria [6, 11–13] and that two of the second-generation glycopeptides in clinical development, dalbavancin and oritavancin [18, 19], contain N-decanoyl and N-aryl substituents, respectively. The ability to decorate teicoplanin and vancomycin aglycone scaffolds with combinations of acyl-CoA and NDP-L- and NDP-D-hexoses (deoxy- and amino-) via action of Atfs and Gtfs should provide routes to new variants in the lipoglycopeptide class.

Significance

The vancomycin and teicoplanin group of natural product antibiotics share related crosslinked heptapeptide scaffolds that are produced by nonribosomal peptide synthetases and then become glycosylated by dedicated tailoring enzymes. One distinction between teicoplanin and vancomycin is that teicoplanin is a lipoglycopeptide, bearing a long-chain acyl group on the 2-aminoglucosyl moiety. We have now charac-
Cloning, Expression, and Purification of tGtFA

The tGtFA gene was PCR amplified from the plasmid pTA9 using the following primer pair: 5'-AAAAACATCATGCCTGCTTTC
CGTCTACT-3' and 5'-AAAAACTACCTTCGCGGAAACGGAGCATCTT
3', in which the Ndel and HindIII sites are underlined. The PCR product was cloned into the pET22b expression vector (Novagen), and the open reading frame of tGtFA was confirmed by DNA sequencing before it was transformed into BL21(DE3)-competent cells (Invitrogen). The tGtFA protein was expressed and purified using a previously described method [22].

Chemoenzymatic Synthesis of Glycopeptide Scaffolds

Vancomycin aglycone (AGV) 1 and teicoplanin aglycone (AGT) 3 were synthesized as previously described [14, 15]. These aglycones were then enzymatically glycosylated [3] with UDP-glucose, UDP-2-aminoglucose, or UDP-6-aminoglucose using the vancomycin biosynthetic glycosyltransferase GtfE to yield the monoglycosylated scaffolds 2 and 4-6 as shown in Figure 2A. The reaction conditions were as follows: 75 mM Tricine (pH 9.0), 8 mM MgCl₂, 2.5 mM TCEP, 1 mg/mL BSA, 10% (v/v) DMSO, 500 μM UDP-sugar, 500 μM aglycone, and 450 μM GtfE. The reactions were monitored by HPLC, and additional aliquots of GtfE were added twice daily until the reaction proceeded to 50% completion (up to 3 days). The resulting monoglycosylated scaffolds were purified by preparative HPLC (Vydac C₄ column, 0%-30% acetonitrile, 0.1% TFA in 25 min) and were verified by LCMS. For the synthesis of 9, the N terminus of 3 was Alloc protected to generate compound 7 as described previously [6]. The protected aglycone was then glycosylated with UDP-6-aminoglucose to generate glycopeptide 8. Alloc-protected glycopeptide 8 was then acylated with a decanoyl chain and deprotected to generate 9 as described for the corresponding 2-NH-decanoyl analog previously synthesized [6]. The NDP-sugars used for the enzymatic glycosylations were obtained as follows: UDP-glucose was commercially available, UDP-2-aminoglucose [23] and TDP-L-vancosamine [24] were synthesized as previously described, and UDP-6-aminoglucose, 14, was synthesized as described below (see Figure 2B).

**Experimental Procedures**

**General**

All chemicals were purchased from Aldrich or Sigma and used without further purification. Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on aluminum plates precoated with silica gel (250 μm, Sigma), with detection by UV and/or spraying with H₂SO₄ 70% (v/v) with heating. Flash chromatography was carried out on silica gel (60 Å, nus of Sigma) with detection by UV and spraying with a solution of aqueous phosphoric acid (1%) and nitroblue tetrazolium (0.01%) in 95% (v/v) ethanol. Solvents were reagent grade and were used until the reaction proceeded to 50% completion (up to 3 days). The products were analyzed by 12% SDS-PAGE. The desired fractions were syrup (3.9 g, 73%). The lactol (3.3 g, 10 mmol) and tetrazole (2.8 g, 10 mmol) were analyzed by LCMS. For the synthesis of 138, the lactol (3.3 g, 10 mmol) and tetrazole (2.8 g, 10 mmol) were analyzed by LCMS. For the synthesis of 138, the lactol (3.3 g, 10 mmol) and tetrazole (2.8 g, 10 mmol) were analyzed by LCMS.
aq, Na₂SO₄ (10%, 100 ml), the mixture was extracted with CH₂Cl₂ and the organic phase was washed with satd. aq, NaHCO₃ (2x) and brine. The organic phase was dried (MgSO₄), evaporated, and purified by flash chromatography on silica gel (petroleum ether/EtOAc 1:6, then pure EtOAc) to give the its percentage of the total peak area. The resulting initial velocity

\[
\text{Hz, H-3), 3.52 (d, 1 H, H-2), 4.40–4.30 (m, 4 H, OCH₂), 4.22–4.16 (m, 1 H, 5-H), 3.45 (dd, 1 H, H-6a, 2.2–2.1, 1 H, H-6b, 2.88–2.80 (m, 4 H, CH₂CN), 2.09, 2.05, 2.02 (3 s, noyl-CoA) using the assay conditions outlined above allowing di-

\[
\text{Hz, H-3), 5.63 (dd, 1 H, 1,2 = 3.5, J,5,6b = 9.9 Hz, H-1, J,1,2 = 3.5, J,5,6b = 9.9 Hz, H-1), netic parameters } k_{cat} \text{ and } K_m \text{. Full kinetic characterization of tAtf was not performed, and instead an estimate of the } k_{cat} \text{ for tAtf was determined by incubating 40 nM tAtf with saturating amounts of both substrates (700 mM 2-aminoglucosyl AGT and 500 mM octanoyl-CoA) using the assay conditions outlined above allowing di-

6-(2-Trimethylsilylhexoxybenzyl)-Amino-6-Deoxy-α-D-Glucopyranosyl Phosphate (13)

To a solution of phosphate 12 (725 mg, 1.4 mmol) in MeOH (20 ml) was added NaOMe (7 ml, 0.5 M in MeOH, 3.5 mmol), and the mixture was stirred at 55°C for 30 min. After evaporation of the solvent, the residue was dissolved in H₂O (30 ml). The solution was neutralized with Amberlite IRC50, filtered, and extracted with Et₂O (20 ml). Pd-C (400 mg) was then added, and the suspension was stirred under H₂ (1 atm) for 3 hr, filtered, and evaporated to a final volume of 10 ml. After the addition of Teoc succinimide (540 mg, 2.1 mmol) and Et₃N (0.5 ml), the mixture was stirred for 16 hr and evaporated. The residue was purified by flash chromatography on silica gel (CH₂Cl₂/MeOH/H₂O/Teoc 16:4:0.5:0.3, then 16:8:2:0.5) to afford phosphate 13 (630 mg, 80%; R₂ = 0.2, CH₂Cl₂/MeOH/H₂O/Teoc 16:4:0.5:0.3) as the triethylammonium salt (1.6 eq.). 1H NMR (CD₃OD, 400 MHz): δ 5.47 (dd, 1 H, J,1,2 = 3.5, J,1,2 = 6.9 Hz, H-1), 4.13–4.05 (m, 2 H, OCH₂), 3.85–3.78 (m, 1 H, H-3), 3.66 (t, 1 H, J,1,2 = 9.6 Hz, H-3), 3.53 (d, 1 H, J,1,2 = 12.9 Hz, H-6a), 3.36–3.32 (m, 1 H, H-2), 2.22–2.12 (m, 2 H, H-4, H-6b), 1.27 (t, 2 H, CH₂SiMe₃), 0.02 (s, 9 H, SiMe₃) 37P NMR (D₂O, 162 MHz): δ 1.40; MS (ESI) for C₁₂H₂₄N₅O₁₁P: 516 [M–H]⁻.

Uridine 5′-(6-Amino-6-Deoxy-α-D-Glucopyranosyl Diphosphate) (14)

Phosphate 13 (590 mg, 1.05 mmol) and triocytamine (0.47 ml, 1.05 mmol) were coevaporated with pyridine (3x). UMP morpholinate (1.15 g, 1.67 mmol) was added, and the mixture was coevaporated with pyridine (3x). The residue was dissolved in pyridine (8 ml), tetracile (250 mg, 3.2 mmol) was added, and the mixture was stirred for 2 days. Following evaporation, the residue was dissolved in H₂O containing 0.1% NH₄HCO₃ (18 ml), extracted with Et₂O, and purified by preparative reversed-phase HPLC to give the protected UDP derivative (420 mg, 56%). This compound was dissolved in DMF (5 ml), Bu₄NBF₄ (1 M in THF, 5 ml) was added, and the solution was stirred for 16 hr. After removal of the solvents, the residue was dissolved in H₂O containing 0.1% NH₄HCO₃ (10 ml), filtered, and purified by preparative reverse-phase HPLC to afford the UDP sugar 14 (265 mg, 80%) as its ammonium salt. 1H NMR (D₂O, 400 MHz): δ 7.98 (dd, 1 H, J,1,2 = 8.1 Hz, uridine Cnb-H), 5.99–5.95 (m, 2 H, uridine Cnb-H, H-1 [ ribose]), 5.63 (dd, 1 H, J,1,2 = 3.5, J,1,2 = 6.9 Hz, Hn-1), 4.40–4.20 (m, 5 H, H-2, H-3, H-4, H-5 [ ribose]), 4.12 (dt, 1 H, J,1,2 = 9.8 Hz, J,2,3 = 2.7 Hz, H-5), 3.78 (t, 1 H, J,1,2 = 9.8 Hz, H-3), 3.60 (dt, 1 H, J,1,2 = 9.8 Hz, J,2,3 = 5.3, J,3,4 = 9.8 Hz, H-4), 3.48 (dd, 1 H, J,1,2 = 9.8 Hz, J,2,3 = 2.7, J,3,4 = 13.2 Hz, H-6a), 3.36 (1 H, J,1,2 = 9.8, J,2,3 = 9.8 Hz, H-4), 3.11 (dd, 1 H, J,1,2 = 9.9, J,2,3 = 13.2 Hz, H-6b), 3.03 NMR (D₂O, 162 MHz): δ -10.4, -12.1; MS (ESI) for C₂₃H₂₆N₅O₁₃P₂: 565 (M+H)⁻.

Initial Kinetic Characterization of tAtf

Octanoyl-CoA (25–200 µM) and either 2-aminoglucosyl AGT (70–700 µM) or 2-aminoglucosyl AGV (90–900 µM) were incubated at 37°C with 40 nM tAtf in 25 µl of reaction buffer (75 mM Tris [pH 7.0], 8 mM MgCl₂, 2.5 mM TCEP, 1 mM mg BSA, and 10% [v/v] DMSO) for 2 min, after which the reactions were quenched by the addition of 50 µl methanol. The quenched reactions were centrifuged for 2 min at 13,000 rpm, and 20 µl of the supernatant was injected into a Vydac analytic C₄ HPLC column. Reaction mixtures were eluted using a gradient from 10% to 75% acetonitrile and 0.1% TFA in 20 min, and products were monitored at 280 nm. Negative control reactions were performed for all assays where enzyme was left out to verify there was no detectable nonenzymatic reaction. Molecular weights of substrates and products were verified by LCMS. The peaks for the acylated products and the unacylated substrates were integrated, and the product concentration was deduced from its percentage of the total peak area. The resulting initial velocity data were fit to the Michaelis-Menten equation to generate the ki-

Characterization of Glycopeptide Acyltransferases

Att Acyl Chain Specificity

Att and tAtf were incubated with 100 µM of various acyl-CoA sub-

pH Dependence of tAtf Activity

The pH dependence of tAtf activity was determined using 2-aminoglucosyl-, 6-aminoglucosyl-, or glucose) at pH 9.0. tAtf was incubated with 50–2000 mM scaffold and 1 mM octanoyl-CoA at 37°C in 25 µl of reaction buffer (75 mM Tris [pH 9.0], 8 mM MgCl₂, 2.5 mM TCEP, 1 mM mg BSA, and 10% [v/v] DMSO). In the 2-aminoglucosyl AGT reactions, 40 nM tAtf was used with 2 min reaction times, while in the 6-aminoglucosyl AGT and glucosyl AGT reactions, 5 µM tAtf was used with 10 min reaction times. The reactions were quenched and HPLC analyzed as described above.

Synthesis of Novel Lipoglycopeptides Using Gfts and Atfs

To make compound 16 (see Figure 5A), 500 µM 6-aminoglucosyl AGT 6, 1 mM decanoyl-CoA, and 1 µM tAtf were incubated in 100 µl of reaction buffer A (50 mM Tris [pH 9.0] and 1 mg/ml BSA) at 37°C for 60 min, generating compound 15. Two byproducts were

formed, the 2-O-decanoyl-6-aminoglucosyl AGT and a doubly acylated product as shown in Figure 5B. 40 µl of the resulting mixture was adjusted to a pH of 7.0 and further incubated with 500 µM TDP-L-vancomycin and 2 µM GtfD at 37°C for 3 hr, resulting in compound 16. To make compound 19, 500 µM 6-aminoglucosyl AGT, 1 mM UDP-GlcNAc, and 5 µM TdtA were incubated in 500 µl of reaction buffer B (50 mM Tris [pH 7.5] and 2.5 mM TCEP) at 37°C for 12 hr to generate compound 17. Compound 17, purified by RP-HPLC using a VyDAC C18 small-pore column, was incubated with 1 mM decanoyl-CoA in the presence of 1 µM TafT in 100 µl of reaction buffer A at 37°C for 60 min, generating compound 18. Half of the resulting reaction mixture was incubated with 500 µM TDP-L-vancomycin and 2 µM GtfD at 37°C for 3 hr to produce compound 19. All reaction mixtures were analyzed by both HPLC and LCMS, and when available, products were confirmed by coinjection with authentic chemically synthesized standards.

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**References**


