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Research Paper

Direct interaction of a vancomycin derivative with bacterial enzymes involved in cell wall biosynthesis

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Abstract

Background: The glycopeptide antibiotic vancomycin complexes D-Ala-D-Ala termini of bacterial cell walls and peptidoglycan precursors and interferes with enzymes involved in murein biosynthesis. Semisynthetic vancomycins incorporating hydrophobic sugar substituents exhibit efficacy against D-Ala-D-Lac-containing vancomycin-resistant enterococci, albeit by an undetermined mechanism. Contrasting models that invoke either cooperative dimerization and membrane anchoring or direct inhibition of bacterial transglycosylases have been proposed to explain the bioactivity of these glycopeptides.

Results: Affinity chromatography has revealed direct interactions between a semisynthetic hydrophobic vancomycin (DCB-PV), and select *Escherichia coli* membrane proteins, including at least six enzymes involved in peptidoglycan assembly. The *N*(*4*)-vancosamine substituent is critical for protein binding. DCB-PV inhibits transglycosylation in permeabilized *E. coli*, consistent with the observed binding of the PBP-1B transglycosylase-transpeptidase.

Conclusions: Hydrophobic vancomycins interact directly with a select subset of bacterial membrane proteins, suggesting the existence of discrete protein targets. Transglycosylase inhibition may play a role in the enhanced bioactivity of semisynthetic glycopeptides.

Keywords: Affinity chromatography; Glycopeptide; Peptidoglycan; Transglycosylase; Vancomycin

1. Introduction

Vancomycin is the antibiotic of choice in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections [1]. The glycopeptide forms high affinity complexes with terminal D-Ala-D-Ala dipeptides in nascent peptidoglycan and peptidoglycan precursors (lipid II) [2], which sterically interferes with the assembly of the bacterial cell wall [3,4], resulting in osmotic lysis and cell death. Three decades after its introduction, resistance to vancomycin was reported in certain enterococci [5], caused by the replacement of D-Ala-D-Ala moieties in the peptidoglycan with D-Ala-D-Lac [6]. This modification results in a 1000-fold decrease in the affinity of the glycopeptide for the depsipeptide substrate [7], with a concomitant loss of bioactivity. Vancomycin is the last line of defense in the treatment of infections caused by multi-drug-resistant bacteria, and the emergence of vancomycin-resistant enterococci (VRE) is viewed with trepidation, given the possibility of resistance transfer to β-lactam-insensitive staphylococci and streptococci [8,9].

Recent reports of *N*-alkylated hydrophobic glycopeptides that retain potency against vancomycin-resistant bacteria [10–12] offer promise in the development of novel antimicrobials for clinical use against resistant strains. The molecular logic that governs the bioactivity of these semisynthetic glycopeptides (including biphenyl chloroeremomycin [13] and chlorobiphenyl vancomycin (CBP-V) [14]), however, remains a matter of debate. Williams and co-workers have proposed that glycopeptide dimerization and membrane anchoring by the lipophilic substituent act

Abbreviations: CBP-V, chlorobiphenyl vancomycin; DCB, 3,4-dichlorobenzyloxybenzyl; DCB-PV, 3,4-dichlorobenzyloxybenzyl putrescinyl vancomycin; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MS, mass spectrometry; NHS, *N*-hydroxysuccinimide; PV, putrescinyl vancomycin; PVDF, polyvinylidene fluoride; PBP, penicillin binding protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VRE, vancomycin-resistant enterococci

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cooperatively [15] to create a chelate effect [16] that increases the affinity for D-Ala-D-Lac in resistant bacteria [17,18]. Alternatively, Kahne and co-workers have suggested that certain hydrophobic glycopeptides exhibit a second mode of action, independent of dipeptide/depsipeptide complexation [14,19]. In their studies, CBP-V and other vancosamine-*N*(*4*)-substituted derivatives inhibited the transglycosylase activity associated with glycan polymerization in ether-permeabilized *Escherichia coli*, whereas vancomycin itself inhibited the subsequent transpeptidation (cross-linking) step [14]. The substituted vancomycins were proposed to interact directly with specific bacterial enzymes, a phenomenon that is distinct from the steric interference afforded by vancomycin complexed to the cell wall [3]. We have now utilized affinity chromatography to test this hypothesis, and have screened solubilized *E. coli* membrane proteins for potential protein targets using immobilized glycopeptide ligands.

2. Results

2.1. Construction of glycopeptide affinity columns

Vancomycin was derivatized with a putrescine linker at the C-terminus (putrescinyl vancomycin, PV, Fig. 1) to facilitate immobilization on an acid-derivatized solid support for affinity chromatography. The bioactivity of PV against sensitive Gram-positive strains was not adversely affected by the presence of this linker (Table 1). The *N*(*4*)-alkylated 3,4-dichlorobenzyloxybenzyl (DCB) derivative of glycopeptide PV (DCB-PV, Fig. 1) was adopted as a prototypic hydrophobic vancomycin for investigating potential interactions with specific bacterial proteins. As summarized in Table 1, this semisynthetic vancomycin, which incorporates a hydrophobic aryl substituent at the *N*(*4*) position of the vancosamine sugar (R_2 in Fig. 1) exhibits efficacy against a broad range of Gram-positive bacteria, including VRE (both teicoplanin-resistant VanA and teicoplanin-sensitive VanB strains), methicillin-sensitive *S. aureus*, and MRSA (Table 1). The DCB-PV glycopeptide was immobilized for affinity chromatography by coupling the C-terminal linker to *N*-hydroxysuccinimide (NHS)-activated Sepharose 4B. A control affinity column

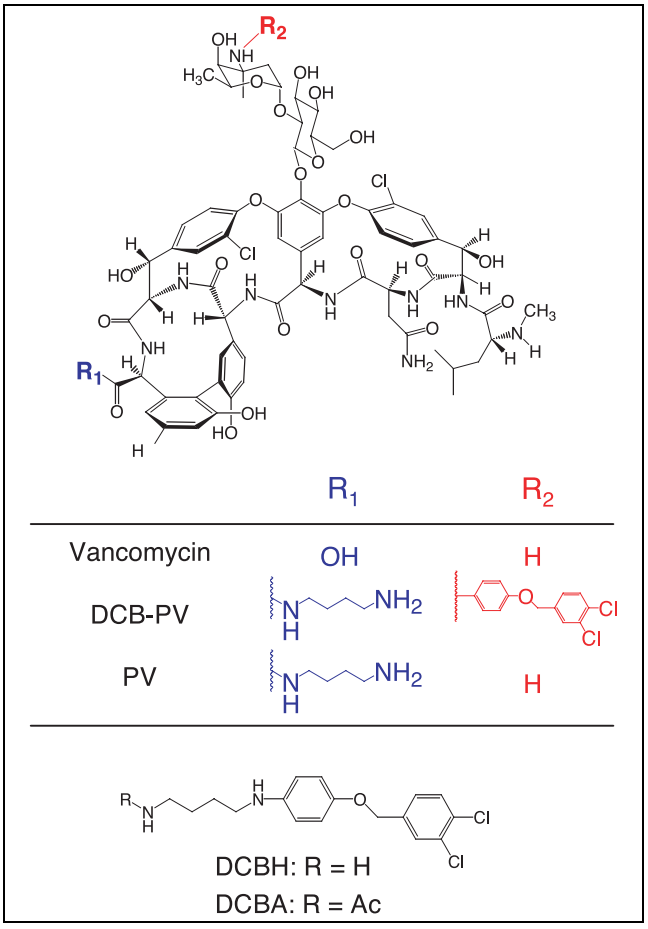


Fig. 1. Chemical structure of vancomycin and its derivatives used in this study. The putrescine linker was appended to the carboxy-terminus of glycopeptides DCB-PV and PV to facilitate immobilization on a solid support. The DCB moiety was similarly immobilized for affinity chromatography using the putrescinyl derivative DCBH. The corresponding *N*-acetylated derivative (DCBA) mimics this *N*(*4*) substituent.

was similarly constructed using glycopeptide PV, which has an unsubstituted disaccharide core and is ineffective against VRE (Table 1).

2.2. Affinity chromatography of E. coli membrane proteins

The search for potential protein targets of hydrophobic vancomycins in *E. coli* focused on membrane preparations, since glycopeptide antibiotics do not penetrate the

Table 1
Minimum inhibitory concentrations (MICs, μg/ml) of the various glycopeptides and side chain analogs used in this study

Compound	VSE	VanA	VanB	MSSA	MRSA
Vancomycin	2	2048	2048	1	1
Teicoplanin	0.5	>1024	≤0.5	0.25	1
DCB-PV	≤0.03	2	2	≤0.03	0.5
PV	0.5	>64	>64	1	1
DCBA	32	32	64	64	64

The compounds were tested against vancomycin-sensitive enterococci (VSE, *E. faecium* strain RLA1), vancomycin-resistant enterococci of the VanA and VanB subtypes (*E. faecium* strain CL5053 and *E. faecalis* strain CL4877, respectively), methicillin-sensitive *S. aureus* (MSSA, strain MB2985), and methicillin-resistant *S. aureus* (MRSA, strain COL).

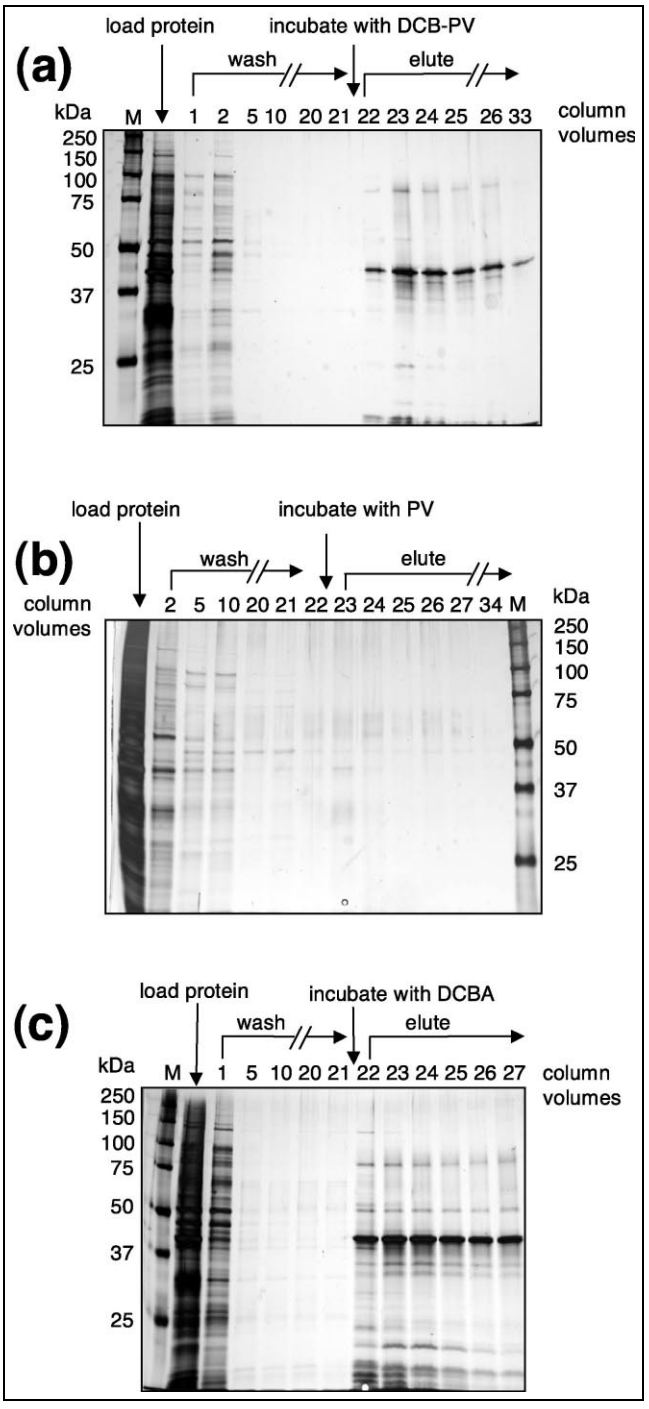


Fig. 2. Affinity chromatography of semisynthetic vancomycins and the DCBH ligand. (a) SDS–PAGE (10% polyacrylamide, silver-stained) of select fractions (identified by column volumes) collected during the passage of solubilized *E. coli* membrane proteins through the DCB-PV affinity column. (b) A similar analysis of representative fractions from the corresponding PV affinity column. (c) SDS–PAGE analysis of fractions from the DCBH affinity column, which mimics the *N*(*4*) substituent of DCB-PV.

outer cell wall [20]. The use of a hydrophobic vancomycin as ligand in affinity chromatography, however, raised the possibility of non-specific glycopeptide–protein interactions. In addition, the limited quantities of glycopeptide available necessitated modifications to the elution protocols typically employed in such experiments. Both issues were addressed in the design of the chromatography protocols. *E. coli* K12 membranes prepared by differential centrifugation were solubilized with a non-ionic detergent (1% (w/v) Triton X-100) and high salt (1 M NaCl) in buffer A (20 mM sodium phosphate buffer, pH 7.0). In a typical chromatography experiment, solubilized membrane proteins (500 µl, containing 3–8 mg of total protein, and clarified by ultracentrifugation) were loaded on the DCB-PV affinity column (7–12 µmol capacity, 1 ml bed volume). Since hydrophobic interactions increase with ionic strength and are dependent on the chemical nature of the salt present in the mobile phase, these parameters were optimized to minimize potentially indiscriminate protein binding by the glycopeptide ligands.

The addition of salts increases the surface tension of water and favors the structural rearrangements of solvent molecules surrounding interacting hydrophobic groups, which is thought to provide the driving force for hydrophobic interactions (the 'salting out' effect) [21]. Cations and anions are ranked in the Hofmeister series (for a review, see [22]), as per their molal surface tension increment (σ) in water. Salts with high σ values are commonly used to increase protein adsorption in hydrophobic interaction chromatography. For example, ammonium sulfate ($\sigma = 2.16$) produces a molal salting-out constant for hemoglobin of 3.86 m^{-1} compared to only 0.62 m^{-1} with sodium chloride ($\sigma = 1.64$) [22,23]. We therefore specifically avoided salts such as ammonium sulfate, and instead, adjusted the ionic strength of the mobile phase with NaCl to minimize the potential for non-specific adsorption of hydrophobic proteins to the glycopeptide ligands.

Representative fractions (1 ml each) that were collected from the DCB-PV affinity column were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% polyacrylamide gel, which was silver-stained to maximize the sensitivity of protein detection (Fig. 2a). An extensive wash of the charged DCB-PV affinity column with 20 column volumes of buffer A containing 400 mM NaCl, 0.05% Triton X-100 was most effective in eluting non-specifically bound proteins. Due to the moderate ionic strength of the mobile phase, any salting-out effect was minimal, and non-specific electrostatic (polar) glycopeptide–protein interactions were also suppressed. As shown in Fig. 2a, proteins bound with low affinity were rapidly stripped from the DCB-PV affinity column during the wash (column volumes 1–5). The absence of protein in fractions corresponding to the latter half of this 'high stringency' wash (column volumes 10–20) confirmed the elimination of most low affinity glycopeptide–protein interactions under these optimal conditions. In contrast, the utilization of a wash buffer with high ionic strength (1 M NaCl) resulted in the extensive salting-out of hydrophobic proteins, which continuously leached from the column, indicative of low affinity inter-

actions (data not shown). The elution of these proteins was not facilitated by the addition of free glycopeptide, consistent with non-specific protein binding by the immobilized DCB-PV ligand under these high salt conditions.

A small subset of *E. coli* membrane proteins (approximately 3–4% of the total protein mass loaded on the column) were retained by the DCB-PV affinity column after the high stringency (400 mM NaCl) wash, which selects for high affinity glycopeptide–protein interactions. Elution of these proteins by the addition of free ligand to the mobile phase (a standard protocol in affinity chromatography) was precluded by the limited quantities of glycopeptide available. Attempts to perturb the glycopeptide–protein interactions by washing the column with a mobile phase of drastically reduced ionic strength (buffer A, 1% Triton X-100) led to inefficient release of bound proteins ($\sim$40%). Instead, the column was incubated overnight with a low concentration of DCB-PV (100 μM) in the low ionic strength buffer. Released proteins were subsequently flushed from the column with the same buffer, except that no glycopeptide was present in the mobile phase. This scheme, necessitated by the limited quantities of free glycopeptide available for competitive elution, inevitably resulted in a broad elution profile ($\sim$12 column volumes, Fig. 2a). Nevertheless, protein recoveries using this strategy were quite acceptable ($>$90%).

To determine the contributions of the *N*(*4*) aromatic substituent in DCB-PV to protein binding, the affinity chromatography experiment was repeated with immobilized PV, which contains an unsubstituted disaccharide core. No proteins eluted from this affinity column after incubation with a 100 μM solution of PV (Fig. 2b), indicating that the unalkylated glycopeptide does not significantly interact with proteins. In a second control experiment, no *E. coli* membrane proteins were retained by an ethanolamine-derivatized Sepharose 4B column under identical chromatography conditions (data not shown), confirming that the column matrix itself does not significantly bind any protein.

2.3. DCB-PV interacts with penicillin binding proteins involved in peptidoglycan biosynthesis

Aliquots of the eluate (200 μl) from both glycopeptide affinity columns were treated with 50 μM Bocillin-FL™, a fluorescent β-lactam that covalently labels penicillin binding proteins (PBPs) [24]. After a brief incubation (30 min, 25°C), the samples were concentrated by acetone precipitation and resolved by SDS–PAGE on a 10% polyacrylamide gel. The gel was scanned with a fluorimager (λ_{ex} = 480 nm, λ_{em} = 530 nm) to reveal any PBPs that may be present. As shown in Fig. 3, several *E. coli* PBPs (PBPs 1B, 2, 3, 5, and 6, each identified by molecular mass, see below) were specifically eluted from the DCB-PV column. No PBPs were present in the elution fractions

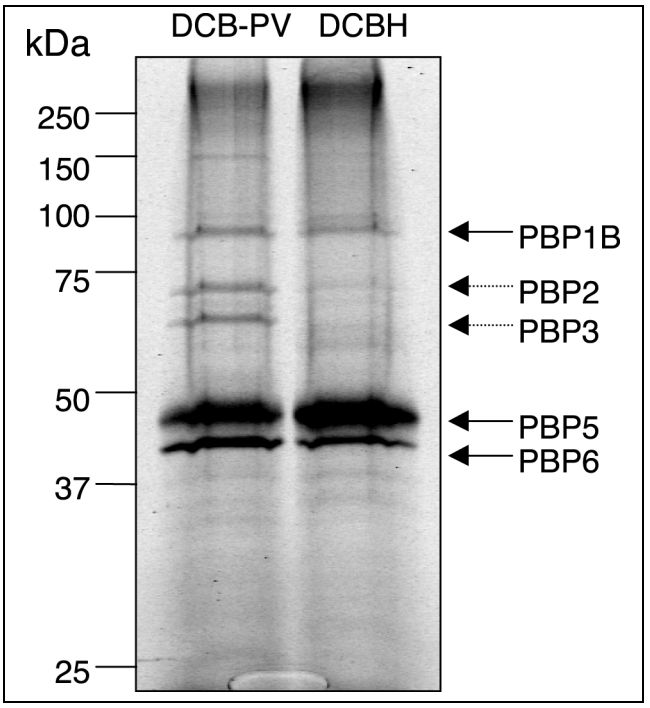
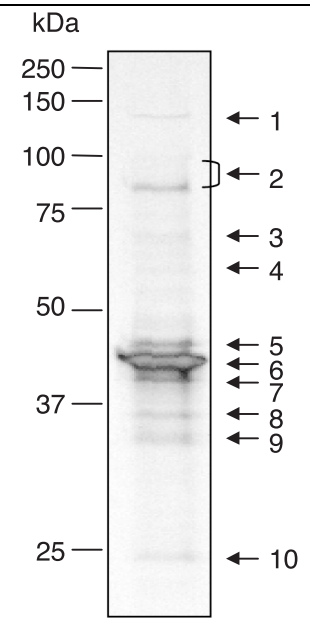


Fig. 3. Comparison of PBPs eluted from the DCB-PV and DCBH affinity columns. The eluate from each affinity column (column volume 22, Fig. 2a,c) was treated with a fluorescent β-lactam (Bocillin-FL), resolved by SDS–PAGE, and scanned with a fluorimager. Five PBPs that interact with DCB-PV were identified by molecular mass, including a bifunctional transpeptidase transglycosylase (PBP-1B), two transpeptidases (PBPs 2,3), and two endopeptidases (PBPs 5,6). Of these proteins, PBPs 2 and 3 are not significantly retained by the DCBH affinity column.

from the PV column (data not shown), consistent with the absence of any significant protein binding by this glycopeptide.

2.4. Mass spectrometric identification of membrane proteins that bind DCB-PV

Proteins eluting from the DCB-PV affinity column were concentrated by acetone precipitation, resolved by SDS–PAGE, and stained with Gelcode Blue™. Ten discrete protein-containing bands (Fig. 4) were excised from the gel and digested in situ with trypsin. The resulting proteolytic fragments were extracted from the gel slices and subjected to liquid chromatography-tandem mass spectrometry (MS/MS) analysis. The MS/MS spectra of tryptic fragments were subjected in batch mode to SEQUEST [25], a direct database correlation algorithm. Identified proteins were validated by manual inspection of the sequence ions present in each spectrum. Several analyzed bands were observed to contain more than one protein. As summarized in Fig. 4, the lipophilic glycopeptide DCB-PV interacts with at least 13 diverse membrane proteins, including redox enzymes and components of transporter complexes. Significantly, a majority of the identified proteins play a role in peptidoglycan biosynthesis and turnover (highlighted in Fig. 4), although their relative abun-



Band	Protein	Accession #
1	No data obtained	
2	PBP-1B	P02919
	DMSO reductase	P18775
3	PBP-2	P08150
	Rotamase D	P77241
4	PBP-3	P04286
5	Glucose permease IIBC	P05053
6	PBP-5	P04287
	Acriflavin resistance protein A	P31223
7	PBP-6	P08506
8	Lytic murein transglycosylase A	P46885
9,10	Proteins of unknown function	

Fig. 4. Mass spectrometric identification of *E. coli* membrane proteins that interact with DCB-PV. Proteins eluting from the affinity column were resolved by SDS–PAGE. Major protein-containing bands (numbered 1–10) were excised and digested in situ with trypsin. Proteins were identified on the basis of tryptic fragments, which were analyzed by tandem electrospray mass spectrometry and SEQUEST database correlation. The NCBI accession number for each identified protein is listed. Proteins highlighted in red are enzymes involved in cell wall biosynthesis and turnover.

dance in the eluate cannot be ascertained by the MS analysis. The detection of PBPs 1B, 2, 3, 5, and 6 by MS confirmed the results of the earlier Bocillin-FL labeling experiment. Unfortunately, the MS analysis of high molecular weight bands (≥100 kDa) was hindered by poor signal-to-noise ratios, probably due to the hydrophobicity of the corresponding membrane proteins. No useful MS data could be collected for band 1, and PBP-1B was only intermittently detected by MS in band 2, even though the Bocillin labeling was consistently reproducible.

2.5. *Protein binding by DCB-PV is mediated by the vancosamine-N(4) aryl substituent*

Since an unsubstituted vancomycin (PV) does not significantly interact with bacterial membrane proteins, the vancosamine-*N*(*4*) substituent of DCB-PV was immobilized to evaluate its contribution to protein binding. The DCB moiety was coupled to NHS-activated Sepharose via a putrescine linker (ligand DCBH, Fig. 1), which mimics the *N*-alkyl connectivity of this side chain to the glycopeptide. Affinity chromatography was performed as described for the glycopeptide ligands, and bound protein was displaced after preincubating the column with a 100 μM solution of DCBA (the *N*-acetylated derivative of DCBH, Fig. 1). Since the chromatography protocols were optimized to minimize non-specific interactions, a protein elution profile similar to that obtained with the DCB-PV glycopeptide indicated that the immobilized DCB moiety itself exhibits a high affinity for a limited number of *E. coli* membrane proteins (Fig. 2c). Bicyclic aryl moieties such as DCB may be especially effective in this regard, since phenyl Sepharose (a prototypic hydrophobic interaction chromatography medium) did not bind any proteins under these chromatography conditions (data not shown). These data suggest that the *N*(*4*) substituent of DCB-PV may function as a protein binding determinant. Significant differences in protein binding were observed, however, between the DCB-PV and DCBH affinity columns, especially in the pattern of interacting PBPs. Bocillin-FL labeling revealed that PBP-2 and PBP-3 were not significantly retained by the DCBH column (Fig. 3), although the other PBPs that interact with DCB-PV (PBPs 1B, 5, and 6) were present.

2.6. *DCB-PV inhibits transglycosylase activity in permeabilized E. coli*

The identification of at least six enzymes involved in peptidoglycan synthesis as proteins that interact with DCB-PV prompted us to investigate the effects of this glycopeptide on late stage peptidoglycan biosynthesis (reviewed in [26]) in a permeabilized-cell 'site of inhibition assay' described previously [14]. Briefly, radiolabeled UDP-*N*-acetylglucosamine ([U-^{14}C]UDP-GlcNAc) and UDP-MurNAc-pentapeptide (bacterial cell wall precursors, Fig. 5) were added to ether-permeabilized *E. coli* in the presence of increasing concentrations of glycopeptide. The extent of radioactivity incorporated into cytoplasmic lipid intermediates (extractable into *n*-butanol, BuOH), immature glycan (insoluble in dimethyl sulfoxide and retained on a polyvinylidene fluoride (PVDF) membrane),

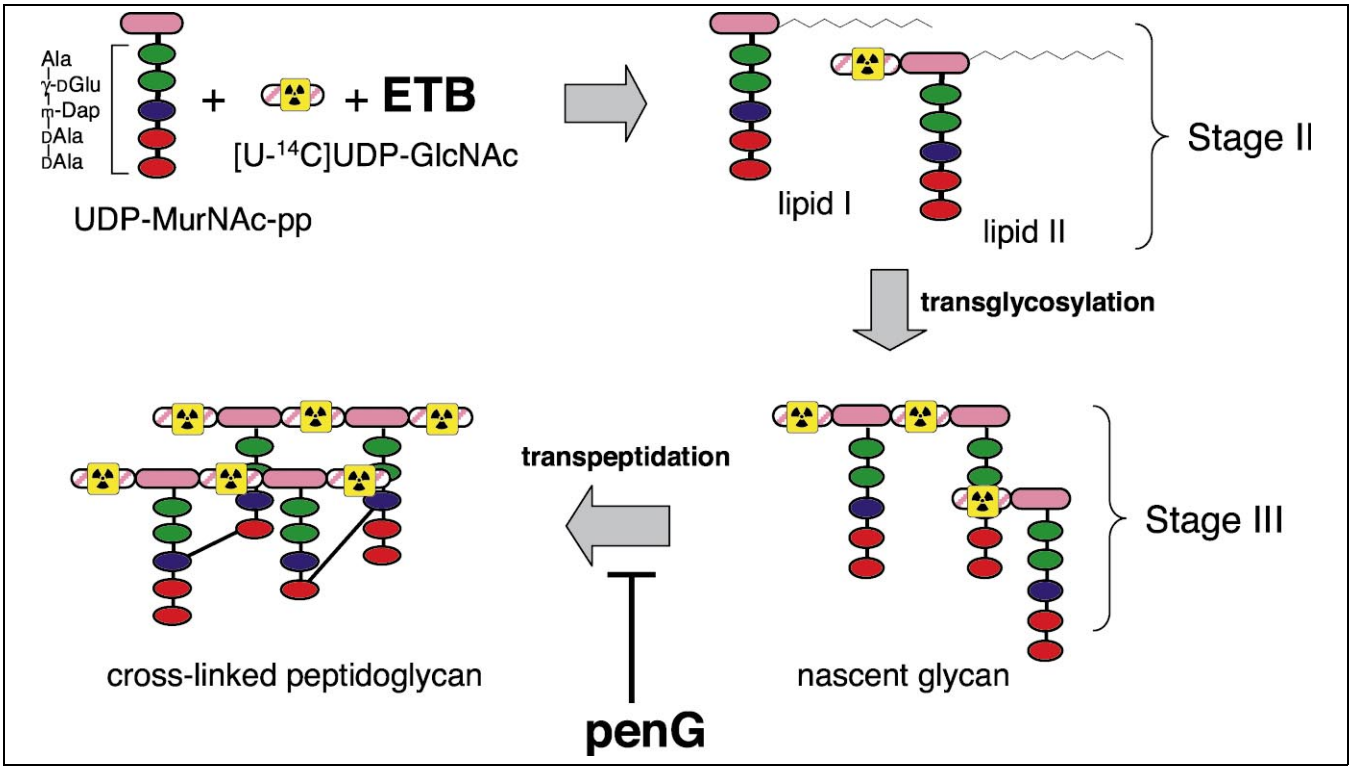


Fig. 5. Late stage peptidoglycan biosynthesis monitored using the ether-treated bacteria (ETB) bioassay. Radiolabeled [U-^{14}C]UDP-GlcNAc and UDP-MurNAc-pentapeptide (UDP-MurNAc-pp) were added to ether-permeabilized *E. coli* (ETB). Incorporation of the radiolabel into lipids (extracted into *n*-butanol) and mature cross-linked peptidoglycan (insoluble in hot 4% SDS) was monitored in the presence of titrated compounds, to evaluate potential inhibition of lipid intermediate formation in stage II, and transglycosylation/transpeptidation in stage III, respectively. The assay was repeated in the presence of penicillin G (penG), which inhibits the cross-linking (transpeptidation) step, and allows for measurement of radiolabel incorporation into nascent glycan (insoluble in dimethyl sulfoxide and retained by a PVDF filter). Transglycosylase inhibitors are identified under these conditions, and distinguished from compounds that affect only the subsequent transpeptidation step.

and mature (cross-linked) peptidoglycan (insoluble in hot 4% SDS) was measured to determine the step at which peptidoglycan synthesis is inhibited. Since transglycosylation precedes transpeptidation in *E. coli* (Fig. 5), these temporally distinct processes in late stage peptidoglycan biosynthesis were delineated by repeating the assay in the presence of penicillin G to inhibit the cross-linking process and exclusively monitor the formation of immature (non-cross-linked) peptidoglycan. The permeabilized-cell bioassay reveals only the earliest step in late stage peptidoglycan biosynthesis that is inhibited by a particular compound. Concomitant effects on subsequent steps cannot be ruled out by this assay.

The amount of hot SDS-insoluble [^{14}C]GlcNAc-labeled material formed in the ether-treated bacteria assay in 60 min at 30°C was ~700 pmol in the absence of inhibitors. Based on the extent of radiolabel incorporated, the ratio of lipid I/II to nascent glycan to cross-linked peptidoglycan formed during this interval was approximately 1:2.4:5.7. The effects of control compounds (ramoplanin, moenomycin, cefoxitin, and vancomycin) that disrupt specific stages in peptidoglycan synthesis were consistent with the identification of stated intermediates detected in this assay (data not shown). Incorporation of radiolabel into the BuOH-soluble fraction (lipid intermediates, Fig. 5) was inhibited only by ramoplanin (a stage II inhibitor). Both ramoplanin and moenomycin (a transglycosylase inhibitor) inhibited incorporation of label into the penicillin-resistant fraction (immature glycan, retained by PVDF), whereas transpeptidase inhibitors (cefoxitin and vancomycin) did not affect this step. Predictably, all four compounds inhibited incorporation of radiolabel into cross-linked peptidoglycan (the hot SDS-insoluble fraction).

As illustrated in Fig. 6a, DCB-PV inhibits the incorporation of radioactive label into nascent glycan (and subsequently into mature peptidoglycan), with the concomitant accumulation of radiolabeled lipid intermediates. DCB-PV therefore interferes with early Stage III glycan polymerization (IC_{50} = 4 μM), consistent with the specific inhibition of transglycosylases responsible for the incorporation of lipid II into existing glycan (Fig. 5). In contrast, the unsubstituted glycopeptide PV inhibits the incorporation of radioactivity into mature (cross-linked) peptidoglycan (Fig. 6b, IC_{50} = 62 μM), resulting in the accumulation of immature glycan and lipid intermediates produced in the preceding steps of cell wall assembly. These observations are consistent with PV interfering with the transpeptidases responsible for cross-linking of the nascent peptidoglycan. The *N*(*4*) substituent of DCB-PV (3,4-di-

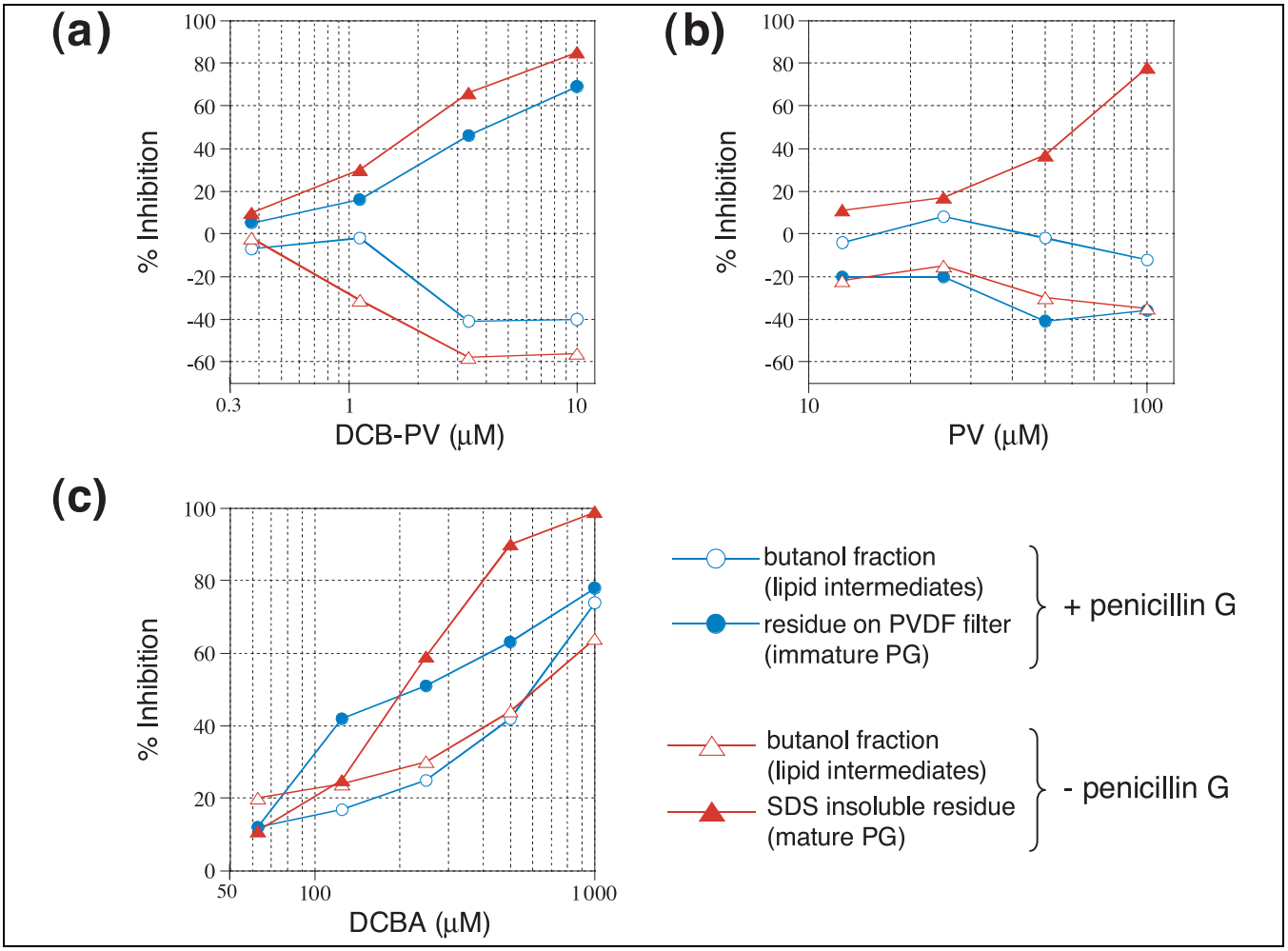


Fig. 6. Inhibition of late stage peptidoglycan biosynthesis by glycopeptides DCB-PV and PV, and by the *N*(*4*) substituent (DCBA) in ether-permeabilized *E. coli*. Inhibition of a particular step in peptidoglycan biosynthesis precludes subsequent processes, and causes the accumulation of intermediates in preceding steps (reflected as 'negative' inhibition). In the absence of any inhibitor, the amount of radiolabel incorporated into each fraction after 60 min at 30°C was as follows: lipid intermediates, 110 pmol (−penG), 136 pmol (+penG); cross-linked peptidoglycan, 704 pmol (−penG); immature glycan, 296 pmol (+penG). (a) Inhibition of transglycosylation by DCB-PV prevents the formation of nascent (and mature) peptidoglycan, and causes the accumulation of lipid intermediates. (b) PV inhibits the subsequent transpeptidation step instead (formation of cross-linked mature glycan), albeit at 10-fold higher concentrations, causing the accumulation of nascent glycan and lipid intermediates. (c) The hydrophobic *N*(*4*) substituent of DCB-PV (DCBA) inhibits the formation of stage II lipid intermediates at high concentrations (>100 μM), which also affects subsequent transglycosylation and transpeptidation.

chlorobenzyloxybenzyl derivative DCBA) also inhibited peptidoglycan synthesis in ether-permeabilized *E. coli*, but only at concentrations that were approximately 100-fold *higher* than that of the parent DCB-PV glycopeptide (Fig. 6c). Whereas DCB-PV inhibits transglycosylation, DCBA itself inhibited the preceding step (formation of Stage II lipid intermediates) in the bioassay.

2.7. *Affinity chromatography with the glucosamine-acyl substituents of teicoplanin*

Teicoplanin, an amphiphilic glycopeptide antibiotic that is related to vancomycin, is a complex of five major components (TA_2-1–5) that differ only in the structure of the *N*-acyl substituent at the glucosamine sugar (Fig. 7a) [27]. In contrast to hydrophobic vancomycins such as DCB-PV, teicoplanin exhibits poor efficacy against DAla-DLac-containing VRE (Table 1). Fatty acids analogous to the acyl side chains of teicoplanin were immobilized and investigated by affinity chromatography to compare protein binding by these aliphatic substituents with that by the *N*(*4*)-DCB substituent of DCB-PV. Affinity columns ($\sim$10 μmol capacity each) were prepared by immobilizing 8-methylnonanoic acid (analogous to the TA_2-2 acyl chain), 10-methylundecanoic acid (one methylene unit longer than the TA_2-5 side chain), and *n*-decanoic acid (corresponding to the TA_2-3 substituent) on EAH Sepharose 4B as per the manufacturer's instructions.

Affinity chromatography of solubilized *E. coli* membranes revealed weak and varying degrees of protein binding by the aliphatic ligands (10-methylundecanoic acid $\ll$ 8-methylnonanoic acid $<$ *n*-decanoic acid). Unlike

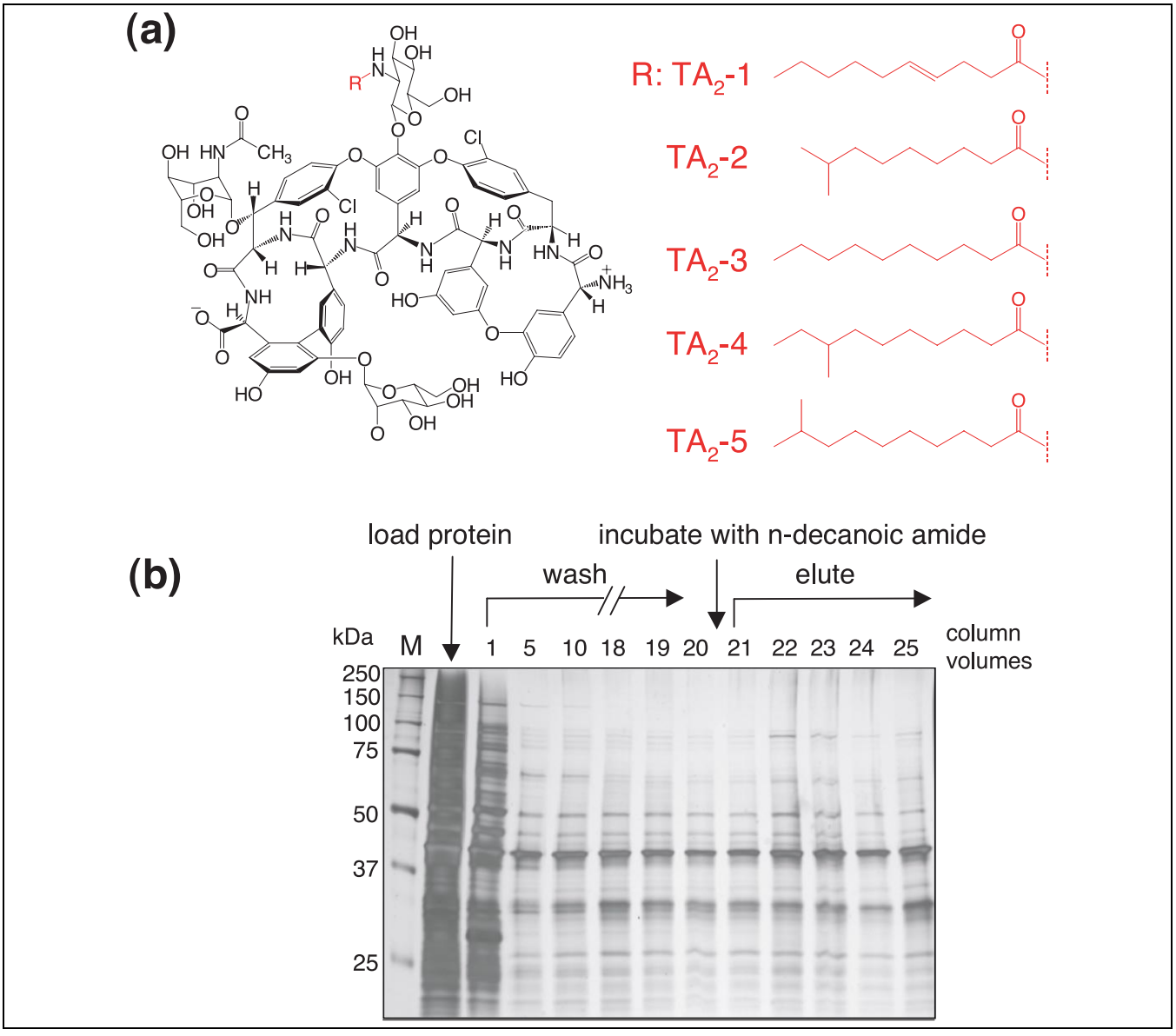


Fig. 7. Affinity chromatography with the acyl substituents of teicoplanin. (a) Chemical structures of glycopeptides TA_2-1–5 that comprise the major components of the teicoplanin complex. (b) SDS–PAGE of representative fractions (identified by column volume) collected during the passage of solubilized *E. coli* membrane proteins through the *n*-decanoic acid affinity column, which mimics the TA_2-3 substituent.

the high affinity interactions observed with the DCB-PV and DCBH affinity columns, the continuous leaching of multiple proteins from the acyl chain columns during the high stringency wash step (Fig. 7b) suggested low affinity protein binding by the teicoplanin substituents. Protein binding by the branched C_9 and C_{10} ligands was too weak to allow for the detection of interacting PBPs by Bocillin-FL labeling. PBP-6 and PBP-1B were detected in fractions eluting from the *n*-decanoic acid column (data not shown), but these proteins were also present in the wash fractions, indicative of non-specific binding. Incubation of these affinity columns with free ligand (a 100 μM solution of the corresponding long chain carboxamide or methyl ester derivatives) did not significantly facilitate the release of proteins, also suggestive of non-specific protein binding by the acyl substituents of teicoplanin.

3. Discussion

In this work, we have utilized affinity chromatography to determine whether direct glycopeptide–protein interactions play a role in the bioactivity of semisynthetic hydrophobic vancomycins. In vitro, vancomycin inhibits either transglycosylation or transpeptidation, depending on whether it sequesters the D-Ala-D-Ala dipeptide in lipid II intermediates or in the immature peptidoglycan, respectively. Ether-permeabilized *E. coli* are moderately susceptible to the antibiotic, which enables the mode of action of

semisynthetic vancomycins to be studied in a Gram-negative strain where the major enzymes responsible for murein assembly are genetically and biochemically characterized.

The observation that DCB-PV interacts with a small subset of *E. coli* membrane proteins is, to our knowledge, the first such report of direct glycopeptide–protein interactions. The high stringency wash step included in the chromatography protocols suggests that the selectively retained proteins are most likely bound with high affinity to the glycopeptide affinity column. Displacement of a limited number of proteins ($\sim$13) from the column upon incubation with free glycopeptide reflects modest specificity in protein binding by DCB-PV. The significance of this observation is highlighted by the complete absence of protein binding by PV, and by the weak non-specific interactions exhibited by the acyl substituents of teicoplanin; both glycopeptides are ineffective against DAla-DLac-containing VRE. The bioactivity of teicoplanin against these strains is reported to be enhanced up to 500-fold in disubstituted semisynthetic derivatives containing *N*-alkyl/aryl substituents at the glucosamine sugar and at the amino-terminus [28]. Whether the enhanced VRE activity of these semisynthetic teicoplanins is due to effective binding of protein targets remains to be established, although increased membrane anchoring of these glycopeptides at the cell surface may also play a role.

Since a majority of the *E. coli* enzymes involved in peptidoglycan assembly belong to the PBP superfamily (targeted by the β-lactam antibiotics) [29], fractions eluting from the affinity columns were first treated with a fluorescent β-lactam that labels PBPs with a detection limit of $\sim$20 ng. Five PBPs that interact with DCB-PV were identified by this method. The most abundant PBP in *E. coli* (PBP-5, 791 molecules/cell [30]) was also the major protein retained by the DCB-PV affinity column. Five other proteins were identified by MS analysis of their tryptic fragments; these include the membrane-bound lytic murein transglycosylase A (murein hydrolase A, MltA), which is involved in cell wall remodeling [31], and components of transporter complexes AcrA [32] and EIIB-Glc [33]. Two proteins corresponding to open reading frames of unknown function were also identified in the MS analysis, as were the PBPs previously detected by covalent labeling.

The identification of multiple PBPs that interact with DCB-PV is noteworthy for several reasons. First, most of these proteins are transpeptidases or endopeptidases involved in cell wall assembly, and some of these enzymes play an essential role in the morphological changes that accompany bacterial cell growth (PBP-2) and division (PBP-3) [34]. Second, the high molecular weight PBP-1B protein detected in the DCB-PV eluate is a dual function transglycosylase/transpeptidase that catalyzes up to 90% of the peptidoglycan synthesis in *E. coli* [35,36]. This enzyme is therefore a putative target for the inhibition of transglycosylase activity by DCB-PV that was measured in the *E. coli* bioassay. Third, the elution of multiple PBPs and a lytic transglycosylase may reflect interactions of the DCB-PV glycopeptide with multienzyme complexes that are proposed to be involved in murein synthesis [37,38]. Such interactions could also contribute to the inhibition of peptidoglycan biosynthesis. Consistent with these hypotheses, DCB-PV effectively inhibits transglycosylation in a permeabilized-cell bioassay. The relevance of these observations (made in ether-treated *E. coli*) to the Gram-positive activity of substituted vancomycins is supported by a recent report describing the inhibition of transglycosylation in VRE by chlorobiphenyldesleucyl vancomycin, a glycopeptide similar to DCB-PV, but lacking the DAla-DAla binding pocket [39].

Although our experiments suggest that DCB-PV, a hydrophobic vancomycin, interacts directly with bacterial transglycosylases such as PBP-1B and MltA, the exact mechanism of transglycosylase inhibition by this glycopeptide in permeabilized *E. coli* remains to be established. Protein binding alone is not sufficient, since the *N*(*4*)-DCB substituent (DCBA), which also binds PBP-1B, is 100-fold less potent in the bioassay, and inhibits an earlier step (the formation of lipid intermediates). Potential nonspecific hydrophobic effects on transglycosylation due to the *N*(*4*)-vancosamine substituent cannot explain the bioactivity of DCB-PV, given the poor antimicrobial activity of DCBA (MIC $\geq$32 μg/ml, Table 1) and the high concentrations of this compound required to inhibit the formation of lipid intermediates in the permeabilized-cell bioassay. Protein binding, while not sufficient, does appear to be necessary for transglycosylase inhibition by DCB-PV, since the unsubstituted PV glycopeptide inhibits transpeptidation instead, and only at 10-fold higher concentrations (consistent with a prerequisite for stoichiometric binding to DAla-DAla moieties). DCB-PV, if bound to a target transglycosylase, should also be capable of sequestering DAla-DAla/DLac dipeptide/depsipeptide moieties in proximal glycosyl donor and acceptor substrates, which could further interfere with transglycosylation and glycan assembly. Such bidentate chelation would be energetically favored since the complexation is effectively intramolecular, analogous to the chelate effect invoked in alternate models of glycopeptide VRE activity [15].

Our demonstration of interactions between an *N*(*4*)-vancosamine-substituted vancomycin derivative and key bacterial enzymes involved in peptidoglycan biosynthesis lends support to the hypothesis of direct transglycosylase inhibition, but does not rule out the possibility for additional modes of action. Depending on the structure and connectivity of the hydrophobic substituent, the identity of the glycopeptide core, and perhaps even the composition of the bacterial cell wall, either or both of the proposed mechanisms (direct enzyme inhibition versus cooperative glycopeptide dimerization and membrane anchoring) may govern the bioactivity of a particular substituted glycopep-

tide. Indeed, Kerns et al. have recently reported that the VRE activity of glucose-C(6)-substituted hydrophobic vancomycins is critically dependent on the structural integrity of the D-Ala-D-Ala binding pocket, unlike that of the corresponding *N*(*4*)-substituted derivatives [19]. Hence, the challenge of establishing whether a particular mechanism is more relevant to VRE activity lies ahead, as does an investigation to determine the possible involvement of cytoplasmic protein targets or transglycosylases that may have escaped detection in this study (e.g. the monofunctional transglycosylases recently reported in *E. coli* [40,41] and in other bacteria [42–44]). The presence of several proteins of undocumented function in the eluate from the DCB-PV affinity column is of particular interest in this regard, since these proteins may represent novel glycopeptide targets in *E. coli*. In conclusion, the implications of direct protein interactions should clearly be considered in future mechanistic studies of hydrophobic glycopeptide derivatives.

4. Significance

In this work, we have used affinity chromatography to demonstrate that DCB-PV, a 3,4-dichlorobenzyloxybenzyl vancomycin derivative incorporating the aromatic substituent at the *N*(*4*) position of the vancosamine sugar, interacts directly with a small subset of membrane-associated *E. coli* proteins, the majority of which are involved in cell wall biosynthesis and turnover. In contrast, the corresponding *N*(*4*)-unsubstituted glycopeptide, PV, does not significantly bind any particular protein. The retention of known *E. coli* transglycosylases (PBP-1B, MltA) on a DCB-PV affinity column, together with the inhibition of transglycosylase activity in ether-permeabilized bacteria by this semisynthetic glycopeptide, is consistent with the hypothesis that specific glycopeptide–protein interactions play a role in the bioactivity of *N*(*4*)-alkylated hydrophobic vancomycins. The *N*(*4*)-vancosamine substituent was identified as a likely protein binding determinant, although the glycopeptide moiety is also critical for bioactivity. These studies provide the first evidence for direct glycopeptide–protein interactions in bacteria and lay the foundation for investigating the role of protein binding in the VRE activity of hydrophobic vancomycins.

5. Materials and methods

The semisyntheses of DCB-PV and PV will be reported elsewhere [45]. *E. coli* wild type K12 strain MB4113A and all Gram-positive bacterial strains were obtained from the Merck Culture Collection. NHS-activated Sepharose 4B Hi-Trap columns and EAH Sepharose 4B resin were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Bradford reagent and SDS–PAGE molecular mass standards were from Bio-Rad Laboratories (Hercules, CA, USA). Bocillin-FL™ was purchased from Molecular Probes (Eugene, OR, USA). Gelcode Blue™ stain was obtained from Pierce (Rockford, IL, USA). 8-Methylnonanoic acid, 10-methylundecanoic acid, and *n*-decanoic acid were from Sigma (St. Louis, MO, USA). The permeabilized-cell 'site of inhibition' transglycosylase assay has been described in detail elsewhere [14]. [U-^{14}C]UDP-GlcNAc was obtained from NEN Life Science Products (Boston, MA, USA) and UDP-MurNAc-pentapeptide was prepared from vancomycin-treated *Bacillus megaterium* MB410 based on the methods of Moore et al. [46].

5.1. MIC determination

The MIC of each strain was determined by broth microdilution according to NCCLS guidelines [47], except that brain–heart infusion broth (Difco Laboratories, Detroit, MI, USA), which supports optimal growth of enterococci, was the test medium and the inoculum was 5–10 times higher than the recommended $3–7\times10^5$ CFU/ml. Cloned strains maintained at 4°C on brain–heart infusion agar (Difco) slants and grown overnight in trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 35°C with shaking at 220 rpm served as the inoculum source. The MIC is defined as the lowest antibiotic concentration that resulted in no visible growth after incubation at 35°C for 22 h.

5.2. Immobilization of glycopeptides

DCB-PV (5.4 μmol) or PV (5.4 μmol) was coupled to NHS-activated Sepharose 4B (10 μmol capacity) in a 1 ml Hi-Trap column using a modification of the manufacturer's protocols. Due to the limited aqueous solubility of the glycopeptides, the couplings were performed in *N*,*N*-dimethyl formamide buffered with three equivalents of triethylamine (4°C, 12 h). The amount of free glycopeptide remaining after each coupling reaction was quantified by C18-reversed phase high pressure liquid chromatography to calculate coupling efficiencies for DCB-PV (75%) and PV (82%). Residual NHS groups on the solid support were deactivated with ethanolamine as recommended by the manufacturer. The 3,4-dichlorobenzyloxybenzyl putrescine derivative DCBH was coupled to NHS-activated Sepharose using the same protocols.

5.3. Preparation of solubilized E. coli membranes

E. coli strain MB4113A was grown to early stationary phase (A_{600} = 1.5). Cells from a 4 l culture were harvested by centrifuging at $8000\times g$ for 10 min, and washed once with 200 ml of 20 mM sodium phosphate buffer, pH 7.0. The cell pellet was resuspended in 60 ml of the same buffer and lysed by passage through a French pressure cell (twice). Membranes were obtained by differential centrifugation of the lysate ($8000\times g$ for 10 min, $100\,000\times g$ for 90 min). The pellet was resuspended in the same buffer (~8 mg/ml total protein concentration as measured in a Bradford assay with bovine serum albumin as standard), and stored at −80°C until further use. Membranes were solubilized by the addition of an equal volume of 20 mM sodium phosphate pH 7.0 buffer containing 2% (w/v) Triton X-100, 2 M NaCl, followed by gentle shaking at 4°C for 2 h.

5.4. Affinity chromatography

The affinity chromatography experiments were performed at 4°C. Solubilized *E. coli* membranes (3–8 mg total protein) were loaded on the glycopeptide/substituent affinity columns (1 ml bed volume, 7–12 μmol binding capacity) at a flow rate of 1 ml/h. The columns were washed with 20 mM sodium phosphate, pH 7.0, 400 mM NaCl, 0.05% (w/v) Triton X-100 (20 column volumes, 30 ml/h). The columns were subsequently incubated for 12 h with 1 ml of elution buffer (20 mM sodium phosphate pH 7.0, 1% Triton X-100), containing the appropriate free ligand (100 μM). The columns were developed with elution buffer alone (12–13 column volumes, 30 ml/h) and 1 ml fractions were collected in each case. Aliquots (7–10 μl) of select fractions were analyzed by SDS–PAGE (10% polyacrylamide gel) and proteins were visualized by silver staining.

5.5. In situ tryptic digest and mass spectrometry of eluted proteins

The SDS–PAGE gels containing protein bands eluted from the affinity columns and stained with Gelcode Blue™ were washed extensively in water. Bands of interest were excised using disposable scalpels. Gel fragments from individual bands were placed in 0.65 μl microfuge tubes. Gel fragments were destained by washing with 50:50 acetonitrile:water followed by acetonitrile, and then 50:50 100 mM ammonium bicarbonate:acetonitrile. At each wash step, the tubes were vortexed for 10 min and the wash solution was discarded. Volumes of each wash step were sufficient to cover the gel fragments. After the last wash step, the gel fragments were dried completely under vacuum. Gel pieces were reconstituted in reduction buffer (10 mM tris(2-carboxyethylphosphine) hydrochloride in 100 mM ammonium bicarbonate). After 30 min, alkylation buffer was added (55 mM iodoacetamide in 100 mM ammonium bicarbonate). After a further 30 min incubation, the solution was removed. The gel fragments were washed with 50:50 100 mM ammonium bicarbonate:acetonitrile and then dried completely under vacuum. Reduced and alkylated gel fragments were reconstituted in digestion buffer (6 ng Promega modified trypsin per μl of 100 mM ammonium bicarbonate). The fragments were incubated at 37°C for 10 h. Following digestion, the supernatant was removed and placed in clean microfuge tubes. The gel fragments were extracted with equal volumes of 20% formic acid and 80% acetonitrile (30 min sonication). The extract was combined with the supernatant and the volume was reduced to 10–20 μl under vacuum. Approximately half of the sample was loaded onto a 100 nm C18 self-packed capillary HPLC column that was equilibrated with 0.1 M acetic acid. An Applied Biosystems 130A HPLC was used to deliver a linear gradient of 0.1 M acetic acid (solvent A) and acetonitrile (solvent B) to the column (0–80% B). The column was directly in line with a home-built micro-electrospray unit mounted on a ThermoQuest LCQ electrospray mass spectrometer. The spectrometer was operated under data-dependent conditions to allow automatic acquisition of MS/MS spectra for any peptide ion signal above a set intensity threshold. Upon completion of the gradient, data acquisition was terminated. The raw data file was then submitted for direct database correlation using a home-constructed web-based interface to SEQUEST as previously described [48].

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