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A Multivalent Approach to Drug Discovery for Novel Antibiotics

Daniel D. Long, James B. Aggen, Burton G. Christensen, J. Kevin Judice, Sharath S. Hegde, Koné Kaniga, Kevin M. Krause, Martin S. Linsell, Edmund J. Moran, John L. Pace

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Abstract The design, synthesis and antibacterial activity of novel glycopeptide/β-lactam heterodimers is reported. Employing a multivalent approach to drug discovery, vancomycin and cephalosporin synthons, **A** and **B** respectively, were chemically linked to yield heterodimer antibiotics. These novel compounds were designed to inhibit Gram-positive bacterial cell wall biosynthesis by simultaneously targeting the principal cellular targets of both glycopeptides and β-lactams. The antibiotics **8a**∼**f** displayed remarkable potency against a wide range of Gram-positive organisms including methicillin-resistant *Staphylococcus aureus* (MRSA). Compound **8e** demonstrated excellent bactericidal activity against MRSA (ATCC 33591) and initial evidence supports a multivalent mechanism of action for this important new class of antibiotic.

Keywords multivalent, multivalency, bifunctional, β-lactam, glycopeptide, antibiotic, MRSA

Introduction

β-Lactam and glycopeptide antibiotics are commonly used to treat many Gram-positive bacterial infections. However, the incidence and spread of antibiotic resistant bacteria poses an increasingly significant concern to the healthcare community. Virulent Gram-positive pathogens have emerged that exhibit high-level resistance to β-lactams and/or glycopeptide antibiotics. Multi-drug resistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermeditate susceptible *Staphylococcus aureus* (VISA), vancomycin-resistant enterococci (VRE) and penicillin-resistant *Streptococus pneumoniae* (PRSP) have prompted an intensified search for novel agents [1]. MRSA is the predominant nosocomial Gram-positive pathogen and is now emerging as a serious threat in the community setting as well as in hospitals [2].

β-Lactam (penicillins, cephalosporins and carbapenems) and glycopeptide antibiotics derive their antimicrobial activity by interrupting processes critical to the formation of the mature cell wall peptidoglycan. Specifically, β-lactams are selective mechanism-based inhibitors that form covalent complexes with the active-site serine of the transpeptidase domain of penicillin binding protein (PBP). Glycopeptides derive their biological activity principally by forming a tight complex with D-alanyl-D-alanine motifs that are present on both lipid intermediate II

D. D. Long (Coresponding author)**, J. B. Aggen**[†]**, B. G. Christensen, J. K. Judice**[†]**, S. S. Hegde, K. Kaniga**[††]**, K. M. Krause, M. S. Linsell**[†]**, E. J. Moran, J. L. Pace**[†††]**:** Theravance, Inc., 901 Gateway Blvd., South San Francisco, CA 94080, USA, E-mail: dlong@theravance.com

[†] Present address: Achaogen, 7000 Shoreline Court, Suite 371, South San Francisco, CA 94080

[††] Present address: Johnson and Johnson Pharmaceutical Research and Development, LLC, 1010 Joaquin Road, M4-2A, Mountain View, CA 94043

[†††] Present address: StratoPharm, LLC, 1 Innovation Way, Suite 100, Newark, DE 19711

[*N*-acetylglucosamine-β-(1,4-undecaprenyl diphospho-*N*-acetylmuramyl-L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala)] and on immature peptidoglycan that has been polymerized through the action of transglycosylase, but not yet cross-linked through the action of transpeptidase. The inhibitory effect of both these mechanisms disrupts the synthesis and cross-linking of peptidoglycan which results in osmotic instability, cell rupture and death. Bacterial resistance to both drug classes has evolved [3] and approaches to circumvent the mechanisms of resistance have focused upon modification of existing glycopeptides and β-lactams. This is exemplified by the addition of lipophilic groups to the saccharide units of glycopeptides [4] (for example, telavancin®) [5], and by the addition of polar, lipophillic substituents at the C3 and C7 positions of a cephalosporin core [6]. Of course, novel antibiotics that target bacterial cell functions other than peptidoglycan biosynthesis are also an area of intense research. The protein synthesis inhibitors dalfopristin/quinupristin (Synercid®), tigecycline (Tygacil®) and linezolid (Zyvox®), and the membrane-targeting lipopeptide daptomycin (Cubicin®) [7] are welcome additions to the antibiotic armamentarium.

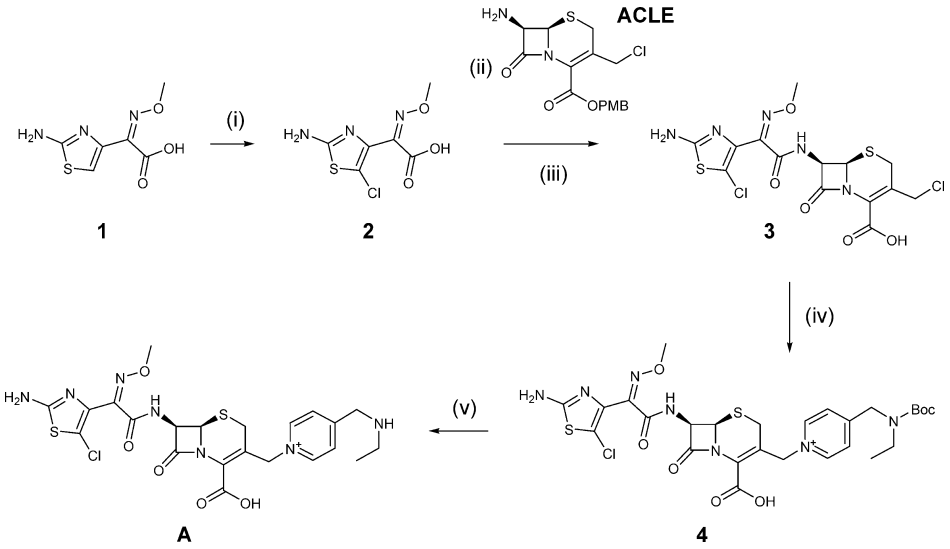
Application of a multivalent approach to drug discovery offers an opportunity for the design of novel antibiotic compounds with the potential for enhanced efficacy, duration and reduced bacterial resistance. Multivalency is a phenomenon whereby multiple, simultaneous, energetically coupled ligand-receptor interactions enhance the overall affinity and selectivity of binding [8]. Multivalency is common in natural systems; for example, in controlling cell-cell interactions and in the adhesion of a virus particle to an erythrocyte [9]. Recent literature suggests that multivalency may also find applicability in the design of small molecule ligands [10]. Homodimers and oligomers of vancomycin [11], paclitaxel [12] and opioids [13] have all been reported to enhance binding relative to their constitutive monomeric components [14]. In addition to enhanced efficacy it has been proposed that multivalent molecules may also offer physicochemical advantages and allow coupling of pharmacophores with distinct mechanisms of action [15]. Due to the physical proximity of the principal cellular targets of glycopeptides (lipid intermediate II) and β-lactams (transpeptidase domain of PBP), and their sequential role in the cell-wall biosynthetic pathway, we reasoned that a single molecule that inhibited both targets in a bifunctional manner may provide enhanced potency and bactericidal activity. In this paper, we present the first examples of glycopeptide/β-lactam heterodimers and report their extraordinary antibacterial activity against multi-drug resistant Gram-positive pathogens.

Multivalent Design

The design of single-molecule glycopeptide/β-lactam heteromers focused on bifunctional constructs of vancomycin and a cephalosporin. Several cephalosporins display potent anti-MRSA activity and both the chemistry and manipulation of cephalosporins is less complex than that of other β-lactams, such as the penicillins and carbapenems [16]. We selected a semi-synthetic cephalosporin core **A** (Scheme 1) that included i) a chloro-substituted aminothiazolyl(syn-methoxyimino)acetyl moiety at the C7 position, and ii) a methyl aminoethyl substituted, pyridinium motif at the C3-position. The former offered chemical stability towards several broad spectrum β-lactamases [16] and in addition, conferred additional potency against MRSA relative to non-chlorinated analogues (in-house unpublished data). The choice of a pyridinium motif at C-3 provided the molecule with a zwitterionic character, a property that has previously been shown to promote better access to immature peptidogylcan and more efficiently inhibit PBPs [17]. The methyl amino moiety provided a convenient attachment point through which to link the cephalosporin core *via* an amide bond. For the glycopeptide core, vancomycin was chosen because it is the best understood and most clinically used agent of this class. We utilized a modified vancomycin synthon **B** (Scheme 2) with a sterically unhindered ethyl primary amino moiety attached to the vancosamine nitrogen. This allowed linkage through an amide bond to vancomycin whilst preserving the basicity at the vancosamine position. This basic site has been shown to be optimal for hydrophobic vancosamine-substituted derivatives of both vancomycin and chloroeremomycin [5, 11, 18]. A variety of amide-linked heterodimers were prepared to establish proof of principle and explore the optimal length of simple alkyl linkages.

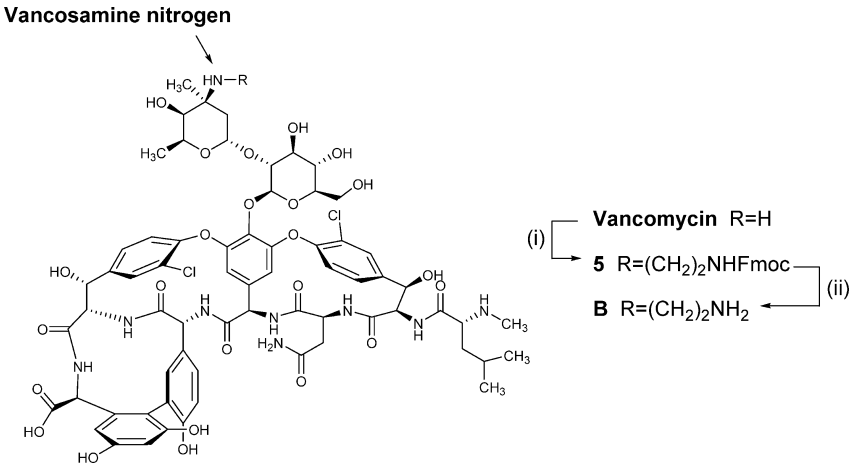
Chemistry

The cephalosporin monomer core **A** was prepared from 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride salt (ACLE) (Scheme 1). Chlorination of aminothiazolyl(syn-methoxyimino)acetic acid **1** was effected with *N*-chlorosuccinimide (NCS) in AcOH at 70°C and the resulting acid **2** coupled to ACLE with *N*-[3-dimethylaminopropyl]-*N*′-ethylcarbodiimide hydrochloride (EDCI) in the presence of collidine. The *p*-methoxybenzyl (PMB) protecting group was removed by treatment with TFA/anisole and the chloro leaving group at C-3 of the resultant acid **3** was displaced with *N*-Boc-*N*-ethyl-4-(aminomethyl)-pyridine. Boc deprotection of the pyridinium cephalosporin **4** with TFA afforded the desired β-lactam core **A**.



Scheme 1

(i) NCS, AcOH, 70°C (ii) EDCI, HOAt, 2,4,6-collidine, DMF, 0°C (iii) TFA, anisole, 1,2-DCE (iv) *N*-Boc-*N*-ethyl-4-(aminomethyl)-pyridine, 2,4,6-collidine, CH_3CN, DMF (v) TFA, anisole.



Scheme 2

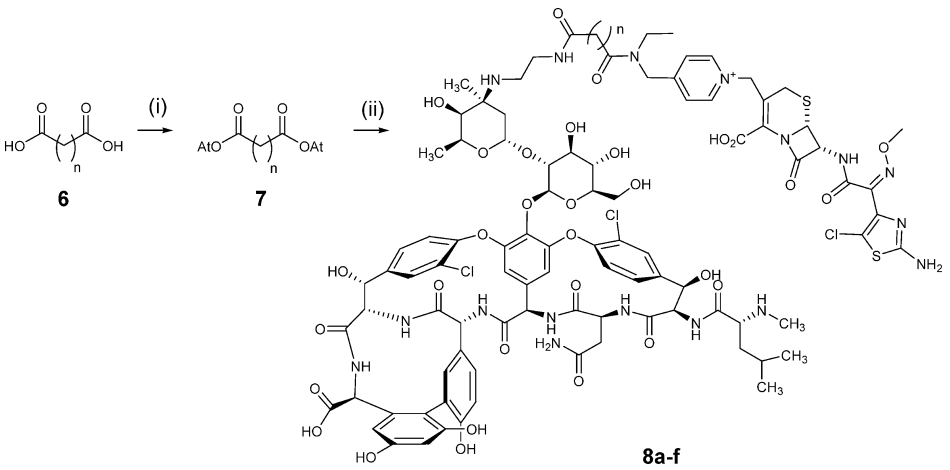
(i) *N*-Fmoc-glycinal, DIPEA, DMF then TFA, $NaCNBH_3$, MeOH (ii) Quinuclidine, DMF.

The vancomycin synthon **B** was prepared in two steps by reductive amination of vancomycin [19] with fluorenylmethoxycarbonyl (Fmoc) protected glycinal, followed by quinuclidine-mediated deprotection of intermediate **5** (Scheme 2). The regioselective nature of the reductive amination was confirmed by mass spectrometry. The daughter fragment corresponding to loss of aminoethyl vancosamine ($m/z=1305$) was observed. No mass related to loss of unsubstituted vancosamine ($m/z=1348$) was apparent.

The two monomer core pieces **A** and **B** were covalently linked by an amide bond at each terminus of a di-carboxylic acid moiety. The diacids **6** were first activated as their 1-hydroxyazabenzotriazole (HOAt) esters **7** and then treated *in situ* sequentially with the β-lactam core **A** and the glycopeptide core **B**, in the presence of collidine (Scheme 3). The products were purified by reverse phase-HPLC to afford the glycopeptide/β-lactam heterodimers **8a**~**f**.

Results and Discussion

The glycopeptide/β-lactam heterodimers were screened against a panel of bacterial strains, representing both the



Scheme 3

(i) HOAt, DIC, DMF (ii) (a) **A**, 2,4,6-collidine, DMF (b) **B**, 2,4,6-collidine, TFA, DMF.

Table 1

Compound	*n*	*m/z* (calcd)[a]	*m/z* (obsd)[b]	MIC (μg/ml)						
				MSSA	MRSA	VISA	EFSVS	EFSVA	EFMVA	PRSP
8a	3	2155.5/862.8	2155.6/862.6	0.012	0.05	0.05	≤0.05	3.13	>50	ND
8b	4	2169.5/869.8	2169.4/870.1	0.012	0.1	0.1	≤0.05	3.13	>50	0.1
8c	6	2197.6/883.9	2197.0/884.3	0.012	0.05	0.05	≤0.05	6.25	>50	≤0.05
8d	8	2225.6/897.9	2225.5/898.4	0.012	0.05	0.05	≤0.05	1.56	>50	≤0.1
8e	10	2253.7/911.9	2252.8/912.2	0.024	0.05	0.05	0.05	3.13	>50	0.024
8f	14	2309.8/940.0	2309.6/940.4	0.2	0.39	1.56	0.1	6.25	25	≤0.1
A	—	567.1	567.2	3.13	12.5	6.25	3.13	6.25	>50	1.56
B	—	1493.3	1493.7	0.78	1.56	6.25	1.56	>50	>50	ND
Vancomycin	—	—	—	0.78	0.78	6.25	0.78	>50	>50	0.78

MSSA, methicillin-susceptible *S. aureus* strain ATCC 13709; MRSA, methicillin-resistant *S. aureus* strain ATCC 33591; VISA, vancomycin-intermediate *S. aureus* strain HIP-5836; EFSVS, vancomycin-susceptible *E. faecalis* strain ATCC 29212; EFSVA, vancomycin-resistant *E. faecalis* strain MGH-01 (VanA phenotype); EFMVA, vancomycin-resistant *E. faecium* strain KPB-01 (VanA phenotype); PRSP, penicillin-resistant *Streptococcus pneumoniae* strain SU-10. [a] Calculated and [b] observed mass-to-charge ratios for singly charged parent ions [M^+] for **8a**∼**f**/**A**, [$M+H^+$] for **B**, and mass-to-charge ratios for doubly charged daughter ions ([M−cephalosporin (430.9)+H^+]$^+$) for **8a**∼**f**. ND, not determined.

major Gram-positive human pathogens (staphylococci, enterococci and streptococci) and important drug resistance phenotypes. MICs for compounds **8a**∼**f**, the monomeric cores **A** and **B**, and vancomycin are presented in Table 1.

The heterodimers **8a**∼**e**, displayed highly potent antibacterial activity (0.012∼0.1 μg/ml) against many of the representative Gram-positive organisms (MSSA, MRSA, VISA, EFSVS and PRSP) and were often significantly (>30 fold) more potent than either of their monomeric components, β-lactam **A** and glycopeptide **B**, and in addition, vancomycin. Interestingly, in the instance where the organism (EFSVA) was resistant to one parent molecule (**B**, >50 μg/ml) the heterodimer MIC was of the same order (1.56∼6.25 μg/ml) as that of the active parent (**A**, 6.25 μg/ml). When the bacterial strain (EFMVA) was resistant to both parent antibiotic components **A** and **B** (>50 μg/ml) all the heterodimers were additionally observed to be ineffective (>50 μg/ml). These observations imply that the selection of the appropriate β-lactam and glycopeptide component is crucial to determining the antibacterial profile of the resultant heterodimer. There were only minor SAR between differing alkyl linkages from n=3 to 10 (**8a**∼**e**). However, for n=14 (**8f**) a general increase in MIC of >7 fold, against the staphylococci

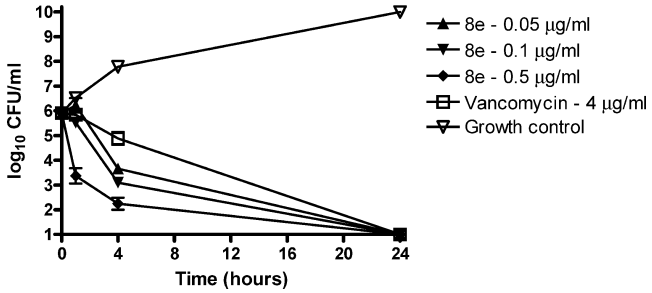


Fig. 1 Bactericidal activity of **8e** against *S. aureus* ATCC 33591 (MRSA).

strains was observed.

Compound **8e** was rapidly bactericidal, achieving 3-log CFU/ml reduction after only 4 hours at a concentration of 0.5 μg/ml against *S. aureus* ATCC 33591 (MRSA) (Fig. 1). This is in contrast to the bactericidal activity of vancomycin which achieves a similar CFU/ml reduction against *S. aureus* ATCC 33591 (MRSA) after 24 hours at a concentration of 4.0 μg/ml.

Evidence for the bifunctional nature of the glycopeptide/β-lactam heterodimers mode of inhibition was investigated by combination experiments. Against MRSA, the combination of β-lactam **A** and vancomycin resulted in no observable synergy and a MIC value of 1.56/1.56 μg/ml for each component, respectively [20]. In addition, checkerboard assay of the monomeric substructures tested in combination resulted in a fractional inhibitory concentration index indicative of an indifferent effect. Therefore, the potent activity of the heterodimers cannot be reproduced by their individual components tested either alone or in combination; covalent linkage of the component moieties is required for the potent activity in accordance with a multivalent mode of action.

In summary, we have designed and synthesized a set of novel glycopeptide/β-lactam heterodimers to simultaneously target the principal bacterial cellular targets of both glycopeptides (PBP peptidoglycan substrates) and β-lactams (transpeptidase domain of PBP). The compounds **8a**～**e** have been shown to display high potency against a range of important Gram-positive bacteria including MRSA and VISA. Compound **8e** demonstrated excellent bactericidal activity against a strain of MRSA (ATCC 33591). Initial evidence supports the hypothesis that the heterodimers act *via* a multivalent mechanism. Further efforts to modulate the potency and physical properties of these important molecules and to elucidate the multivalent mechanism of action will be reported in due course.

Experimental

General Methods

Reagents and solvents were used as received from commercial suppliers and all reactions were carried out at room temperature and without rigorous exclusion of ambient atmosphere unless otherwise noted. Ion-spray mass spectra (IS-MS) were obtained on a PE Sciex API 150EX mass spectrometer operating in positive ion mode. NMR spectra were recorded at 300 MHz. Chemical shifts (δ) are reported in ppm downfield of TMS. Abbreviations for reagents: dichloroethane (DCE), diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIPEA), *N*-[3-dimethylaminopropyl]-*N*′-ethylcarbodiimide hydrochloride (EDCI).

HPLC Methods

Analytical: Reactions were monitored by analytical reversed-phase HPLC (RP-HPLC) with an HP1100 instrument using a 2.1 mm×50 mm, 3.5 μm C14 Zorbax Plus Bonus-RP column with UV detection at 214 nm. For the analytical separations, a 0.5 minutes isocratic period was followed by a 4.5 minutes gradient of 2～90% CH_3CN in water with 0.1% TFA.

Preparative: Compounds were purified by preparative RP-HPLC on a Varian ProStar system using 2.5 or 5.0 cm×25 cm Rainin Dynamax columns and flow rates of 15 or 50 ml/minute, respectively. Separations were accomplished with a gradient of 5～60% CH_3CN in H_2O containing 0.1% TFA over 90 minutes.

Intermediate A

N-Chlorosuccinimide (27.9 g, 0.21 mol) was added to a stirred solution of 2-amino-α-(methoxyimino)-4-thiazoleacetic acid **1** (40 g, 0.20 mol) in AcOH (500 ml) and the reaction mixture heated to 70°C for 75 minutes. The dark solution was concentrated *in vacuo* to afford crude **2** and then dissolved in DMF (700 ml). ACLE (81.1 g, 0.20 mol) and 1-hydroxyazabenzotriazole (27.0 g, 0.20 mol) were added and the reaction mixture cooled to 0°C. 2,4,6-Collidine (26.7 g, 0.22 mmol) and EDCI (42.2 g, 0.22 mol) were susbsequently added and the reaction mixture stirred for 5 hours. The reaction mixture was diluted with EtOAc/diethyl ether (1 : 1) and washed with H_2O. The aqueous phase was extracted with EtOAc (×2) and the total combined organic extracts washed with 0.5 M citric acid, H_2O, saturated sodium hydrogen carbonate, water and brine. The organic phase was dried ($MgSO_4$), filtered and concetrated *in vacuo*. The crude residue was dissolved in a minimum of CH_2Cl_2 and poured into diethyl ether. The

resulting precipitate was filtered and dried *in vacuo* to afford PMB-protected cephalosporin acid (48.5 g, 42%). ^{1}H-NMR (DMSO-d_6) δ 9.54 (d, 1H), 7.33 (s, 2H), 7.28 (d, 2H) 6.85 (d, 2H), 5.77 (dd, 1H), 5.15 (m, 3H), 4.46 (d, 1H), 4.37 (d, 1H), 3.79 (s, 3H), 3.67 (s, 3H), 3.62 (d, 1H), 3.46 (d, 1H). IS-MS, calcd. *m*/*z* for [M+H$^+$]: 586.04; obsd. 586.2.

TFA (300 ml) was added to a stirred solution of PMB-protected cephalosporin acid (48.5 g, 83 mmol) and anisole (40 ml) in 1,2-DCE (400 ml). After 40 minutes, the reaction mixture was concentrated to approx. 300 ml volume *in vacuo* and added to diethyl ether (1 liter). The resultant precipitate was filtered, washed with diethyl ether and dried *in vacuo* to afford crude **3** (42.5 g, >100%). *N*-Boc-*N*-ethyl-4-(aminomethyl)-pyridine (15.2 g, 64.5 mmol) was added to a stirred solution of crude **3** (25.0 g, 53.6 mmol) and 2,4,6-collidine (10.1 g, 83.3 mmol) in CH_3CN/DMF (1 : 1, 200 ml). After 3.5 hours, the reaction mixture was added to diethyl ether (1 liter) and the resultant precipitate filtered, washed with diethyl ether and dried *in vacuo*. The crude residue (26 g, 73%) was purified by RP-HPLC to afford **4**·TFA salt as an off-white powder. ^{1}H-NMR (DMSO-d_6) δ 9.36 (d, 1H), 8.91 (d, 2H), 8.73 (s, br, 3H), 7.95 (d, 2H), 7.24 (s, br, 1H), 5.83 (m, 1H), 5.50 (m, 2H), 5.17 (d, 1H), 4.66 (s, 2H), 3.83 (s, 3H), 3.52 (d, 1H), 3.30 (m, 3H), 1.37 (s, 9H), 1.08 (t, 3H).

TFA (8.0 ml) was added to a stirred solution of **4**·TFA salt (610 mg, 0.78 mmol) in anisole (1.0 ml). After 20 minutes, the reaction mixture was added to diethyl ether (100 ml) and the resultant precipitate filtered, washed with diethyl ether and dried *in vacuo* to afford **A**·2TFA salt as a light yellow solid. ^{1}H-NMR (DMSO-d_6) δ 9.56 (d, 1H), 9.44 (bs, 1H), 9.10 (d, 2H) 8.21 (d, 2H), 7.38 (s, br, 1H), 5.86 (m, 1H), 5.54 (m, 2H), 5.15 (d, 1H), 4.51 (s, 2H), 3.80 (s, 3H), 3.40 (m, 2H), 3.30 (m, 2H), 1.22 (t, 3H). IS-MS, calcd. *m*/*z* for [M$^+$] : 567.1; obsd. 567.2.

Intermediate B

N-(9-fluorenylmethoxycarbonyl)glycinal (6.5 g, 23 mmol) was added to a stirred suspension of vancomycin hydrochloride (34.1 g, 23 mmol) and DIPEA (8.5 ml, 46 mmol) in DMF (340 ml) at room temperature. The reaction mixture was stirred for 2 hours and then MeOH (340 ml) and sodium cyanoborohydride (4.3 g, 69 mmol) were added followed by TFA (5.2 ml, 69 mmol). The reaction mixture was stirred for a further 1 hour and then concentrated *in vacuo* to remove the MeOH. The remaining solution was added to CH_3CN (3.5 liters) and the resultant precipitate filtered, washed with diethyl ether (2 liters) and dried. The residue was purified by RP-HPLC to afford intermediate **5**·2TFA salt. IS-MS, calcd. *m*/*z* for [M+H$^+$]: 1715.6; obsd. 1715.2.

Quinuclidine (820 mg, 7.4 mmol) was added to a stirred solution of intermediate **5**·2TFA salt (4.75 g, 2.4 mmol) in DMF (25 ml) at room temperature and stirred for 5 hours. The reaction mixture was added to diethyl ether (200 ml) and the suspension centrifuged. The solution was decanted and the solid washed with diethyl ether (150 ml), centrifuged and decanted and this process repeated with CH_3CN (3×150 ml). The solid was dried to afford **B**·quinuclidine salt (4.2 g, 100%). IS-MS, calcd. *m*/*z* for [M+H$^+$]: 1493.3; obsd. 1493.7.

Representative Example: Glycopeptide-β-lactam Heterodimers 8a~f

Diisopropylcarbodiimide (31 μl, 0.2 mol) was added to a stirred solution of diacid (0.1 mmol) and 1-hydroxy-azabenzotriazole (34 mg, 0.25 mmol) in DMF (0.5 ml) at 0°C. The reaction mixture was stirred overnight and then cooled to 0°C and a solution of **A**·2TFA salt (16 mg, 20 μmol) and 2,4,6-collidine (8.2 μl, 60 μmol) in DMF (0.1 ml) added. After 2 hours, the reaction mixture was added to diethyl ether (14 ml) and the suspension centrifuged. The solution was decanted and the solid dissolved in DMF (0.5 ml) and added to diethyl ether (14 ml). The suspension was again centrifuged, the solution decanted and the solid residue dried. To the solid was added a solution of **B**·quinuclidine salt (50 mg; 30 μmol), TFA (6.7 μl; 90 μmol) and 2,4,6-collidine (24 μl; 0.18 mmol) in DMF (0.5 ml) at 0°C. The reaction mixture was stirred at 0°C for 1 hour and then quenched with TFA (14.3 μl, 0.2 mmol) and concentrated *in vacuo*. The residue was purified by RP-HPLC to afford the glycopeptide-β-lactam heterodimers **8a**, **b**, **c**, **d**, **e**, **f**·3TFA salt (yield typically approx. 20%). IS-MS, Table 1.

Determination of *in Vitro* Antimicrobial Activity

MIC susceptibility determinations were performed using a standard broth microdilution assay according to the recommendation of the National Committee for Clinical Laboratory Standards [21].

Determination of Bactericidal Activity

Time-kill assays were performed in broth media. Log-phase cultures of bacteria with a final density of approximately 10^6 CFU/ml were tested. Immediately after inoculation, the first samples (100 μl) were taken out and constituted the time point 0 hour. Test tubes were incubated in ambient air at 35°C with constant agitation for 24 hours and were sampled at various time points. Samples were diluted in saline and colony counts were performed by applying 100 μl of samples directly on solid media. Colonies were

counted after 24 hours incubation.

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