Synthesis of rigidly-linked vancomycin dimers and their *in vivo* efficacy against resistant bacteria†

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A novel and efficient avenue for the preparation of dimeric vancomycins is described, and the dimers exhibited excellent antibacterial activities in the murine infection model.

The emergence of multidrug-resistant gram-positive pathogens represented by MRSA (methicillin resistant *S. aureus*) is of concern in clinical institutions. The emergence of vancomycin-resistant bacteria (VRE:[1] vancomycin-resistant *enterococci*; VRSA:[2] vancomycin-resistant *S. aureus*) is of particular concern,[3] because vancomycin is the last resort for the treatment nosocomial infections by multi-resistant gram-positive bacteria. Currently, Zyvox (Linezolid; Pfizer), Synercid (Monarch and King), and Tygacil (Wyeth) are available for VRE infections, but have problems such as side effects and drug resistance.[4] Here we describe the synthesis of novel vancomycin dimers with excellent antibacterial activity both *in vitro* and *in vivo*.

Vancomycin binds to a bacterial cell wall intermediate by recognizing the D-Ala–D-Ala terminus of lipid intermediates, and thus exhibits antibacterial activities against Gram-positive bacteria.[5] The dipeptide terminal of lipid II is transformed to D-Ala–D-lactate in VREs (VanA and VanB phenotypes) and VRSAs (Van A phenotypes), and this lowers the binding affinity of vancomycin toward the mutated lipid II.[6]

The hypothesis that the associated dimer of vancomycin plays important roles in the recognition events of the lipid intermediates[7] prompted synthetic chemists to design covalently-linked glycopeptide dimers. It has been shown that the choice of the linker structure significantly affects the antibacterial profiles.[8]

In order to develop lead-drug compounds based on the dimerization strategy, advances in novel synthetic protocols are necessary. The polyfunctionalized nature of vancomycin limits the reactions applicable for dimerization. It should be possible to link bulky vancomycin molecules without tedious protection/deprotection procedures over a short distance. Most of the reported dimers have long flexible linkers, and some include biologically unstable disulfide bonds in the linker portion.

To overcome these problems, we focused our attention on the use of natural privileged templates found in actinomycin[9] (Fig. 1).

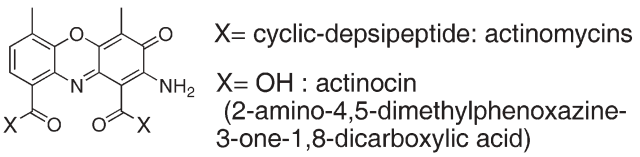

Fig. 1 Actinocin, a natural privileged scaffold for dimeric peptidic ligands.

Two cyclic depsipeptide subunits of actinomycin were arranged in parallel on a rigid tricyclic actinocin template. This led to the design of novel vancomycin dimers (Fig. 2). It should be noted that the actinocin-type linkers are quite rigid. Since flexible linkers (*e.g.* $-(CH_2)_n-$) are thought to reduce the entropic advantages in dimeric binding modes,[10] the rigid nature of the linker was expected to minimize the entropic loss arising from the linker unit in dimeric binding events. There are only four rotatable bonds between the linked vancomycin molecules in **1** (Fig. 2). Molecular modeling studies (data not shown) revealed that two vancosamine nitrogen atoms should exist approximately 7 angstroms apart in **1**.[11,12]

With the biosynthetic pathway to actinocin nuclei in mind, an alkoxynitrobenzaldehyde **6** was introduced to vancomycin by

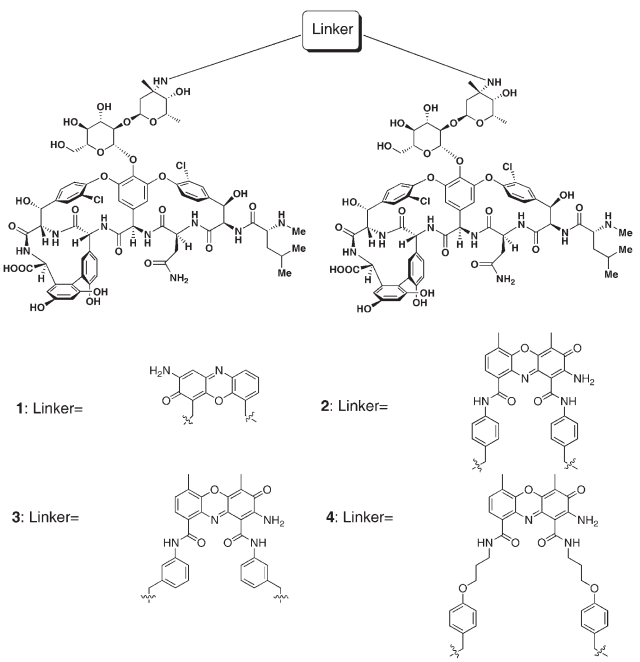

Fig. 2 Structures of vancomycin dimers with actinocin-based linkers.

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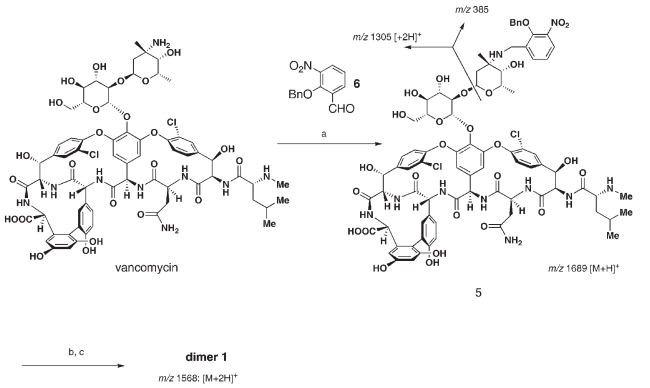

Scheme 1 Synthesis of vancomycin dimer **1** by mild oxidative coupling of an amino-phenol. *Reagents and conditions*: (i) aldehyde **6**, DMF–MeOH (1 : 1), *i*-Pr_2NEt, 70 °C, 2 h; $NaBH_3CN$ (4 eq.), 70 °C, 20 h (59%); (ii) Pd–C/H_2, MeOH; (iii) *p*-quinone, MeOH, RT, 20 h (68%).

reductive alkylation. The regioselectivity of the alkylation was proven by fragmentation analysis of ESI mass spectra (Scheme 1). Conversion of a nitro group to an amine and removal of benzyl ether were achieved in a one-pot reaction by catalytic hydrogenation. The obtained aminophenol intermediate was unstable, and thus was employed directly in the dimerization step. Treatment of the aminophenol in methanol by quinone furnished a pink-colored dimer at room temperature. After HPLC purification (Develosil ODS HG-5, ϕ 20 mm × 250 mm, CH_3CN : H_2O : TFA 33 : 66 : 0.1, flow rate 2 mL min^{-1}, t_R = 21.82 min) and lyophilization, dimer **1** was isolated in 68% yield as a TFA salt. Various reactions, such as amide formation, disulfide coupling, and cross metathesis, have been employed for the synthesis of vancomycin dimers. Our oxidative coupling to actinocin-linked dimers has notable advantages, including mild reaction conditions, high yield and selectivity and giving stable products in the *in vivo* test. Dimers **2**, **3**, and **4** were also prepared.

The antibacterial activities of the actinocin-based dimers were evaluated with a set of vancomycin-susceptible and -resistant bacterial strains (Table 1). The synthetic dimers were found to be active against VREs. No clear correlation was found between the linker structures and the antibacterial activities. We next selected **1** and **3** and conducted further investigations. The potencies of dimers for vancomycin-susceptible *S. aureus* were somehow lower than those for *enterococci*, but the problem could be solved by changing the counter anion of the dimers **1** and **3** from trifluoroacetate. The minimum inhibitory concentrations (MICs) of dimer-HCl salt are shown in parentheses (Table 1 and 2). The antibacterial activities of these dimer-HCl salts were enhanced 2 to 32-fold, not only for *S. aureus* but also for other strains including vancomycin-resistant strains. The dimers thus possessed balanced and practical *in vitro* antibacterial activities against both vancomycin-sensitive and vancomycin-resistant strains.

We next conducted investigations with the second set of bacteria, including vancomycin-resistant *S. aureus* (VRSA: Table 2). VRSA and VanA-type VRE share the same vanA-type resistant gene. However, the pathogenic and more harmful nature of the parent *S. aureus* strain than those of *enterococci* leads to VRSA being a cause of serious concern in clinics. The dimers were found to be quite active against VRSA.

Table 1 The first set of *in vitro* evaluations of vancomycin dimers with different linker lengths[a]

	Dimer **1**	**2**	**3**	**4**	Vancomycin
S. aureus FDA209P JC-1	2 (N.T.)	2	2 (N.T.)	1	0.5
S. aureus SR3637 (MRSA)	16 (8)	32	16 (2)	32	1
S. aureus ATCC700787 (VISA:Mu50)	32 (32)	64	16 (4)	32	1
E. faecalis ATCC29212	2 (N.T.)	2	4 (N.T.)	1	2
E. faecalis SR7914 (VRE:VanA)	8 (4)	2	8 (4)	8	>64
E. faecalis SR23630 (VRE:VanB)	2 (1)	4	4 (0.5)	2	32
E. faecium NCTC7171	0.5 (N.T.)	1	2 (N.T.)	0.5	0.5
E. faecium SR7917 (VRE:VanA)	32 (N.T.)	2	8 (N.T.)	32	>64
E. faecium SR23598 (VRE:VanB)	1 (0.25)	1	2 (0.5)	1	>64

[a] Minimum inhibitory concentration (MIC) in micrograms per millilitre as determined by microdilution broth assay *in vitro*. TFA salts of dimers **1**–**4** were employed for the evaluation. Antibacterial activities of dimers **1** and **3** were also examined with their HCl salts, and the MIC values are shown in parentheses.

Table 2 The second set of *in vitro* evaluations of antibacterial activity[a]

	1	**3**	Vancomycin
S. aureus VRSA-2 (VCM-resistant)	8 (4)	32 (1)	>64
E. faecium SR7940 (VRE:VanA)	8 (4)	8 (4)	>64
S. aureus Smith	16 (4)	16 (2)	1
S. pneumoniae Type1	0.125 (⩽0.063)	1 (0.25)	0.5
S. pneumoniae SR23928 (PRSP)	⩽0.063 (⩽0.063)	1 (0.5)	0.5
S. pneumoniae tupelo (VTSP)	0.125 (⩽0.063)	1 (0.125)	0.25

[a] Minimum inhibitory concentration in micrograms per millilitre as determined by microdilution broth assay *in vitro*. The TFA salts and HCl salts of dimers **1** and **3** were employed for the evaluation. Antibacterial activities of the HCl salts are shown in parentheses.

These promising antibacterial results prompted us to examine the efficacy of the selected dimers *in vivo* (Table 3).[13] A test was conducted with *S. pneumoniae*-infected mouse, which is resistant to penicillin, using **1**, **3**, and linezolid because the treatment of upper

Table 3 *In vivo* efficacy in murine lung infection model with *S. pneumoniae*[a]

Compounds	Two-log kill dose/mg kg^{-1}	Three-log kill dose/mg kg^{-1}	MIC/μg mL^{-1}
1	4.49	6.54	⩽0.063
3	34.8	72.2	0.125
Linezolid	21.3	33.1	0.5

[a] Animal: CBA mouse (6 weeks old, N = 5). Infection: *S. pneumoniae* SR11031 (PRSP: 6 × 10^5 cfu $mouse^{-1}$) was administered intranasally (i.n.). Compounds (HCl salt) were administered by the intravenous route (i.v.) 17 h and 24 h post infection. Viable count per lung was carried out at 48 h postinfection. A 2-log kill dose was defined as the dose required to produce a decrease in titer of 2 log CFU g^{-1} from pretreatment controls. Similarly, 3-log kill doses were defined as the doses required to produce decreases in titer of 3 log CFU g^{-1}.

and lower respiratory tract infections by multi-resistant *S. pneumoniae* has become a therapeutic problem. Among the tested compounds, **1** exhibited the most promising bactericidal effect. Dimer **1** at 10 mg kg^{-1} dose yielded undetectable bacterial counts (*i.e.* <50 CFU mL^{-1}, data not shown). Pharmacokinetic profiles greatly affect the *in vivo* potency, which includes the protein binding, and the tissue penetration of the drug showing that there are significant barriers to attaining good *in vivo* activity. It is noteworthy that **1** showed a preliminary but promising curative effect *in vivo*.

At present, it is not clear how actinocin-based vancomycin dimers exhibit their enhanced antibacterial activities. Biochemical studies are underway to investigate the mechanism of these actions. The answer appears to be more complex than simple cooperative binding to transformed peptidoglycan intermediates.[8b]

The novel and efficient synthesis route presented here should be applicable to the preparation of highly potent dimeric vancomycins that are effective in the animal infection model.

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Notes and references

1 (*a*) R. E. Leclerq, R. E. Derlot, J. Duval and P. Courvalin, *N. Engl. J. Med.*, 1988, **319**, 157–161; (*b*) A. H. C. Uttley, C. H. Collins, J. Naidoo and R. C. George, *Lancet*, 1988, **1**, 57–58.

2 D. M. Sievert, M. L. Boulton, G. Stoltman, D. Johnston, M. G. Stobierski, F. P. Downes, P. A. Somsel, J. T. Rudrik, W. Brown, W. Hafeez, T. Lundstrom, E. Flanagan, R. Johnson, J. Mitchell and S. Chang, *Morbid. Mortal. Weekly Rep.*, 2002, **51**, 565–567.

3 D. Kahne, C. Leimkuhler, W. Lu and C. Walsh, *Chem. Rev.*, 2005, **105**, 425–448.

4 As Zyvox, Synercid and Tygacil inhibit the protein synthesis of bacteria, all differ from vancomycin in their mechanisms of action.

5 (*a*) D. H. Williams, M. P. Williamson, D. W. Butcher and S. J. Hammond, *J. Am. Chem. Soc.*, 1983, **105**, 1332–1339; (*b*) P. Reynolds, *Eur. J. Clin. Microbiol. Infect. Dis.*, 1989, 943–950.

6 (*a*) C. T. Walsh, S. L. Fisher, I. S. Park, M. Prahalad and Z. Wu, *Chem. Biol.*, 1996, **3**, 21–28; (*b*) T. D. H. Bugg, G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin and C. T. Walsh, *Biochemistry*, 1991, **30**, 10408–10415; (*c*) R. Xu, G. Greiveldinger, L. E. Marenus, A. Cooper and J. A. Ellman, *J. Am. Chem. Soc.*, 1999, **121**, 4898–4899.

7 D. H. Williams and B. Bardsley, *Angew. Chem., Int. Ed.*, 1999, **38**, 1172–1193.

8 (*a*) U. N. Sundram, J. H. Griffin and T. I. Nicas, *J. Am. Chem. Soc.*, 1996, **118**, 13107–13108; (*b*) J. H. Griffin, M. S. Linsell, M. B. Nodwell, Q. Chen, J. L. Pace, K. L. Quast, K. M. Krause, L. Farrington, T. X. Wu, D. L. Higgins, T. E. Jenkins, B. G. Christensen and J. K. Judice, *J. Am. Chem. Soc.*, 2003, **125**, 6517–6531; (*c*) D. R. Stack. and R. G. Thompson, *Eur. Pat.* EP 0801075 A1, 1997; (*d*) K. C. Nicolaou, R. Hughes, S. Y. Cho, N. Winssinger, C. Smthurst, H. Labischinski and R. Endermann, *Angew. Chem., Int. Ed.*, 2000, **39**, 3823–3828; (*e*) K. C. Nicolaou, R. Hughes, S. Y. Cho, N. Winssinger, H. Labischinski and R. Endermann, *Chem.–Eur. J.*, 2001, 3824–3843.

9 (*a*) S. A. Waksman and H. B. Woodruff, *Proc. Soc. Exp. Biol. Med.*, 1940, **45**, 609–614; (*b*) H. Brockmann, *Angew. Chem.*, 1954, **66**, 1–10.

10 Whitesides also pointed out that a flexible linker should reduce the entropic advantages in dimeric binding modes;M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794.

11 Recognition of the D-Ala–D-Ala motif by the associated vancomycin dimer in the crystal structure seemed to be a good starting point for the rational design of covalently linked vancomycin dimers. Amino groups of vancosamine sugars were shown to be located at distances ranging from 7 to 11 angstroms with the "back-to-back dimers" and located at shorter distances than the "face-to-face dimers"; P. J. Loll and P. H. Axelsen, *Annu. Rev. Biophys. Biomol. Struct.*, 2000, **29**, 265–289.

12 In 2000, Nitanai and Aoki deposited the crystal structure of diacetyl-Lys–D-Ala–D-Ala complexed with vancomycin with the Protein Data Bank (code: 1FVM). The distance of vancosamine amino groups in associated back-to-back dimers was, at the shortest, 7.8 angstroms.

13 In all animal studies, experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Shionogi Discovery Research Laboratories.