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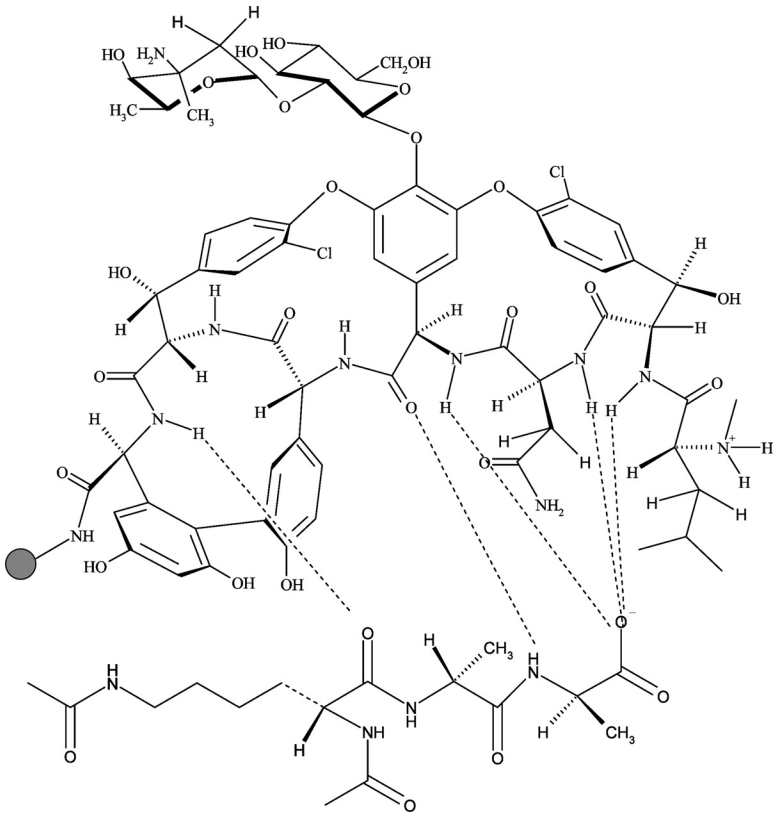

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Conformational Studies of Resin-Bound Vancomycin and the Complex of Vancomycin and Ac_2-L-Lys-D-Ala-D-Ala

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The molecular target of vancomycin, a commonly used glycopeptide antibiotic, is the D-Ala-D-Ala dipeptide subunit on the bacterial cell wall. The molecular basis of interaction between vancomycin and D-Ala-D-Ala in solution is well-known. However, there is no structural data on vancomycin, and its interaction with D-Ala-D-Ala when the drug is tethered to a solid support. In this Article, vancomycin was directly coupled onto TentaGel or PEGA resin through its C terminus. High-resolution magic angle spinning NMR studies indicated that conformation of PEGA bead-bound vancomycin is identical to that of the free drug. Broadening and shifts of the same proton resonances were observed in solution-phase vancomycin or PEGA-bound vancomycin when complexed with Ac_2-L-Lys-D-Ala-D-Ala. This study demonstrates that bead-bound molecules can behave the same as solution-phase molecules in terms of molecular interaction with its target molecule, thus validating the on-bead screening approach of the "one-bead−one-compound" combinatorial library method.

Introduction

Solid-phase combinational chemistry has been developed and successfully utilized for drug lead discovery and basic research.[1,5] The "one-bead−one-compound" (OBOC) combinatorial libraries generated by the "split-mix" synthesis approach enables one to rapidly synthesize millions of compounds.[2,3] Using on-bead screening methodology,[3−5] literally millions of chemical compounds can be screened in parallel within a day or two. However, one of the concerns of on-bead screening is that the resin and the linker may directly interfere with the interaction between the ligand and the target molecule.[5] This is particularly true if the linker is tethered to the part of the ligand that is important for binding to the target molecule. Another concern is that when a ligand is tethered to a solid support via a linker, it may behave very differently from ligands in aqueous solution.[9,10] Although these are valid concerns, in the last 14 years, we were able to successfully use the OBOC combinatorial library method to identify ligands for a large number of macromolecular receptors or substrates for protein kinases and proteases.[5−8] We hypothesize that if the solid support and linkers are chosen appropriately, these potential problems can be minimized. We have routinely used TentaGel resin (Rapp Polymere, Tubigen, Germany), polystyrene resin grafted with poly(ethylene glycol) linker, as solid support to construct our OBOC combinatorial libraries. Since the poly(ethylene glycol) linker is relatively long (optimal PEG chains are above 3000 Da), we believe under aqueous conditions, the library compounds will be highly solvated as if they were in solution. With the advent of high-resolution magic angle spinning NMR (HR/MAS NMR) techniques, we can now experimentally determine if our assertion is correct.

Vancomycin, a glycopeptide antibiotic isolated from *Streptomyces orientali*,[11] is of great interest because (i) it is the last line of defense against a serious clinical pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA),[12,13] and (ii) its interaction with a small peptide component of the bacteria cell wall can provide a useful model for studying substrate−receptor interactions.[14,15] Vancomycin binds strongly to muropeptidic precursor terminating in -L-Lys-D-Ala-D-Ala of the bacterial cell wall. This prevents the transpeptidase from completing the cross-linking of the bacterial cell wall during the biosynthesis process, leading to a cessation of growth and eventual destruction of the bacterial cell by lysis.[16,17] Vancomycin is a tricyclic molecule consisting of vancosamine, glucose, and seven unusual amino acid residues, which are constrained by several phenolic oxidative cross-links.[18] On the basis of NMR studies of vancomycin/Ac-D-Ala-D-Ala and vancomycin/Ac_2-L-Lys-D-Ala-D-Ala complexes, the molecular basis of interaction between the antibiotic and the cell wall component has been determined (Figure 1). A series of hydrogen bonds is formed between the carboxylate anion and amides of D-Ala-D-Ala and the amides of vancomycin. These intermolecular hydrogen bonds are further stabilized by hydrophobic interactions between the hydrocarbon portion of vancomycin and the alanine methyl groups of the dipeptide.[19,20]

HR/MAS NMR has been successfully applied to study the conformation of peptides bound to different resins.[9,10,23−25] Recently, we reported the use of HR/MAS NMR to monitor the O-glycosylation of amino acids covalently attached to TentaGel resin.[22] In this article, we describe the use of HR/

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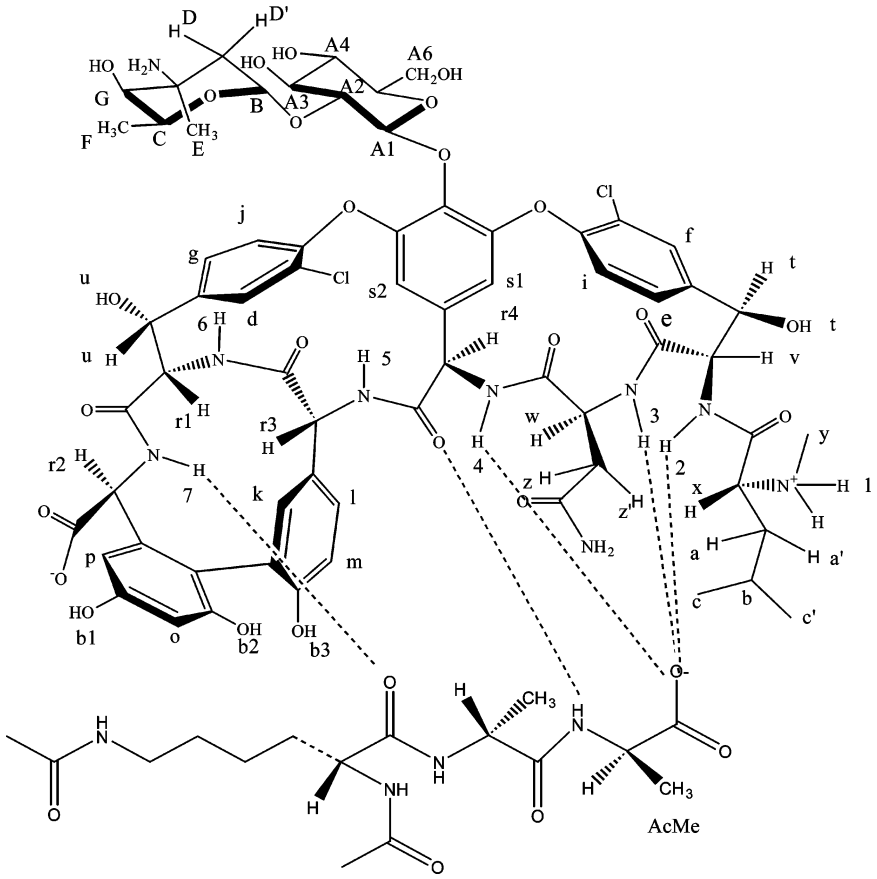

Figure 1. Vancomycin and the Ac_2-L-Lys-D-Ala-D-Ala complex showing the proton nomenclature. Dashed lines indicate hydrogen bonds.

MAS NMR to characterize the conformation of vancomycin that was tethered to PEGA or TentaGel resin. Its interaction with a tripeptide (Ac_2-L-Lys-D-Ala-D-Ala) was also determined.

Results and Discussions

Direct Coupling of Vancomycin to Solid Support. In on-bead screening, the ligands are still covalently attached to solid support, and the assays involve either a binding assay (direct binding of molecular target to the bead-bound ligand) or a functional assay (detection of functional properties of the bead bound ligand, such as protein kinase substrates).[5] Ideally, the resins should swell well in both organic and aqueous condition, be uniform in both size and substitution, and be nonsticky. Both TentaGel resin[26] and PEGA resin[27,28] fit several of these criteria and have been used by others and us in OBOC combinatorial libraries. Therefore, we have chosen these two resins as the solid support in this study. Since the cations of both the N-terminal and vancosamine amino of vancomycin play the role of attracting the carboxylate anion of the peptide substrate (D-Ala-D-Ala) into the binding site,[29,30] we decided to tether vancomycin to amino-TentaGel or PEGA resin through its C terminus (Figure 2). Given the fact that the vancosamine amino group is protected by cation formation and the methylated amino group of D-leucine at the N terminus is a weaker nucleophile than the primary amino group on the resin, acylation occurs only on the resin to give vancomycin−resin conjugate. Vancomycin self-acylation was not observed.

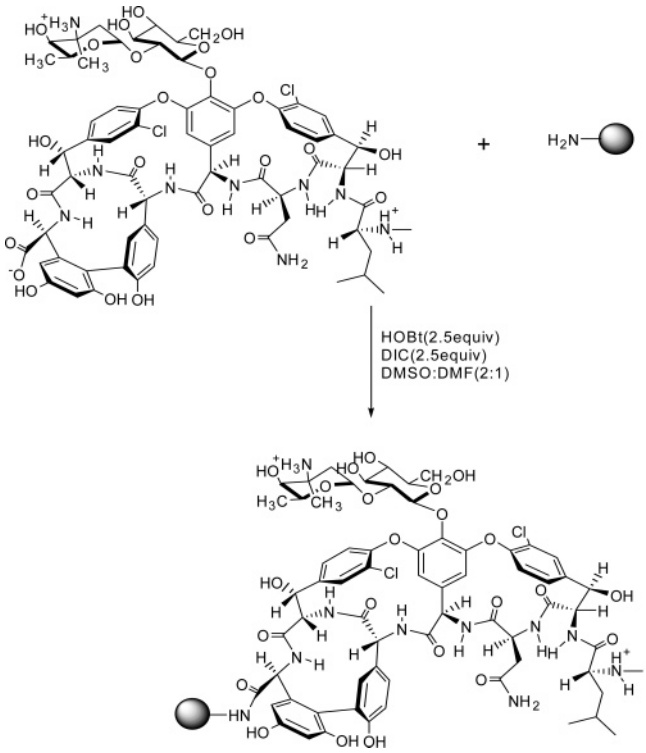

Figure 2. Synthetic route of coupling vancomycin onto amino-TentaGel resin or amino-PEGA resin.

The NMR Spectral Comparison of Resin-Bound Vancomycin with Free Vancomycin. Previously, we have studied the influence of resin, temperature, and deuterated solvents on the resolution of HR/MAS NMR spectra.[21] The best resolved spectra were obtained by swelling TentaGel or PEGA beads in DMSO or DMF. To directly compare the

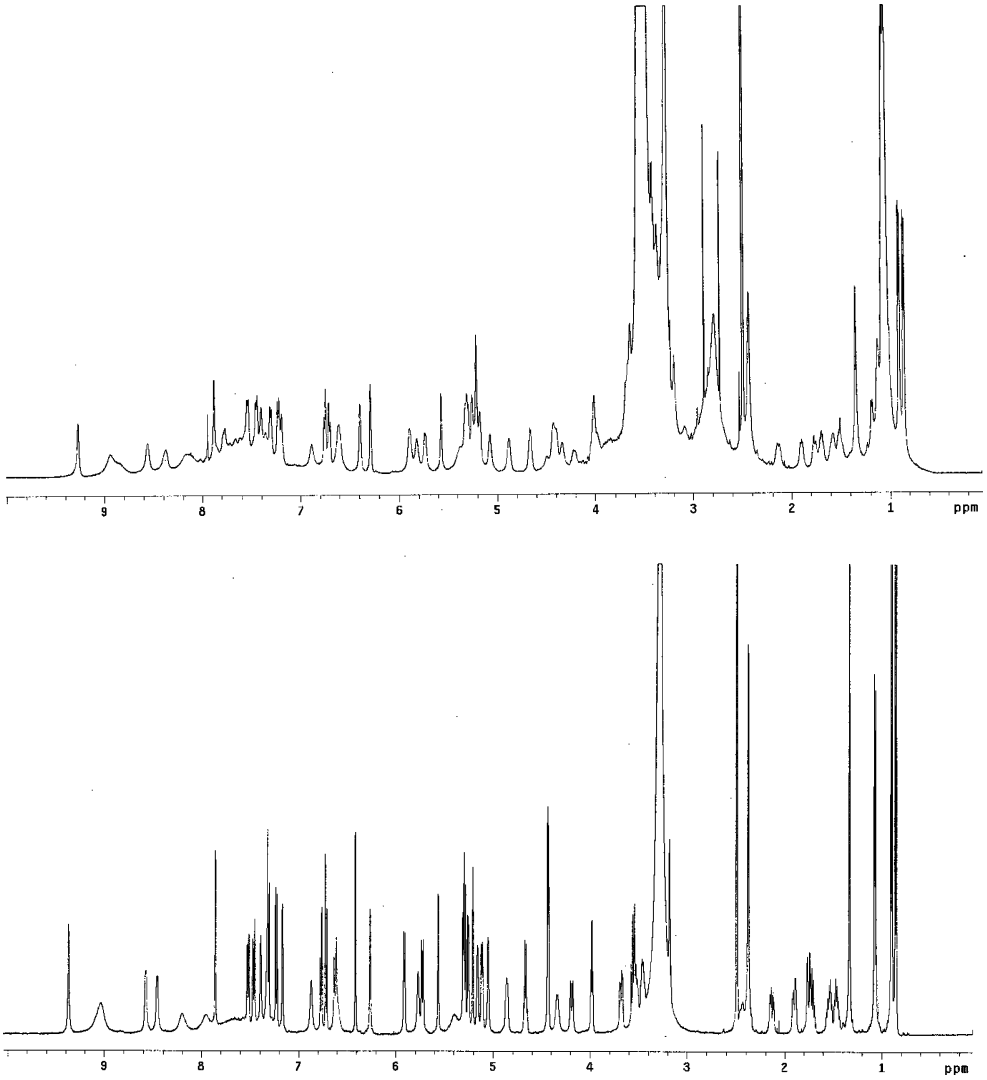

Figure 3. (Top) ^{1}H HR/MAS NMR spectrum of PEGA-bound vancomycin swollen in DMSO-d_6 at 313k with a presaturation at 3.55. (Bottom) ^{1}H HR/MAS NMR spectrum of free vancomycin in DMSO-d_6 at 313 K.

NMR spectra of resin-bound vancomycin with free vancomycin in DMSO, 1.5 mg of PEGA was swollen in 40 μL DMSO-d_6, and the HR/MAS NMR analysis was performed at 313 K. The high-resolution ^{1}H NMR of PEGA-bound vancomycin is shown in Figure 3. 2D NMR (^{1}H−^{1}H COSY, NOESY 1D, NOESY) allowed the full assignment of vancomycin linked to PEGA. Since the chemical shift is a very sensitive probe for 3D environment, we compared the NMR spectra of PEGA-bound vancomycin with that of the free drug (Figure 3.). The excellent agreement of proton assignments between the two spectra (Table 1) indicates that the PEGA-bound vancomycin has adopted the same conformation as the free drug (nomenclature in Figure 1.). In the NOESY1D spectrum, N7-r1, N7-d−u-r3, N7-d−k-u−r3 correlations were observed (Figure 4). Additionally, in the NOESY spectrum, d-u-r_1, r_1-r_3-k, N_7-d-k proton correlations were also observed. These results suggest that d, u, r_1, r_3, k and N_7 must be on the same side of the molecule, and the C-terminal region of PEGA-bound vancomycin showed rigid conformation. Nevertheless, in the N terminus, only one cross-peak between t and v was found. This implies that t and v should be on the same side of molecule, and the N-terminal region is more flexible.

Temperature coefficient has been recognized as an excellent criterion to determine the extent of exposure of the NH group to the solvent environment. The "exposed" amides (solvent interacting) usually exhibit a larger temperature coefficient than "internal" amides (buried or intramolecularly H bonded).[31,32] To further confirm if any influence is exerted by the resin on the conformation of PEGA-bound vancomycin, a series of ^{1}H NMR spectra was recorded as a function of temperature (298−313 K). The temperature coefficients of NH protons of PEGA-bound vancomycin and the free drug are illustrated in Figure 5A and B, respectively. Accordingly, the NH proton temperature coefficients of PEGA-bound vancomycin are the same as those of the free drug in DMSO. This result further demonstrates that PEGA-bound vancomycin displays the nativelike conformation.

TentaGel resin is the most popular resin used in on-bead screening because of mechanical stability and is relatively uniform in substitution and size.[26] With lower loading capacity of TentaGel resin (0.27 mmol/g), 5.5 mg of TentaGel-bound vancomycin was swollen in 40 μL of DMSO-d_6. The HR/MAS NMR analysis was performed at 313 K. Although we obtained a highly qualitative ^{1}H NMR

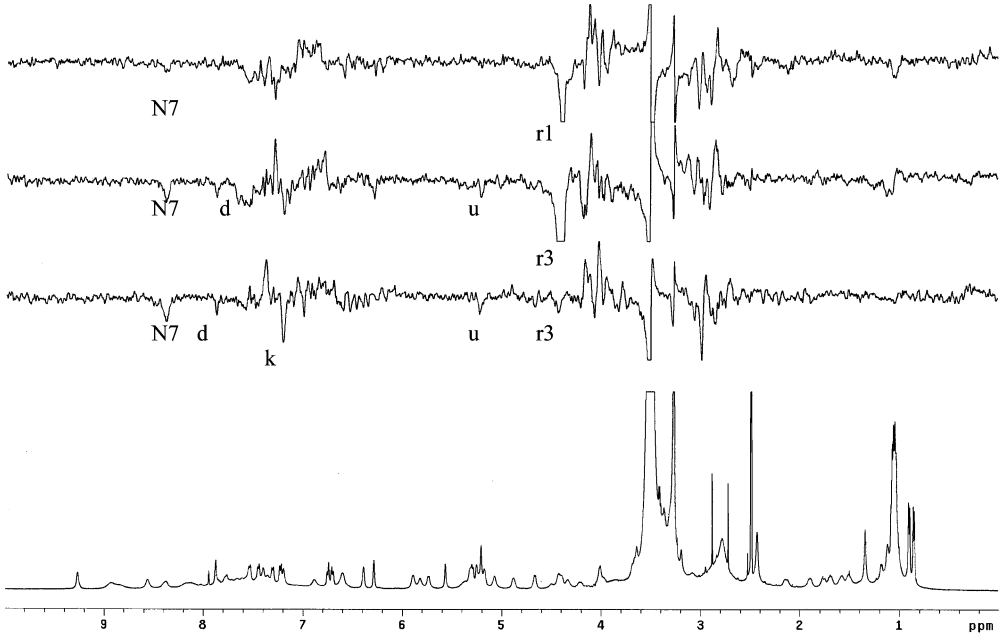

Figure 4. NOESY 1D spectrum of PEGA-bound vancomycin swollen in DMSO-d_6 at 313 K.

Table 1. ^{1}H NMR Chemical Shifts of PEGA-Bound Vancomycin (**1**)[a] and Free Vancomycin (**2**)[b]

^{1}H	1	2	^{1}H	1	2
OH b1	9.46	9.37	B	5.25	5.24
OH b2	8.93	9.03	s2	5.20	5.20
N5	8.56	8.57	t	5.17	5.15
N7	8.37	8.45	u	5.07	5.11
N4	8.16	8.21	v	4.87	4.85
N2	8.01	7.95	C	4.66	4.66
d	7.87	7.85	r2	4.42	4.42
e	7.53	7.51	r3	4.42	4.42
g	7.45	7.45	w	4.00	3.97
f	7.40	7.39	r1	4.20	4.18
j	7.30	7.31	G	overlap	overlap
i	7.22	7.23	x	overlap	overlap
k	7.19	7.16	y	2.35	2.37
l	6.75	6.76	z	2.13	2.13
m	6.70	6.71	z′	1.89	1.89
N3	6.88	6.86	D	1.75	1.75
N6	6.60	6.61	D′	1.69	1.68
o	6.39	6.40	b	1.69	1.71
p	6.28	6.26	E	1.34	1.32
u-OH	5.89	5.92	F	overlap	1.06
t-OH	5.82	5.77	c	0.90	0.90
r4	5.73	5.72	c′	0.86	0.85
s1	5.56	5.56	a	1.58	1.52
A1	5.30	5.29	a′	1.50	1.45

[a] 1.5 mg in DMSO-d_6, T = 313 K. [b] 13.5 M in DMSO-d_6, T = 313 K.

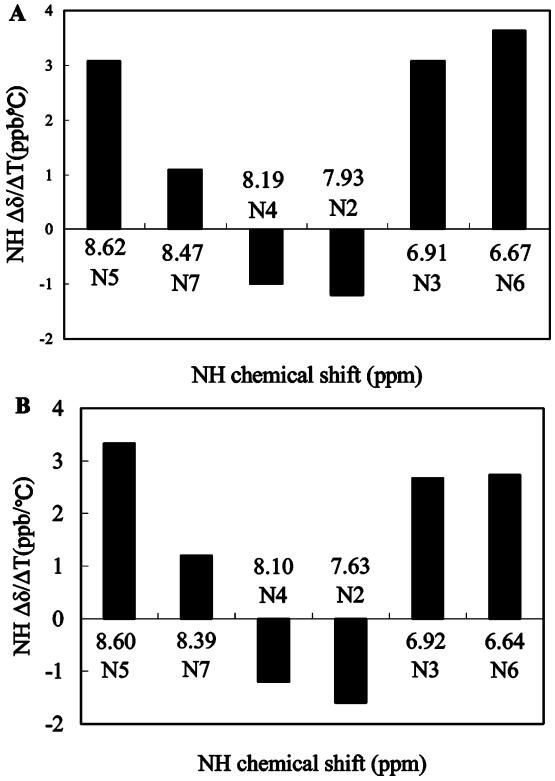

Figure 5. Temperature coefficients of the amide NH of PEGA-bound vancomycin (A) swollen in DMSO-d_6 and free vancomycin (B) in DMSO-d_6, measured in the range 298−313 K. (A positive sign shows an upfield shift)

spectrum of TentaGel-bound small molecular compounds,[22] it was difficult to discern clearly full assignments of TentaGel-bound vancomycin by a ^{1}H NMR spectrum (Figure 6). Furthermore, both ^{1}H-^{1}HCOSY and NOESY of TentaGel-bound vancomycin also gave very little contact. This intrinsic problem, which does not allow full assignment of TentaGel-bound vancomycin, lies probably in the limitation of the mesh of the polystyrene matrix, the poor dispersion of the resonance due to the anisotropic magnetic susceptibility induced by the aromatic rings that constitute the polystyrene matrix,[33] and the presence of signals originating from the resin.[34]

The Complex of PEGA-Bound Vancomycin and Ac$_2$-L-Lys-D-Ala-D-Ala. PEGA-bound vancomycin was swollen in 40 μL of DMSO-d_6 containing 1.5 mmol equiv of Ac$_2$-L-Lys-D-Ala-D-Ala and analyzed by HR/MAS NMR at 298 K (Figure 7). The formation of PEGA-bound vancomycin

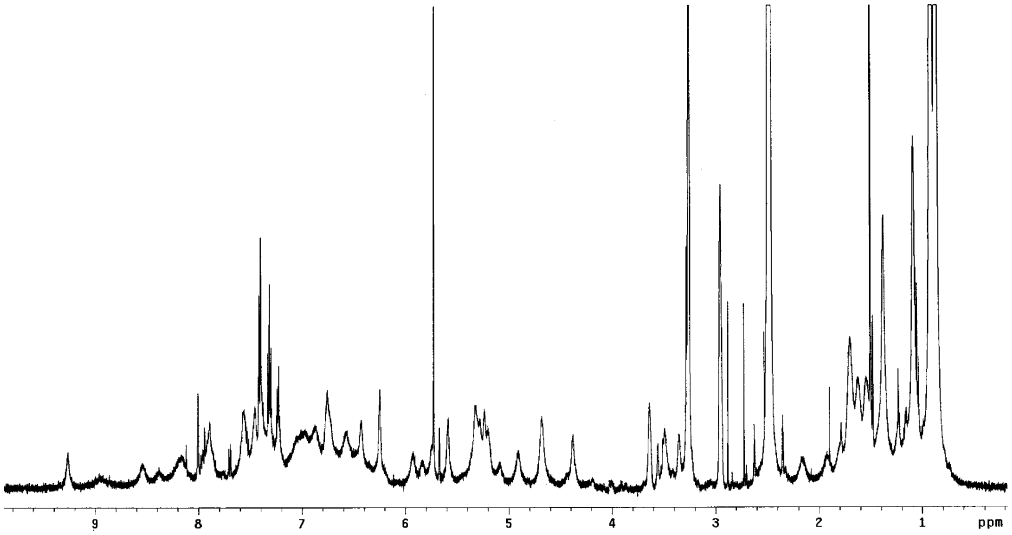

Figure 6. ^{1}H HR/MAS NMR spectrum of TentaGel-bound vancomycin swollen in DMSO-d_6 at 313 K with a presaturation at 3.55.

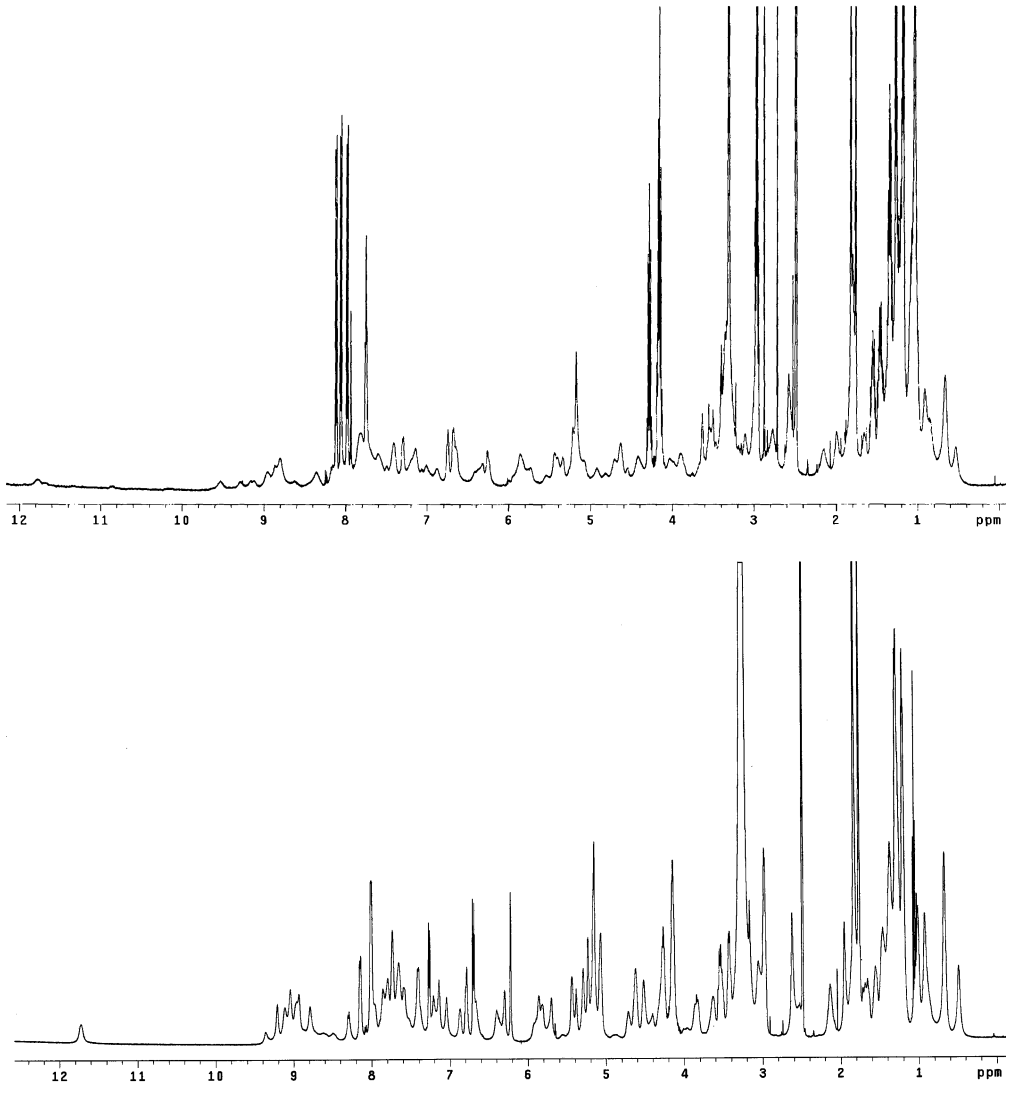

Figure 7. (top) ^{1}H HR/MAS NMR spectrum of the complex of PEGA-bound vancomycin and Ac_2-L-Lys-D-Ala-D-Ala in DMSO-d_6 at 298 K. It was presatured at 3.55. (bottom) ^{1}HNMR spectrum of the complex of Vancomycin and Ac_2-L-Lys-D-Ala-D-Ala in CCl_4/DMSO-d_6 (v:v 1:3) at 281 K.

and the Ac_2-L-Lys-D-Ala-D-Ala complex was evident from selective broadening and shifts of specific proton resonance, which was in excellent agreement with vancomycin and the Ac_2-L-Lys-D-Ala-D-Ala complex in DMSO-d_6. Williams

Table 2. Assignment of ^{1}H Chemical Shifts in the Free and in the Complex PEGA-Bound Vancomycin (**1**) and Free Vancomycin (**2**)

	1	**1** + dipeptide	2	**2** + dipeptide
HN2	8.01	11.77	7.95	11.76
HN3	6.88	8.35	6.86	8.33
HN4	8.16	8.86	8.21	8.96
HN7	8.37	9.15	8.45	9.14
AcMe	1.26	0.53	1.26	0.50

reported the detailed binding sites of the complex at 278 K in DMSO-d_6 containing 30% carbon tetrachloride[35] on the basis of the slow dissociation rate at this temperature. However, a comparable accurate elucidation of the PEGA-bound complex was not obtained at ~278 K. This might be caused by the solution's viscosity and resin's lower swelling profiles at such lower temperature, although 30% CCl_4 was added into DMSO. This did not allow us to acquire the highly qualitative 2D NMR spectrum, such as ^{1}H−^{1}H COSY and NOESY. However, the chemical shifts of major protons of PEGA-bound vancomycin and Ac_2-L-Lys-D-Ala-D-Ala complex are still assigned (Table 2) in comparison with the assignments of previous reports in solution phase[36−38] (Figure 7, bottom). A very large downshift (3.76 ppm) of the PEGA-bound complex indicates that HN2 is involved in formation of the strongest hydrogen bond between PEGA-bound vancomycin and Ac_2-L-Lys-D-Ala-D-Ala. HN3, HN4, and HN7 moving downfield by 1.47, 0.70, and 0.78 ppm, respectively, also suggests the presence of hydrogen bonds at those positions. Additionally, a remarkable upfield shift by 0.73 ppm of AcMe of Ac_2-L-Lys-D-Ala-D-Ala (nomenclature in Figure 1) indicates that AcMe is probably shielded by an aromatic ring and is located at the "upper" face of that aromatic ring.

Conclusion

Vancomycin was successfully coupled to PEGA and TentaGel resin. Using the HR/MAS NMR technique, we were able to demonstrate that the conformation of bead-bound vancomycin is identical to that of the free drug. Furthermore, we have found that Ac_2-L-Lys-D-Ala-D-Ala interacts with PEGA bead-bound vancomycin the same way that it interacts with free vancomycin. This study provides good evidence that ligands tethered to an appropriate solid support could still retain their native conformation and their binding capacity to a target receptor. This validates the use of the on-bead screening method to evaluate our OBOC combinatorial libraries.

Experiment Section

TentaGel resin (loading 0.27 mmol/g, 1% DVB cross-linked, 90 μm) was purchased from Rapp Polymere (Tübingen, Germany). PEGA resin (loading 0.4 mmol/g, 1% DVB cross-linked, 50−100 mesh) was purchased from Chem-Impex International, Inc. (Wood Dale, IL). Vancomycin hydrochloride was purchased from Fluka and was used without any further purification. Ac_2-L-Lys-D-Ala-D-Ala was purchased from Bachem Bioscience Inc. (King of Prussia). *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were distilled from appropriate drying agents.

Coupling Procedure for the Preparation of Resin-Bound Vancomycin. TentaGel resin (100 mg) or PEGA resin (60 mg) were swollen in 3 mL of DMF at room temperature in a reaction column fitted with a frit filter. After 30 min, the solvent was removed by vacuum. Vancomycin hydrochloride (2.5 equiv), HOBt (2.5 equiv) and DIC (2.5 equiv) then were mixed in 3 mL of DMSO/DMF (2:1, v:v) for 15 min and added to the reaction vessel containing TentaGel or PEGA resin. The final mixture was agitated in a shaker for at least 24 h at room temperature until a Kaiser test became negative. The resin was thoroughly washed with DMF (×3), DCM (×3), MeOH (×3), and DCM (×3) and, finally, lyophilized prior to performing the HR/MAS NMR experiment.

NMR Spectroscopy Experiments. All NMR experiments were carried out on aVarian Unity INOVA-500 spectrometer equipped with a 4-mm 1H-observe Nano NMR probe. The spin rate was ~2 K Hz for all samples. A 1.5-mg portion of PEGA-bound vancomycin beads was transferred into a Nano NMR tube, and 40 μL of DMSO-d_6 was then added. The spectra were acquired in a temperature range from 298 to 313 K with presaturation at 3.55. In the case of the complex, a ration of 1:1.5 PEGA-bound vancomycin or free vancomycin and Ac_2-L-Lys-D-Ala-D-Ala was employed. The solvent cocktail of DMSO-d_6 containing ~30% CCl_4 was used to prevent freezing at 281 K. A 5.5-mg portion of TentaGel-bound vancomycin was tested in the same way as the PEGA-bound vancomycin. A 0.8-mg portion of Vancomycin hydrochloride in 40 μL of DMSO-d_6 was used in the HR/MAS NMR experiment. One-dimensional spectra were obtained with spectral widths of 4000−5000 Hz and 32K of data points. Two-dimensional experiments (^{1}H−^{1}H COSY and NOESY) were performed by using the standard pulse sequences. NOESY spectra were recorded in phase-sensitive mode and with mixing times of 0.15 and 0.2s. The 2D data matrix consisted of 2K × 256 spectra, which yielded, after zero-filling in the F1 dimension and Fourier transformation, a 2K × 2K matrix. Phase-sensitive data were subjected to Lorentzian−Gaussian manipulation, and other data, to sine bell manipulation.

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Supporting Information Available. NMR analysisis data. This information is available free of charge via the Internet at http://pubs.acs.org.

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