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PEPTIDE-BINDING ANTIBIOTICS: A SOLID-PHASE ASSAY FOR SCREENING LIBRARIES OF VANCOMYCIN MIMICS FOR SELECTIVE *d*-ALA-*d*-ALA BINDING

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Abstract: Due to recent increases of vancomycin-resistant bacterial infections, research on more effective antibiotics has intensified. As part of such research, screening of combinatorial libraries is a valuable approach for discovering new lead antibiotics. A screening method is presented here that uses a solid state library and a fluorescent label to detect RCO-*d*-Ala-*d*-Ala binding in aqueous media. © 1997 Elsevier Science Ltd.

Introduction

Vancomycin has been the premier antibiotic used against streptococcal and staphylococcal infections that are resistant to ß-lactam antibiotics.[1] Its mode of action involves binding *d*-Ala-*d*-Ala termini on the bacterial cell surface, which then obstructs further attachment of free peptidoglycan.[2] The subsequent reduction of crosslinking peptidoglycan strands decreases cell wall strength, thus eventually causing the bacteria to become susceptible to osmotic lysis. Due to the advent of vancomycin-resistant bacterial strains[3] characterized by *d*-Ala-*d*-Lac peptidoglycan termini, research is underway for development of even more effective antibiotics.

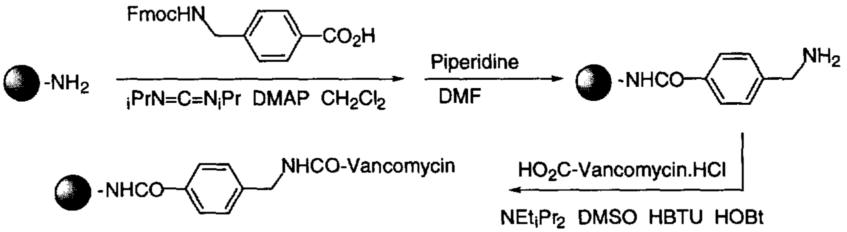
One of the more promising tools for drug discovery, combinatorial libraries (attached to solid support), potentially offer a rapid means for identifying molecules that show binding propensity towards target molecules in aqueous solution. By conjugating a visualising moiety (e.g., a fluorescent dye) to the target molecule, it is possible to find particular compounds from a combinatorial library on solid-phase beads that have significant binding activity for the target.[4-6] Isolation of thus-visualised binding beads allows subsequent ECGC tag decoding[4] for library member structure determination.

In the *d*-Ala-*d*-Ala binding screen presented here, a bead-immobilised combinatorial library interacts with a labeled target molecule under aqueous, physiologically relevant conditions. Since an aqueous solvent medium is more polar than the binding region of a molecule immobilised on a solid support, we attempted to develop a visualisation strategy based on the differing dielectric constants of these two environments. Certain fluorescent molecules such as dansyl sulphonamides show negative fluorosolvatochromism whereby their excited state becomes more stabilised with respect to their ground state as solvent polarity decreases. Effectively, a probe molecule with a fluorescent label demonstrating this property would be expected to fluoresce more intensely than in free aqueous solution when bound to an immobilised molecule on solid support. Previous work has shown such a fluorescence increase with a fluorescent *d*-Ala-*d*-Ala conjugate upon binding vancomycin in solution and found it to provide more accurate binding constants than traditional difference-UV or ^1H NMR spectroscopy,[7,8] especially at low concentrations where aggregation is minimized.[9]

In our work, vancomycin is immobilised on TentaGel solid support beads and treated in pH 7.0 aqueous buffer with fluorescent [*d,d*], [*d,l*], [*l,d*], [*l,l*] dansyl-Gly-Ala-Ala-OH dipeptide diastereomers **1**, **2**, **3**, **4**, respectively, and the depsipeptide [*d,d*] dansyl-Gly-Ala-Lac-OH, **5**. In order to evaluate the sensitivity of this screening method, the fluorescence of individual beads was measured and compared to known vancomycin/ Ac-Ala-Ala dipeptide binding constants. Our results show that CCD-measured fluorescence intensities of the individual probe-bound vancomycin beads are good semiquantitative indicators of binding strength.

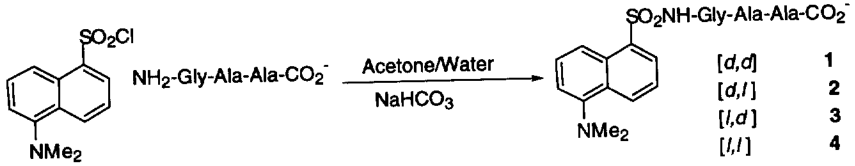
Methods

Vancomycin was immobilized on poly(ethyleneglycol)-polystyrene (TentaGel) beads by attachment through its carboxyl terminus. This was accomplished by intermediate formation of its hydroxybenzotriazole active ester by HBTU and subsequent carboxamide formation with TentaGel-NHCO-*p*-benzylamine (Scheme 1). Previous studies have shown that self-condensation of vancomycin does not occur under these conditions, due most likely to the relatively hindered nature of its amino moieties. The loading of vancomycin on our TentaGel solid support was found to be similar to that reported previously (~0.1 mmol/g).[10]

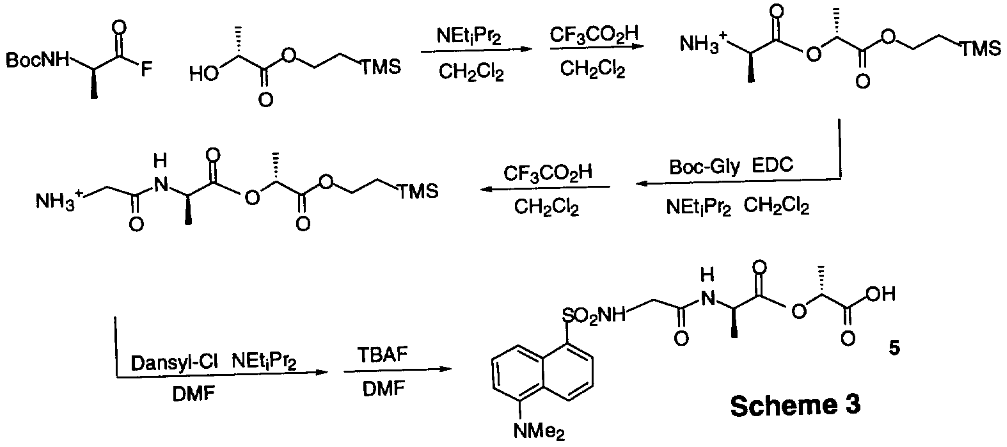


Scheme 1

The Gly-Ala-Ala peptides of the probe molecules were obtained from *d*- and *l* -alanines and glycine by the usual *N*-Fmoc/O_tBu ester strategy. The fully deprotected tripeptide zwitterion was then coupled to dansyl-Cl under aqueous basic conditions to form the fluorescent sulphonamide[9] (Scheme 2). Due to the sensitivity of the Gly-*d*-Ala-*d*-Lac ester linkage to the basic Fmoc deprotection conditions, this depsipeptide was prepared via an *N*-Boc/trimethylsilylethyl ester strategy. Thus, the Gly-*d*-Ala-*d*-Lac depsipeptide was obtained by first esterifying the Boc-*d*-alanyl acid fluoride with the *d*-lactate trimethylsilylethyl ester followed by *N*-deprotection (TFA), and subsequent carbodiimide coupling of Boc-Gly. To avoid problems with the base-labile lactate ester, the dansyl-Cl coupling was conducted in DMF with H-Gly-*d*-Ala-*d*-Lac-$OCH_2CH_2SiMe_3$ which was then subsequently deprotected with fluoride (Scheme 3).



Scheme 2



Scheme 3

Our binding screen was performed by exposing the bead-immobilised vancomycin to a 2.0 μM solution of fluorescent peptide probe (**1**–**5**) in 0.1 N phosphate buffer at pH 7.0 for 30 min at 25 °C. Twice the molar amount of probe was used with respect to that of the theoretical maximum loading of vancomycin on the TentaGel. The beads were then washed twice for 30 min with the same volume of 0.1 N phosphate buffer. The fluorescence intensity of individual solid support beads were measured at 340 nm excitation and 420 nm emission using a fluorescent microscope equipped with a CCD camera and image processing software. From each assay, intensities of a half dozen different beads were measured and averaged to give the reported values. In all cases, fluorescence measurements of individual beads within an assay group were within 5% of one another, thus demonstrating the consistency of our solid-phase assay system.

Results and Discussion

We began our solid-phase binding studies by testing TentaGel beads carrying no vancomycin for nonspecific binding to our probes. Thus TentaGel amine and its acetamide were assayed for binding to *d*-Ala-*d*-Ala **1** under the same conditions to be used with the TentaGel-immobilised vancomycin. Binding results for the TentaGel acetamide beads showed little detectable fluorescence (relative intensity 24) after exposure to 2.0 μM **1** (Figure 1a). With TentaGel amine, a more purposeful control due to probable ionic interaction with the probe carboxylate, we did observe a slight fluorescence increase (relative intensity 71) due to nonspecific ionic binding (Figure 1b). However, with vancomycin-conjugated TentaGel beads and 2.0 μM probe **1**, we found fluorescence of considerably higher intensity (relative intensity 184) than that of the aforementioned control beads (Figures 1a and 1b). Additionally, we found this screen to be sensitive enough to detect relatively weak binding as shown by the binding results with the more weakly binding probes **2**–**5** (Table 1, Figures 2–6). Thus while nonspecific binding to the fluorescently labeled probes can be observed, it provides a background fluorescence that is considerably weaker than the fluorescence due to specifically bound probe molecules.[11]

Fluorescent Probe	**TentaGel amine** Relative Intensity of Bead-Bound Fluorescent Probe	**TentaGel Vancomycin** Relative Intensity of Bead-Bound Fluorescent Probe
Dansyl-Gly-*d*-Ala-*d*-Ala	74	185
Dansyl-Gly-*d*-Ala-*l*-Ala	72	109
Dansyl-Gly-*l*-Ala-*d*-Ala	76	130
Dansyl-Gly-*l*-Ala-*l*-Ala	77	90
Dansyl-Gly-*d*-Ala-*d*-Lac	74	89

Table 1: Comparison of fluorescence intensities of various dansyl-labeled probes bound specifically (vancomycin functionalized) and nonspecifically (amine functionalized).

Fluorescent Probe	**Relative fluorescence** due only to Vancomycin-Probe binding	**Ligand Binding Constants** K_A(mM^{-1})	**Ligands** solution binding determination with Vancomycin
Dansyl-Gly-*d*-Ala-*d*-Ala	111	16.0[9]	*N*-Ac-*d*-Ala-*d*-Ala
Dansyl-Gly-*d*-Ala-*l*-Ala	37	1.1[9]	*N*-Ac-*d*-Ala-*l*-Ala
Dansyl-Gly-*l*-Ala-*d*-Ala	54	1.3[9]	*N*-Ac-*l*-Ala-*d*-Ala
Dansyl-Gly-*l*-Ala-*l*-Ala	13		*N*-Ac-*l*-Ala-*l*-Ala
Dansyl-Gly-*d*-Ala-*d*-Lac	15	<0.02[12]	*N*-Ac-*d*-Ala-*d*-Lac

Table 2: Comparison of binding fluorescence on solid phase surface to binding constants calculated in solution.[7]

Figure 1a

Figure 1b

Figure 1a: Dansyl-Gly-*d*-Ala-*d*-Ala (**1**) bound to both TentaGel-vancomycin beads and TentaGel acetamide beads shown on same field.

Figure 1b: Dansyl-Gly-*d*-Ala-*d*-Ala (**1**) bound to both TentaGel-vancomycin and TentaGel amine.

Figure 2

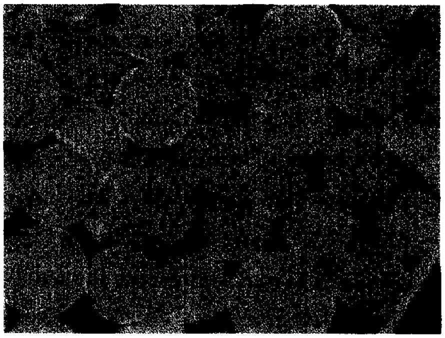


Figure 3

Figure 2: Dansyl-Gly-*d*-Ala-*l*-Ala (**2**) bound to TentaGel amine (control).
Figure 3: Dansyl-Gly-*d*-Ala-*d*-Ala (**1**) bound to TentaGel vancomycin.

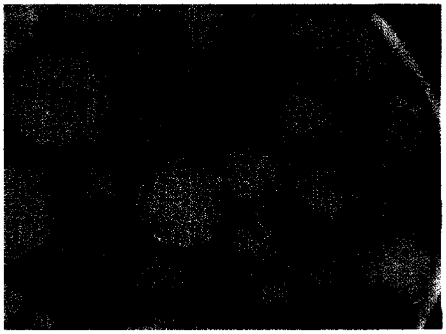


Figure 4

Figure 5

Figure 4: Dansyl-Gly-*d*-Ala-*l*-Ala (**2**) bound to TentaGel vancomycin.
Figure 5: Dansyl-Gly-*l*-Ala-*d*-Ala (**3**) bound to TentaGel vancomycin.

Figure 6

Figure 7

Figure 6: Dansyl-Gly-*l*-Ala-*l*-Ala (**4**) bound to TentaGel vancomycin.
Figure 7: Dansyl-Gly-*d*-Ala-*d*-Lac (**5**) bound to TentaGel vancomycin.

Comparison of the solid-phase binding fluorescence data from the various probes shows that this screen can discriminate binding of varying strengths. Thus our fluorescence measurements show a clear trend in the vancomycin-binding abilities of the probes as **1** > **3** > **2** > **4** ≈ **5**, and correlate well with their reported binding constants (Table 2). Of particular note is the observed distinct binding preference for [*l,d*] **3** over [*d,l*] **2**. This finding correlates well with ^{1}H NMR evidence supporting the *C*-terminal alanine as having a stronger binding interaction than that of the *N*-terminal.[13] The screening method described here assesses the binding strengths of various probe molecules (here peptides) in solution to a particular binding molecule (here vancomycin) immobilised on solid support. However, the converse method, whereby a combinatorial library of *d*-Ala-*d*-Ala- or *d*-Ala-*d*-Lac-binding candidates on solid support particles are screened with a single peptidic probe (e.g., **1** or **5**) should prove useful in visually (or mechanically with a fluorescently activated cell sorter) selecting those candidates having the strongest binding to the probe sequence. Thus the solid-phase screening method outlined here should be valuable for efficient screening of solid-phase combinatorial libraries to find new compounds having vancomycin-like peptide- or depsipeptide-binding properties.

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