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Binding of Vancomycin Group Antibiotics to D-Alanine and D-Lactate Presenting Self-Assembled Monolayers

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Abstract—Peptides terminating in -Lys-D-Ala-D-Ala, -Lys-D-Ala-L-Ala and -Lys-D-Ala-D-Lactate were covalently coupled via an N-terminal aminohexanoic acid linker to a self-assembled monolayer of $HS(CH_2)_{15}CO_2H$ on a thin gold film. Binding of the glycopeptide antibiotics vancomycin and chloroeremomycin to these surfaces was then measured using a surface plasmon resonance biosensor. Both antibiotics bound with micromolar affinity to the D-Ala-terminating surface and with millimolar affinity to the D-Lactate-terminating surface. Increasing density of these covalently attached peptides on the surface had no effect on the resultant affinities of either antibiotic for the surface. In contrast, when the lipid-anchored peptide *N*-α-docosanoyl-ε-acetyl-Lys-D-Ala-D-Ala was inserted into a supported lipid monolayer, the affinity of the strongly dimerizing antibiotic chloroeremomycin for the surface showed a dependence on ligand density. This was not the case with the weakly dimerizing antibiotic vancomycin. The lipid monolayer surface, which is a more realistic model of the surface of a bacterium, was thus better suited for the study of the cooperative binding interactions that occur between dimeric glycopeptide antibiotics and surface-bound ligands.

Introduction

The vancomycin group of glycopeptide antibiotics is active against a wide range of Gram-positive bacteria, particularly those staphylococci and enterococci responsible for post-surgical infections. The activity of the vancomycin group antibiotics arises from their ability to bind peptidoglycan precursors terminating in the sequence -Lys-D-Ala-D-Ala (-KDADA).[1] We have shown previously that with the exception of teicoplanin, glycopeptide antibiotics dimerize in aqueous solution[2] and that dimerization plays an important role in their biological activity.[3,4] Unfortunately, resistance to vancomycin is now increasing rapidly,[5] and the accompanying increase in the number of deaths from bacterial infections has given new urgency to the study of these antibiotics. The resistance is the result of a deceptively simple change of an amide linkage to an ester linkage in the growing bacterial cell wall.[6] The change, conferred by substitution of the terminal D-alanine of the cell wall precursors with D-lactate (D-Lac), results in a repulsive interaction within the binding pocket of the antibiotic, and a consequent approximately 1000-fold decrease in affinity (in aqueous solution) which renders the antibiotic therapeutically useless (Fig. 1).[6]

In this study, we have employed an SPR biosensor to analyze binding of two glycopeptide antibiotics to peptides present in the peptidoglycan of Gram-positive bacteria which terminate in the sequences -KDADA and -KDADLactate. The former is found in the peptidoglycan precursors present in vancomycin-sensitive bacteria and the latter in those precursors in VanA and VanB vancomycin-resistant bacteria.[6] A peptide terminating in -L-Ala was also included in the study to probe the chiral selectivity of vancomycin for its substrate at a surface.[1]

Results and Discussion

H_2N-Ahx-L-Lys(εAc)-D-Ala-D-Ala-OH (Ahx-KDADA) and H_2N-Ahx-L-Lys(εAc)-D-Ala-L-Ala-OH (Ahx-KDALA) were synthesized by solid phase methodology using commercially available pre-loaded Wang-D-Ala and Wang-L-Ala resins and standard Fmoc-protecting group chemistry. H_2N-Ahx-L-Lys(εAc)-D-Ala-D-Lactate-OH (Ahx-KDADLac) was synthesized using 2-

Abbreviations: SPR, surface plasmon resonance; RU, response units; HPA chip, hydrophobic association sensor chip; CE, chloroeremomycin (LY264826); doc-KAA, *N*-α-docosanoyl-lysyl(*N*-ε-acetyl)-D-alanyl-D-alanine; ac-KAA, *N*,*N*-diacetyl-lysyl-D-alanyl-D-alanine; SUV, small unilamellar vesicle; PC, L-α-phosphatidylcholine; MIC, minimum inhibitory concentration.

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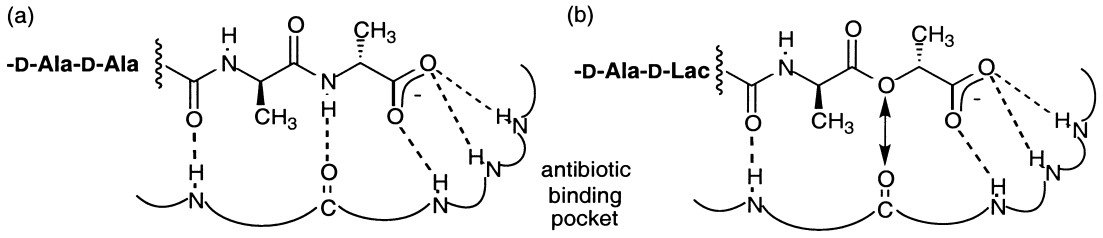

Figure 1. Hydrogen bonding interactions[6] (dashed lines) between the binding pocket of vancomycin and peptides terminating in (a) -D-Ala-D-Ala and (b) -D-Ala-D-Lactate. In (b) the repulsive interaction is shown with a double-headed arrow.

chlorotrityl resin by an analogous method to that described by Cho et al.[7] The aminohexanoic acid linker was used to space the peptide away from the site of attachment to a self-assembled monolayer (SAM) of $HS(CH_2)_{15}CO_2H$ on a commercially available thin gold film SPR sensor chip (Fig. 2). A mixture of *N*-hydroxysuccinimide (100 mM) and *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (400 mM) was injected across the second flow cell of the sensor chip. This was followed immediately by an injection of Ahx-KDADA resulting in the immobilization of 120 response units (RU) of peptide. As the correlation between RU and absorbed mass is known,[8] this corresponds to 0.11 ng/mm^2 or 0.25 pmol/mm^2 of peptide, equivalent to ca. 10% surface coverage. Such low levels of peptide were initially coupled to the surface to minimize any mass transport limitation on binding of the antibiotic from the bulk solution to the surface, and to ensure that no limitation was placed on binding by steric crowding at the surface.[9] Residual NHS esters were then capped by an injection of ethanolamine (1 M at pH 8.0). In a similar manner, Ahx-KDADLac and Ahx-KDALA were deposited on the third and fourth flow cells of the sensor chip. The first flow cell of the sensor chip was activated with EDC/NHS and immediately capped with ethanolamine to provide a control surface.

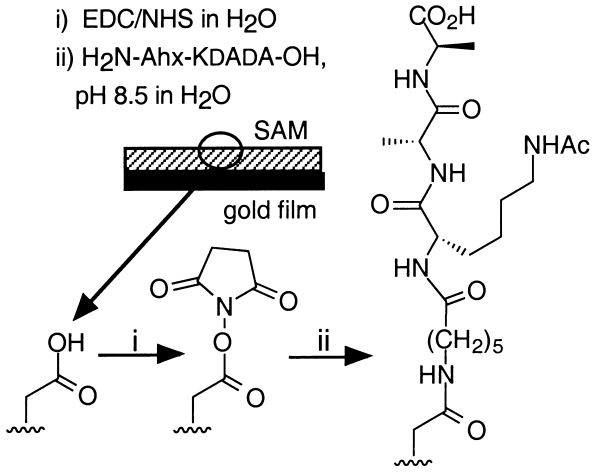

Figure 2. Representation of the surface chemistry for coupling of peptides to a carboxylic acid-presenting self-assembled monolayer.

Binding of the glycopeptide antibiotics vancomycin and chloroeremomycin to the prepared surfaces was then assayed by injecting phosphate buffered solutions of each antibiotic across the sensor chip and observing the change in the SPR signal with time. At the end of the injection, the antibiotic solution was replaced by buffer and the antibiotic–peptide complex allowed to dissociate (Fig. 3). Regeneration of the free surface-coupled peptides between binding assays was effected by a 30 s injection of a solution of 10 mM hydrochloric acid. The

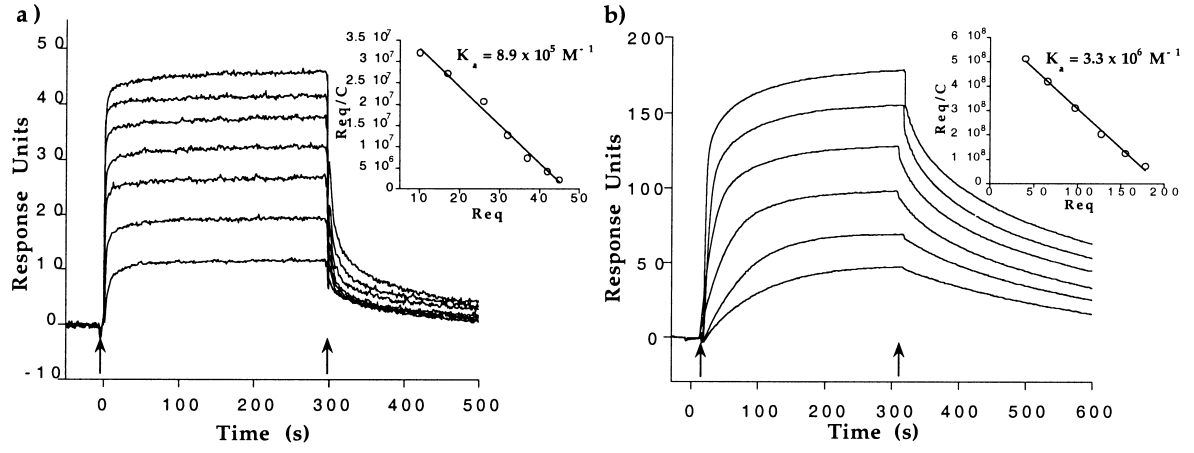

Figure 3. Serial 2-fold dilutions of (a) vancomycin (20–0.31 μM), and (b) chloroeremomycin (2.5–0.04 μM), binding to a KDADA-derivatized SAM corrected for bulk refractive index changes (see text). Arrows indicate the beginning and end of the injections. The insets are Scatchard plots of the data: R_{eq} is the response in response units when the binding reaches equilibrium and C is the concentration of antibiotic. A plot of R_{eq} versus R_{eq}/C has a slope of $-K_a$.

antibiotics did not bind to the control surface that had been derivatized with only ethanolamine. To correct for bulk refractive index changes between the antibiotic sample plug and the running buffer, these data were subtracted from data for binding of vancomycin to the peptide-derivatized surfaces.[9] No association of the antibiotics with the Ahx-KDALA surface was observed. This marked preference for the D-amino acid-containing sequence over the L-amino acid sequence at a surface mirrors that first observed by Nieto and Perkins in free solution.[1] In all other cases examined, binding occurred to the surface-bound peptides and depsipeptides, and affinities could be calculated by Scatchard analysis of the equilibrium binding levels attained at varying antibiotic concentration (Fig. 3).

The calculated affinities of vancomycin and chloroeremomycin for the Ahx-KDADA-derivatized surface were similar to those affinities reported for binding of the antibiotics to $N^{\alpha,\varepsilon}$-diacetyl-KDADA (ac-KDADA) in solution determined by UV difference spectroscopy and by affinity capillary electrophoresis (Table 1). Using *N*-acetyl-KDADA coupled via the lysine side chain to mixed carboxylic acid-terminating SAMs, the group of Whitesides has also recently reported vancomycin surface binding affinities similar to those affinities in solution ($K_a = 2.7\times10^6$ M^{-1} with 15% surface peptide coverage[10] and 9.1×10^5 M^{-1} with 50% surface peptide coverage[11]). In our hands using up to 50% surface coverage of peptide, the binding constants for vancomycin and chloroeremomycin were also not significantly higher than those obtained in solution (Table 1). A key question raised by these data is: why do the calculated affinities for the strongly dimerizing chloroeremomycin ($K_a = 3.3\times10^6$ M^{-1}) not reflect the enhanced surface binding ($K_a = 2.1\times10^7$ M^{-1}) which is observed[12] for binding of this antibiotic to N^{α}-docosanoyl-N^{e}-acetyl-KDADA (doc-KAA) anchored in a lipid monolayer? It is likely that this is because covalent attachment of peptides to the SAM surface does not allow the adjustment of the surface position of the peptides necessary for cooperative binding of a dimeric antibiotic. In contrast, peptide which has been inserted into a lipid monolayer can migrate within the plane of the lipid to allow formation of a complex in which antibiotic dimer is simultaneously bound to two peptides with an attendant enhancement of surface binding.[12]

The affinities of vancomycin and chloroeremomycin for the Ahx-KDADLac-derivatized surface were between 1.5- and 10-fold higher than those affinities reported for binding of the antibiotics to $N^{\alpha,e}$-diacetyl-KDADLac (ac-KDADLac) in solution, depending on which literature value was taken for comparison (Table 1). In the light of the variation in the reported solution binding constants to ac-KDADLac, these results are consistent either with no cooperativity of binding to -KDADLac depsipeptide that is covalently attached to the surface, or with a small cooperativity. We have previously observed between a 20- and 10,000-fold enhancement in surface binding over solution binding of chloroeremomycin using the alkylated D-Lactate-terminating peptides inserted into lipid vesicles.[13,14] Thus, the very large cooperative effects that can be observed when these peptides are membrane bound are not observed when the peptides are covalently attached to the surface.

To investigate this point further, the influence of the surface density of doc-KAA in a supported lipid monolayer on the resultant affinities of vancomycin and chloroeremomycin was examined. Supported lipid monolayers were formed from extruded phosphatidylcholine vesicles on a hydrophobic self-assembled monolayer resulting in the deposition of ca. 2000 RU or 1.84 ng/mm^2 or 2.3 pmol/mm^2 of lipid.[15] Coverage of the surface with lipid was confirmed by the lack of nonspecific binding of bovine serum albumin, which binds strongly to the hydrophobic self-assembled monolayer alone. Increasing amounts of doc-KAA were then inserted into the lipid monolayer by injection across lipid surfaces as dilute solutions. A lipid monolayer containing no added doc-KAA provided a control surface to correct for bulk refractive index changes and signal drift as described earlier. Vancomycin and chloroeremomycin were then exposed to the surfaces containing increasing amounts of doc-KAA and the affinity constants were determined by Scatchard analysis of the equilibrium response levels attained at varying concentrations of the antibiotics. The results show that the affinity of vancomycin did not vary with increasing concentration of doc-KAA in the lipid layer (Fig. 4). In contrast, the affinity of chloroeremomycin varied with doc-KAA density, reaching a maximum at 11 mol% of doc-KAA in the lipid monolayer.

Table 1. Binding constants (M^{-1}) of vancomycin and chloroeremomycin for peptidoglycan analogues in free solution, and coupled to a self-assembled monolayer

	ac-KDADA (Solution)	SAM-Ahx-KDADA Surface)[e]	ac-KDADLac (Solution)	SAM-Ahx-KDADLac (Surface)[e]
Vancomycin	1.5×10^6 [a] 4.1×10^5 [d]	$8.9\pm0.8\times10^5$	410[d]	1900±500
Chloroeremomycin	1.3×10^6 [b]	$3.3\pm0.7\times10^6$	240[c] 1600[d]	2400±600

[a]Determined by UV difference spectroscopy (ref 1).
[b]Determined by UV difference spectroscopy (ref 25).
[c]Determined by UV difference spectroscopy (ref 26).
[d]Determined by affinity capillary electrophoresis (ref 22).
[e]Determined by SPR with standard deviations for $n = 2$.

The invariant affinity of vancomycin for increasing densities of doc-KAA indicates that the changes observed for binding of chloroeremomycin cannot be due solely to an increased extent of mass transport limitation of binding at different ligand densities. Rate-limiting mass-transport from the bulk solution to a surface can adversely affect experimental data in biosensor flow cell systems and usually occurs when massive molecules bind with rapid association rates.[9] At the concentrations employed in this work, vancomycin ($K_{dim} = 700\ M^{-1}$) will be present essentially all as monomer,[3] and probably associates with the surface as such. In contrast, chloroeremomycin (K_{dim} reported in the range 16,000 to 180,000 M^{-1} according to the method of determination)[3,16] will be significantly populate dimer at the range of antibiotic concentrations used. This dimer binds more strongly in solution to ac-KDADA than does the monomer by a factor of 8, thus the binding of dimer to the surface can reasonably be expected in this case.[3] The observed enhancement in the surface affinity for chloroeremomycin can be partitioned into two distinct factors. Firstly, a simple concentrating factor arising from an increase in local concentration of ligand when it is located in the two-dimensional plane of the lipid.[17] Secondly, a factor arising from the cooperative interaction of species mutually bound to the surface. The initial binding of doc-KAA to one half of the chloroeremomycin dimer is inter-molecular, whereas binding to the other half of the dimer at the surface is effectively intra-molecular and results in an affinity constant much greater than that observed in solution.[12]

At doc-KAA densities greater than 11 mol% the affinity of chloroeremomycin for the surface decreased. This may be due to steric crowding at the surface that leads to an increase in the proportion of ligand bound to antibiotic monomer with lower affinity. If the antibiotic contacts the lipid surface at the face bearing the sugar residues (for which there is supporting evidence[27]), then the appropriate cross-section of the antibiotic dimer has an area of ~500 Å^2. The cross-sectional area[19] of 20 phospholipid molecules (i.e. the number which would be covered if antibiotic dimer bound to two doc-KAA molecules present at 10 mol% concentration in lipid) is ~1300 Å^2. Thus, as the cross-sectional area of the antibiotic dimer approaches that of the covered surface area, the possibility of surface crowding at higher doc-KAA levels appears plausible. Studies of bivalent antibody binding at surfaces[18] have also shown that measured affinities depend upon the antigen surface density, giving rise to bell-shaped curves similar to that seen in Fig. 4. These observations have important implications for experimental design in the analysis of binding of dimeric or polyvalent molecules using model membrane systems in general. We have previously used fluorinated peptides together with ^{19}F NMR spectroscopy to examine the effect of increasing ligand density on the affinity of chloroeremomycin for a peptidoglycan analogue that was inserted into a phosphatidylcholine vesicle.[17] Using this model system, it was also found that the affinity of chloroeremomycin increased with increasing ligand density in the vesicle, reaching a maximum at a ligand density of 10%, a value which is almost identical to that found in this study using a supported lipid monolayer.

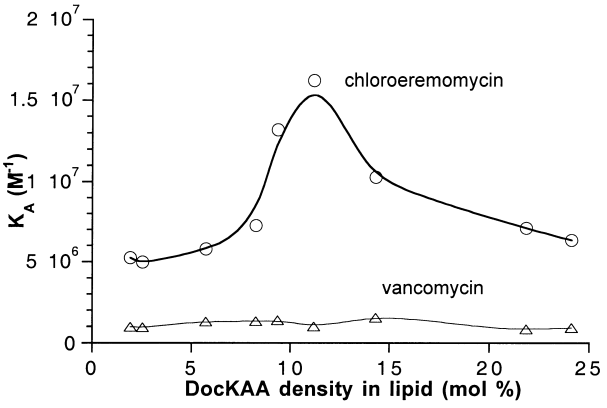

Figure 4. Affinities of vancomycin (Δ) and chloroeremomycin (○) for increasing concentrations of doc-KAA inserted into a supported phosphatidylcholine monolayer determined by surface plasmon resonance.

The total number of peptidoglycan precursors (lipid I and lipid II) present on the surface of a single Gram-positive bacteria has been calculated[19] to be of the order of 2×10^5. The cross-sectional surface area of a phospholipid has been determined[20] by continuous X-ray scattering to be 66 Å^2. Thus the number of lipid molecules in the outer leaflet of a Gram-positive bacterial membrane which has a diameter of 1 μm and which typically contains 70% w/w protein[21] can be estimated to be of the order of 2×10^6.[†] Hence the mole percentage of peptidoglycan precursors in lipid on the surface of the bacteria is ca. 10%. This ratio is very close to the value at which maximal binding of chloroeremomycin to ligand occurs on both a supported lipid monolayer and on a vesicle. In other words, the surface area of antibiotic dimer appears to be close to the optimum area required to roughly fill the available surface of a bacterium if all the available peptidoglycan precursors were bound to antibiotic. It seems plausible that evolutionary pressures would lead to such a situation.

Examination of the data presented in Fig. 3 raises a second question: why is it that chloroeremomycin associates with, and dissociates from, the surface much more slowly than does vancomycin? The binding pockets of the antibiotics are identical: they differ only in that chloroeremomycin possesses an additional 4-epi-vancosamine residue distant from the binding pocket which enhances dimerization. If it is accepted that vancomycin binds initially as a monomer and chloroeremomycin as a dimer as proposed earlier, then the slower kinetics of chloroeremomycin binding might be attributed to an increased structural rigidity of the peptide backbones of the dimer. There are four hydrogen bonds and two salt bridges between each half of the

[†]The surface area of a bacterium of diameter 1 μm is $4\pi(500\ nm)^2 = 3.1 \times 10^6\ nm^2$. Lipids are 30% w/w of the membrane which, to a first approximation, is 45% of the surface area, which equals $1.4 \times 10^6\ nm^2$. Thus the number of lipids in the outer leaflet is $1.4 \times 10^6 / 0.66\ nm^2$.

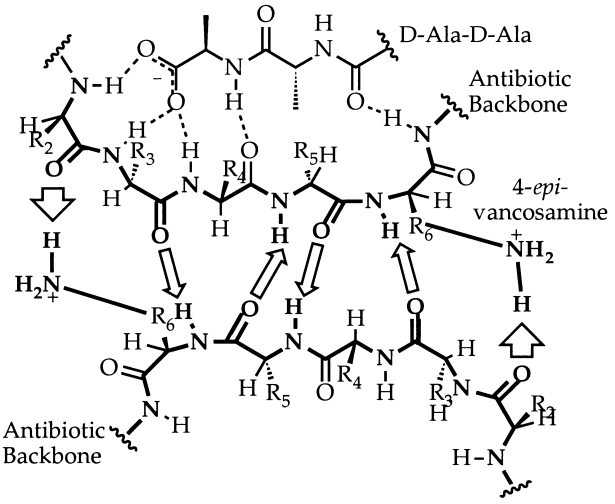

Figure 5. Interactions formed in chloroeremomycin between the antibiotic backbone and the peptide D-Ala-D-Ala. Hydrogen bonds are shown as dashed lines at the antibiotic–peptide binding interface and arrows at the antibiotic–antibiotic dimer interface. Salt bridges to the epi-vancosamine residues are represented by the thicker arrows.

chloroeremomycin dimer (Fig. 5), which we have shown previously act to reduce the conformational mobility of the peptide binding pocket,[16] leading to the expectation of slower binding kinetics. It is notable that studies have previously shown that dimeric single chain antibody fragments dissociate from surface-bound ligand with slower kinetics than their monomeric counterparts.[28]

Conclusion

This work demonstrates the dramatic differences in the affinity of vancomycin and chloroeremomycin between D-Ala, L-Ala and D-Lactate terminating peptides immobilized on a surface. The large surface cooperativities that are observed for membrane anchored bacterial cell wall precursors are not observed when precursors are covalently attached to a surface. Thus, to mimic the enhanced binding that is found at the surface of bacteria,[4] or bacterial protoplasts,[22] membrane anchoring of cell wall precursors in a model membrane should be employed. In particular, in conjunction with earlier work,[12] it is also illustrated that strongly dimerizing antibiotics associate with, and dissociate from, KDADA-containing surfaces with slower rates than weakly dimerizing antibiotics. This is in accord with the anticipated less dynamic peptide backbones of the dimeric antibiotic structure.

Experimental

General

Solvents used were freshly distilled from appropriate drying agents, or provided dry, in 'sure seal' bottles from the Aldrich Chemical Company. Octyl D-glucoside, bovine serum albumin and egg L-α-phosphatidylcholine were purchased from Sigma-Aldrich (UK) and were used without further purification unless otherwise stated. Solid phase synthesis resins Fmoc-D-Ala-Wang resin, Fmoc-L-Ala-Wang resin and 2-chlorotrityl chloride resin were purchased from NovaBiochem (UK). Mercaptohexadeconoic acid was prepared as described previously.[29] The synthesis of the acylated peptidoglycan analogue *N*-α-docosanoyl-*N*-ε-acetyl-lysyl-D-alanyl-D-alanine has been described previously.[23] The SPR instrument was a BIACORE 2000 (Biacore AB, UK) used with: (a) custom made Au-SIA thin gold film sensor chips coated for 22 h with a 1 mM ethanolic solution of mercaptohexadeconoic acid, and (b) with a hydrophobic association (HPA) sensor chip which was composed of octadecane-thiol, a self-assembled monolayer on a thin gold film. Each sensor chip contained four flow cells of dimensions 2.4×0.5×0.05 mm (l×w×h) with a probing spot for the SPR signal of ca. 0.26 mm^2 for each flow cell. All experiments were carried out at 25 °C with data points taken every 0.5 s.

Synthesis of H_2N-ε-Ahx-Lys-(εAc)-D-Ala-D-Ala-OH. Fmoc-D-Ala-Wang resin (1 mmol, 2 g, 100–200 mesh size) was swelled in DMF (50 ml) for 10 min and any fine particles were removed by decanting. The process was repeated three times, then the resin was washed with DMF (6×15 mL). A solution of piperidine in DMF (20% v/v, 10 mL) was added and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was washed with DMF (6×15 mL) and DCM (6×15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was then washed with DMF (6×15 mL), and a solution of Fmoc-D-Ala-OH (0.94 g, 3.0 mmol) and HBTU (1.14 g, 3.0 mmol) in DMF (10 mL) was added and resin agitated under argon for 10 min before addition of DIEA (0.76 mL, 6.0 mmol). The resulting solution was agitated under argon overnight. The resin was washed with DMF (6×15 mL) and DCM (6×15 mL) to afford Fmoc-D-Ala-D-Ala-Wang. A negative Kaiser test indicated the absence of free NH_2 groups. The Fmoc group was removed by addition of a solution of piperidine in DMF (20% v/v, 10 mL) and agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL) and DCM (6×15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was washed with DMF (6×15 mL). A solution of Fmoc-D-Lys(εAc)-OH (1.04 g, 3.0 mmol) and HBTU (1.14 g, 3.0 mmol) in DMF (10 mL) was added to resin and agitated under argon for 10 min before addition of DIEA (0.76 mL, 6.0 mmol). The resulting solution was agitated under argon overnight. The solution was then filtered and the resin was washed with DMF (6×15 mL) and DCM (6×15 mL) to afford Fmoc-Lys(εAc)-D-Ala-D-Ala-Wang. A negative Kaiser test indicated the absence of free NH_2 groups. The Fmoc group was removed by addition of a solution of piperidine in DMF (20% v/v, 10 mL) and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL) and DCM (6×15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The

resin was washed with DMF (6×15 mL). A solution of Fmoc-D-εAhx-OH (1.06 g, 3.0 mmol) and HBTU (1.14 g, 3.0 mmol) in DMF (10 mL) was added to resin and agitated under argon for 10 min before addition of DIEA (0.76 mL, 6.0 mmol). The resulting solution was agitated under argon overnight. The solution was then filtered and the resin was washed with DMF (6×15 mL) and DCM (6×15 mL) to afford Fmoc-εAhx-Lys(εAc)-D-Ala-D-Ala-Wang. A negative Kaiser test indicated the absence of free NH_2 groups. The Fmoc group was removed by addition of a solution of piperidine in DMF (20% v/v, 10 mL) and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL), DCM (6×15 mL), EtOAc (6×15 mL) and Et_2O (6×15 mL). The resin was then re-suspended in DCM (15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was drained and just enough DCM added to swell the resin. Phenol (a few grains in 1 mL DCM) was added and drained through the resin. TFA (99 + % pure) was added to resin to re-suspend it and the reaction was left for 2 h. The TFA was then drained into a clean flask and evaporated to dryness. The resultant yellow oil was flooded with ether and cooled at 4 °C overnight. A white precipitate formed which was dissolved in distilled water and lyophilized to give a white powder (430 mg, 97%). δ_H ppm (250 MHz, DMSO): 8.08 (1H, d, $J=7.9$, Lys-αNH), 8.00 (1H, d, $J=7.5$, AlaNH), 7.93 (1H, d, $J=7.5$, AlaNH), 7.77 (1H, t, Lys-εNH), 7.64 (1H, bs, $AhxNH_3$), 4.29 (1H, quin, Ala-αCH), 4.18 (2H, quin, Ala-αCH + quin Lys-αCH), 2.98-2.94 (2H, q, Lys-εCH), 2.74 (2H, m, $AhxCH_2$), 2.10 (2H, t, $J=6.8$, $AhxCH_2NH_3$), 1.75 (3H, s, acetyl CH_3), 1.65 (1H, m, Lys-βCH), 1.55 (1H, m, Lys-βCH), 1.47 (6H, m, $AhxCH_2$), 1.33 (2H, m, 2×Lys-δCH), 1.25 (3H, d, $J=7.3$, $AlaCH_3$), 1.17 (3H, d, $J=7.2$, $AlaCH_3$); *m/z* (ESMS) 444 (100%, M^+); HRMS (+ve FAB) 444.28490, $C_{20}H_{38}N_5O_6$ requires 444.28220.

Synthesis of H_2N-ε-Ahx-Lys-(εAc)-D-Ala-L-Ala-OH. Synthesis was carried out in a similar manner to that described for H_2N-ε-Ahx-Lys-(εAc)-D-Ala-D-Ala-OH, but using 1 g (0.69 mmol, 100-200 mesh size) of Wang-L-Ala resin. On cleavage from the resin a white precipitate formed which was dissolved in distilled water and lyophilized to give a white powder (306 mg, 99%). δ_H ppm (250 MHz, DMSO): 8.08 (1H, d, $J=7.5$, Lys-αNH), 8.00 (1H, d, $J=7.8$, AlaNH), 7.92 (1H, d, $J=7.6$, AlaNH), 7.77 (1H, t, Lys-εNH), 7.64 (1H, bs, $AhxNH_3$), 4.28 (1H, quin, Ala-αCH), 4.13 (2H, quin, Ala-αCH + quin Lys-αCH), 2.99–2.94 (2H, q, Lys-εCH), 2.73 (2H, m, $AhxCH_2$), 2.10 (2H, t, $J=6.8$, $AhxCH_2NH_3$), 1.76 (3H, s, acetyl CH_3), 1.63 (1H, m, Lys-βCH), 1.55 (1H, m, Lys-βCH), 1.49 (6H, m, $AhxCH_2$), 1.34 (2H, m, 2×Lys-δCH), 1.26 (3H, d, $J=7.3$, $AlaCH_3$), 1.18 (3H, d, $J=7.2$, $AlaCH_3$); *m/z* (ESMS) 444 (100%, M^+); HRMS (+ve FAB): 444.27920, $C_{20}H_{38}N_5O_6$ requires 444.28220.

Synthesis of H_2N-ε-Ahx-Lys-(εAc)D-Ala-D-Lac-OH. All solvents, glassware and syringes were pre-dried overnight. Lithium-D-Lactate (750 mg, 7.8 mmol) was dissolved in a mixture of DCM:DMSO (1:1 v/v, 20 mL) and added to 2-chlorotrityl resin (2.6 mmol, 2 g resin). The resulting mixture was agitated under argon overnight at room temperature. The resin was then filtered and washed with DMSO (4×15 mL) to give D-Lac-trityl resin. Fmoc-D-Ala (2.4 g, 7.8 mmol) was dissolved in DMF (10 mL) and added to the resin. The mixture was cooled to 0 °C, then DIC (984 mg, 7.8 mmol) and DMAP (48 mg, 0.40 mmol) were dissolved in DMF (8 mL) and the resulting solution was added dropwise at 0 °C to the resin over 30 min. The resin was then agitated under argon overnight. The resin was then filtered and washed with DMF (6×15 mL) and DCM (6×15 mL). A solution of piperidine in DMF (20% v/v, 10 mL) was added and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL) and DCM (6×15 mL) to afford H-D-Ala-Lac-trityl resin. A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was washed with DMF (6×15 mL), then a solution of Fmoc-D-Lys(εAc)-OH (6.40 g, 15.6 mmol) and HBTU (5.96 g, 15.6 mmol) in DMF (20 mL) was added to resin and agitated under argon for 10 min before addition of DIEA (0.52 mL, 4.14 mmol). The resulting solution was agitated under argon overnight, then filtered and the resin was washed with DMF (6×15 mL) and DCM (6×15 mL) to afford Fmoc-Lys(εAc)-D-Ala-Lac-trityl resin. A negative Kaiser test indicated the absence of free NH_2 groups. The Fmoc group was removed by addition of a solution of piperidine in DMF (20% v/v, 10 mL) and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL) and DCM (6×15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was washed with DMF (6×15 mL), then a solution of Fmoc-D-εAhx-OH (2.76 g, 7.8 mmol) and HBTU (2.96 g, 7.8 mmol) in DMF (20 mL) was added to resin and agitated under argon for 10 min before addition of DIEA (1.98 mL, 15.6 mmol). The resulting solution was agitated under argon overnight, then filtered and the resin was washed with DMF (6×15 mL) and DCM (6×15 mL) to afford Fmoc-εAhx-Lys(εAc)-D-Ala-Lac-trityl resin. A negative Kaiser test indicated the absence of free NH_2 groups. The Fmoc group was removed by addition of a solution of piperidine in DMF (20% v/v, 10 mL) and the resin was agitated under argon for 5 min. The piperidine–DMF was removed by filtration and the process repeated twice. The resin was then thoroughly washed with DMF (6×15 mL), DCM (6×15 mL), EtOAc (6×15 mL) and Et_2O (6×15 mL). The resin was then re-suspended in DCM (15 mL). A positive Kaiser test result indicated the presence of free NH_2 groups. The resin was drained and just enough DCM added to swell the resin. Phenol (a few grains in 1 mL DCM) was added and drained through the resin. TFA (99 + % pure) was added to resin to re-suspend it and the reaction was left for 2 h. The TFA–DCM was then drained into a clean flask and evaporated to dryness. The resultant yellow oil was then flooded with ether and cooled at 4 °C overnight. A white precipitate formed which was dissolved in distilled water and lyophilized to give a

white powder (406 mg, 35%). δ_H ppm (250 MHz, DMSO): 8.17 (1H, d, $J=7.9$, Lys-αNH), 7.99 (1H, d, $J=6.0$, AlaNH), 7.77 (1H, t, Lys-εNH), 7.64 (1H, bs, $AhxNH_3$), 4.23 (1H, quin, Lac-αCH), 4.17–4.11 (2H, quin, Ala-αCH + quin Lys-αCH), 2.97 (2H, q, Lys-εCH), 2.75 (2H, m, $AhxCH_2$), 2.10 (2H, t, $J=8.0$, $AhxCH_2NH_3$), 1.76 (3H, s, acetyl CH_3), 1.64 (1H, m, Lys-βCH), 1.53 (1H, m, Lys-βCH), 1.48 (6H, m, $AhxCH_2$), 1.33 (2H, m, 2×Lys-δCH), 1.24 (3H, d, $J=7.2$, $AlaCH_3$), 1.18 (3H, d, $J=7.0$, $AlaCH_3$); *m/z* (ESMS) 445 (100%, MH^+); HRMS (+ve FAB): 445.26880, $C_{20}H_{37}N_4O_7$ requires 445.26620.

Coupling of peptides to self-assembled monolayers. A BIACORE™ Au-SIA sensor chip was immersed for 22 h in a 1 mM ethanolic solution of $HS(CH_2)_{15}CO_2H$, rinsed thoroughly with ethanol and then blown dry under a stream of nitrogen. The sensor chip was docked in a BIACORE™ 2000 biosensor and all four flow cells were cleaned with an injection of a solution of 100 mM glycine and 0.3% Triton X-100 at pH 12 (100 μL, 20 μL/min). Equal volumes of NHS (50 μL, 100 mM in water) and EDC (50 μL, 400 mM in water) were mixed together, and 50 μL of this solution injected at 10 μL/min across all four flow cells. This was followed immediately by injections of Ahx-Lys(εAc)-D-Ala-L-Ala, Ahx-Lys(εAc)-D-Ala-D-Lactate and Ahx-Lys(εAc)-D-Ala-D-Ala (70 μL, 20 mg/mL, pH 10) across flow cells 2, 3 and 4 respectively, resulting in the immobilization of ca. 120 RU of each peptide. All four flow cell surfaces were then inactivated with an injection of ethanolamine (50 μL, 1 M, pH 8.0).

Formation of peptide-containing supported lipid monolayers. Small unilamellar vesicles of egg L-α-phosphatidylcholine were prepared in phosphate buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) by extrusion.[24] The surface of an HPA sensor chip was cleaned by a 10 min injection of 40 mM octyl D-glucoside at a flow rate of 10 μL/min. The injection needle was cleaned by predipping in water, then the small unilamellar vesicles (20 μL, 500 μM) injected immediately at a flow rate of 2 μL/min. The lipid layer was then washed at 100 μL/min with sodium hydroxide (10 mM, 20 μL). The degree of coverage of the surface was determined from the amount of lipid bound at a stable level (after the sodium hydroxide wash) and from the extent of non-specific binding of BSA (0.1 mg/mL in phosphate buffer, 5 min injection).

SPR binding assay

Serial 2-fold dilutions (20–0.16 μM and then 12.5–0.097 mM) of vancomycin in phosphate buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) were injected serially (100 μL, 20 μL/min) across the underivatized control surface of the sensor chip, then the surface derivatized with Ahx-Lys(εAc)-D-Ala-L-Ala, followed by the surface derivatized with Ahx-Lys(εAc)-D-Ala-D-Lactate and finally the surface derivatized with Ahx-Lys(εAc)-D-Ala-D-Ala with the BIACORE™ instrument in multi-detection mode. The antibiotic–peptide complexes were allowed to dissociate for 4 min, then the surfaces regenerated by injection of hydrochloric acid (10 μL, 10 mM). Five dummy runs of binding and regeneration were performed before data acquisition. All assays were carried out at 25 °C in duplicate with data points taken every 0.5 s.

SPR data analysis

Data were prepared for analysis by adjusting the response prior to injection to zero and adjusting the time of each injection to zero. Data from the underivatized flow cell was subtracted from corresponding data obtained from the peptide-containing flow cells to correct for bulk refractive index changes. Affinities were calculated from analysis of equilibrium binding levels at varying antibiotic concentration. In the SPR flow cell, the antibiotic is continually added to and removed from the system so the concentration remains at the initial value, C. The total amount of ligand present is expressed in terms of R_{max}, the maximum possible response. The amount of complex formed is proportional to R_t, the observed response. Thus after a time, t, the concentration of antibiotic will still be C, and the amount of free ligand will be given by $R_{max}-R_t$.

$$\begin{array}{lccc} & A + B & \overset{k_a}{\underset{k_d}{\rightleftharpoons}} & AB \\ t=0: & C & R_{max} & 0 \\ t=t: & C & R_{max}-R_t & R_t \end{array} \quad (1)$$

The association and dissociation rates, k_a and k_d, for formation of a homogeneous binary complex of analyte A and ligand B in the SPR flow cell are thus given by:

$$\frac{dR}{dt} = k_a C(R_{max} - R_t) - k_d R_t \quad (2)$$

By measuring the resonance units attained at equilibrium (when by definition dR/dt is zero), as a function of analyte concentration, affinities can be determined from a Scatchard analysis using the equation:

$$\frac{R_{eq}}{C} = K_a R_{max} - K_a R_{eq} \quad (3)$$

where R_{eq} is the response at equilibrium and K_a is the association constant (M^{-1}). A plot of R_{eq}/C versus R_{eq} thus has a slope of $-K_a$.

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