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Design and evaluation of analogues of the bacterial cell-wall peptidoglycan motif L-Lys-D-Ala-D-Ala for use in a vancomycin biosensor

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Abstract—Four small molecular receptors of vancomycin have been designed to make part of a novel biosensor device based on the FTIR-ATR detection: *N*-Boc (**2a**) or *N*-Ac (**2b**)-6-aminocaproyl-D-Ala-D-Ala and *N*-Boc (**3a**) or *N*-Ac (**3b**)-6-aminocaproyl-D-Ala-D-Ser. Using an original microbiological approach to assess the competition of compounds with the natural target of vancomycin in bacteria, EC_{50} values of $6.3–8.0 \times 10^{-5}$ M (**2a**–**b**) and $7.1–9.3 \times 10^{-4}$ M (**3a**–**b**) were determined. Vancomycin:**2b** complex was characterized by MS.

Immobilisation of bioactive molecules on solid supports has gained a growing interest because the resulting devices can be used in various detection systems called biosensors.[1] These allow the specific recognition of a free analyte (the ligand) by a target (the receptor) which is tightly bound to the device surface. Sensors based on the molecular recognition of biomolecules have already attracted intensive interest in many fields such as environmental analysis, monitoring of biotechnological processes, and medical diagnosis and control.[2] Among the different surface-sensitive techniques applied to detect ligand–receptor interaction, the FTIR-ATR method (Fourier transform infrared spectroscopy in the attenuated total internal reflection mode) is of particular interest since it allows for high-sensitive label-free detection.[2]

In the course of a programme devoted to the development of biosensors based on the FTIR-ATR spectroscopy detection,[3] we are engaged in the detection and quantification of the glycopeptide antibiotic vancomycin in human fluids. Vancomycin is of large clinical importance as it is currently the most often recommended antibiotic for treating infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals. Yet, it requires repeated blood level monitoring and rapid delivery of the results to the clinician to ensure optimal efficacy and avoid undue toxicity.[4]

The antibiotic activity of vancomycin results from strong non-covalent interactions (five hydrogen bonds) between the drug and the C-terminal motif of the pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala present in the cell wall peptidoglycan precursor of procaryotes.[5] Recently, vancomycin has been used in single-molecule force spectroscopy to detect and image the localization of free D-Ala-D-Ala termini on the surface of bacteria.[6] In bacteria in which this bonding motif is terminated by D-Lac or D -Ser, the vancomycin affinity is markedly reduced, resulting in resistance to this antibiotic.[7] The usual simplified model to study the interaction of

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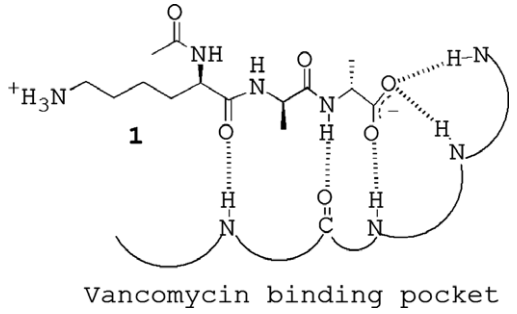
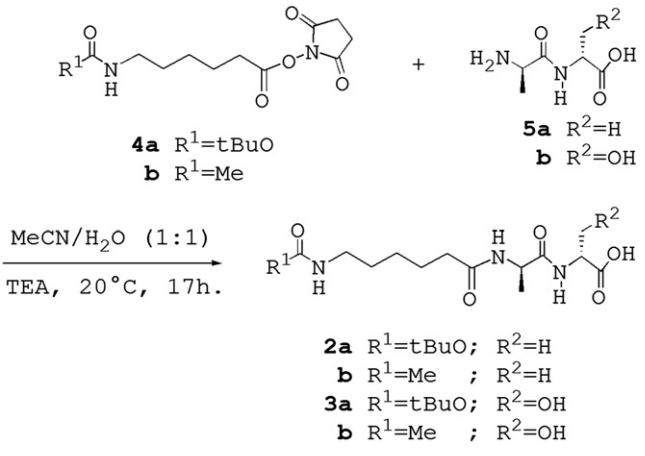
vancomycin and its bacterial target is the *N*-α-Ac-L-Lys-D-Ala-D-Ala tripeptide (**1**) for which the molecular basis of vancomycin affinity is well established (Scheme 1)[1c] and which has been used, after immobilisation on agarose, to purify vancomycin from fermentation broth.[8]

In view of immobilising vancomycin binding motifs (receptors) on an ATR optical element, two key questions need to be addressed, namely: (i) the possibility of replacing L-Lys with 6-aminocaproic acid (this structural modification has the advantage of suppressing a chiral centre and simplifying the synthesis of the biosensor); (ii) the possibility of recycling the biosensor thanks to the formation of weaker complexes than the one formed with L-Lys-D-Ala-D-Ala.

In this communication, we have selected four potential synthetic targets (**2a**–**b** and **3a**–**b**) ending, respectively, with D-Ala or D-Ser (Scheme 2). Before their immobilisation on solid supports via the NH_2-aminocaproyl ending, those molecules have been subjected to HPLC (High Performance Liquid Chromatography), MS (Mass Spectrometry) and microbiological studies to determine their ability to bind to vancomycin in comparison with compound **1**. Since neither the α-*N*-Ac nor the ε-NH_2 groups of **1** are considered critical in its binding to vancomycin, we speculated that the first group could be removed and that the second one could be used for surface anchoring. This has been modelled here by masking the ε-NH_2 with *t*-butyloxycarbonyl or acetyl groups.



Scheme 1. Interaction between vancomycin and **1**.



Scheme 2. Different 6-aminocaproyl mimics of the D-Ala-D-Ala receptor.

The target molecules **2**–**3** were prepared as usual in peptide chemistry (Scheme 2). Briefly, the amine function of 6-aminocaproic acid was protected with *tert*-butyloxycarbonyl group (Boc_2O, NaOH (1 M), dioxane/H_2O (2:1), 0–20 °C, 17 h.) and the acid function was activated as *N*-hydroxysuccinimidyl ester (NHS, DMAP, DCC, CH_2Cl_2, 0–20 °C, 17 h.). This ester **4a** was reacted with commercial D-Ala-D-Ala using PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) as coupling reagent.[9] The resulting *N*-Boc-6-aminocaproyl-D-Ala-D-Ala peptide **2a** was purified by chromatography. D-Ala-D-Ser (**5b**) was obtained by coupling commercial H-D-Ser-(O*t*-Bu)-O*t*-Bu with Boc-D-Ala-OH (PyBOP, TEA, MeCN, 20 °C, 2 h.) followed by hydrolysis of the *tert*-butyl esters (TFA/CH_2Cl_2 (1:1), 20 °C, 1 h.). *N*-Boc-6-aminocaproyl-D-Ala-D-Ser (**3a**) was prepared in a same manner as for **2a**.

From commercially available 6-acetamidocaproic acid (**4b**), the two analogous mimics **2b** and **3b** were obtained in the same conditions.[10]

HPLC (RP C18 column and UV detection)[11] was used to follow the formation of complexes between vancomycin (obtained as Vancocin® 500 from GlaxoSmithKline, Genval, Belgium) and selected target compounds. Vancomycin (67 μM final concentration) was mixed with increasing concentrations of compounds **1** or **2a** in aqueous solution (pH 7.4). A 52.2% and a 73.9% reduction of the free vancomycin concentration were observed at a vancomycin:target compound molar ratio of 1:25 for **1** and **2a**, respectively. We, however, were not able to identify or to isolate the corresponding vancomycin complexes by (semi-preparative) HPLC. Yet, direct injection of the mixtures in a mass spectrometer led to unambiguous evidence of complex formation (analysis performed with electrospray ionization [ESI]) using the negative ion mode (data processed by Excalibur™ version 1.2 software). Figure 1 shows a typical collision-induced dissociation spectrum of a vancomycin:**2b** mixture (1:25 molar ratio). A similar finding was made with a vancomycin:**1** mixture.[12]

A microbiological approach was then used to directly assess the competition of compounds **2a**, **2b**, **3a** and **3b** with the natural target of vancomycin in susceptible bacteria. For this purpose, 10^6 viable bacteria/mL (colony forming units [CFU]) of a fully sensitive *S. aureus* (ATCC 25923) were exposed to a constant concentration of vancomycin (1 mg/L [0.69 μM] corresponding to its minimal inhibitory concentration as determined by broth microdilution technique[13]) and increasing concentrations of the target compounds (molar ratios 1:1 to 1:100,000). The mixtures were then incubated for 5 h at 37 °C in Mueller–Hinton cation-adjusted broth. Bacterial killing or growth was then evaluated by colony counting[13] (in this system, vancomycin alone caused a 1 log CFU decrease, whereas cultures made in the absence of vancomycin showed a 2 log CFU

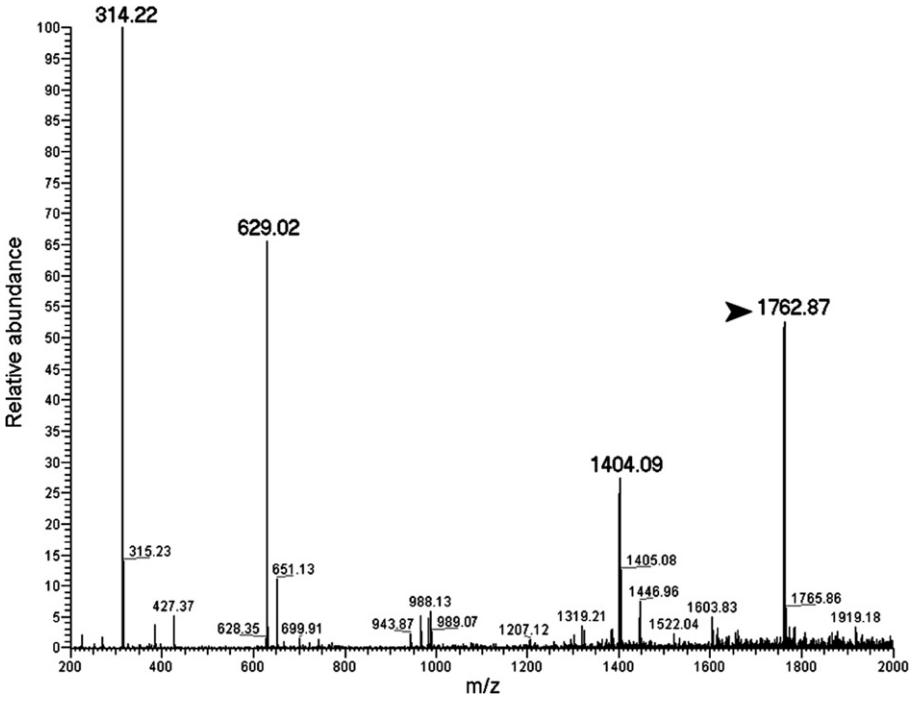


Figure 1. Fragmentation spectrum of the monoanionic vancomycin + **2b** at 1:25 molar ratio. The arrowhead points to an m/z value consistent with a complex vancomycin + **2b**-2H (1762.87).

increase; the diluent used to solubilize compounds **2a** and **2b** [DMSO 1% final concentration] was without influence).

Results are shown in Figure 2 and Table 1.[14] Compound **1** proved competitive with an apparent EC_{50} (half maximal effective concentration) value of 6.6×10^{-6} M, in agreement with previous, independent measures of its affinity constant with vancomycin (2.1×10^{-6} M).[15] In contrast, the dipeptide D-Ala-D-Ala was poorly competitive, demonstrating the critical role of the far-left hydrogen bound (see Scheme 1) in the binding of compound **1** with vancomycin. Replacement of *N*-α-Ac-L-Lys by N-protected-6-aminocaproyl (**2**) increased the EC_{50} of about 1 log, suggesting that the α-*N*-Ac plays a so far undescribed, albeit minor, role in vancomycin binding to its target. Conversely, an acetyl or a more bulky group such as *t*-Boc on the ε-NH_2 group was not critical. Replacing the terminal D-Ala by D-Ser (**3**) further increased the EC_{50} (about one log for **3a/3b** compared to **2a/2b**) in accordance with published values for the difference in affinities between *N*-α-Ac-L-Lys-D-Ala-D-Ala and *N*-α-Ac-L-Lys-D-Ala-D-Ser.[16]

This simple and inexpensive competition assay thus provided useful information for the selection of the candidate targets. Our microbiological method could be routinely used to determine the binding affinity of synthetic small molecules towards vancomycin.

In conclusion, we found that *N*-protected-6-aminocaproyl-D-Ala-D-Ala (**2**) still binds vancomycin with an apparent dissociation constants of the order of 10^{-5} M. As the plasma concentrations of vancomycin

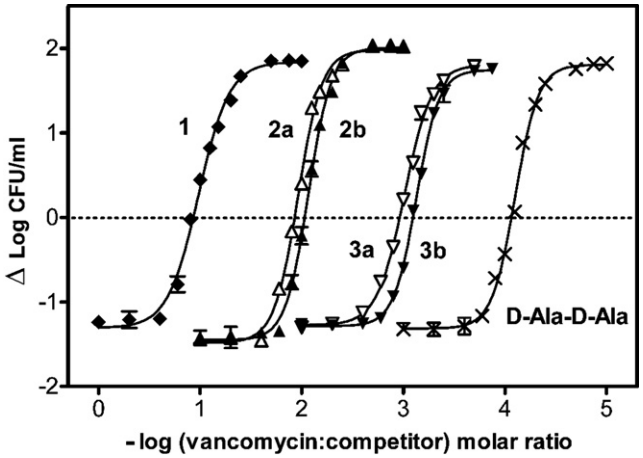


Figure 2. Competition between the vancomycin natural target in *S. aureus* and the target analogues **1, 2a/2b**, **3a/3b**, or the dipeptide D-Ala-D-Ala.

Table 1. EC_{50} values of the compounds tested in competition with the vancomycin natural target in *S. aureus*

Compound	EC_{50}	
	Observed log (vancomycin:target) molar ratio	Calculated target concentration (M)[a]
1	0.984 ± 0.014	6.6×10^{-6}
2a	1.964 ± 0.011	6.4×10^{-5}
2b	2.066 ± 0.007	8.0×10^{-5}
3a	3.011 ± 0.010	7.1×10^{-4}
3b	3.129 ± 0.004	9.3×10^{-4}
D-Ala-D-Ala	4.101 ± 0.009	8.7×10^{-3}

[a] $10^{\log(\text{vancomycin:target molar ratio})} \times 0.69 \times 10^{-6}$.

that need to be monitored in patients (15–50 mg/L)[17] are within the same molar range, these analogues could be used as potential receptors for FTIR-ATR-based detection of vancomycin in the clinics. 6-Aminocaproyl-D-Ala-D-Ser derivatives (**3**) could provide an alternative if a less tight binding was necessary to obtain faster and more complete dissociation in washout-phases. Since the Boc-compounds are easily *N*-deprotected for further linking to germanium devices,[3] they are currently used for the effective development of a vancomycin biosensor for medical applications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.08.055.

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10. Spectral characteristics of final products. (**2a**) ^{1}H NMR (300 MHz, D_2O) δ 1.09–1.67 (m, 21H); 2.27 (t, J = 7.5 Hz, 2H); 3.04 (t, 2H, J = 7.5 Hz); 4.18 (q, J = 7.5 Hz, 1H); 4.29 (q, J = 7.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.4; 179.3; 176.7; 160.9; 83.3; 52.8; 51.9; 42.3; 37.7; 30.2; 27.9; 27.4; 22.4; 19.7; 19.1. HRMS (ES^+) calcd for $C_{17}H_{31}N_3O_6$Na $[M+Na]^+$ 396.2111: found 396.2107. (**3a**) ^{1}H NMR (300 MHz, D_2O) δ 1.20–1.66 (m, 18H); 2.28 (t, J = 7.5 Hz, 2H); 3.03 (t, J = 7.5 Hz, 2H); 3.86 (t, J = 4.7 Hz, 2H); 4.29–4.41 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ 179.4; 177.4; 177.3; 160.9; 83.3; 64.2; 58.6; 52.0; 42.3; 37.8; 31.1; 30.2; 27.9; 27.4; 19.2. HRMS (ES^+) calcd for $C_{17}H_{31}N_3O_7$Na $[M+Na]^+$ 412.2060: found 412.2049. (**2b**) ^{1}H NMR (300 MHz, D_2O) δ 1.17–1.61 (m, 12H); 1.95 (s, 3H); 2.27 (t, J = 6.9 Hz, 2H); 3.11 (t, J = 6.9 Hz, 2H); 4.14 (q, J = 5.8 Hz, 1H); 4.32 (q, J = 5.4 Hz, 1H). ^{13}C NMR (75 MHz, D_2O) δ 180.0; 177.6; 175.0; 174.7; 51.3; 50.3; 40.1; 36.0; 28.7; 26.0; 25.7; 22.7; 18.1; 17.4. HRMS (ES^+) calcd for $C_{14}H_{25}N_3O_5$Na $[M+Na]^+$ 338.1692: found 338.1690. (**3b**) ^{1}H NMR (300 MHz, D_2O) δ 1.23–1.69 (m, 9H); 1.96 (s, 3H); 2.28 (t, J = 7.5 Hz, 2H); 3.14 (t, J = 7.3 Hz, 2H); 3.79–3.96 (m, 2H); 4.29–4.45 (m, 2H). HRMS (ES^+) calcd for $C_{14}H_{25}N_3O_6$Na $[M+Na]^+$ 354.1641: found 354.1642.
All the experimental protocols, for intermediates and final products, with structural characterisations and figures of selected ^{1}H and ^{13}C NMR spectra are available as Supporting Information.
11. We used Waters equipment (HPLC system 2690, diode array detector 996, Millenium 32® program) and Symmetry Shield™ RP C18 column from Waters (4.6 × 100 mm; 5 μm). The analysis conditions are as follows: mobile phase = acetonitrile and ammonium acetate buffer, 70 mM, pH 5.0; gradient from 5% CH_3CN + 95% aqueous buffer to 95% CH_3CN + 5% aqueous buffer; flow rate = 1 mL/min; T = 20 °C; injected volume = 10 μL; analysis time = 20 min; UV detection = 280 nm.
12. Vancomycin:**2b** complex: m/z = 1762.87 (vancomycine + **2b**-2H = $C_{80}H_{100}Cl_2N_{12}O_{29}$); 1404.09 (vancomycine–$CO_2$–H); 943.87 (trimer of **2b**); 629.02 (dimer of **2b**); 314.22 (**2b**) (see Fig. 1).
Vancomycin:**1** complex: m/z = 1776.96 (Vanco + **1**-2H = $C_{80}H_{100}Cl_2N_{13}O_{29}$); 1318.83 (tetramer of **1**); 988.93 (trimer of **1**); 659.17 (dimer of **1**); 329.26 (**1**) (spectrum not shown).
13. Samples were plated on tryptic soy agar (TSA) after appropriate dilutions, and incubated for 24 h at 37 °C. Colonies were enumerated using a Gel Doc 2000 apparatus (Bio-Rad laboratories, Hercules, CA) operated with Quantity One software (Bio-Rad Laboratories). The actual number of colonies counted was typically between 200 and 800, and was never less than 80. See Carryn, S.; Van de Velde, S.; Van Bambeke, F, Mingeot-Leclercq, M.-P.; Tulkens, P.M. J. Infect. Dis. 2004, 189, 2101 and Barcia-Macay, M.; Lemaire, S.; Mingeot-Leclercq, M. P.; Tulkens, P. M.; Van Bambeke, F. *J Antimicrob Chemother* 2006, *58*, 1177 for details and validation.
14. The ordinate shows the variation in the number of viable bacteria after 5 h of incubation compared to the original inoculum (horizontal dotted line). Negative values indicate bacterial killing due to the action of vancomycin (the values of the negative plateaus are similar to what was observed with vancomycin alone [not shown]). Positive values correspond to bacterial growth due to the decreased efficacy of vancomycin in the presence of the tested compounds (the values of the positive plateaus are similar to what was observed in the absence of vancomycin [not shown]. Curves were generated by fitting a variable slope sigmoidal dose-response equation using GrapPad Prism (version 4.03, GraphPad Software Inc., San Diego, CA) to the experimental points. EC50 values (shown on Table 1)

and slope factors (spanning from 2.83 to 3.92) were calculated with the same software for each compound. Each point represents the arithmetic mean of triplicates ±SD and the experiment was repeated twice with similar results.

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