Synthesis and evaluation of vancomycin target analogues as potential biosensors for on-line therapeutic drug monitoring

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Abstract

Background : On-line monitoring of AB could offer significant advantages over laboratory-based methods when dosage readjustments must be quickly implemented. We develop immobilized biosensors for "on-line" quantitative and specific AB detection using Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy (PCT WO 02/056018A1). We have evaluated the recognition properties of molecules intended as VAN sensor, starting from its natural target L-Lys-D-Ala-D-Ala, but replacing L-Lys by a non-chiral spacer that allows immobilization to suitable surfaces.

Methods: Compounds **2a**, **2b**, **3a**, **3b** (see Table) were synthesized by peptide chemistry methods (Hernout et al., Bioorg & Med. Chem Lett, in press). *S. aureus* (ATCC 25923) in cation-adjusted MH broth were exposed to 1 x MIC VAN and increasing concentrations of these analogues or of alpha-N-Ac-Lys-D-Ala-D-Ala or D-Ala-D-Ala. Changes in CFU were measured after 5h incubation and EC50 values determined by non-linear regression. Complex formation was independently examined by HPLC-ESI-MS.

Results:

Competitor	EC_{50} (M)
alpha-N-Ac-Lys-D-Ala-D-Ala (**1**)	6.6×10^{-6}
N-(*t*-butyloxycarbonyl)-6-aminocaproyl-D-Ala-D-Ala (**2a**)	6.4×10^{-5}
N-acetyl-6-aminocaproyl-D-Ala-D-Ala (**2b**)	8.0×10^{-5}
N-(*t*-butyloxycarbonyl)-6-aminocaproyl–D-Ala-D-Ser (**3a**)	7.1×10^{-4}
N-acetyl-6-aminocaproyl-D-Ala-D-Ser (**3b**)	9.3×10^{-4}

The EC_{50} of **1** is in agreement with its known affinity constant for VAN (Biochem. J. 1971,123:789). **2a** and **2b** (L-Lys replaced by caproyl) have EC_{50} about 1 log lower, close to VAN molar concentrations in serum of treated patients (10-50 mg/L [0.7-3.10-5 M]). The formation of complexes was confirmed by examination of collision-induced dissociation spectra of mixtures at a 1:25 molar ratio. Replacing the terminal D-Ala by D-Ser increased the EC_{50} about 10-fold, as anticipated from the known effect of this substitution on VAN susceptibility in bacteria expressing the vanC resistance phenotype.

Conclusion: Alkyl-aminocaproyl-D-Ala-D-Ala has an affinity for VAN that makes it suitable detection of clinically-meaningful concentrations of VAN. This molecule can, therefore, be used for sensor manufacturing.

Introduction and Aims

Immobilisation of bioactive molecules on solid supports allows for the design of biosensors, which allows for specific recognition of free analytes. In a program based on the FTIR-ATR spectroscopy,[1] we wished to detect and quantify vancomycin "*on line*" in serum and human fluids, as a mean to facilitate its monitoring in difficult-to-treat patients.

Vancomycin binds to the terminal motif of the pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala present in procaryotes (Figure 1).[2] The N-α-acetyl-L-Lys-D-Ala-D-Ala tripeptide has been used, after immobilization on agarose, to purify vancomycin from fermentation broth.[3]

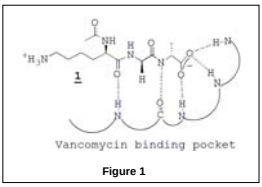


Figure 1

In view of immobilizing vancomycin binding motifs on an ATR optical element, we examined the following questions:

- can we replace L-Lys with α-aminocaproic acid (to suppress a chiral center and, thereby, simplifying thereby the synthesis of the biosensor) ?;
- can we use weaker complexes than the one formed with L-Lys-D-Ala-D-Ala to allow the recycling of the biosensor ?

Methods

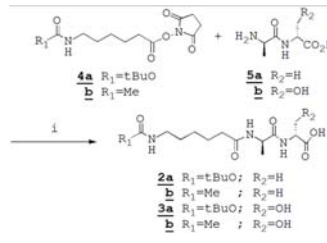
Four potential synthetic targets (**2a-b** and **3a-b**) ending respectively with D-Ala or D-Ser were prepared as usual in peptide chemistry (see scheme 1).

Briefly, the amine function of α-aminocaproic acid was protected with *t*-butoxycarbonyl group (BOC_2O, NaOH (1M), dioxane/H_2O (2:1), 0°C to 20°C, 17h) and the acid function was activated as N-hydroxysuccinimidyl ester (NHS, DMAP, DCC, CH_2Cl_2, 0°C to 20°C, 17h). The ester was reacted with commercial D-Ala-D-Ala using PyBOP as coupling reagent. The resulting Boc-aminocaproyl-D-Ala-D-Ala peptide **2a** was purified by chromatography. D-Ala-D-Ser was obtained by coupling H-D-Ser-(OtBu)-OtBu with Boc-D-Ala-OH (PyBOP, TEA, MeCN, 20°C, 2h.) followed by hydrolysis of the *t*-butyl esters (TFA/CH_2Cl_2 (1:1), 20°C 1h). Boc-aminocaproyl-D-Ala-D-Ser (**3a**) was prepared in a same manner as for **2a**.

Binding of target molecules to vancomycin in comparison with compound **1** was measured as follows:

- **HPLC-MS:** HPLC-ESI-MS was used to follow the formation of complexes between vancomycin (obtained as Vancocin® from GlaxoSmithKline, Genval, Belgium) and compounds **1** or **2b**, using the negative ion mode with data processed by Excalibur™ version 1.2 software. Aliquots of 100 mg/L stock solution of vancomycin were mixed with **1** or **2b** in aqueous solution (pH 7.4) to yield a constant concentration of vancomycin (67 µM) and molar ratios from 1:1 to 1:25.
- **Microbiology:** 10^6 colony forming units/mL of *S.aureus* (ATCC 25923; MIC=1 mg/L) was exposed to a constant concentration of vancomycin (1 mg/L) and increasing concentrations of the target compounds at molar ratios spanning from 1:1 to 1:100,000, and incubated for 5h at 37°C in Mueller-Hinton cation-adjusted broth (2 $\log_{10}$ growth in the absence of vancomycin; 1 log decrease in the presence of vancomycin alone). Bacterial killing or growth was evaluated by colony counting. The solvent used to solubilize compounds **2a** and **2b** (DMSO 1% final concentration) was without influence.

Results



Scheme 1: Synthesis of the Boc-aminocaproyl mimics of the D-Ala-D-Ala binding motif. Conditions (i): MeCN/H_2O (1:1), TEA, 20°C, 17h.

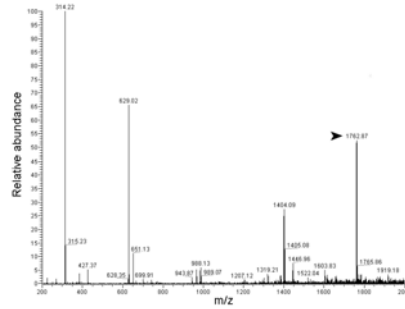


Figure 2: Collision-induced dissociation spectrum of a mixture vancomycin - **2b** at a 1:25 molar ratio. The arrowhead points to an m/z value consistent with a complex vancomycin + **2b** - 2H (1762.87). A similar complex was evidenced for a mixture vancomycin - **1**.

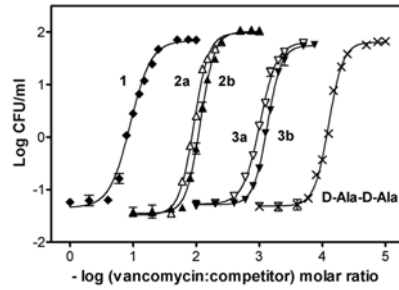


Figure 3: Competition between vancomycin natural target in *S. aureus* and target analogues (**1**, **2a**/**2b**, **3a**/**3b**) and the dipeptide D-Ala-D-Ala. The 0 value in the ordinate corresponds to the original inoculum. Negative values indicate bacterial killing due to the action of vancomycin. Positive values correspond to bacterial growth due to decreased efficacy of vancomycin in the presence of the target analogues. All curves correspond to best fitting functions using the Hill equation (with slope factors spanning between 2.83 and 3.92). EC_{50} (M) values **1**, 6.6×10^{-6}; **2a**, 6.3×10^{-5}; **2b**, 8.0×10^{-5}; **3a**, 7.1×10^{-4}; **3b**, 9.3×10^{-4}; D-Ala-D-Ala, 8.7×10^{-3}.

Main points demonstrated by this study

- Compound **1** proved competitive with an apparent EC_{50} value in agreement with previous, independent measures of its affinity constant with vancomycin (2.1×10^{-6} M).[3]
- 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 the L-Lys by a caproyl 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, a methyl- or a more bulky group such as *t*-Boc on the ε-NH_2 group was not critical.
- Replacing the terminal D-Ala by a D-Ser further increased the EC_{50} (about 1 log compared to **2a**/**2b**) in accordance to published values for the difference in affinities between Acetyl-D-Ala-D-Ala and Acetyl-D-Ala-D-Ser.[4]

Conclusions

- Alkyl-aminocaproyl-D-Ala-D-Ala (which can be covalently linked to surfaces by removing the alkyl protective group) still maintains affinity for vancomycin, with apparent dissociation constants of about 10^{-5} - 10^{-6} M.
- Since the plasma concentrations of vancomycin that need to be monitored (10-50 mg/L) are within the 10^{-5} molar range, these analogues, once linked to surfaces, could be used as potential sensors for FTIR-ATR-based detection of vancomycin in patients. The alkyl-aminocaproyl-D-Ala-D-Ser could provide an alternative if less tight binding was necessary to obtain faster and more complete dissociation in washout-phases.
- These sensors can now be developed for on-line monitoring of vancomycin in patients.

References

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