Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 22, pp. 2699-2704, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00493-3

SYNTHESIS, ANTIMICROBIAL ACTIVITY AND BINDING PROPERTIES OF CALIX[4]ARENE BASED VANCOMYCIN MIMICS

Alessandro Casnati, Massimo Fabbi, Nicola Pelizzi, Andrea Pochini, Francesco Sansone and Rocco Ungaro*

Dipartimento di Chimica Organica e Industriale, Viale delle Scienze 78, I - 43100 Parma, Italy[§]

Enza Di Modugno and Giorgio Tarzia[†]

Glaxo Research Laboratories, Via A. Fleming 4, I - 37138 Verona, Italy

Abstract. Biologically active Vancomycin antibiotic mimics have been synthesized by linking two opposite aromatic nuclei of a calix[4]arene derivative in the cone conformation with a polifunctional bridge containing D- or L- alanine units and a diethylentriamine segment.

The development of biologically active molecules based on Molecular Recognition is an attractive and challenging research topic in Medicinal and Supramolecular Chemistry.[1]

An interesting class of biologically active molecules, which explicate their action through a relatively simple molecular recognition process, is the Vancomycin group of antibiotics.[2] They are active against certain aerobic and anaerobic Gram-positive bacteria and have been in the clinical use for more than thirty years. However, glycopeptide resistance has recently been identified among clinical isolates of several Gram-positive species,[3] and the molecular mechanism of this resistance clarified.[4] Resistance to glycopeptide is known to exist in enterococci and to be inducible, transferable and sometimes plasmid-mediated, so the increased use of Vancomycin increases the selection pressure. The molecular basis of the mode of action of this family of antibiotics has been elucidated during recent years.[2,5] It is now clear that the antibiotic binds to the cell wall mucopeptide precursors terminating in the sequence -D-alanyl-D-alanine thus inhibiting the growing of the cell wall and causing the cell lysis.

These findings have stimulated much research in the field of peptide recognition by synthetic receptors,[6] although no data on biological activity of the newly synthesized Vancomycin mimics have been reported.

Calixarenes have been extensively used in the last few years as molecular platforms to attach binding groups for the selective recognition of several guest species.[7] We have also recently reported the synthesis of calixsugars, bearing carbohydrate units both at the upper rim (aromatic nuclei) and at the lower rim (phenolic OH groups).[8]

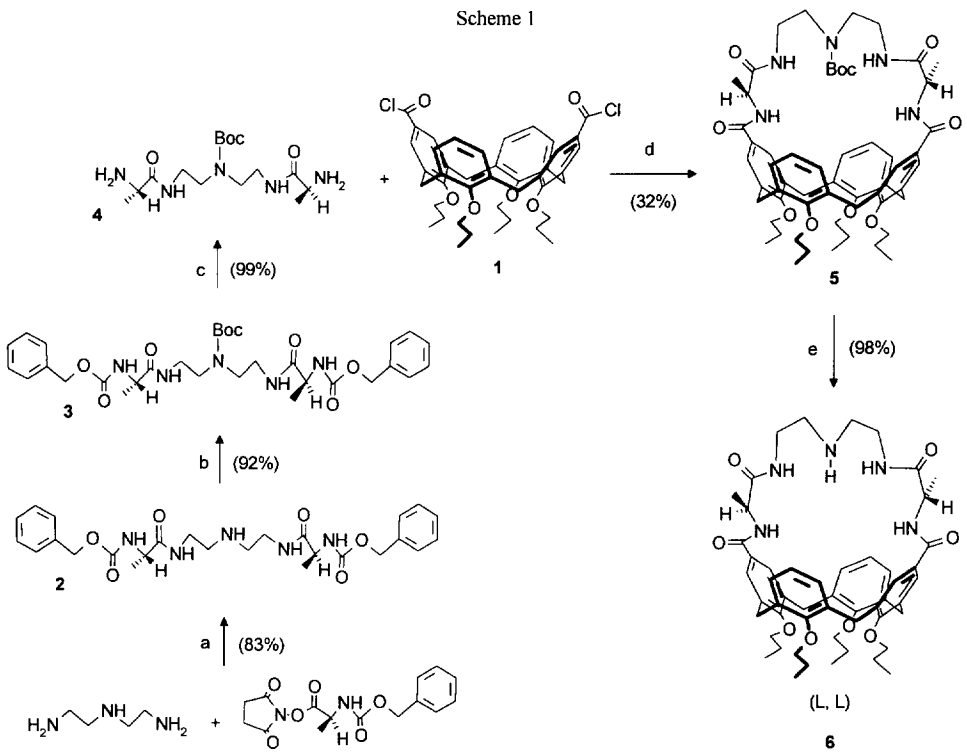
[§] E-mail: chimorg@ipruniv.cce.unipr.it; fax: +39 521 905472.
[†] Present address: Istituto di Chimica Farmaceutica, Università di Urbino, Piazza Rinascimento 6, Urbino 61029, Italy.

We report in this paper our preliminary results on the synthesis, antimicrobial activity and binding properties of new macrobicyclic peptido calixarenes which were designed as synthetic receptors of diacetyl-lys-D-ala-D-ala and of N-acetyl-D-ala-D-ala and might act as Vancomycin mimics. The carboxylate-binding pocket of Vancomycin group antibiotics is created by several aromatic units linked together with aryl-aryl, ether and peptide bonds where three amide NH groups interact with the guest carboxylate anion and two additional hydrogen bonds occur between carbonyl and NH groups of the host and guest. A primary ammonium ion on the periphery of the antibiotic, helps to orient correctly the guest peptide chain in the binding pocket.[2,5]

Synthesis: To mimic this situation we have designed a series of macrobicyclic ligands (e.g. **6**, **7** and **9**) which belong to the class of upper rim bridged calix[4]arenes in the cone conformation.

The synthesis of the short bridged (L, L) compound **6** is illustrated in Scheme 1.

Scheme 1

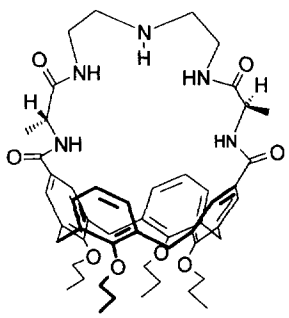


a) Et_3N, CH_2Cl_2; b) $[(CH_3)_3COCO]_2O$, tBuOH; c) Pd/C, H_2, EtOH; d) Et_3N, CH_2Cl_2; e) CF_3COOH, CH_2Cl_2

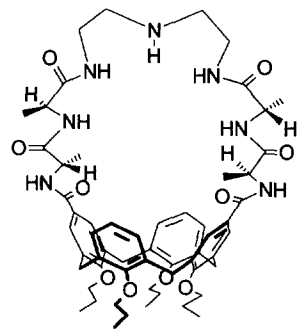
Diethylentriamine has been first reacted with Cbz-protected L-alanine-N-hydroxysuccinimide active ester[9] to give compound **2** which has been further protected with Boc group to give compound **3**.

Deprotection of compound **3** with catalytic hydrogenation (Pd/C, 10%) gives compound **4**, which is condensed in high dilution conditions, with calix[4]arene diacylchloride **1** to give the N-Boc protected macrobicyclic compound **5**. Final removal of the Boc group gives the receptor **6**. Using Cbz-D-alanine-N-hydroxysuccinimide ester as starting material, the enantiomeric receptor **7** has been obtained with overall yields comparable to that of **6**.

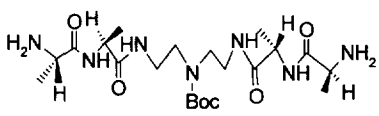
The longer bridge receptor **9** has been synthesized in 18% overall yield by condensation of diacylchloride **1** and segment **8,** followed by Boc deprotection.



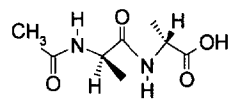
7



9



8



10

The structure of all new compounds synthesized has been established on the basis of mass spectra, elemental analysis, ^{13}C NMR, mono- and bidimensional ^{1}H NMR.

Microbiological studies: A preliminary evaluation of the antibacterial activity of ligands **5**, **6**, **7** and **9**, in comparison with Vancomycin was conducted against selected Gram-positives (including penicillin and methicillin resistant strains). The *in vitro* activity was assessed by determination of minimum inhibitory concentration (MIC) values, utilizing standard broth dilution methods according to the technical procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[10] The MIC is defined as the lowest concentration that gives no visible growth after 16 to 20 h.

As reported in Table 1 the compounds tested show an anti Gram-positive activity from moderate to good although slightly inferior to Vancomycin. Ligands **6** and **7** are the most active compounds of the series in terms of potency, including a MRSA strain.

Both enlarging the peptide bridge as in compound **9** or protecting the basic nitrogen of **6** and **7** with Boc, as in compound **5**, cause a substantial reduction of the antibacterial activity (Table 1). Control experiments show that peptide precursors **2**, **3** and **4** and their (D,D) analogues are inactive towards the same bacteria (MIC > 128 mg/l).

Given the promising antibacterial profile obtained for ligands **6** and **7** further investigations against selected bacteria have been carried out. As shown in Table 2, both compounds **6** and **7**, like Vancomycin, are inactive against fungi and yeast. Likewise, Vancomycin and compounds **6** and **7** are inactive (MIC > 128 mg/l) against a cell wall lacking bacteria, *Acholeplasma laidlawii*, suggesting that the antibacterial target should be involved in the cell wall biosynthesis. Moreover, like Vancomycin, both ligands are completely excluded by the intact outer membrane of Gram-negative bacteria (MIC > 128 mg/l against *E. coli 1852*) and show a weak activity when tested against sensitized *E. coli* obtained by addition of polymyxin B nonapeptide (PMBN at 3 mg/l as final concentration).

The minimum bactericidal concentration (MBC) has been determined from the MIC plates by a transfer of 5 μl to the appropriate antibiotic-free medium. The MBC is defined according to NCCLS recommendations,[10] as the lowest antibiotic concentration that gives no visible growth after 16 to 20 h at 35°C.

MBC determined for ligands **6** and **7** (not shown) are the same as or twice the MIC, suggesting that both compounds are bactericidal rather than static agents.

Table 1. *In vitro* activities of peptido calixarenes and Vancomycin against selected Gram-positives.

	MIC (mg/l)				
Organism	**Vancomycin**	**5**	**6**	**7**	**9**
S. aureus 663	2	64	8	8	32
S. aureus 853	2	>64	16	8	32
S. aureus 1131	2	64	4	4	32
S. epidermidis	2	32	4	8	32
B. cereus	2	NT	16	NT	NT

S. aureus 663 = penicillin sensitive, S. aureus 853 = penicillin resistant, S. aureus 1131 = methicillin resistant strain; NT = not tested

Table 2. *In vitro* activities of peptido calixarenes and Vancomycin against selected strains.

	MIC (mg/l)		
Organism	**Vancomycin**	**6**	**7**
E. coli 1852 + PMBN	0.12	32	64
S. cerevisiae	>128	>128	>128
C. albicans	>128	>128	>128
A. laidlawii	>128	>128	>128

PMBN = polimyxin B nonapeptide at 3 mg/l as final concentration.

Cytotoxicity assays: In order to address potential toxicity of the compounds, ligands **6** and **7** have been tested in COS cell cultures at 10 and 20 μg/ml. COS cells (approximately $5x10^4$ cells/ml) have been incubated in DMEM medium in the absence of serum for 4 h with both ligands and Vancomycin as non toxic control.[11] For both concentrations tested there is no significant difference between cells incubated with ligands **6**, **7** or Vancomycin and cells incubated with media only, used as reference control (the % of viable cells after 4 h of incubation with the investigated compounds are above 90% compared to the untreated cells).
This strongly suggests that ligands **6** and **7**, like Vancomycin, are not acting as a general toxin but are acting specifically as bactericidal compounds.

Binding studies: The results of the *in vitro* antibacterial tests prompted us to investigate the binding properties of ligand **6** towards the classical model for cell wall peptidoglycan termini, namely N-acetyl-D-alanyl-D-alanine (**10**).
By stirring a 10^{-3}M solution of ligand **6** in $CDCl_3$ with solid **10**, a soluble 1:1 complex is obtained. In this complex several signals of the host **6** are shifted compared with the free ligand. Particularly significant are the downfield shifts experienced by the NH and by the methylene groups of the diethylentriamine bridge (Δδ=0.25-0.8 ppm). Dilution of the $CDCl_3$ solution of the complex down to 10^{-5}M does not induce significant variations in the spectrum, thus indicating that the complex has an association constant $>10^5 M^{-1}$.
To obtain more insight into the complexing mode of ligand **6** we have investigated its behavior towards $CDCl_3$ soluble acidic substrates of increasing complexity. Addition of picric acid (pKa=0 in CH_3OH and DMSO)[12] to a $CDCl_3$ solution of **6** causes downfield shifts of the NH and CH_2 protons of the diethylentriamine bridge similar to those observed with N-acetyl-D-alanyl-D-alanine. Moreover the UV-Vis spectrum of the complex shows two maxima at λmax=348nm (Log ε = 4.11) and 418nm (Log ε = 3.87), which are almost identical to those of cyclohexylammonium picrate in $CHCl_3$, λmax=348nm (Log ε = 4.20) and 414nm (Log ε = 3.97). These data are consistent with the occurrence of a proton transfer from picric acid to amine, possibly followed by ion-pair association.
Addition of lauric acid causes more regular shifts; a plot of chemical shift changes versus concentration shows a saturation behavior, which has been analyzed by non linear-least square method[13] to give an association constant of 990±40 M^{-1}.
Similar titration with N-lauroyl-D-alanine gives an association constant of 11,000±1,000 M^{-1}, thus indicating that beside the primary interactions with the carboxylate binding cleft other hydrogen bonds are involved, which cause a tenfold increase in association constant. In agreement with this trend and with the qualitative solid-liquid extraction of N-acetyl-D-ala-D-ala in $CDCl_3$ *(vide supra)* the homogeneous titration of host **6** with N-lauroyl-D-ala-D-ala gives association constants too high ($>10^5$ M^{-1}) to be measured accurately in this

medium, indicating that additional hydrogen bonds stabilize the complex with the dipeptide. However the exact structure of the complex is not known so far, and we are trying to obtain informations through X-ray and Molecular Modeling studies.

In conclusion we have shown in this work that the combination of designed electrostatic, hydrogen bonding and possibly other weak interactions can stabilize complexes between the synthetic host and amino acids and small peptides, leading to synthetic biologically active Vancomycin antibiotic analogs. We are currently modifying the basic structure of receptors **6** and **7** to improve their binding to N-acetyl-D-alanyl-D-alanine and, as a consequence, their antibiotic activity towards Gram positive bacteria.

Acknoledgments
We thank Glaxo Company for a Ph. D. fellowship to M. Fabbi.

References and Notes:

1. *Design and Synthesis of Organic Molecules Based on Molecular Recognition from Chemistry to Biology* (Ed.: van Bist, G.), Springer, Berlin, **1986**.
2. Williams, D. H.; Westwell, M. S. *Chem. Tech.* **1996**, 17 and references therein. Reynolds, P. E., *Eur. J. Clin. Microbiol. Infect. Dis.* **1989**, *8*, 943.
3. Woodford, N.; Johnson, A. P.; Morrison, D.; Speller, D. C. E. *Clinical Microbiology Review* **1995**, *8*, 585.
4. Walsh, C.; Fisher, S. L.; Park, I. S.; Prahaland, M.; Wu, Z. *Chemistry and Biology* **1996**, *3*, 21; Arthur, M.; Molinas, C.; Courvalin, P. *J. Bacteriol.* **1992**, *174*, 2582; Billot-Klein, D.; Gutmann, L.; Sablé, S.; Guittet, E.; van Heijenoort *J. Bacteriol.* **1994**, *176*, 2398.
5. Chatterjee, A. N.; Perkins, M. R. *Biochem. Biophys. Res. Commun.* **1966**, *24*, 489; Perkins, H. R. *Biochem. J.* **1971**, *124*, 845; Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332.
6. For recent review articles see: Hamilton, A. D.; Pant, N.; Muehldorf, A. *Pure & Appl. Chem.* **1988**, *60*, 533; Schneider, H.-J. *Angew. Chem.* **1993**, *105*, 890; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 848; Still, W. C. *Acc. Chem. Res.* **1996**, *29*, 155.
7. For recent review articles on calixarenes see: Böhmer, V. *Angew. Chem.* **1995**, *107*, 785; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 713; Pochini, A.; Ungaro, R. *Comprehensive Supramolecular Chemistry*, Vol. 2, Pergamon Press, **1996**, in press.
8. Marra, A.; Scherrmann, M.-C.; Dondoni, A.; Casnati, A.; Minari, P.; Ungaro, R. *Angew. Chem.* **1994**, *106*, 2533; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 2479.
9. Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839.
10. National Committee for Clinical Laboratory Standards **1993**; Methods for diluition antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. approved standard M7-A3, vol. 10, no.8. National Committee for Clinical Laboratory Standards, Villanova, Pa.
11. COS cell viability was measured using MTT assay system (3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolim bromide) based on the reduction by mitochondrial dehydrogenasis to a purple formazan; see: Mosmann, T. *J. of Immunological Methods* **1983**, *65*, 55.
12. Bordwell, F. G. *Acc. Chem. Res.* **1988**, *21*, 456.
13. Connors, K. A. *Binding Constants*; Wiley Interscience: New York, **1987**.

(*Received in Belgium* 12 *July* 1996; *accepted* 14 *October* 1996)