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Bio-inspired Synthetic Receptor Molecules Towards Mimicry of Vancomycin

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Abstract—A 512-member library of bio-inspired synthetic receptor molecules was prepared featuring a triazacyclophane scaffold. The purpose of this scaffold was to orient three (identical) peptide 'binding arms' in order to mimic an antibiotic binding cavity as is present in the vancomycin antibiotics. The library was screened with D-Ala-D-Ala and D-Ala-D-Lac containing ligands, which are present in the cell wall precursors of pathogenic bacteria. Screening and validation led to identification of a synthetic receptor capable of binding these ligands. © 2001 Elsevier Science Ltd. All rights reserved.

The antibacterial activity of glycopeptide antibiotics such as vancomycin probably largely results from binding to the terminus of the bacterial cell wall precursor, namely D-alanine-D-alanine, thereby inhibiting formation of the cell wall in growing bacteria.[1,2,3] In view of the increasing number of bacteria resistant against vancomycin antibiotics, it is imperative to uncover new molecules capable of binding D-alanine-D-alanine and/or D-alanine-D-lactic acid, which is the terminus of the cell wall precursor present in resistant bacteria.[4]

We are involved in two programs towards realizing this goal. In the first program of *biomimetic* synthetic receptor molecules, we aim at vancomycin mimics in which analogues of crucial parts of the vancomycin structure are prepared to dissect and further improve the biological activity due to these parts.[5,6] In the second program of *bio-inspired* synthetic receptor molecules, completely different molecules are constructed.[7] However, in developing these molecules, we are guided—if not inspired—by the basic characteristics of vancomycin: (1) It is a natural *receptor molecule* for the terminus of the bacterial cell wall precursor. (2) It derives its rigidity by covalent control of its shape. (3) Its building blocks are amino acids.

To construct these synthetic receptor molecules, we have chosen for covalent control by using a scaffold, to which three peptide arms can be attached and oriented in space. The tripodal hinge for these receptor molecules was based on the best hinge we have recently found for tweezer-like receptors.[8] A bridge between the two nitrogen atoms containing an additional nitrogen atom then leads to a triazacyclophane scaffold to which three binding arms can be attached (Fig. 1).

Thus, starting from bisbromide **1**,[9] triazacyclophane scaffold **3** was prepared by alkylation with sulfonamide **2**[10] in 64% yield. Removal of the *o*-nitrobenzenesulfonyl and Boc protecting groups was followed by introduction of a spacer by alkylation with 3-bromo *N*-Boc propylamine leading to ester **4**.[11] This spacer facilitates the introduction of buildings blocks of the binding arms (Scheme 1).

Following saponification of the methylester, coupling to a solid-phase resin containing glycine[12] and removal of the Boc protecting groups, a library was prepared by the split-mix method.[13] To facilitate screening, a representative set consisting of eight, both non-functionalized and functionalized amino acids, namely Gly, Ala, Val, Leu, Phe, Lys(Boc), Glu(O*t*-Bu) and Ser(*t*-Bu) was used. Although this set is limited, it reflects the amino acid diversity to a considerable extent. The peptidic binding arms consisted each of three amino acid residues that were introduced in three subsequent BOP-

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coupling reactions, leading to a library of 8^3 (=512) synthetic receptors (Scheme 2). Finally, Fmoc, Boc and *tert*-butyl protecting groups were removed by piperidine and TFA, to give the deprotected library.

Screening of this library was carried out by incubating 10 mg of the library (ca. 10^4 beads) with a chloroform solution of Ds-Gly-D-Ala-D-Lac-OH **8** or Ds-Gly-D-Ala-D-Ala-OH **7**[17] and visual inspection of the beads using a Leica fluorescence stereomicroscope. At concentrations >50 μM, no selectivity of binding was observed and $>50\%$ of the beads were fluorescent.

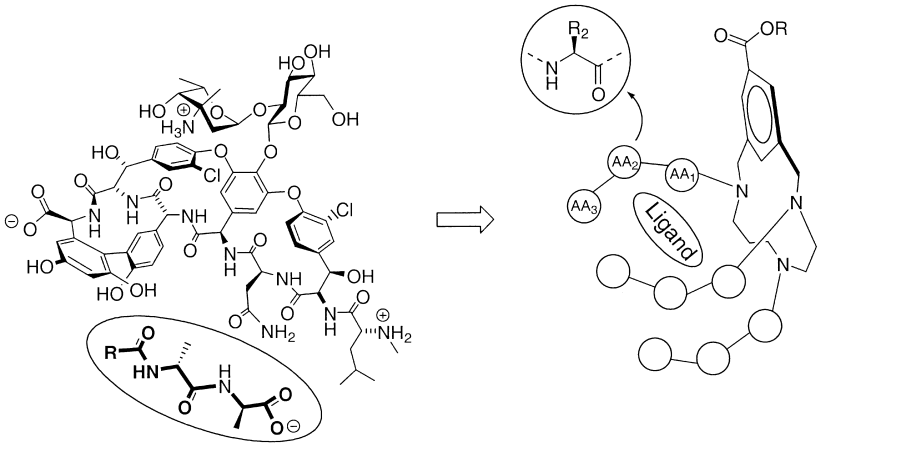
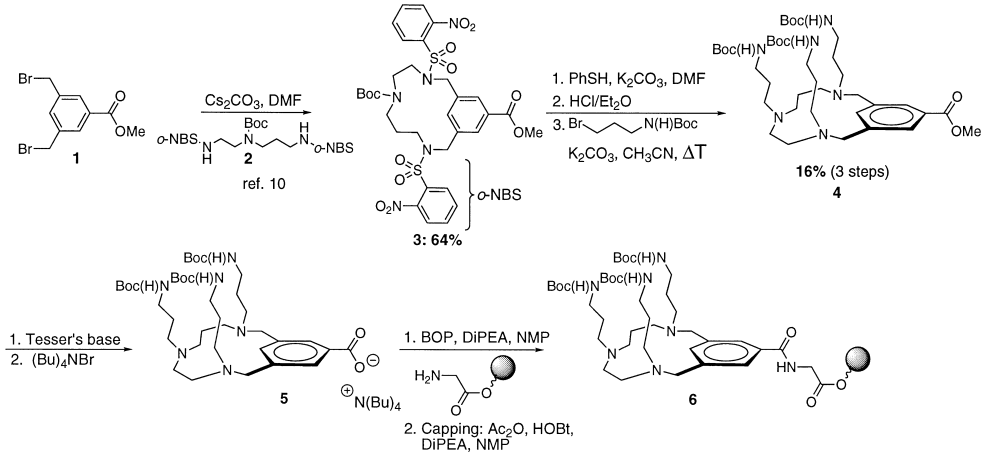
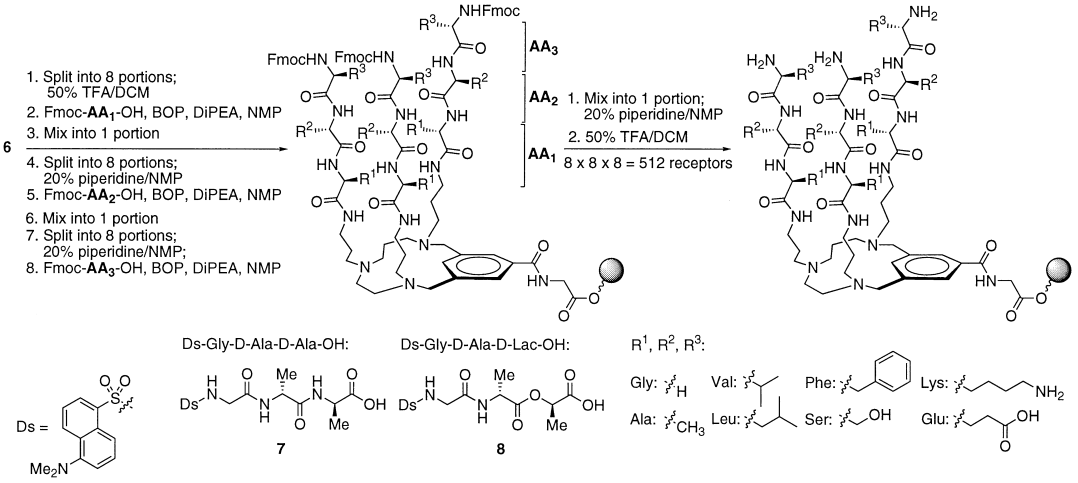


Figure 1. Vancomycin and bioinspired synthetic receptor molecules having a triazacyclophane scaffold.



Scheme 1. Synthesis and solid-phase attachment of the triazacyclophane scaffold.



Scheme 2. Preparation of the 512-member synthetic receptor library.

However, using concentrations of 10 μM resulted in selective binding and <10% of the beads were fluorescent. In general, the observed fluorescence intensity was higher for the D-lactate-containing ligand **8** as compared to the D-alanine-containing ligand **7** which might be a qualitative indication of stronger binding. After incubation with the ligands three fluorescent beads were selected from each incubation and subjected to Edman degradation. The identity of the peptide arms of the corresponding synthetic receptors, attached to the solid-phase resin bead, is shown in Table 1.

Table 1.

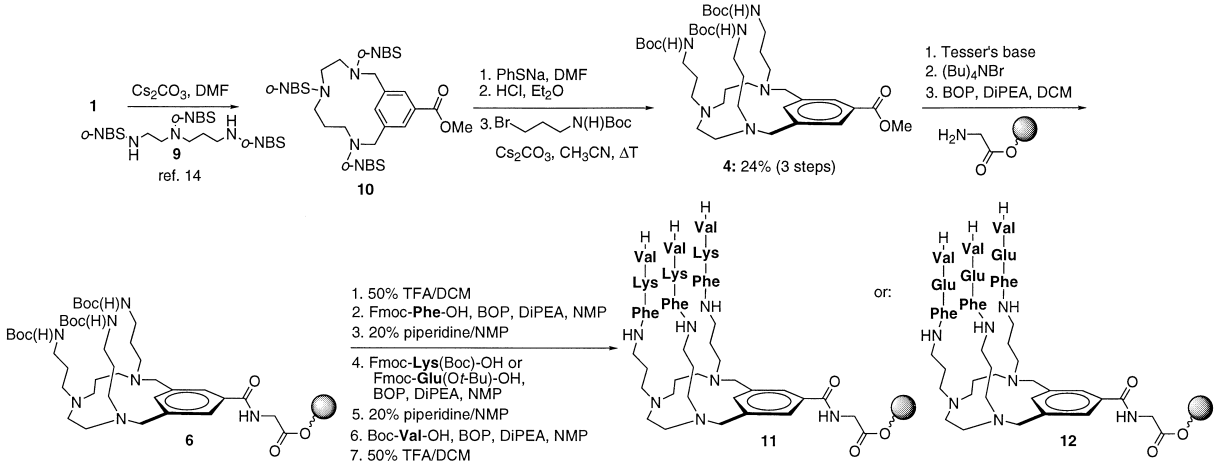
Screening with:	Sequence found by Edman degradation: AA_3-AA_2-AA_1	Resynthesized synthetic receptor
Ds-Gly-D-Ala-D-Ala-OH **7**	Val-Glu-Phe Phe-Lys-Ser Glu-Gly-Gly/Phe[a]	**12**
Ds-Gly-D-Ala-D-Lac-OH **8**	Val-Lys-Phe Lys-Lys-Gly Lys-Leu-Val	**11**

[a]The sequencing cycle was not completely unambiguous with respect to this amino acid residue.

Validation of 'hits' by resynthesis of the selected library members and reproducing—in a more accurate way—the properties for which the library was screened is essential in any combinatorial approach towards finding compounds with desired properties. In these screenings, it was found that the peptidic arms of a synthetic receptor library member capable of binding the fluorescent D-Ala-D-Ala ligand **7** consisted of Val-Glu-Phe, whereas the peptidic arms in a library member capable of binding D-Ala-D-Lac **8** had the sequence Val-Lys-Phe. The differences between the other library members capable of binding D-Ala-D-Ala or D-Ala-D-Lac were greater, but even judging from these small sets, it seemed that binding of D-Ala-D-Ala involved an acidic amino acid (Glu, two out of three selected library members), whereas binding of D-Ala-D-Lac involved a basic amino acid (Lys, three out of three selected library members). The differences of only one amino acid between the former library members in determining the selectivity prompted us to resynthesize these library members and determine in detail the binding characteristics.

Resynthesis was carried out analogously to the synthesis depicted in Scheme 1 except for the use of trisulfonamide **9**[14] instead of disulfonamide **2**. The former is more easily accessible, in higher yields, and more suitable for synthetic receptor molecules containing three identical arms[15] (Scheme 3). Furthermore, Boc-Val-OH was used instead of Fmoc-Val-OH, thereby avoiding an additional Fmoc-deprotection step. After resynthesis the protected compounds were cleaved from the resin for characterization. Electron spray mass spectrometry was in agreement with the identity of the compounds and TLC showed that the purity was quite good considering the number of steps (10) which were carried out for the solid-phase synthesis of these compounds.

For establishing the binding characteristics, it is of utmost importance to determine the binding constants as accurately and rigorously as possible. To this end, aliquots of resin containing the deprotected receptors **11** and **12**, respectively, were accurately weighed and incubated with varying concentrations of dansylated ligand (Ds-Gly-D-Ala-D-Ala-OH **7** or Ds-Gly-D-Ala-D-Lac-OH **8**) in chloroform. Around 10 concentrations were chosen in order to obtain a number of evenly distributed data points for the generation of a good regression line. After incubation overnight in the dark and filtration of the beads, fluorescence intensity was measured in the filtrate, thereby allowing determination of the concentration of the unbound ligand. A calibration curve was used for this purpose since at higher concentrations fluorescence is quenched, giving rise to a non-linear relationship. The obtained values were also corrected for volume loss due to the evaporation of the solvent. For establishing the concentration of synthetic receptors on the resin ([R_0]), it was important to correct the mass of the weighed resin for the mass of the synthetic receptor, which at the particular loading of the resin (ca. 0.3 mmol g^{-1}), amounts to ca. 30%!



Scheme 3. Resynthesis of two members, i.e. **11** and **12** of the synthetic receptor library.

Furthermore, the loading, which is ultimately the number of synthetic receptors, has to be determined precisely, since the binding constant K_a is very sensitive to variations in this number.[16] As a reference we used resin containing an acetylated glycine residue, that is Ac-Gly-O-Argogel™ which is used as a linker for attachment of the synthetic receptors (Scheme 2). The values for the amount of binding obtained with this resin—referred to as non-specific binding—were always subtracted from the values obtained with the resin containing the synthetic receptors.

Resynthesis of resin containing synthetic receptor **11** having the amino acid sequence Val-Lys-Phe in its peptidic binding arms and which in the screening experiment was selected as a binder of Ds-Gly-D-Ala-D-Lac-OH (**8**), followed by determination of the binding constant resulted in quite a satisfactory K_a of 10,100 M^{-1}. However, incubating this receptor with Ds-Gly-D-Ala-D-Ala-OH (**7**) gave the even better K_a of 26,600 M^{-1}, although this synthetic receptor was not identified upon screening the library of synthetic receptors with Ds-Gly-D-Ala-D-Ala-OH! Even more surprisingly were the results found after resynthesis of resin containing synthetic receptor **12** having the amino acid sequence Val-Glu-Phe in its peptidic binding arms followed by incubation with ligand Ds-Gly-D-Ala-D-Ala-OH used in the screening to uncover this synthetic receptor. No binding was detected whatsoever; binding to the reference resin Ac-Gly-*O*-Argogel™ was in fact better than binding to the synthetic receptor! It seemed that the presence of the synthetic receptor led to an increasing incliness to bind less ligand. Clearly no K_a could be determined. Also, no binding was detected with the other ligand Ds-Gly-D-Ala-D-Lac-OH **8**. Although at first glance these data appeared difficult to explain, they may point at subtle differences between screening conditions and determination of the binding constants, thereby underlining the importance of validating any hits which are obtained after screening. Determination of the binding characteristics was carried out on the deprotected synthetic receptor, which was obtained after deprotection by acidolysis with TFA to remove Boc and *t*-Bu protective groups. To remove residual acid, the resin bound deprotected receptor was rigorously washed with triethylamine, followed by washings to remove excess of triethylamine. However, washing with triethylamine will convert the side-chain carboxylic acid moieties of glutamic acid residues into the carboxylates. The resulting negatively charged residues may discourage binding of carboxylate-containing ligands such as **7** and **8**. This may explain the observed absence of binding by these synthetic receptor containing resins and in fact a higher level of binding by the uncharged reference resin. It was found that binding to the synthetic receptor was observed when the pH was lowered, for example by adding acetic acid (data not shown). It was therefore assumed that screening of the library of receptors, which led to selection of the resynthesized receptor **12**, had been carried out under slightly acidic conditions either because not entirely acid-free chloroform was used or the deprotected synthetic library was not completely neutralized. Be this as it may, these results emphasize the absolute requirement for validation of hits found by screening (Table 2).

Table 2. Determination of the binding constant K_a of the resynthesized receptors **11** and **12**

	Val-Lys-Phe (**11**)	Val-Glu-Phe (**12**)
Ds-Gly-D-Ala-D-Ala-OH **7**	26,600 M^{-1}	—
Ds-Gly-D-Ala-D-Lac-OH **8**	10,100 M^{-1}	—

In conclusion, we have prepared a library of bio-inspired receptors aimed at mimicry of vancomycin. Although screening indicated that we had selectivity for binding to the cell wall precursor present in resistant bacteria, validation of the hits showed that our synthetic receptor was even better in binding D-Ala-D-Ala present in non-resistant bacteria. Nevertheless, affinity constants for binding by synthetic receptor **11** of either one of both cell wall precursors are very promising. Therefore, we think that this approach is an attractive one to pursue further in order to find synthetic receptors with binding properties in more polar solvents including water as well as for uncovering synthetic receptors capable of binding other ligands.

Acknowledgements

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16. This is apparent from the used equation:

$$1/\left([L_0] - \left[L_{eq}\right]\right) = 1/\left(\left[L_{eq}\right][R_0]K_a\right) + 1/[R_0]$$

in which $[L_0]$ is the initial concentration of ligand **7** or **8**; $\left[L_{eq}\right]$ is the equilibrium concentration of the ligand in solution and $[R_0]$ is the 'concentration' of synthetic receptors on the resin.
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