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Synthesis and Ion-binding Properties of an Immobilized bis-Cysteine Peptide

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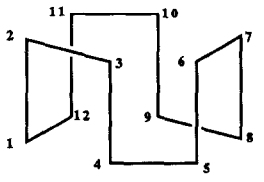
Abstract: *Techniques of solid-phase peptide segment coupling have been applied for the first time to the unambiguous grafting of a preformed ion complexing peptide to a solid support. The immobilized peptide forms complexes with sodium ions in trifluoroethanol with high affinity and considerable selectivity.*

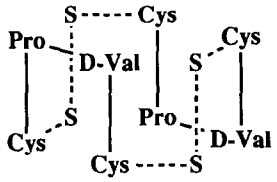
The approach of attaching chemically a well defined ligand with ion-binding properties to a solid support represents an important technological goal with multiple and important applications. So far, efforts to accomplish such chemical bonding involved the formation of hydrocarbon, ether or amide linkages between the macrocycle and the solid-matrix.[3,4] The long synthetic sequences performed on resins to accomplish the attachment of the ligand resulted in many cases in chelating resins with low structural purity.[5,6] Furthermore, determination of the capacity of these resins is not straightforward.

Natural and synthetic cyclic peptides have shown a wide range of complexing properties in their binding of many molecules.[7] To the best of our knowledge, the idea of attaching one of these ionophore peptides to a solid support and determining their binding properties has so far not been reported. Such an approach takes advantage of the methodology involved in the "convergent solid-phase peptide strategy,"[8] used for the synthesis of large peptides by coupling of different purified protected peptides on a solid support. Furthermore, this strategy is compatible with a full range of solid supports, including microporous and macroporous polystyrenes, microporous or encapsulated polyamides, controlled pore glasses, and polyethylene glycol- and Kel-F-grafts.[9] Finally, the capacity of the chelating-resin, easily determined by amino acid analysis, can be modulated by the level of functionalization of the initial resin.

In the present work, we describe the unambiguous preparation and ion-binding properties of a bis-cysteine peptide grafted by an amide bond to a solid-support. The cyclic peptide attempts to mimic the bracelet conformation of the valinomycin complex with potassium.[10] The two monomers can adopt a type II β-turn and the disulfide bonds can hold them in a relative orientation suitable for metal ion complexation.



VALINOMYCIN:K^+ COMPLEX



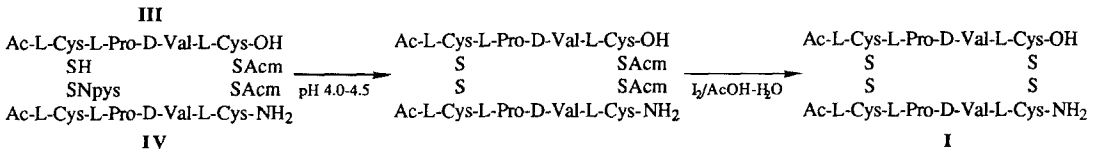
CYCLIC PEPTIDE

The sequence of **I** and **II** was chosen in light of the theoretical prediction of Venkatachalam[11] and Wilmot & Thornton[12] so that it had a high probability of adopting a β-turn conformation. Preliminary results by nuclear magnetic resonance and circular dichroism indicate that the dimer **II** presents solvent and temperature dependent conformations as in the case of valinomycin and forms complexes with alkaline and alkaline-earth cations.[13]

Ac-L-Cys-L-Pro-D-Val-L-Cys-OH
S S
S S
Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2
I

Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2
S S
S S
Ac-L-Cys-L-Pro-D-Val-L-Cys-NH_2
II

In the synthesis of **I** (Scheme 1), we employed a previously described strategy[14] that involves the use of two different monomers and three different cysteines protecting groups. One of the monomers provides the *C*-terminal carboxylic acid for establishing the amide bond between the dimer and the solid support. The formation of the first disulfide bond is directed by the use of an activating group such as 3-nitro-2-pyridylsulfenyl (Npys)[15] in front of a free thiol. The second disulfide bond is obtained upon treatment of the linear precursor with iodine.

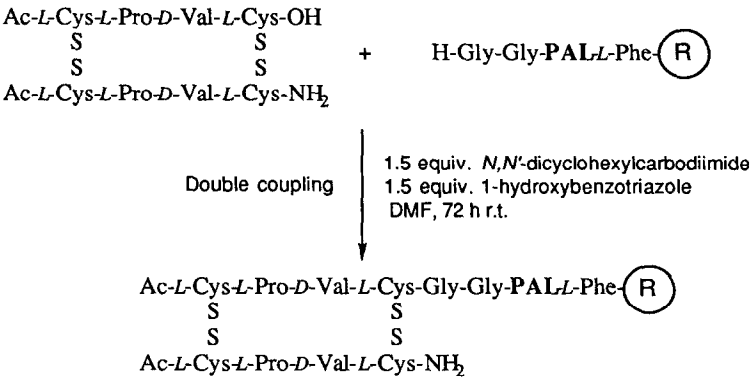


Scheme 1

Chain assembly was carried out manually using a conventional strategy[16] on a chloromethyl-resin for the synthesis of Ac-L-Cys-L-Pro-D-Val-L-Cys(Acm)-OH (**III**) and a *p*-methylbenzhydrylamine for establishing the *C*-terminal peptide amide of Ac-L-Cys(Npys)-L-Pro-D-Val-L-Cys(Acm)-NH_2 (**IV**). For **III**, the first amino acid was anchored onto the resin according to the cesium salt method[17] and the side chain of the *N*-terminal cysteine was protected with the *p*-methylbenzyl group (pMeBzl) which was removed in the cleavage step. In both syntheses, the *tert.*-butyloxycarbonyl (Boc) group was used for the temporary protection of N^{α}-amino groups. Once chain assembly was completed, the two peptide-resins were cleaved with HF–*p*-cresol (9:1, v/v) and purified by reversed-phase medium pressure liquid chromatography (MPLC).[18] Equivalent amounts of both peptides were mixed in an aqueous solution at pH 4.0-4.5. After completion of the reaction (6 h), the mixture was liophylized and the crude peptide purified by MPLC (86 % overall yield from the starting material). The second disulfide bond was formed upon treatment of the bis-*S*-acetamidomethyl(Acm)-peptide with iodine (10 equiv.) in acetic acid-water (4:1, v/v), 2 h, r.t. Subsequent purification by MPLC (66 % overall yield) gave peptide **I**. The final product **I** and all intermediates shown a single peak in high performance liquid chromatography (HPLC) and were characterized by amino acid analysis, fast atom bombardment mass spectrometry (FAB-MS), and nuclear magnetic resonance.[19]

Before the incorporation of the cyclic peptide to the solid support, we checked by analytical HPLC the stability of the disulfide bridge to the coupling conditions.[20] In accordance with previously reported data,[21] the disulfide bonds of **I** were completely stable to the standard coupling conditions. The cyclic peptide **I** was covalently grafted onto an aminomethylpoly(styrene-*co*-1% divinylbenzene)-resin (Scheme 2) using *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole with an overall yield of 74 % as determined by amino acid analysis. The handle 5-(4-(9-fluorenylmethyloxycarbonyl)-aminomethyl-3,5-dimethoxyphenoxy)-

valeric acid (PAL)[22] and the two residues of glycine[23] were used as spacers between the peptide and the resin to increase the spatial mobility of the ionophore. After the coupling reaction, the free amino groups were capped with acetic acid and DCC. Amino acid analysis of an aliquot of resin gave a capacity of 0.17 mmol of ionophore per gram of resin.



Scheme 2

The binding constant of the ion pair to the peptide-matrix was calculated from the spectrophotometric determination of the free picrate salt after equilibration with the immobilized peptide in trifluoroethanol. For the set of picrate salts studied (Li^+, Na^+, and K^+), the peptide-matrix only showed affinity for the sodium picrate salt. The binding constant K_{Na^+}= 5200 M^{-1} (T= 20° C) was obtained from a rearranged form of the Langmuir adsorption isotherm previously described[24] assuming a 1:1 complex. No affinity for either of the above salts was observed with the acetyl derivative of the aminomethylresin Ac-Gly-Gly-PAL-Phe-resin which was used as a blank.

$$1 / R = 1 / n + 1 / (nKA)$$

where $1 / R = P_o/P$, A is the free picrate concentration in solution which is measured by UV, K is the binding constant, and $1 / n$ denotes the number of peptide units in the complex. P_o is the total amount of peptide and P corresponds to the amount of complex peptide.

The selectivity observed falls far the original expectation from the solution studies of **II** in acetonitrile (Li^+: log K=2.60±0.04; Na^+: log K=2.11±0.04; K^+ : no affinity was detected).[13] Additional factors beyond the polarity of the solvent or the counterion seem to be responsible for this difference in selectivity which is quite common in other immobilized ion extractants.[6]

In summary, we have devised a new approach for the unambiguous preparation of bound macrocycles with binding properties. This involves solid-phase synthesis of protected peptides and, after purification, their assembly on a new solid support. Thus, new and general possibilities are presented for the synthesis and application of separation systems that incorporate ionophore peptides.

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16. Cycles for incorporation of Boc-amino acids comprised deprotection with CF_3COOH-CH_2Cl_2 (3:7), neutralization with $^iPr_2EtN$-CH_2Cl_2 (1:19), and single coupling (2.5 fold) mediated by DCC in CH_2Cl_2; all couplings were ninhydrin or chloranil negative within two hours. Acetylation was carried out with acetic acid and DCC once peptide chain was assembled .
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18. Yields: 77 % and 78 % for the chromatography of **III** and **IV**, respectively.
19. For final product **I**, FAB-MS: 920 [$M+H^+$], 942 [$M+Na^+$], 953 [$M+Cl^-$]; amino acid analysis: Pro 1.96, Val 2.04, Cys, 3.50; HPLC: t_R, 14.6 min [Nucleosyl C_{18} reversed phase column (0.4 x 25 cm) and eluting with a linear gradient from 5% to 70% of B in A, where A is H_2O/0.045% TFA and B acetonitrile/0.036% TFA, at a flow rate of 1.0 mL/min. UV absorbance at 220 nm].
20. Cyclic peptide I was incubated for three days with 1.5 equiv. of DCC and 1.5 equiv. of 1-hydroxybenzotriazole in *N,N*-dimethylformamide (DMF). See HPLC conditions in ref.19
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23. Cycles for incorporation of Fmoc-amino acids comprised deprotection with piperidine-DMF (3:7) and single coupling (3.0 fold) mediated by DCC in DMF; all couplings were ninhydrin negative within two hours. The resin contained phenylalanine as internal reference.
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