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Synthetic Approaches to Macromolecular Models for Ion Channel Proteins

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Abstract: The synthesis of a branched peptide is described using a ligation strategy to couple two peptides one of which is fully deprotected while the other is in a protected form. The ligation step involves formation of a thioether bond by using the thiol of a cysteine on the deprotected sequence to displace bromide from a bromoacetyl moiety on the second peptide. © 1998 Elsevier Science Ltd. All rights reserved.

Molecular mechanisms which underlie cation transport across biological membranes are of fundamental importance. In one method of transport, the "channel" mechanism, the protein spans the membrane as a conduit and the ion passes through[1]. Since the protein is membrane-bound it is not easily studied on a molecular basis; its structure depends on the membrane matrix and the structural integrity is lost once the membrane is removed. One method of studying the molecular mechanism of ion transport is to use synthetic protein systems[2] where the structure can be stabilised by chemical bonds rather than by physiological interactions with a membrane. As part of our on-going studies on the structure and ion transport properties of δ-toxin[3] we have been studying the synthesis of model systems with potential helix bundle topology by covalently linking a number of peptide units to a template sequence.

Here we report on a general strategy for macromolecular construction based on chemoselective formation of thioether bonds for the attachment of unprotected peptides to a protected template sequence which is compatible with the Fmoc-t-butyl method of solid phase peptide synthesis. Our approach combines Mutter's TASP (Template Assembled Synthetic Protein) technique[2a] with selective fragment coupling methodology which was pioneered by the groups of Kent[2b] and Tam[2c].

The sequence Phe-Thr-Lys-Lys-Cys (**1**), consisting of the C-terminal tetrapeptide Phe^{23}-Lys^{26} of δ-toxin plus a key cysteine residue, was covalently linked to the side chain of Lys^2 of the nonapeptide template Boc-Lys(Boc)-Lys($COCH_2Br$)-Lys(Boc)-Pro-Gly-Lys(Boc)-Glu(OBu^t)-Lys(Boc)-Gly (**2**) by using the thiol of the cysteine to displace the bromide from the bromoacetyl moiety at the target ligation site[4] (Fig. 1). Due to the presence of a multitude of functional groups (amino, hydroxyl, carboxyl) this pentapeptide appears to be the ideal prototype for studying the chemoselectivity of this ligation mode.

Both peptides were assembled using standard Fmoc-t-butyl solid-phase chemistry[5] on a Novasyn TG resin bearing a 4-hydroxymethyl-3-methoxyphenoxyacetyl linker. The ε-amino group of the target ligation site in the template sequence was protected with a Dde group[6] to enable selective derivatisation. Derivatisation was a two-step process: Dde removal followed by bromoacetylation. Thus the fully protected resin-bound template peptide was treated with a 3% solution of hydrazine in DMF for 15 min followed by washing with DMF,

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MeOH and Et_2O. Freshly prepared bromoacetic anhydride[7] was then added, as a solution in CH_2Cl_2, to the partially protected resin-bound template and the acylation was found to be complete after 1 h[8]. The protected and derivatised template was then removed from the resin using 1% TFA in CH_2Cl_2. The cysteine thiol of (**1**) was protected as the Acm derivative during solid-phase assembly of the pentapeptide and this group was removed with H_2S immediately prior to the coupling step.

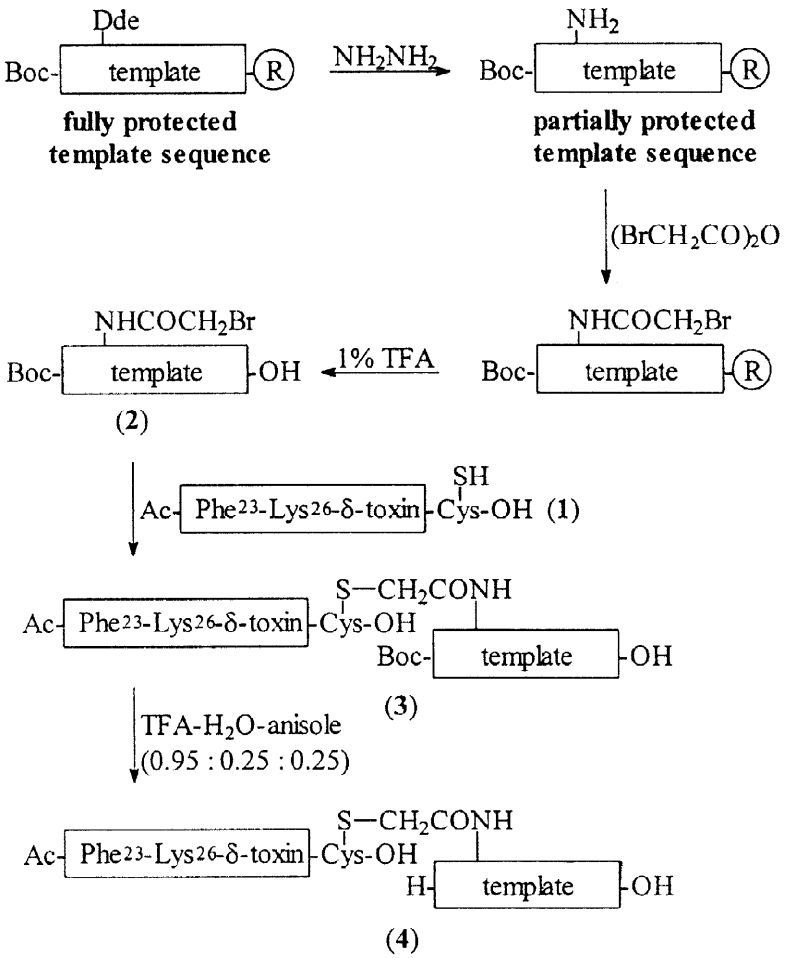
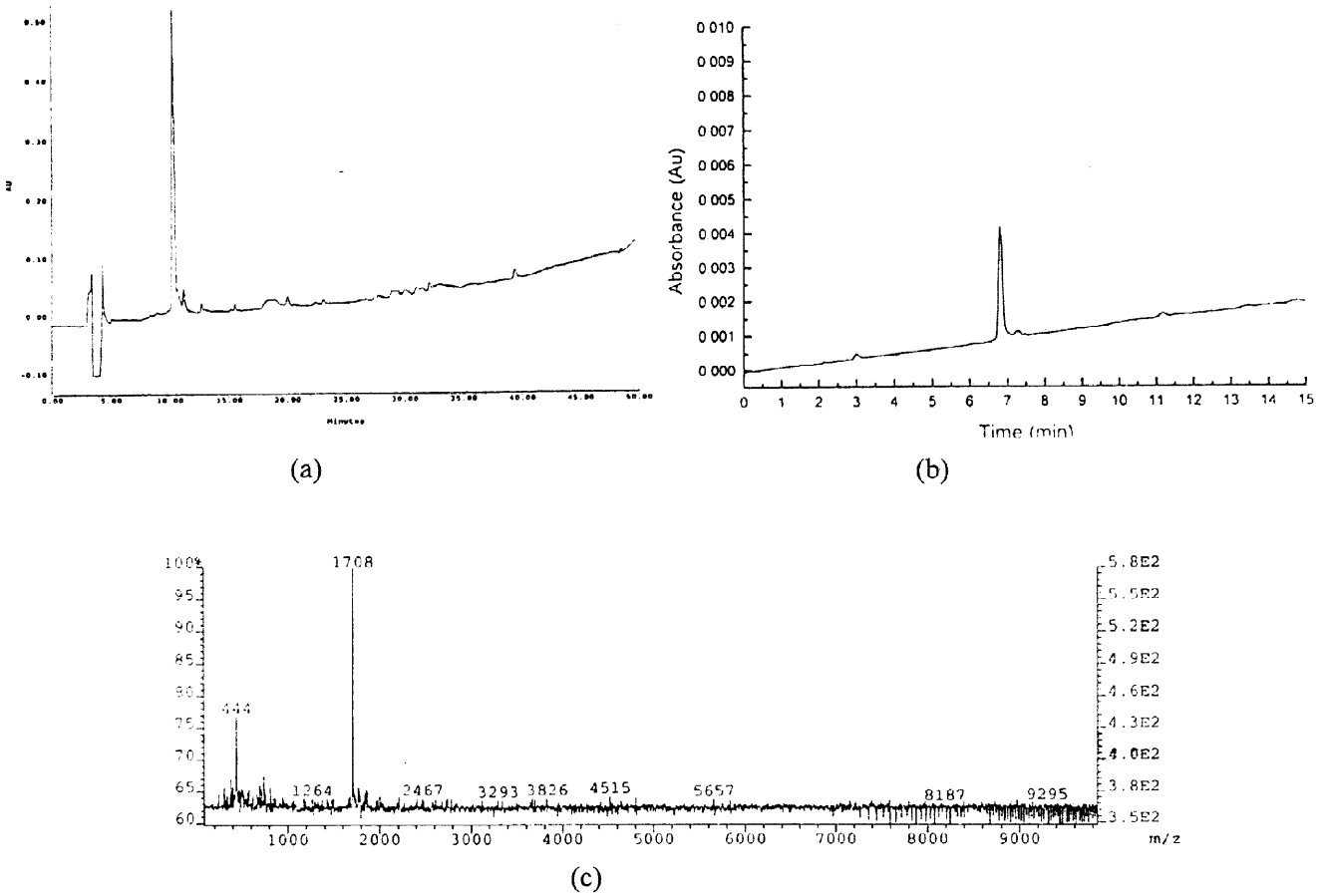


Fig. 1: Chemoselective ligation of the deprotected pentapeptide to the protected template *via* thioether bond formation

The bromoacetylated template (**2**) was dissolved in DMF containing a trace of DIPEA and to this approximately 1.5 molar equivalents of pentapeptide (**1**), as a solution in DMF, was added. The resulting mixture was left stirring at room temperature for 24 h and the solvent was then evaporated *in vacuo*. The crude product was analysed by RP-HPLC[9] on a C-18 column and contained the desired compound (**3**) as the major product at a retention time of 28 min with the only significant by-product being the pentapeptide dimer at 10 min (see Fig. 2a for elution conditions). Mass spectral analysis confirmed the presence of the protected coupled product (**3**) with a molecular ion $[M+Na]^+$ at 2284 ($C_{105}H_{179}N_{21}O_{31}S$ requires $[M+Na]^+$ 2287)[11] being observed.

The coupled product was purified by RP-HPLC and the remaining protecting groups on the template moiety were then removed by treating (3) with a standard cleavage cocktail (TFA-H_2O-anisole, 9.5:0.25:0.25) for 45 min at room temperature. RP-HPLC (Fig. 2a) and CZE (Fig. 2b) showed the deprotected coupled product (4) as a single species and a strong molecular ion MH^+ at 1708 ($C_{76}H_{131}N_{21}O_{21}S$ requires MH^+ 1708) was observed in the mass spectrum (Fig 2c).

Studies into the attachment of multiple pentapeptides to the template sequence are in progress and will be described in a future report.



(a) (b)

(c)

Fig 2:Characterisation of the deprotected coupled product (4). (a) HPLC: the C_{18} column was eluted isocratically for 2 min with 10% B and then with a linear gradient of 10-90% B over 40 min; (b) CZE chromatogram[10]; (c) MALDI-TOF Mass Spectrum[11]

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7. Anhydride preparation: Bromoacetic acid and dicyclohexylcarbodiimide (2:1 mol ratio) were dissolved in CH_2Cl_2 and the resulting mixture was stirred at room temperature for 20 min. The precipitated dicyclohexylurea was filtered off and the anhydride solution was immediately added to the resin-bound template.
8. As determined by the Kaiser test: Kaiser, E.; Colescott, R.L.; Bossinger, C.D.; Cook, P.I. *Anal. Biochem.* **1970**, *34*, 595-598.
9. Waters 616 HPLC system equipped with a Waters 486 detector. Peptide samples were analysed on a Vydac C-18 reverse phase analytical column (4.6mm x 250mm) and purified on an Aquapore RP-300 semi-preparative column (7mm x 250mm). Samples for semi-preparative HPLC were filtered (0.45μm membranes) prior to purification. Peptides were eluted with gradients composed of 0.1% TFA in H_2O (eluent A) and 0.1% TFA in CH_3CN (eluent B). Elution was at a flow rate of 1ml/min and 2.5ml/min for the analytical and the semi-preparative columns respectively. The eluent from the column was monitored at 220 nm.
10. Capillary zone electrophoresis (CZE): analysis carried out on a Beckman P/ACE 5000 series instrument using a 50cm (75μmID) eCAP capillary. The separation was driven by a 15kV potential and a pH 8.35 buffer (0.4% boric acid , 0.3% sodium borate) and the eluent was monitored at 214nm.
11. Recorded on a VG Tofspec with Matrix Assisted Laser Desorption Time of Flight Instrument (MALDI-TOF) mass spectrometer using a matrix of α-cyanocinnamic acid. An error of ± 1 in 1000 units is quoted by the manufacturer.