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Synthesis of a Template-associated Peptide Designed as a Transmembrane Ion Channel Former

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Abstract: We describe the design and the Fmoc/*t*Bu solid phase synthesis of a 20 residue long peptide containing five regularly distributed lysines. Cyclization of this peptide was achieved using BOP as coupling agent. After side-chain deprotection, all the basic residues were iodoacetylated and then allowed to react either with a *C*-terminal free COOH peptide or with peptides bearing a cysteamide group. The final pentameric templates were identified by mass and amino acid analysis which gave data compatible with the expected values. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: template assembled; SPPS; cyclic peptide; pentameric association

INTRODUCTION

Recently, we have shown that a primary amphipathic peptide resulting from the association of a signal peptide with a nuclear localization sequence (SP-NLS) can act as carrier for intracellular delivery of covalently linked drugs [1,2]. The sequence of the vector peptide is: Ac-M-G-L-G-L-H-L-L-V-L^{10}-A-A-A-L-Q-G-A-K-K-K^{20}-R-K-V-Cya, where Ac is an acetyl *N*-terminal blocking group and Cya a *C*-terminal cysteamide function which allows post-synthesis modifications into conjugates. However, this peptide was also cytotoxic when used at concentrations above 5×10^{-6} M [3]. This toxicity was later attributed to the peptide being able to induce formation of ionic channels when incorporated into either *Xenopus laevis* membranes or into planar lipid bilayers [4]. Although the channels formed appear to be cation selective, several types of single channel conductances could be detected suggesting that various degrees of association may occur, leading to channels of different sizes and therefore probably to various absolute selectivities. Furthermore, this peptide was shown to adopt either a non-ordered structure when dissolved in water or a β-type or α-helical conformation when in lipid containing media depending on the 'lipid/peptide' ratio [5]. The purpose of the work described here was to build a peptide containing a well-defined number of monomers in order to specify the type of aggregates engaged in the channel structure.

The approach we used has been based on previous work reported principally by Mutter and colleagues called template-assembled synthetic proteins (TASPs) and which was applied to several channel-forming peptides [6–10]. In this strategy, the functional peptide is covalently linked to the template which can be a true cyclic peptide or a 'pseudo' cyclic one which is locked through a disulfide bridge. In addition, the linkage between the

Abbreviations: AEDI, aminoethyldithio 2-isobutyric acid; Boc, *t*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; *t*Bu, *t*-butyl; Cya, cysteamine; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GAE, glycolamide ester linker; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo(4,5b)pyridin-1-ylmethylene]-*N*-methyl methanaminium-hexafluorophosphate *N*-oxide; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; IMC, *N*-acyl imidazolidine-2-one linker; NEM, 4-ethylmorpholine; Pbf, 2,2,4,6,7 pentamethyldihydrobenzofuran-5-sulfonyl; TCEP, tris (2-carboxyethyl) phosphine; TFA, trifluoroacetic acid; Trt, triphenylmethyl.

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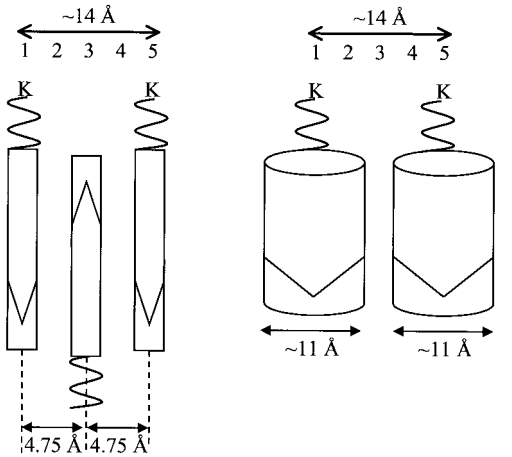

Figure 1 Schematic representation of the positioning of the peptides chains when linked to the cyclic template. Left panel: antiparallel β-type association. Right panel: association of α-helices.

template and the peptide occurs through the use of lysine side chains which are strategically distributed through the template. However, for the TASPs reported so far, the successive lysine residues facing the same side of the template are separated by only one residue. Therefore, the maximum distance between two successive lysines is at maximum of 7.2 (which appears too short for accommodating α-helices with bulky side chains and this in such a way that the template itself cannot participate in the channel structure. Our work describes the design and synthesis of a template which can easily accommodate either antiparallel β-structure or α-helices in a parallel arrangement.

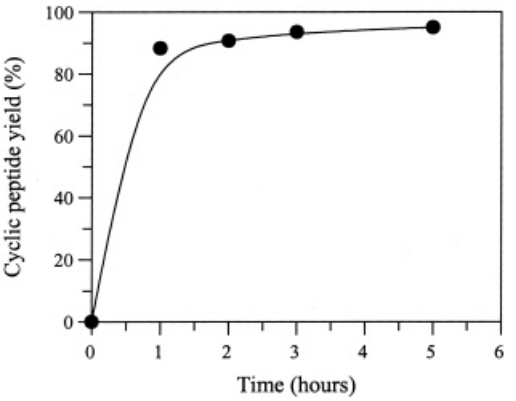

Figure 2 Time course of the cyclization of peptide **1**.

DESIGN OF THE TEMPLATE

According to the possible formation of a β-type structure and owing to the fact that the SP-NLS peptide leads to channel formation, we first hypothesized that the channel could be similar to that formed by porins and we assumed the presence of ten antiparallel peptide strands. Owing to the antiparallel positioning of the peptide chains, it must contain five functional groups where the peptide forming the barrel will be covalently linked. These functional groups must be separated by at least 4.75×2 Å where 4.75 Å corresponds to the interchain

Table 1 Chemical Sequences of All the Peptides Described in this Work

H_2N-G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A-OH
1

G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A
2

G-A-K-A-P-G-K-A-G-A-K-A-P-G-K-A-G-A-K-A
3

G-A-K(IAc)-A-P-G-K(IAc)-A-G-A-K(IAc)-A-P-G-K(IAc)-A-G-A-K(IAc)-A
4

Ac-M-G-L-G-L-W-L-L-V-L[10]-A-A-A-L-Q-G-A-K-K-K[20]-R-K-V-Cya
5

H_2N-W-K-K-K-R-K-V-Cya
6

(Boc)W-K(Boc)-K(Boc)-K(Boc)-R(Pbf)-K(Boc)-V-OH
7

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distance of peptides assembled in a β-structure. A distance of 9.5 Å is too long to accommodate a dipeptide unit for the template. Therefore, taking into account the fact that two successive peptides linked to the template must point toward the same direction, the template must be based on a tetrapeptide unit, i.e. functional side chains in positions i and $i+4$. In the extended conformation, two successive functional side chains of the template will thus be separated by about 14 Å. Besides the fact that such a distance can accommodate an antiparallel β-structure as schematically illustrated on Figure 1 (left panel), such a distance has also the advantage of being appropriate for α-helix formation. However, for steric reasons, only a parallel arrangement of the α-helices can be considered (see right panel of Figure 1). In addition to the above mentioned requirements, to favour the cyclization procedure, the other residues of the template were selected on the basis of their ability to induce flexibility and/or turns in the peptide chain as found for Pro or Gly containing peptides or also by the use of D-residues.

Taking into account all the above requirements, we synthesized a cyclic template with the following sequence:

G^1 A K A P G K A G A^{10} K A P G K A G A K A^{20}

Expecting to improve the cyclization yield we also synthesized an analogue where Ala^2 was replaced by a residue of the opposite configuration.

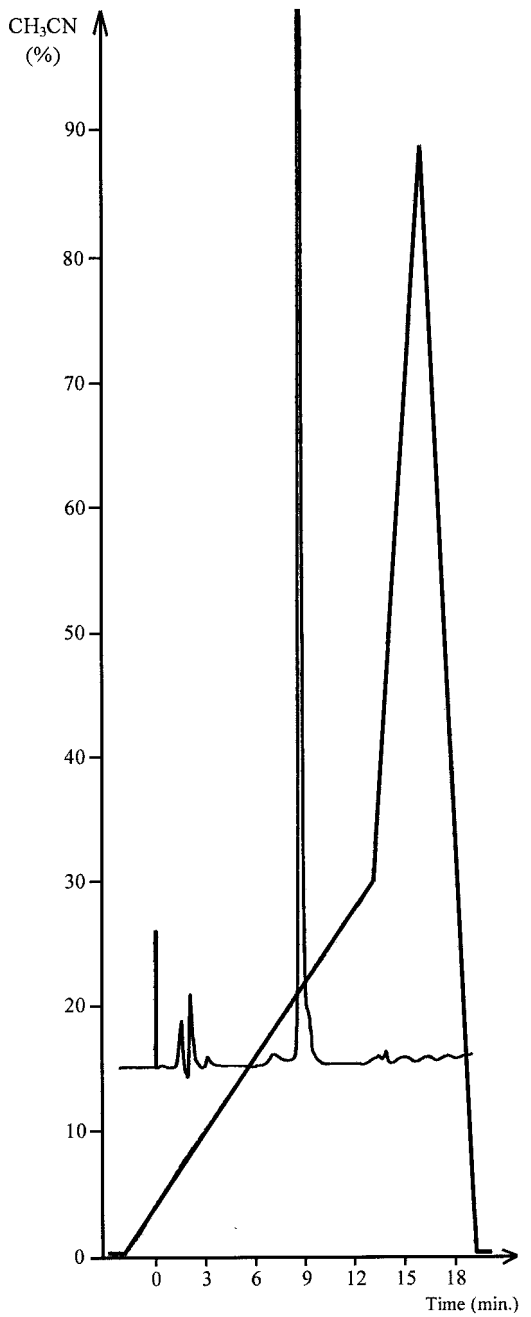

Figure 3 Reversed-phase HPLC analysis of purified deprotected cyclic peptide **3** with a linear elution gradient of CH_3CN/TFA (0.05%) in H_2O/TFA (0.1%) at a flow rate of 1.5 ml/min. The percentage of CH_3CN is shown by the straight lines. Detection was performed at 220 nm.

EXPERIMENTAL SECTION

Materials and Methods

Purex grade DMF stored over 4 Å molecular sieves, HPLC grade acetonitrile, peptide synthesis grade piperidine and pure TFA were purchased from SDS (Peypin, France). Iodoacetic acid, DCC and 4-ethylmorpholine puriss reagents were purchased from Fluka AG (Buchs-Switzerland).

BOP, HBTU, HOBt and Fmoc-Arg(Pbf)-OH were obtained from Senn Chemicals (Gentilly, France). Other Fmoc-amino acids and DMF peptide synthesis grade were obtained from PerSeptive Biosystem (Voisins-le-Bretonneux, France). The following protecting groups were used for side-chain protection: Boc, Trt and Pbf for Lys, Gln and Arg, respectively.

H_2N-AEDI-Expansin (0.547 mmol/g) was prepared as previously described [11]. H_2N-Val-GAE-Expansin (0.581 mmol Val/g) was obtained as in reference [12]. H_2N-Ala-IMC-Expansin (0.250 mmol Ala/g) was prepared according to reference [13].

Analytical Procedures

Analytical HPLC was performed on Waters equipment (Milford, MA, USA) with an Aquapore RP 300

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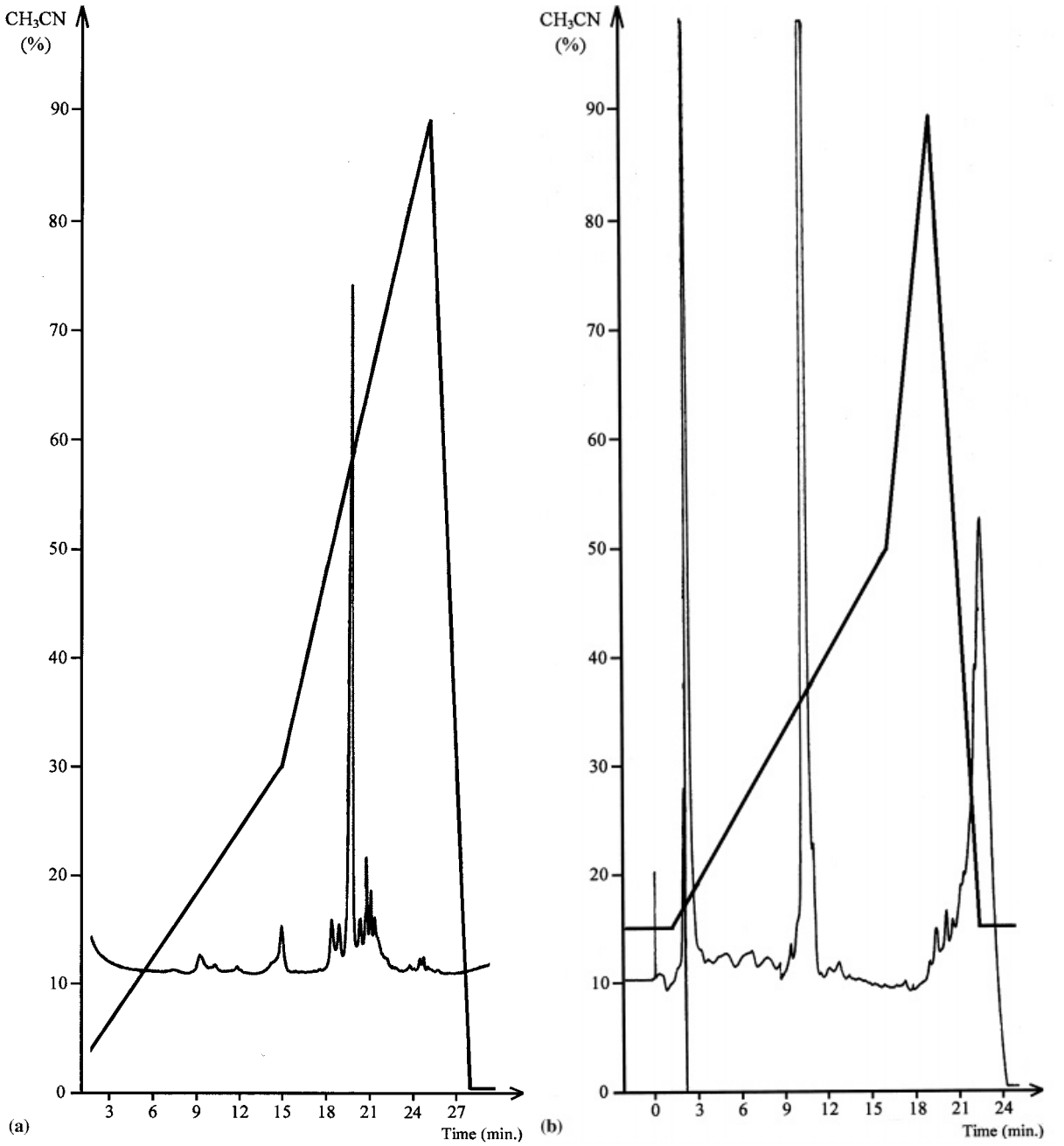

Figure 4 Reversed-phase HPLC analysis of crude (a) and purified (b) peptide **4**. Same conditions as in Figure 3.

column, C8, 7 μm, 220 × 4.6 mm (Brownlee Lab., Applied Biosystems, San José, CA, USA) and semi-preparative HPLC was performed with a Nucleosil 300, C8, 5 μm column, 200 × 20 mm, SFCC (Neuilly-Plaisance, France). The amino acid composition of peptide hydrolysates was determined on a High Performance Analyzer (Model 7300, Beckman Instruments, Fullerton, CA, USA). Electrospray mass analysis was carried out in the positive ion mode using a Trio 2000 VG Biotech Mass spectrometer (Altringham, UK). FAB mass analyses were carried out in the positive ion mode using a double focusing mass spectrometer ZAB-2E (VG Analytical, Manchester, UK). Samples were dissolved in glycerol/thioglycerol containing 1% trichloroacetic acid. Circular dichroism spectra were recorded on a Mark

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V dichrograph (Jobin-Yvon, Paris) using 1 mm thick quartz cells.

General Protocol for Peptide Synthesis

Peptide syntheses were performed with a 9050 pep-synthetizer Milligen (PerSeptive Biosystems) using the Fmoc/*t*Bu system according to manufacturer-specified protocols. Fmoc deprotections were achieved by percolating 20% piperidine in DMF through the reaction column (4.5 ml/min for 4 min). Fmoc amino acids (4 equivalents) were activated by the addition of equimolar amounts of HATU and HOAt diluted to 0.27 M with 6.8% NEM in DMF. Coupling was obtained by recycling this solution through the reaction column for a standard 60 min. Double couplings (30–60 min) were performed at crucial steps according to our own experience, where a single coupling leads to large yield loses.

Synthesis of the Peptidic Template

The protected linear precursor **1** (see Table 1 for all sequences) H_2N-G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A-P-G-K(Boc)-A-G-A-K(Boc)-A-OH was synthesized from H_2N-Ala-IMC-Expansin (0.7 g) according to the general protocol. After *N*-terminal deprotection, the side-chain protected peptidyl resin was washed three times with EtOH, DCM, DMF, Et_2O, MeOH and isopropanol/H_2O (70/30). Peptide release was obtained by calcium promoted hydrolysis of the IMC handle [13] with 15 ml of isopropanol/H_2O (70/30), 0.8 M $CaCl_2$ containing 3.4 equivalents NaOH for 5 h leading to a yield of 99%. The crude peptide was thoroughly purified by semi-preparative reverse-phase HPLC (0.01% TFA/CH_3CN, 9 ml/min). The resulting purified peptide fractions were neutralized with 3 M NaOH, evaporated to remove CH_3CN and then freeze-dried (150 mg). The FAB(+) mass spectrum of **1** was in accordance with the expected structure (MH^+ at $m/z = 2206$; theoretical = 2206).

Table 2 Amino Acid Analysis of the Peptide Resulting from the Coupling of **3** and **7**

Amino acid	Theoretical number of residues	Experimental number of residues
Pro	2	2×1.00
Ala	8	8×1.08
Lys	25	25×0.99
Gly	5	5×1.09
Val	5	5×0.93
Arg	5	5×1.02

Cyclization and Side-chain Deprotection of Peptide 1

Peptide **1** (120 mg, 70 μmol) was allowed to react overnight with BOP (93 mg, 210 μmol), HOBt (105 mg, 700 μmol) and NEM (0.85 ml, 700 μmol) in 1150 ml of peptide synthesis grade DMF. The time course of peptide **1** cyclization is shown in Figure 2. After a 5 h reaction, the cyclization yield was better than 95%, the remainder being essentially composed of the dimethylamide derivative. The FAB(+) mass spectrum of peptide **2** corresponded to the expected value (MH^+ at $m/z = 2188$; theoretical = 2188). The crude peptide was purified and collected as described above. Deprotection of **2** was achieved in 10 ml of TFA for 90 min. TFA was removed by evaporation under vacuum and the deprotected cyclic peptide **3** was dissolved in 5 ml of 0.01 M HCl and filtered through a Sephadex G10 column (113 × 2 cm, 50 ml/min, 0.01 M HCl). Fractions corresponding to peptide **3** were collected and freeze-dried (61 mg). The elution profile of purified **3** is shown in Figure 3 and its electrospray mass spectrum corresponded to the expected value ($M_{exp} = 1688$; theoretical = 1688.9).

Iodoacetylation of 3

Iodoacetic anhydride was prepared from 103.2 mg (0.556 mmol) of iodoacetic acid and 57.1 mg (0.278 mmol) DCC which were dissolved in 1.8 ml DCM. After 30 min at 4°C dicyclohexylurea was removed by filtration and the filtrate evaporated under vacuum and taken up in 2.4 ml DMF.

A total of 25.3 mg of **3** (0.015 or 0.075 mmol of free NH_2) was dissolved in 3 ml DMF containing 26 μl DIEA (0.15 mmol; i.e. 2 equivalents) and 1.3 ml of the above iodoacetic anhydride solution (0.15 mmol) was then added. After 20 h, the solvent was removed and the crude pentaiodoacetylated peptide **3** purified by semi-preparative reverse-phase HPLC (0.01% TFA/CH_3CN, 9 ml/min). The elution profile of the purified compound **4** (13 mg, yield: 30%) is shown in Figure 4 and its FAB(+) mass spectrum corresponded to the expected value (MH^+ at $m/z = 2528$; theoretical = 1738).

Syntheses of the Linear Peptides

Peptides **5** (Ac-M-G-L-G-L-W-L-L-V-L^{10}-A-A-A-L-Q-G-A-K-K-K^{20}-R-K-V-Cya) and **6** (H_2N-W-K-K-K-R-K-V-Cya) were synthesized from H_2N-AEDI-Expansin (0.547 mmol AEDI/g), side-chain deprotected,

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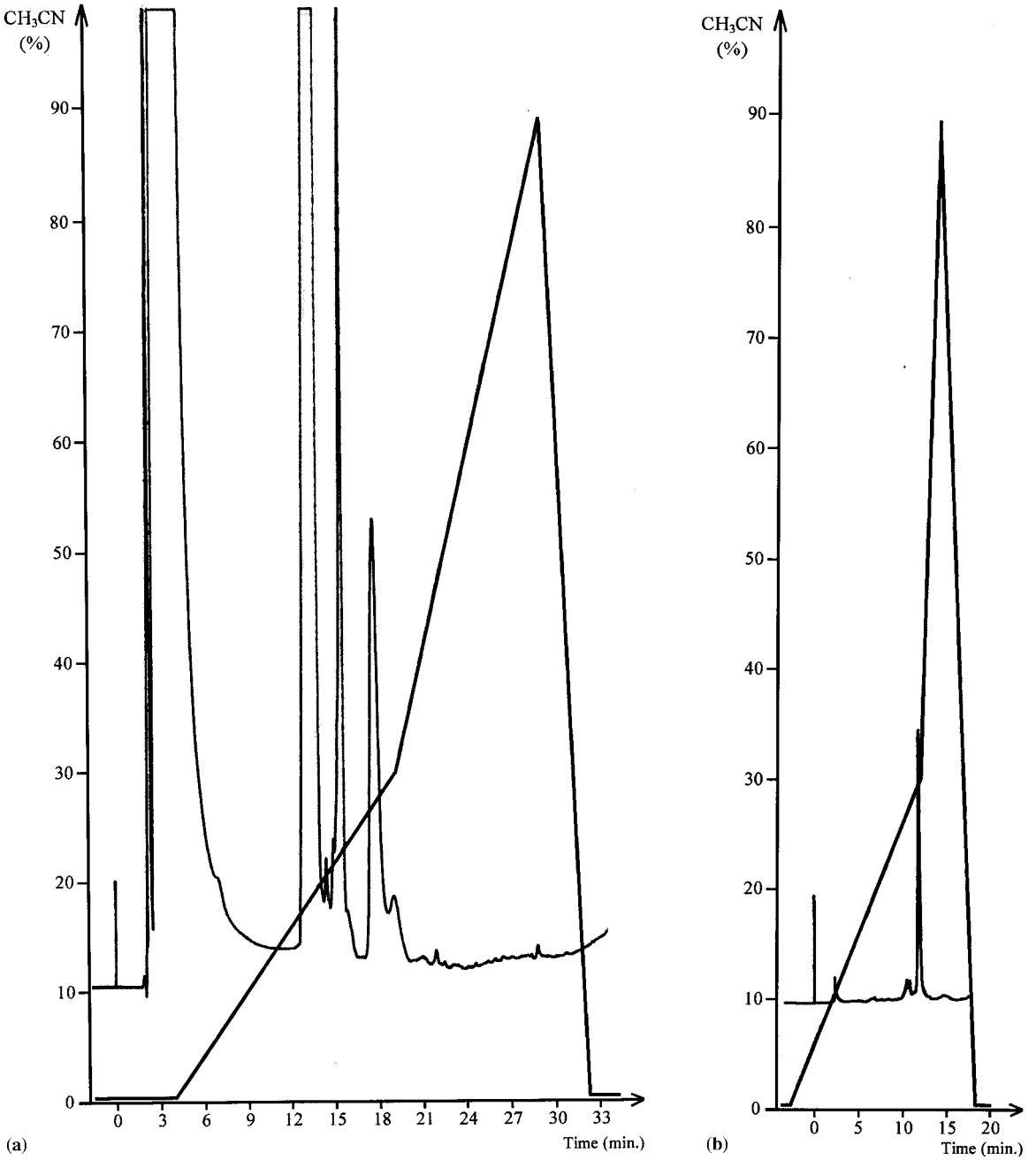

Figure 5 Reversed-phase HPLC analysis of crude (a) and purified (b) template **4** + **6**. Same experimental conditions as in Figure 3 except detection which was performed at 290 nm.

released from the resin and purified as described previously [14].

Electrospray mass spectra of **5** and **6** were as expected ($M_{exp} = 2566.9$ and 1031.4; $M_{theor} = 2566.4$ and 1031.5, respectively).

Peptide **7** {(Boc)W-K(Boc)-K(Boc)-K(Boc)-R(Pbf)-K(Boc)-V-OH} was synthesized from H_2N-Val-GAE-Expansin according to the general protocol. After stepwise synthesis, the protected peptidyl resin was washed three times with EtOH, DCM, Et_2O, MeOH and isopropanol/H_2O (70/30). Peptide release (96%) was obtained by saponification for 16 h of the protected peptidyl resin with 15 ml of isopropanol/H_2O (70/30) containing 4 equivalents NaOH per peptide equivalent. The crude peptide was then purified by SiO_2 column chromatography using DCM/MeOH

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(85/15) as eluent. The resulting purified peptide (359 mg, 0.207 mmol) has the expected FAB(+) mass spectrum (MH^+ at $m/z = 1739$).

Synthesis of the Template-associated Peptides

Amide Bond Linkage: 3 + 7. A total of 4.6 mg of the cyclic peptide **3** (2.72 μmol) was reacted with 36.5 mg of **7** (21 μmol, 7.7 equivalents) in the presence of 3.6 mg of HOAt (26.4 μmol, 9.7 equivalents), 8.0 mg of HATU (21 μmole, 7.7 equivalents) and 6 μl NEM (48 μmol, 17.5 equivalents) in 0.1 ml DMF. After 24 h, 20 μl NEM was added and the reaction was allowed to continue for a further 24 h. The reaction mixture was then evaporated under vacuum and deprotected with 2 ml of TFA containing 90 μl ethanedithiol, 60 μl thioanisole, 30 μl trisisopropylsilane and 20 μl H_2O for 2.5 h. TFA was removed by evaporation under vacuum and the residue was washed twice with diethylether and redissolved in 4 ml water. The crude deprotected mixture was purified by semi-preparative reverse-phase HPLC (0.1% TFA/CH_3CN, 9ml/min). Its amino acid analysis was in very good agreement with that expected (Table 2). Unfortunately, probably due to the large number of lysine residues, electrospray mass analysis led to a very broad spectrum which was unable to be interpreted.

Thioether Linkage

4 + 6. A total of 2.5 mg of the cyclic pentaiodoacetylated peptide **4** (1 or 5 μmol of functional ICH_2CO-) and 10.3 mg of peptide **6** (10 μmol, 2 equivalents) were reacted in 40 μl DMF and 100 μl of sodium phosphate buffer (0.02 M, pH 7.5) for 16 h with stirring after sonication under nitrogen to improve homogeneity. The HPLC profile of the reaction mixture showed three major peaks (Figure 5). Peak 1 corresponds to the initial peptide **6** and peak 2 to its corresponding disulfide form. Indeed, after addition of TCEP (20 μmol for 30 min) to the reaction mixture peak 2 was reduced entirely into peak 1. Peak 3 was purified under analytical conditions, evaporated under vacuum, taken up in 1 ml H_2O and freeze-dried (0.27 mg, 0.039 μmole, yield 3.9%). Its amino acid analysis was in accordance with that expected (Table 3) but again, electrospray mass analysis led to a non-interpretable spectrum.

Table 3 Amino Acid Analysis of the Peptide Resulting from the Coupling of **4** and **6**

Amino acid	Theoretical number of residues	Experimental number of residues
Pro	2	2 × 0.96
Ala	8	8 × 1.04
Lys	25	25 × 0.93
Gly	5	5 × 1.18
Val	5	5 × 0.90
Arg	5	5 × 0.93

4 + 5. A total of 6.5 mg of peptide **4** (2.6 or 13 μmol of functional ICH_2CO-) and 76 mg of peptide **5** (24 μmol, 1.9 equivalents) were treated as above in 0.6 ml DMF and 1 ml sodium phosphate buffer. After 30 min, the solution became very viscous and thus 1 ml of DMF with 1.8 ml buffer were added to the medium. After 18 h stirring, the mixture was acidified to pH 4 with 1 M HCl. As above, the HPLC profile of the reaction mixture showed three major peaks (Figure 6) and peak 3 corresponded to the expected conjugate. Although peak 3 led to a broad semi-preparative HPLC peak, its amino acid composition agreed with that expected (Table 4) and MALDI-TOF mass spectra (Figure 7) confirmed that the pentameric compound with a mass of 14733 Da (theoretical = 14723.5) was the major product.

RESULTS AND DISCUSSION

This paper describes the most effective procedure reported thus far for producing a template associated peptide, but several observations concerning mainly the cyclic template and its linkage to the linear peptide, need to be addressed.

Cyclic Template

Several attempts to obtain the 20 residue cyclic peptide were tried. One, related to the introduction of a D-alanine in position 3 in order to favour a chain reversal structure but we did not notice any improvement of the cyclization yield. On the contrary the yield was 30% lower than that obtained for the all-L peptide. On the second attempt, although maintaining the overall sequence, we changed the linear precursor into AKAGAKAPGKAGAKAPGKAG to have a non-chiral *C*-terminal residue avoiding thus any racemization during cyclization. However, for un known reasons, the corresponding linear peptide was poorly soluble and therefore difficult to handle. The cyclization procedure led to the same cyclic peptide as that described in the current experimental procedure but with a lower yield and the peptide recovery was less efficient.

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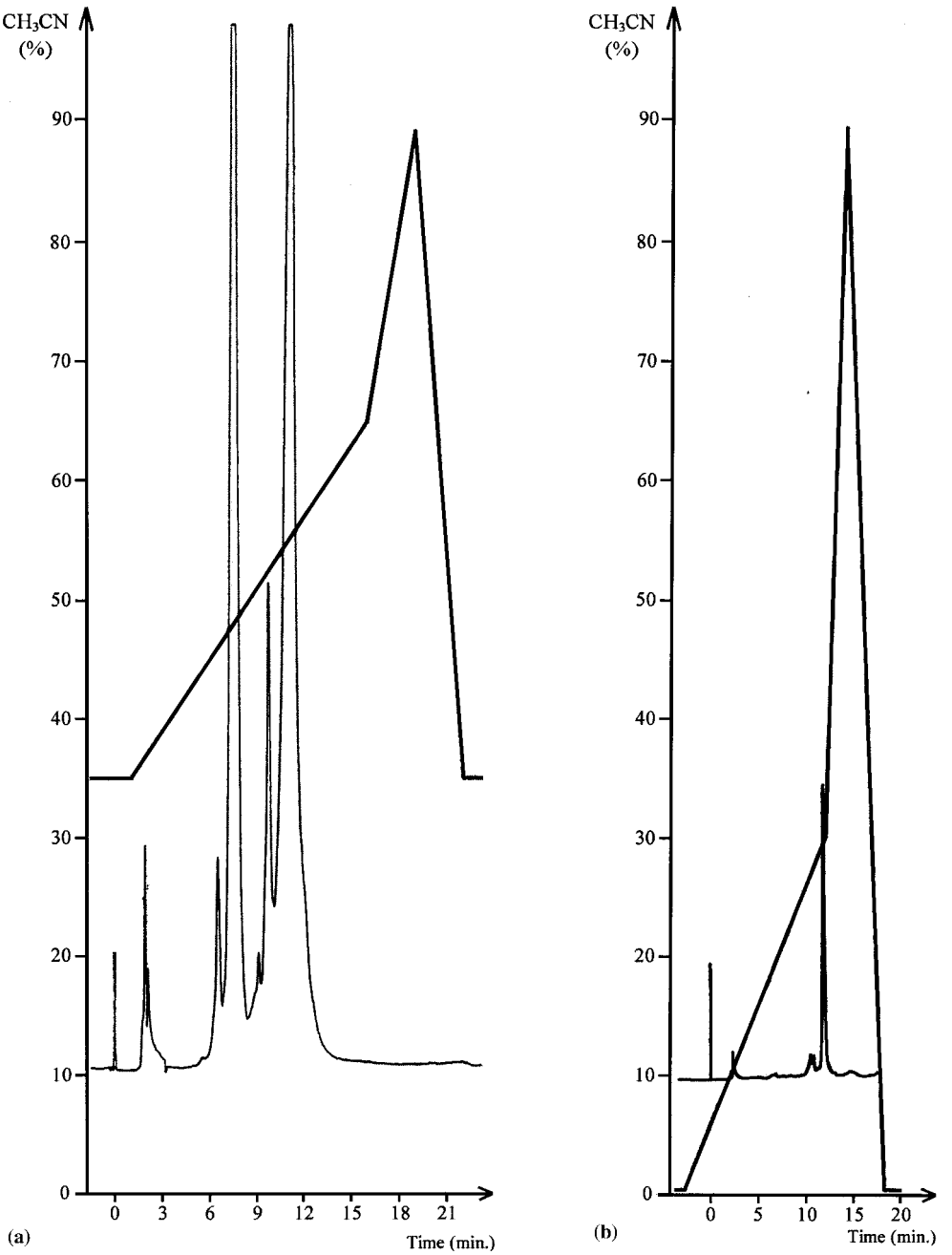

Figure 6 Reversed-phase HPLC analysis of crude (a) and purified (b) template **4** + **5**. Same experimental conditions as in Figure 5.

In addition to the choice of the sequence, we encountered some difficulties at the cyclization step resulting from the choice of the coupling agent and the use of solid phase grade DMF. Indeed, the cyclization procedure requires a very low peptide concentration (10^{-4}) and the use of this type of DMF in association with HATU/HOBt as COOH activator leads to the quantitative formation of the *C*-terminal dimethylamine and *N*-terminal tetramethylguanidine derivative. The use of BOP instead of HATU inhibits the guanidylation but not the amidation process. Only the use of BOP activation in freshly prepared and highly purified DMF (without any trace of dimethylamine as determined by the

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Table 4 Amino Acid Analysis of the Peptide Resulting from the Coupling of **4** and **5**

Amino acid	Theoretical number of residues	Experimental number of residues
Glu	5	5×0.71
Pro	2	2×0.87
Ala	28	28×1.03
Met	5	5×0.96
Lys	25	25×1.05
Trp	5	ND
Gly	20	20×1.06
Val	10	10×0.81
Leu	30	30×0.96
Arg	5	5×1.06

ND, not determined.

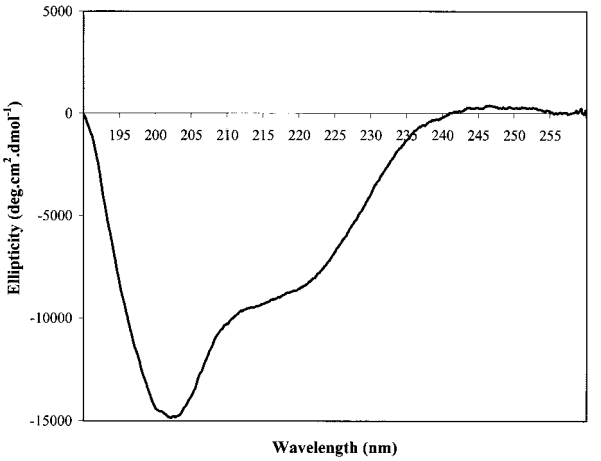

Figure 8 Far UV-CD spectrum of **3** in water. Cell path 1 mm, $c = 0.1$ mg/ml.

bromophenol blue test) leads to a high yield of cyclization.

The last point we wish to address concerning the cyclic template is related to its conformational state. The CD spectra of the cyclic peptide when dissolved in water or in phosphate buffer is shown in Figure 8. It is characterized by a single minimum centered at 200 nm associated with a shoulder at about 225 nm, indicating that the cyclic peptide is rather non-structured in these solutions. This finding is in full agreement with previous observations made on a series of cyclic peptides containing $2n$ residues with $6 \leq n \leq 16$ [15]. In this series those with $2(2n + 2)$ residues are non-structured while the others contain a high amount of sheet structure. The peptide described here falls in the $2(2n + 2)$ series with $n = 4$ and is thus in line with the spectra already reported. The fact that the template is rather non-structured is of major importance for the further

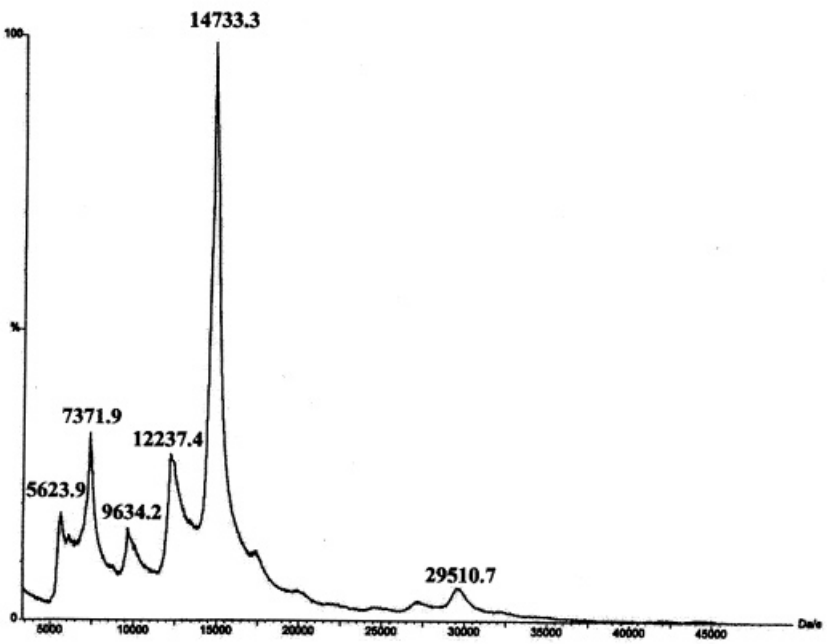

Figure 7 MALDI TOF mass spectrum of the final template molecule. The major peak at 14733 Da corresponds to that of the pentasubstituted cyclic peptide. Note the presence of small amounts of incompletely substituted cyclic peptide (mainly tetra and trisubstituted).

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binding of peptides, especially for α-helical peptides. Again, geometrical considerations indicate that a hydrogen bonding pattern such as that which occurs for 6-, 10-, 14- and 18-mers with an interstrand distance of 4.75 Å as dictated by the hydrogen bond length, cannot accommodate the linkage of α-helices and thus lead to the formation of deleted pentasubstituted templates.

Template Linkage of the Linear Peptides

We have described in a comparative manner the two possibilities of linkage between the cyclic template and linear peptides. The first one is based on binding through an amide bond involving the terminal COOH of the linear peptide and the ε-NH_2 of the template lysines while the second type involves a reaction between an iodoacetylated derivative and the *C*-terminal SH of the cysteamide group. In the case of a short peptide, the finding of very similar yields prompted us to use the latter linking procedure since we had at our disposal large amounts of the long peptide in the cysteamide form. Good yields of pentasubstituted template were obtained but owing to the presence of 25 charged residues (5 Arg and 20 Lys), its identification could not be achieved by electrospray mass analysis but rather required the use of a MALDI-TOF mass spectrometer.

It must be added that attempts to use a bromoacetylated derivative instead of a iodoacetylated one gave very similar results but was less easy to handle because of its lower solubility. Finally, it must be mentioned that the presence of the cysteamide group has also the advantage of introducing a calibrating probe for the amino acid analysis. Indeed, the acidic hydrolysis of the full template issued by linking through a thioether bond leads to formation of S-carboxymethylcysteamine which is stable and can be quantified by amino acid analysis. Its presence and amount thus allows a determination of the amount of linear peptide per cyclic one and also indicates that the linking occurs through a thioether bond and not through a bond involving the side chains, such as the lysine NH_2.

CONCLUSIONS

In conclusion, the synthesis described here confirms that the mercaptoamide strategy introduced in peptide chemistry is a useful technique especially when the peptides must be submitted to post-synthesis modifications. This *C*-terminal function was particularly efficient for the synthesis of a template-assembled synthetic protein built of a 20 residue cyclic peptide and five template-linked peptides. The high yields obtained at each step during the synthesis now allows further physico-chemical investigation involving conformational and pore formation studies.

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