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Solid Phase Synthesis of a Cyclic Peptide Derived from a Curaremimetic Toxin

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Abstract : We synthesized a 18 residues cyclic peptide corresponding to a loop involved in the curaremimetic action of a snake toxic protein. This peptide, which competes with the native toxin for the binding to the nicotinic acetylcholine receptor, includes the β-turn inducing Pro-Asn moiety. The peptide was prepared and cyclized on solid phase, starting from Boc-Asp-OFm linked to the methylbenzhydrylamine resin through the β-carboxylic function. Final treatment by HF converts this C-terminal Asp into Asn.

Curaremimetic proteins (60-74 amino acids) from snake venoms (*Elapidae* and *Hydrophidae*) bind specifically and with high affinity (K_d 10^{-9} to 10^{-11} M) to the nicotinic acetylcholine receptors (AchR) thus inducing flaccid paralysis. The three-dimensional structure of some toxins has been previously determined, revealing that the polypeptide chain is fold into three loops projecting from a core region. Sequence comparisons[1, 2], chemical modifications[1, 3] and mutagenesis studies[4] have been used to determine the residues involved in the toxin/receptor complex formation. Most of residues presently identified (Lys^{27}, Trp^{29}, Asp^{31}, Phe/His/Trp^{32}, Arg^{33}, Lys^{47}) are located mostly in the three-stranded β-sheet structure (Lys^{27}, Trp^{29}, Asp^{31}, Phe/His^{32}, Arg^{33}) on loop 2, and only Lys^{47} belongs to loop 3. During the past few years, a number of synthetic peptides corresponding to portions of curaremimetic toxins were described, either to study their interactions with AchR[5,6,7] or to generate toxin-specific neutralizing antibodies[8]. The role of the secondary and tertiary structures in the biological properties of synthetic loop 2 peptides is now currently addressed in several laboratories[7, 9]. In particular, it was shown that after cyclisation, the immunogenic property of the synthetic loop 2 of *Naja nigricollis* better mimicked those of the native loop 2 than the corresponding linear peptide[8]. Neverless, a recent NMR study revealed that the cyclic peptide possessed in solution a native-like β-turn, but not the expected β-sheet-like structure[9]. In this case, cyclisation was made by introducing a disulfide bridge between the first and the last amino acid positions (Cys^{24} and Cys^{41}).

In an attempt to further reduce the conformational space of the synthetic peptide and with the aim of better initiating the β-sheet structure that the loop 2 adopts in the toxin molecule, we have linked N to C-terminal ends of the selected sequence by the Pro-Asn moiety, which is known to induce β-turns (Pro and Asn present at positions i+1 and i+2 of β-turn, respectively)[10]. In order to respect the alternation and the orientation of potential hydrogen bonds as they exist in the native protein, Cys^{24} and Cys^{41} were replaced by Asn and Pro, respectively.

The selected peptide sequence was therefore :

c(-Asn-Tyr-Lys-Lys-Val-Trp-Arg-Asp-His-Arg-Gly-Thr-Ile-Ile-Glu-Arg-Gly-Pro-)

This paper describes the synthesis of this peptide using the solid phase cyclisation methodology[11-16]. Methods for C-terminal to N-terminal cyclisation by the solid phase strategy have been recently published[14-16]. In these cases[15, 16], linear precursor was assembled starting from the aspartic residue linked to PAM resin through the β-carboxylic function using Boc or Fmoc chemistry. In our solid phase cyclisation, the Asp C-terminal residue was attached to the MBHA resin and the cyclisation was achieved by formation of an amide bond between the C-terminal and the N-terminal residues. Release of the peptide was made using a treatment by HF and simultaneously, the original aspartic residue was converted into an asparagine residue[17]. To our knowledge, the present work is the first example of a solid-phase cyclisation involving the subsequent conversion of an Asp residue into an Asn residue.

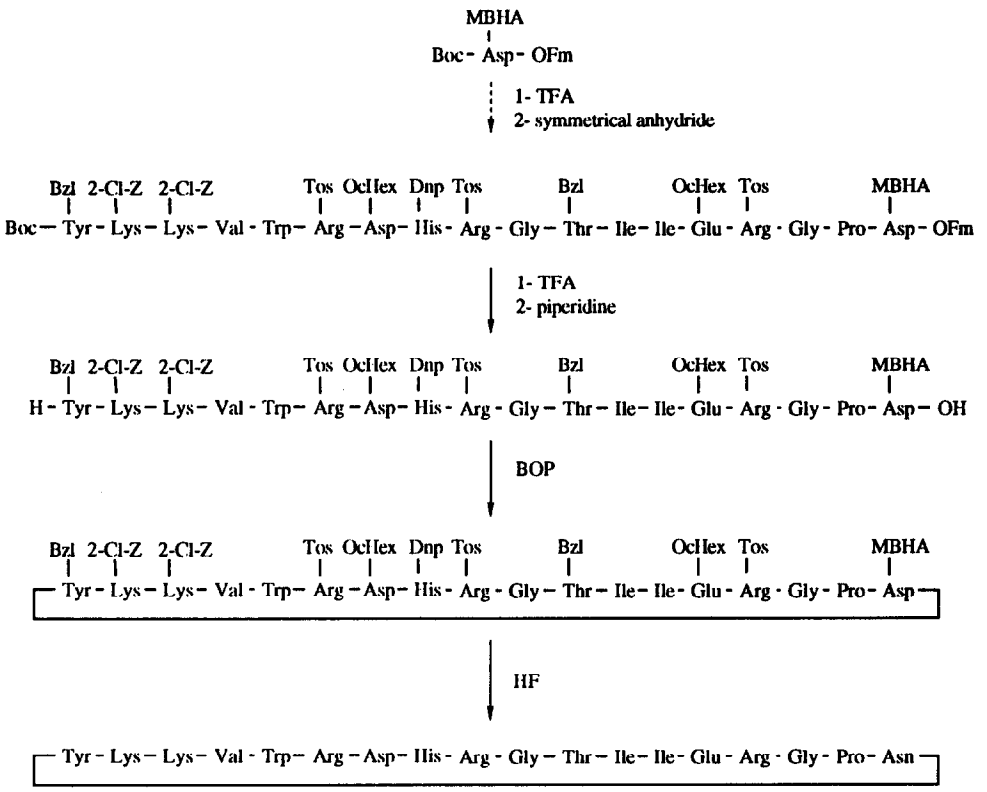
Synthesis was performed using an automatic synthetiser (Applied Biosystems 430 A) starting with 1 g Boc-Asp(β-MBHA resin)-OFm. In order to minimize interchain side-reactions during the cyclisation step, the resin substitution level was only 0.4 mmol/g. The starting compound Boc-Asp(β-MBHA resin)-OFm was

prepared from MBHA resin (Novabiochem, 0.5 mmol/g) and condensed with 0.8 equivalents of Boc-Asp-OFm. The remaining amino group were capped by acetylation.

Chain elongation was carried out according to the classical Boc/benzyl strategy by consecutive addition of the following amino acids as symmetrical anhydride or HOBt esters (His, Glu) :
Boc-Pro-OH, Boc-Gly-OH, Boc-Arg(Tos)-OH, Boc-Glu(OcHex)-OH, Boc-Ile-OH, Boc-Ile-OH, Boc-Thr(Bzl)-OH, Boc-Gly-OH, Boc-Arg(Tos)-OH, Boc-His(Dnp)-OH, Boc-Asp(OcHex)-OH, Boc-Arg(Tos)-OH, Boc-Trp(CHO)-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Tyr(2,6-di ClBzl)-OH.[18]

Removal of the protecting groups was achieved, for Dnp, by treatment with thiophenol, for Boc, with TFA 50% in dichloromethane, for CHO and OFm simultaneously with 20% piperidine in DMF for 2 h at 0°C.

After the appropriate washing step, cyclisation was performed on the resin by treatment with 3 equivalents BOP[19] in NMP containing 6 equivalents DIEA for 2 h at room temperature. The Kaiser test indicated 20% and 0% free amino group after the first and second BOP treatment, respectively.



Side-chain deprotection and cleavage of cyclic peptide from the resin was achieved with HF/m-cresol/ethanedithiol (10/1/0.5) for 1 h at 0°C. The crude product was extracted in aqueous acetic acid (30%) and the resultant solution lyophilized. Analytical RP-HPLC of the peptide on a Vydac C18 column eluted with a linear gradient from 0 to 100% B over 45 min (A : 0.1% TFA H_2O ; B : CH_3CN/H_2O (60/40), 0.1% TFA, flow rate 0.75 ml/min) showed a main peak (retention time 20 min) corresponding to the peptide confirmed by analytical data. Purification was carried out by RP-HPLC on a Vydac C18 preparative column eluted at 9ml/min with the same gradient. Analytical RP-HPLC indicated > 97% purity. Overall yield 10%.

Electrospray MS : 2206.7 (M = 2207.8)
Amino acid analysis : Asp 1.98 (2), Glu 1.2 (1), Gly 2.3 (2), His 0.7 (1), Arg 3.2 (3), Thr 0.9 (1), Pro 1.1 (1), Tyr 0.9 (1), Val 0.7 (1), Ile 1.6 (2), Lys 1.7 (2).

Preliminary experiments revealed that the peptide inhibited the binding of the *alpha*-peroxydase labelled toxin α from *Naja nigricollis* to AchR from the electric organ of *Torpedo marmoratta,* using the previously described methodology[8]. The concentration of peptide that produced 50% inhibition (IC_{50}) was similar to that obtain with nicotine and higher than that of decamethonium.

COMPOUND	SEQUENCE	IC_{50} (mM)
Nicotine		0.2 +/- 0.1
Decamethonium		0.06 +/- 0.03
c(-Asn-α-toxin(25-40)-Pro-)	c(NYKKVWRDHRGTIIERGP)	0.2 +/ 0.1

In conclusion, we have prepared a 18 residues cyclic peptide (N- to C- terminal) including the β-turn-inducing dipeptide Pro-Asn. The residue Asn was introduced as Asp, linked to MBHA resin by its side chain, and liberated as Asn upon treatment by HF. This efficient procedure allows the solid phase synthesis of any cyclic peptides, and is generally applicable to introduce an Asn, or a Gln, at a desired position.

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References and Notes

1. Endo, T. ; Tamiya, N. *Pharm. Ther.* **1987**, *34*, 403-451.
2. Karlson, E. Snake Venoms, in *Handbook of Experimental Pharmacology*, Vol. 52 ; C.Y. Lee Eds. ; Springer Verlag : Berlin, 1979 ; pp. 159-212.
3. Tu, A.T. ; Miller, R.A. *Mem. Inst. Butantan*, **1989**, *51*, 177-193.
4. Pillet, L. *Ingénierie génétique et propriétés immunologiques d'une protéine toxique,* Université Paris XI 1991.
5. Mc Daniel, C.S. ; Manshouri ; T. ; Atassi, M. Z. *J. Prot. Chem.* **1987**, *6*, 455-461.
6. Juillerat, M.A. ; Schwendimann, B. ; Hauert, J. ; Fulpius, B.W. ; Bargetzi, J. P. *J. Mol. Biol.* **1982**, *257*, 2901-2907.
7. Lentz, T.L. *Biochem.* **1991**, *30*, 10949-10956.
8. Léonetti, M. ; Pillet, L. ; Maillère, B. ; Lamthan, H. ; Frachon, P. ; Couderc, J. ; Menez, A. *J. Immunol.* **1990**, *145*, 4214-4221.
9. Cuniasse, Ph. ; Thomas, A. ; Smith, J. ; Gilquin, B. ; Lamthan, H. ; Menez, A. ; F. Toma, submitted for publication.
10. Willmot , C.M. ; Thornton, J.M. *J. Mol. Biol.* **1988**, *203*, 221-232.
11. Schiller, P.W. ; T.M-D. Nguyen ; Miller, J. *Int. J. Peptide Protein Res.* **1985**, *25*, 171-177.
12. Felix, A.M. ; Wang, C-T.; Heimer, E.P. ; Fournier, A. *Int. J. Peptide Protein Res.* **1988**, *31*, 231-238.
13. Plaue, S. *Int. J. Peptide Protein Res.* **1990**, *35*, 510-517.

14. Ösapay, G. ; Profit, A. ; Taylor, J. W. *Tetrahedron Lett.* **1990**, *43*, 6121-6124.
15. Rovero, P. ; Quartara, L. ; Fabbri, G. *Tetrahedron Lett.* **1991**, *32*, 2639-2642.
16. McMurray, J.S. *Tetrahedron Lett.* **1991**, *32*, 7679-7682.
17. Shao, J.S. ; Li, Y-H ; Volter, W. *Int. J. Peptide Protein Res.*, **1990**, *36*, 182-187.
18. Abbreviations : Boc, *ter*-butyloxycarbonyl ; BOP, benzotriazoloxy-tri-(dimethylamino)phosphonium hexafluorophosphate ; Bzl, benzyl ; 2-ClZ, 2-chlorobenzyloxy ; CH_3CN, acetonitrile ; cHex, cyclohexyl ; DCCI, dicyclohexylcarbodiimide ; DMF, dimethylformamide ; 2,6-di ClBzl, 2,6-dichlorobenzyl ; DIEA diisopropylethylamine ; Dnp, dinitrophenyl ; Fmoc, 9-fluorenylmethoxycarbonyl ; HOBt, 1-hydroxybenzotriazole ; MBHA, methylbenzhydrylamine ; NMP, N-methylpyrrolidone ; OFm, fluorenylmethyloxy ; RP-HPLC, reverse phase high performance liquid chromatography ; TFA, trifluoroacetic acid ; Tos, tosyl.
19. Castro, B. ; Dormoy, J.R. ; Evin, G. ; Selve, C. *Tetrahedron Lett.* **1975**, *14*, 1219-1222.

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