CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS VI : SYNTHESIS BY THE FMOC PROCEDURE WITH A MODIFIED PROTOCOL OF TWO PROTECTED SEGMENTS, SEQUENCE 5-17 AND 18-31 OF THE NEUROTOXIN II OF THE SCORPION ANDROCTONUS AUSTRALIS HECTOR.

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Abstract- Synthesis of two protected peptides thirteen and fourteen residues long, sequence 5-17, i.e. Fmoc-Tyr(cHex)-Ile-Val-Asp(Bzl)-Asp(Bzl)-Val-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-Gly-OH, and 18-31, i.e. Fmoc-Arg(Tos)-Asn-Ala-Tyr(cHex)-Cys(Acm)-Asn-Glu(Bz1)-Glu(Bz1)-Cys(Acm)-Thr(Bz1)-Lys(Z)-Leu-Lys(Z)-Gly-OH, of the scorpion neurotoxin II from Androctonus australis Hector, was performed by the solid phase method. The hydroxymethylphenoxymethyl copoly(styrene -1% -divinylbenzene) type resin was used in combination with Fmoc-amino acids for both syntheses. A general protocol minimizing side reactions has been developed for the use of the base labile Fmoc-a-amino protecting group. The time of reac-tion with piperidine (50% in N,N'-dimethylformamide) has been shortened to three times one minute and coupling was performed mainly in methylene chloride with just dicyclohexyl or diisopropyl-carbodiimide. The side chain protecting groups of the Fmoc-a-amino acids were of the hydrogen fluoride labile type, which permitted, after trifluoroacetic acid cleavage of the peptide to resin ester bond, obtainment of protected peptides. The crude segments, precipitated from N,N'-dime-thylacetamide with water, were highly purified by HPLC and chemically characterized for future use in convergent solid phase assembling.

Convergent solid phase stategy has been designed for synthesis of large peptides (1,2,3). It consists of solid phase synthesis of protected segments followed by purification and then solid phase assembling of these segments. For synthesis of the neurotoxin II of the scorpion <u>Androctonus australis Hector</u> (AaH II), hydrogen fluoride (HF) labile side chain protection has been chosen and two different procedures for solid phase synthesis of segments were retained. One is based on the use of a photosensitive linkage between the peptide and the resin which enables to perform the assembling of the peptide with the general protocol of Merrifield and the usual Boc-amino acids. Several segments have thus been obtained (4,5).

The other procedure that has been explored concerns the use of Fmoc-amino acids on a Wang type resin (6). This strategy looked very promising with a good orthogonality (7) between deprotection conditions and both side chain protection and resin linkage, the possibility of facile UV monitoring at the repetitive deprotection steps and the near complete yield of peptide cleavage by trifluoroacetic acid (TFA). In fact, the first segments (1) were obtained with rather great difficulties due mainly to some side reactions that were not studied till then. Three main problems in the use of Fmoc-amino acids have been pointed out in the course of our syntheses. The first problem concerned the purity of the starting Fmoc-amino acids that were obtained by reaction of Fmoc-chloride with a-carboxyl unprotected amino acids. This acylation agent induces formation of amino acid mixed anhydride which condenses to form di-and even tripeptides (8-11). A second problem appeared clearly during incorporation of the first Fmoc-amino acid residue on the Wang type resin. Esterification on this hydroxybenzyl is almost null with just dialkylcarbodiimide, the yield may increase to a high level by addition of 4dimethylaminopyridine (DMAP) but then unstability of the Fmoc group induces autocondensation and again formation of dipeptide up to 9% as described (1). A compromise was found (12,6) by use of amino acid anhydride (13) and by diminishing the ratio of DMAP, which still allowed a yield of about 63% of esterification of the hydroxyl groups and an acceptable amount of dipeptide (less than 0.7%). In this work, addition of 1-hydroxybenzotriazole (HOBt) (14) to these conditions has been tried. A third problem was that piperidine was found to be an extremely efficient catalyst for intramolecular aminolysis reaction yielding diketopiperazine derivatives (15). This side reaction was worth being studied and it was found that shortening of the deprotection step followed by fast coupling gave acceptable results for most of the sequences.

In this paper, syntheses of two protected segments, thirteen and fourteen residues long, are described followed by their characterization. A protocol has been applied to try to minimize side reactions during synthesis, ie, minimal deprotection time with piperidine in N,N'-dimethylformamide (DMF) and direct coupling in methylene chloride (DCM) with just dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIPC). The cycles are thus short, approximately 50-70 min per amino acid, depending on the coupling time.

The two segments are the largest fragments that will be assembled later on in the convergent solid phase synthesis of scorpion toxin AaH II. The respective sequences are 5 to 17 and 18 to 31 (Fig. 1). They are highly protected as 7 out of 13 residues are trifunctionnal for the 13-segment (5-17) and 9 out of 14 for the 14-segment (18-31). The choice of cyclohexyl (cHex) as protection for tyrosine (16) is guided by the fact that the toxin contains seven tyrosines and that 2,6dichlorobenzyl (Dcb) as a protecting group provoques still 5% average side chain alkylation in hydrogen fluoride. The cysteine residues are protected by acetamidomethyl (Acm) groups (17) which are stable to piperidine and also essentially to hydrogen fluoride which may permit eventually to isolate the toxin with all cysteines protected.

cHex Bzl Bzl Acm Bzl cHex Acm Ł 1 1 - 1 Fmoc-TYR-ILE-VAL-ASP-ASP-VAL-ASN-CYS-THR-TYR-PHE-CYS- GLY-OH 5-17 AaH II Bzl Bzl Acm Bzl Z z Tos cHex Acm ł ł 1 Fmoc-ARG-ASN-ALA-TYR-CYS-ASN-GLU-GLU-CYS-THR-LYS-LEU-LYS-GLY-OH 18-31 AaH II

Fig.1- Amino acid sequences of the protected segments 5-17 and 18-31 of AaH II.

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RESULTS AND DISCUSSION.

The 9-fluorenylmethyloxycarbonyl group (Fmoc) has encountered a recent development as a temporary protection for a-amines during solid phase synthesis of peptides, mainly free peptides. It looked attractive to try and apply this methodology to the synthesis of protected segments with hydrogen fluoride labile side chain protection. It turned out, at first, that the protocol for Fmoc-amino acids was certainly not devoid of side reactions.

Since the discovery of the formation of Fmoc-diamino acids resulting from the reaction of Fmoc-chloride on free amino acids (8-11), the commercial Fmoc-amino acids have greatly improved in purity. Our experience is that use of Fmoc-azide (18,9) is very satisfactory. Fmoc-azide is quite easy to obtain in high purity as it crystallizes from the mixture of Fmoc-chloride and sodium azide. The very long reaction time with Fmoc-azide that was described in the early paper (9) is not really necessary. The protection reaction takes a few hours depending on the amino acids, it is sometimes complete in two hours.

Incorporation of the first amino acid has long been subject to side reactions due mainly to use of the catalyst DMAP employed for esterification of Fmoc-amino acids onto the Wang resin. The protocol used in this work (for 1 eq of hydroxymethylresin, 2.5 eq Fmoc-amino acid anhydride, 2.5 eq DCC, 0.8 eq DMAP, 2.5 eq HOBt for one hour at room temperature) gave good results (around 75% yield, 0.7% or less dipeptide formed), but another method avoiding completely use of DMAP is desirable in the future.

Assembling of the Fmoc-amino acids on the resin has been accomplished with a new protocol (19) that has been set up in order to avoid at maximum the side reactions that could occur either during the deprotection step (in presence of piperidine, a good nucleophile) or during coupling in DMF. The deprotection is performed by three one minute reactions with a solution of 50% piperidine in DMF (except for the deprotection of Fmoc-Glu(O-Bzl)-peptide, see further down) and the coupling of the Fmoc-amino acid by just diisopropylcarbodiimide in methylene This protocol has been developed mainly after the investigation concerchloride. ning catalytic aminolysis side reactions with piperidine (15). Use of symmetrical Fmoc-amino acid anhydrides in DMF requires that DMF be very clean in order to avoid Fmoc deprotection and autocondensation reactions. For these reasons, N.N'dimethylacetamide (DMA) was recommended for some time (20). DMF can be purified to great extent by 48 hrs storage over molecular sieves and by overnight nitrogen а worthwhile trying a bubbling. but it seemed coupling reaction with dicyclohexylcarbodiimide in methylene chloride. This new protocol has been employed also for synthesis of usual free peptides (19) with excellent yield and quality. The insolubility of Fmoc-amino acids in DCM is usually not a problem. Excellent coupling yields have been obtained even when the protected amino acids are mixed as a powder with the resin in a minimum of DCM for a few minutes before addition of the carbodiimide.

Assembling of the two peptides 5-17 and 18-31 of the sequence of toxin II of the scorpion Androctonus australis Hector proceeded without noticeable problem. Monitoring by UV determination of total 9-fluorenylmethylpiperidine at each deprotection step (21) showed a slight decrease of functionality over the whole synthesis (around 10%, taking into account the fluctuation of the results at each step that was also near 10% due to experimental errors and also maybe remaining adsorbed amino acid derivative). It should be mentioned here that synthesis of the segment 5-17 has been attempted twice by us using the Boc strategy on a photosensitive 4-bromomethyl-3-nitrobenzamido-copoly(styrene-1%-divinylbenzene). It failed

twice at the level of the aspartic acids (O-benzyl) with a considerable loss of reactive amines. No explanation has been found for this blocking side reaction and decision was taken then to try synthesis of this segment with Fmoc-amino acids. The side reaction described to occur for the benzyl protected dicarboxylic amino acid, Asp, with piperidine in DMF (22) could be kept to a strict and acceptable minimum with this modified protocol. Deprotection of Fmoc-Glu(O-benzyl)-peptide for segment 18-31 under the former conditions (50% piperidine in DCM for 30 min) used in a previous synthesis (23), induced formation of a pyroglutamic derivative, to 30% for each glutamic acid. Changing slightly the deprotection up conditions circumvented this side reaction. Deprotection of Fmoc-Glu(O-Bzl)-peptide in segment 18-31 was performed with 10% piperidine in DMF for two times ten minutes (24). No clear loss of functionalization was detected at these steps. Incorporation of the Fmoc-amino acids was performed in a maximum of DCM with just a little DMF to dissolve some of the amino acids. After 10 minutes of contact amount of between the amino acid (2.5 excess over the amines) and the resin, DIPC was added the coupling left for 15 to 60 minutes. Ninhydrin test (25) was used for and checking completeness of reaction, and when necessary a second coupling (with only 1.25 excess Fmoc-amino acid) was performed. For segment 5-17 two amino acids had to be recoupled due to a slightly positive test: Asp at position 9 and Ile at position 6. For segment 18-31 five amino acids needed a second coupling: Leu-29, Thr-27, Cys-26, Asn-19 and 23. No acetylation was necessary for blocking the amino groups after the second coupling reaction. The amino acid composition of the segments on the resin were as follows: Asp 2.9 (3), Thr 0.8 (1), Gly 1.1 (1), Cys 1.7 (2), Val 1.9 (2), Ile 0.7 (1), Tyr 1.2 (2), Phe 1.1 (1) for segment 5-17, and Asp 1.5 (2), Thr 0.9 (1), Glu 1.9 (2), Gly 1.2 (1), Ala 1.0 (1), Leu 1.2 (1), Tyr Lys 2.2 (2), Arg 0.6 (1) for segment 18-31. As described in an earlier 0.3 (1), paper (4) acid hydrolysis of a segment containing both Tyr(cHex) and Cys(Acm) destroy both amino acids. Addition of phenol helps for the recovery of tyrosine.

Cleavage of the segments by 55% TFA in DCM proceeded well. Removal of TFA was performed under vacuum in three steps in presence of DCM to minimize side reactions as seen by HPLC. The crude peptides were not dried completely but at first recovered by precipitation with water from a concentrated solution. The overall yield was near 95% for both segments.

Purification of the crude segments was done by preparative HPLC on a reverse phase column. Heating the column helped keeping a good resolution when scaling up. Fifty to one hundred milligram amounts of the crude segments could be treated at each run. The purified peptides showed excellent homogeneity as seen by TLC in three different systems, by amino acid analysis, and by analytical HPLC (Fig.2). The results of amino acid analyses of purified 5-17 AaH II are (theoretical value is in parenthesis): Tyr 1.8 (2); Ile 0.9 (1); Val 2.0 (2); Asp 2.9 (3); Cys 1.7 (2); Thr 1.0 (1); Phe 1.1 (1); Gly 1.1 (1).

Purified 18-31 AaH II appears homogeneous by HPLC under our experimental conditions and has the following amino acid content: Arg 0.8 (1); Asp 2.0 (2); Ala 0.9 (1); Tyr 0.8 (1); Cys 1.7 (2); Glu 1.8 (2); Thr 0.9 (1); Lys 1.9 (2); Leu 1.0 (1); Gly 1.0 (1).

The overall yields are of 10% and 19% respectively for segment 5-17 and segment 18-31.

From the work presented in this paper and the preceeding one (4) the synthesis of protected segments by solid phase methodology has become very versatile and almost as convenient as synthesis of free peptides. For convergent solid phase synthesis of large peptides it is not always necessary to build up large segments but, as long as fragment coupling is not a racemization free reaction, the length of the segments is guided by the presence of the two amino acids glycine and

We have shown that protected peptides as large as fourteen residues long proline. can easily be obtained. The seven segments of the sequence of the scorpion toxin II of Androctonus australis Hector are now in the process of solid phase assemand the reoxidation conditions from totally reduced toxin have been studied bling in detail (26).



Fig.2- Purification by HPLC of the two protected segments 5-17 and 18-31 AaH II. a)- Analytical HPLC of the crude segments 5-17 (left) and 18-31 (right) AaH II. b) - Preparative HPLC of the crude segments.
c) - Analytical HPLC of the two purified segments. ъj-

The experimental conditions of the different HPLC's are described in METHODS.

EXPERIMENTAL PROCEDURES

MATERIALS

The p-benzyloxybenzyl alcohol resin (0.7 meq OH /gram) was purchased from Novabiochem (Laufelfingen) and controlled by coupling of a Fmoc-amino acid to check incorporation and also mainly the yield of TFA cleavage that should be quantitative (5).

All solvents and reagents were analytical grade commercial products. DMF and DMA from Merck were stored over 0.4 nm anhydrous molecular beads. DMF was then flushed with N_2 and controlled by UV absorbance at a wavelength of 381 nm after reaction with 1-fluoro-2,4-dinitrobenzene (DNFB). DCM (Merck) was freshly distilled over anhydrous Na₂CO₃ and piperidine (Merck) under N₂ over KOH pellets. TFA (Merck) was distilled prior to use over chromoxyde. DCC was obtained from Peptide Institute Inc. and HOBt from Fluka. N- α -Fmoc-L-amino acids with HF labile side chain protection (except for cysteine and tyrosine), were purchased from Bachem (Bubendorf) and Novabicchem (Laufelfingen). Purity of the starting Fmoc-amino acids was checked by thin layer chromatography (TLC) and their melting point.

METHODS

Synthesis of Fmoc-Tyr(CHex)-OH

H-Tyr(CHex)-OH was prepared according to the literature (15,27). H-Tyr(CHex)-OH (4.64 g, 17.7 mmol) was then solved in 35 ml of 10% Na_2CO_3 aqueous solution at a temperature of $0^{\circ}C$. After addition of 18.5 ml dioxan (peroxide free), a solution of Fmoc-N₃ (4.2 g, 15.9 mmol) in 26 ml dioxan was slowly added for 1 hr at 0°C and left 4 hrs at room temperature. After reaction, 530 ml of ice-water was added and extraction performed with 2x 140 ml of diethyl ether. Aqueous phase was acidified by 12 N HCl to pH 2 at a temperature of 0°C. The precipitate was solubilized with ethyl acetate and the organic phase washed twice with 0.1 N HCl. The organic phase, dried for 2 hrs over MgSO4, was filtered and evaporated under vacuum in a rotative evaporator (Buchi). Crystallization was performed twice with n-hexane-ethyl acetate (1:1). Purity of Fmoc-Tyr(CHex)-OH (4.47 g, 8.94 mmol) was checked by TLC in chloroform-EtOH 3:1 (Rf:0.57). Melting point is 162°C. Elemental analysis : found C, 74.2; H, 6.5; N, 3.1; calculated for C29H2605N : C, 74.2; H, 6.4; N, 2.9.

Esterification of Fmoc-Gly-OH on the p-benzyloxybenzyl ester resin

The Wang resin (2.50 g, 1.75 mmol) was washed several times with DMF, DCM and suspended in DMF. Fmoc-Gly-OH (2.5 excess /OH-resin), DCC (2.5 excess /OH-resin), suspended in DMF. Fmoc-Gly-OH (2.5 excess /OH-resin), DCC (2.5 excess /OH-resin), DMAP (0.8 excess/ OH-resin) and HOBt (2.5 excess/ OH-resin) were added and the suspension shaken for 1 hr at room temperature. The resin was then collected by filtration and thoroughly washed with DMF, DCM, EtOH successively. In order to block unreacted hydroxyl groups on the resin, benzoylation was performed using benzoylchloride. Deprotection value of Fmoc-glycyl resin, obtained after reaction with 50% piperidine in DMF (3 times 1 min) and measurement by spectrophotometer of N-(9-fluorenylmethyl)piperidine (ϵ 301nm = 7,800 M⁻¹.cm⁻¹) gave an incorporation wield of 74% for the first amino acid. Incorporation value was in agreement with yield of 74% for the first amino acid. Incorporation value was in agreement with that obtained by picric acid monitoring (28). Estimation of the amount of Fmocdipeptide formation on the resin was controlled on an aliquot after preliminary cleavage of the amino acid from the resin using 55% TFA-DCM for 1 hr followed by

deprotection with piperidine. The value obtained on the amino acid analyzer (8)

was of 0.7% Fmoc-Gly-Gly-OH compared to Fmoc-Gly-OH.

Assembling of the protected peptides 5-17 AaH II and 18-31 AaH II

Manual assembling of the peptides was performed in a polypropylene syringe fitted with a porous polyethylene disk and a teflon bar for smooth agitation. Elongation of the Fmoc-Glycyl-resin was performed according to the following operational cycle protocol (washings and deprotection were carried out with 50 ml portions of solvents, but each coupling step was in a final volume of about 20 ml):

- 1- washing with DCM (4x 1.5 min) (4x 1.5 min)
- 2- washing with DMF 3- deblocking with 50% piperidine-DMF (3x 1 min) *
- 4- washing with DMF
- (4x 1.5 min) (4x 1.5 min) 5- washing with DCM

measurement at a wavelength of 301 nm of the N-(9-fluorenyl-Absorbance methyl)piperidine

6- Fmoc-amino acid in DCM with DMF to ensure dissolution (10 min) 7- coupling with either DCC or DIPC (30 min-1 hr) **

Ninhydrin test.

For Fmoc-Glu(OBzl)-peptidyl resin : deblocking with 10% piperidine-DMF * (2x 10 min) to avoid a derivatization as pyroglutamic acid. Coupling of Fmoc-Asn-OH was performed as its HOBt-ester in DMF, in order ** to avoid dehydratation of amide to nitrile group.

Cleavage of peptide from resin

of the crude protected segments 5-17 AaH II and 18-31 AaH II was Cleavage performed with 55% TFA-DCM for 1 hr at room temperature. After filtration, resin was washed twice with DCM, DMA and DCM respectively. The filtrate the was concentrated under vacuum on a rotative evaporator after three additions of DCM to a final volume of about 5 ml. Then, the protected peptide was precipitated by addition of deionized quartz-bidistilled water. The crude protected peptide was filtered on a Millipore ultrafiltration system $(0.2\,\mu$ m) and dried under vacuum. The cumulated yield of cleavage of the peptide and precipitation was of 95% as shown by amino acid analysis.

High pressure liquid chromatography

High pressure liquid chromatography (HPLC) was performed with a Waters solvent delivery system, two F6000 pumps, a model 660 solvent programmer, a model 481 UV spectrophotometer and a data module model 730 integrator, on either a preparative column or in analytical conditions.

Purification of crude segments was performed using reverse phase HPLC on a preparative column (2.5 x 25 cm with a special turbulence free head and a divided solvent delivery system (29)) filled with Nucleosil C-18 7μ m. The elution was in isocratic conditions:

- solvent system: DMF-H2O-propionic acid (83:16.5:0.5)
- flow rate: 10 ml /min (7.5 ml pump A, 2.5 ml pump B) temperature of the column: 40°C
- pressure: 4000 psi
- sample injection: 50 mg for crude 5-17 AaH II and 42 mg for crude 18-31 AaH II dissolved in 600 µ1 DMF.

The eluate absorbance was recorded at a wavelength of 270 nm at a detector sensitivity of 2 AUFS

For analytical HPLC of the crude segments on a Lisochrob RP C-18 7 μ m column (0.9 x 25 cm) (Merck), the parameters were the same except flow rate (1.3 ml /min) and sample injection (about 500 μ g of crude peptide in 10 μ l DMF), pressure 1400 psi and detector sensitivity (0.05 AUFS).

For analytical HPLC of the purified segments, the column used was a C-18 $7 \ \mu$ m Merck Spherisorb (25 x 0.4 cm) (Tracer). Elution of purified 5-17 AaH II was performed with a linear gradient of 20 min from 40 to 90% buffer B (buffer A: DMF-H₂O-propionic acid 59.75:39.75:0.50 and buffer B: DMF-CH₃CN-propionic acid 59.75:39.75:0.50). For 18-31 AaH II, the linear gradient used was 60 to 90% buffer B in 15 min. The flow rate was of 1.2 ml /min. The UV monitoring was at a wavelength of 301 nm at a detector sensitivity of 0.05 AUFS for a sample injection of 100 g in 10 1 DMF.

Amino acid analyses

Amino acid analyses were performed on either a Biotronik Bt 7025 or a Beckman model 6300 apparatus, after preparation of the peptide hydrolysates by 6 N HCl treatment in sealed vacuum evacuated tubes for 1 hr (150°C). To obtain better values of tyrosine 1% (w/v) phenol was added before hydrolysis and extracted by chloroform prior to application on the analyzer.

Physico-chemical data

Reichert thermovar apparatus was used for melting point determination. Melting point is m.p. 262° C for segment 18-31 and over 350° C for segment 5-17. Specific rotation was determined on a Perkín Elmer polarimeter (model 141) and proton NMR spectra on a Varian XL 200 spectrometer (200 MHz). Specific rotation $[\alpha]_D = -20 \pm 0.8$ (C 0.5, DMF) for segment 5-17 and $[\alpha]_D = -61 \pm 3.6$ (C 0.12, DMF) for segment 18-31. Ìor

Purity of the segment was also checked by TLC and visualised by a single UV fluorescence spot. TLCs were carried out on precoated RP-18 plates (Merck) in the three systems: A- DMF-MeOH 50:50 ; B- DMF-H₂O-propionic acid 91:8.5:0.5 ; C-DMF-CH₃CN-H₂O-propionic acid 70:20:9.5:0.5 . Results show single spots : B- Rf 0.72; C- Rf 0.77. A- Rf 0.90;

NMR spectra at 200 MHz, in spite of complexicity of the peptides, showed that characteristic signals were as expected and could be assigned.

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