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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS. VII. GOOD YIELDS IN THE COUPLING OF PROTECTED SEGMENTS ON A SOLID SUPPORT

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Protected peptides corresponding to the 64 amino acids of the whole sequence of toxin II of Androctonus australis Hector have been assembled on a solid support. Solid phase sequencing has proved to be extremely useful for the evaluation of the coupling yields, which have all been nearly quantitative.

In spite of the spectacular results achieved with the highly optimised methods of stepwise solid phase peptide synthesis (1), it is evident that a convergent strategy in which peptide "blocks" are assembled is the best alternative to carry out the synthesis and purification of small proteins. In the last few years many authors have reported syntheses in which the coupling step is carried out on a solid support (2-11).

We have developed two different approaches for the synthesis of protected peptide segments on a solid matrix (4, 5) which, after extensive purification, can be assembled to yield a polypeptide or a small protein. This convergent strategy has been applied to the synthesis of toxin II of the North African scorpion Androctonus australis Hector (12), a polypeptide 64 residues long with a C-terminal carboxamide function and four disulphide bridges (13).

Abbreviations: Acm, acetamidomethyl, AcOH, acetic acid; Boc-, t-butoxycarbonyl; t-Bu, t-butyl; Bzl, benzyl; DCC, N,N'-dicyclohexylcarbodiimide; DIEA, N,N'-diisopropylethylamine; DMA, N,N'-dimethylacetamide; DMAP, 4--dimethylaminopyridine; DMF, N,N'-dimethylformamide; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hdroxybenzotriazole; cHex, cyclohexyl; iPrOH, isopropanol; MeOH, methanol; MPLC, medium pressure liquid chromatography; Nbb-, nitrobenzamidobenzyl; NMM, N-methylmorpholine; pMeBHA, p--methylbenzhydrylamine; resin, poly(styrene-co-1%-divinylbenzene; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tos, tosyl; Z, benzyloxycarbonyl. Amino acid symbols denote the L configuration where applicable.

In the preceding papers of this series (14-17) we have described the synthesis and purification of the protected peptide segments originally designed for the convergent synthesis of the toxin, all of them having either a glycine or a proline residue at the C-terminus to minimise the risk of racemisation during the coupling step (Table I), and in this paper we report the assembly of the entire sequence of the toxin mentioned above.

Table I

Sequences of the protected peptide segments of toxin II of AaH

Boc-Val-Lys(Z)-Asp(tBu)-Gly-OH	(1-4)
Fmoc-Tyr(cHex)-Ile-Val-Asp(Bzl)-Asp(Bzl)-Val-Asn-Cys(Acm)-Thr(Bzl)--Tyr(cHex)-Phe-Cys(Acm)-Gly-OH	(5-17)
Fmoc-Arg(Tos)-Asn-Ala-Tyr(cHex)-Cys(Acm)-Asn-Glu(Bzl)-Glu(Bzl)--Cys(Acm)-Thr(Bzl)-Lys(Z)-Leu-Lys(Z)-Gly-OH	(18-31)
Boc-Glu(Bzl)-Ser(Bzl)-Gly-OH	(32-34)
Fmoc-Tyr(cHex)-Cys(Acm)-Gln-Trp-Ala-Ser(Bzl)-Pro-Tyr(cHex)-Gly-OH	(35-43)
Boc-Asn-Ala-Cys(Acm)-Tyr(cHex)-Cys(Acm)-Tyr(cHex)-Lys(Z)-Leu-Pro-OH	(44-52)
Boc-Asp(Bzl)-His-Val-Arg(Tos)-Thr(Bzl)-Lys(Z)-Gly-OH	(53-59)
H-Pro-[2-^{3}H]Gly-Arg(Tos)-Cys(Acm)-His-pMeBHA-resin	(60-64)

Results and discussion.

The C-terminal pentapeptide was synthesised by standard Boc-amino acid methodology on a carbonyl-free p-methylbenzhydrylamine resin obtained according to the method of Hirao et al. (18) as optimised in our laboratory (19), thus avoiding any risk of reaction between amino groups of the growing peptide chain and carbonyl functions present on the polymer. Glycine-61 was introduced as a tritiated residue in order to facilitate the final purification of the toxin, and the tosyl group on the imidazole ring of histidine was removed prior to the incorporation of Boc-Cys(Acm)-OH. An aliquot of peptide-resin was submitted to high HF treatment (HF/p-cresol 9/1, 1 hr, 0°C), and the crude peptide showed a high degree of purity by reversed phase HPLC (20).

The first five protected segments (residues 18-59 inclusive) have been attached to the resin following the same general procedure: the N-terminal amino group of the peptide-resin was first deprotected either by a treatment with trifluoroacetic acid or with piperidine depending upon the type of

N-protecting group. Protected peptides (1.3-2.8 equivalents) were dissolved in the minimum volume of DMF and added to the peptide-resin together with an equimolar amount of HOBt in DMF. After 2 min stirring, DCC (1.3-2.8 equiv.) was added and the mixture was stirred for 2 hr at 0°C and then at room temperature. Aliquot portions of the peptide-resin were removed a few hours after initiation of the coupling reaction and the extent of coupling was determined by amino acid analysis of the acid hydrolysates. If the yield appeared to be less than 95%, the coupling was allowed to continue and if necessary, was repeated (Table II). Assessment of whether or not the reaction was complete was also carried out using the ninhydrin or chloranil tests (21, 22). In all cases couplings were first tested on a small amount of peptide-resin (2-3 μmol) before carrying them out on a large scale.

Table II

Yields of the first five couplings

Protected peptide			Amino component	Time (hr)	Yield[a]
53-59	160 μmol	2.1 equiv.	60-64-resin	20	96%
44-52	96 μmol	1.3 equiv.	53-64-resin	14	84%
				40	91%
	55 μmol	0.7 equiv.		24	quantit.
35-43	112 μmol	1.6 equiv.	44-64-resin	16	90%
	44 μmol	0.6 equiv.		16	quantit.
32-34	115 μmol	1.8 equiv.	35-64-resin	28	94%
	100 μmol	1.6 equiv.		16	quantit.
18-31	156 μmol	2.8 equiv.	32-64-resin	48	80%

[a]Calculated by amino acid analysis of the acid hydrolysates.

Histidine residues of segments 53-59 and 60-64 were both left unprotected in order to avoid the danger of consumption of HOBt during the coupling step, possibly by HOBt-induced deprotection of the His-tosyl group which might then lead to the formation of HOBt-tosylate which in turn could lead to irreversible blockage of the amine groups (23).

Nakagawa et al. have reported an unsuccessful coupling to a peptide-resin with a proline residue at the N-terminus (8). Although the test-coupling on a small scale gave fairly good results, peptide 53-59 was anchored to 60-64-resin by using directly a 2.1 fold-excess of protected

peptide. With all of the segments coupling was first attempted with less than 2 equivalents.

Tripeptide 32-34 was initially prepared with the Fmoc protecting group at the N-terminus (15), but as the synthesis of segment 18-31 (which includes two glutamic acid residues) showed that piperidine treatment of Fmoc--Glu(Bzl)-peptide-resin caused some intramolecular cyclisation to pyroglutamic acid (17), sequence 32-34 was finally N^{α}-protected with a Boc group (16) for coupling to 35-64-resin. Nevertheless, some risk of pyroglutamisation remained for the coupling of segment 18-31 to 32-64-resin, because this side reaction can also be promoted to a considerable extent by exposure to HOBt in long reaction times (24). Segment 18-31 was thus coupled to 32-64 -resin by using an excess much larger than usual (2.8 equiv.), but the solubility of the peptide necessitated carrying out the coupling step in a large volume of solvent with the concomitant decrease in the reaction rate, and this procedure only partially prevented the intramolecular cyclisation (see yield in Table II).

The protected segment 5-17 could not be attached to the peptide-resin following the same protocol, even in the presence of bases (0.25 equiv. of 4-dimethylaminopyridine or 1 equiv. of N-methylmorpholine), urea (100 equiv.), higher temperature (45°C), or by the succinimide ester method. On the other hand, it was possible to incorporate a residue of glycine. Thus, an alternative strategy had to be designed to circumvent the use of peptide 5-17 even though it might have a risk of racemisation: anchoring of glycine, followed by coupling of two protected segments, 11-16 and 5-10. Since our own experience had shown that peptides with less than ten residues could be obtained with a high degree of purity, carrying out the synthesis and purification of two hexapeptides looked more promising than atempting to synthesise and purify a peptide 12 residues long. Moreover, coupling of segment 35-43 onto 44-64-resin, with a N-terminal asparagine at 44, had proceeded without any difficulty. Thus we synthesised and purified two protected peptide segments:

Boc-Tyr(cHex)-Ile-Val-Asp(cHex)-Asp(cHex)-Val-OH (5-10)

Fmoc-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-OH (11-16)

Segment 11-16 was obtained using Fmoc-amino acids on an alkoxybenzyl ester resin, and segment 5-10 was synthesised with Boc-amino acids on a resin with a photolabile anchoring in order to avoid side reactions promoted by the use of piperidine in the deprotection of Fmoc-Asp(Bzl) (25).

Boc-glycine (10 equiv.) was anchored to 18-64-resin *via* a DCC/HOBt mediated coupling over 90 min., in quantitative yield as demonstrated by

amino acid analysis. The last three segments were assembled following the procedure described above with the addition of 1 equivalent of N-methylmorpholine (Table III). The absence of this base led to a very poor coupling yield of these peptides. Excess of acylating component was also increased to obtain good yields.

The determination of the extent of the final couplings by amino acid analysis was much more difficult than for previous couplings, since only one new residue was added (one isoleucine near two valines in sequence 5-10) and the concluding results had to be interpreted from an increase in one residue out of several already present in the peptide-resin, and often in a large number. Furthermore, ninhydrin tests became less sensitive as the length of the peptide chain anchored to the resin increased.

Table III

Yields of the last three couplings

Protected peptide			Amino component	Time (hr)	Yield[a]
11-16	85 µmol	5 equiv	17-64-resin	18	90%
	28 µmol	1.6 equiv		20	quantit.
5-10	85 µmol	5 equiv	11-64-resin	28	91%
	28 µmol	1.6 equiv		16	quantit.
1-4	85 µmol	5 equiv	5-64-resin	24	quantit.[b]

[a]Calculated by amino acid analysis except in the last case (see [b]).

[b]The coupling was demonstrated to be complete by sequencing (see text and Figure I) and a negative ninhydrin test. In this case the amino acid analysis was complicated and did not give reliable information concerning the coupling yield (see Table IV in Experimental Part).

After assembly of the complete sequence, yields were checked by solid phase automatic sequencing on aliquots (5-10 nmoles) of different peptide--resins removed after some of the couplings (26). The results from "one segment preview" agreed very well with those obtained by amino acid analysis (Figure I, compare with results in Tables II and III).

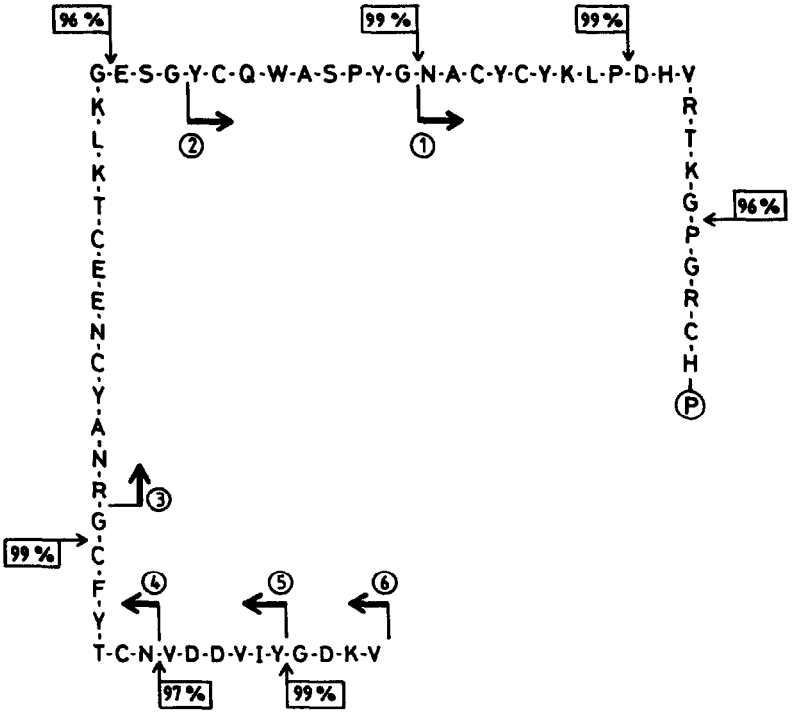
The discrepancy between the 97% yield for the coupling 18-31 + 32-64--resin obtained by solid phase sequencing and the 80% yield determined by amino acid analysis may be explained by the cyclisation of Glu(Bzl) at position 32 forming pyroglutamic acid, which is not detectable by Edman degradation.

Another advantage of peptide sequencing is the evaluation of homo-

geneity of protected peptides after data from "one amino acid preview". This analysis showed that from residues 1 to 16 the accumulated preview was of 1.7%, meaning that the purity of the three segments 1-4, 5-10 and 11-16 was equivalent to that of a peptide obtained with an average yield per cycle (deprotection plus coupling) of 99.9%; for segments 35-43, 44-52 and 53-59 the preview analysis corresponded to an average yield of 99.9%, 99.6% and 99.4% respectively. The 14-residue segment (sequence 18-31) contained a deletion peptide (13%) lacking the asparagine-19 residue. This impurity had not been detected by amino acid analysis nor by HPLC. These results clearly indicate the usefulness of the convergent strategy and that the small peptide segments can be obtained in a very pure state.

Figure 1

Coupling yields as determined by automated solid phase Edman degradation



Numbers in circles correspond to the different aliquots of peptide-resin removed and sequenced.

Conclusions

The present work illustrates the usefulness of the convergent solid phase approach for the assembly of large peptides. Protected peptides obtained after solid phase synthesis and extensive purification have been efficiently coupled on a solid support (all yields are higher than 96%). This methodology helps to solve the drawbacks associated with synthesis in solution, where the growing protected peptide is usually insoluble and thus difficult to purify, and coupling yields are often low due in part to the difficulties associated with working at high concentration. Here, the growing peptide is linked to a solid support and the coupling may be forced to completion by using a large excess of the soluble component, or by repeated couplings under different conditions without loss of the chain on the resin. It has proved to be very important to carry out the reaction in the minimum amount of solvent, and the advantage of a solid phase synthesis with respect to that in a homogeneous phase is that only the acylating component has to be solubilised. Furthermore, the polystyrene solid support seems to favour the coupling by providing a highly solvating microenvironment which may enhance the mobility of the peptide chain (27, 28).

From our experience, it seems advisable to divide a protein into segments of up to 10 residues long, to ensure thorough purification and to obtain high coupling yields by facilitating the solubilisation of the peptide. We have found FAB-MS to be a very useful technique for obtaining good characterisation of protected peptides (29).

Solid phase Edman sequencing has demonstrated its accuracy in the determination of the coupling yields, and its use is highly recommended when amino acid analyses and ninhydrin tests do not give clear results.

Experimental Part.

Protected amino acids and p-benzyloxybenzyl alcohol resin were obtained from Novabiochem AG (Läufelfingen, Switzerland). Boc-[2-^{3}H]Gly-OH was synthesised as described in the literature (30) from [2-^{3}H]glycine (ICN, 2 mCi at 17 Ci/mmol). Bromomethyl-Nbb-resin was prepared as previously described (5). pMeBHA-resin was synthesised by a modified procedure described by us (19) and was free from carbonyl groups as demonstrated by ^{13}C-NMR. Photochemical cleavages were carried out with three H 125 BL eye lamps using an apparatus previously described (5). Peptide syntheses were carried out either in a teflon reaction vessel or in a 50 mL polypropylene syringe both fitted with a polyethylene disc. Amino acid analyses were run on a Biotronik autoanalyser model 6000. Solid phase sequencing was performed on a 475A Applied Biosystems automated sequencer.

Fmoc-Asn-Cys(Acm)-Thr(Bzl)-Tyr(cHex)-Phe-Cys(Acm)-OH

The peptide was synthesised on a p-alkoxybenzyl alcohol resin (1.4 g; 1 mmol) following procedures previously described (15, 17). The first amino acid (Fmoc-Cys(Acm)-OH, 2.5 excess) was anchored to the resin by the DCC--DMAP mediated coupling method in a yield of 92% as calculated by spectrophotometric measurement of N-(9-fluorenylmethyl)piperidine obtained after deprotection with 50% piperidine-DMF. At the end of the synthesis, treatment of the peptide-resin with 55% TFA-CH_2Cl_2 provided 0.66 mmol of crude peptide (cumulated yield from starting resin was 66%). Purification was performed using reverse phase HPLC on a preparative column (2.5x25 cm) with a special turbulence-free head and divided solvent delivery system (31)) filled with 7 µm Nucleosil C-18. The peptide (40 µmol in 0.6 mL of DMF were injected at each run) was eluted with DMF/CH_3CN/H_2O/propionic acid (50:19:31:0.5) at a flow rate of 10 mL/min at 40°C. 0.31 mmol of purified peptide (97% pure by HPLC) were obtained (78% yield) after evaporation, addition of H_2O and lyophilisation. Amino acid analysis: Asp, 0.95; Thr, 0.98; Tyr, 0.83; Phe, 1.07. FAB-MS (thioglycerol matrix), m/z 1308 $(M + Na)^+$, 1286 $(MH)^+$. The most significant signals in the ^{1}H-NMR spectrum of the peptide (DMSO-d_6, 200 MHz) were: δ8.3-8.1 (m, 2xNH), 7.9-7.7 (m, 2xNH), 7.69 (d, NH), 6.95 (dd, Ar Tyr), 6.92 (d, NH Tyr), 4.8-4.5 (m, Asp α-CH, Tyr α-CH), 4.3-4.0 (m Ile α--CH, Val α-CH), 2.9-2.6 (m, Asp β-CH_2), 2.1-1.9 (m, Val β-CH, Ile β-CH), 1.9-1.2 (m, cHex, Ile γ-CH_2), 1.28 (s, t-Bu), 0.9-0.7 (m, Val γ-CH_3, Ile γ--CH_3, δ-CH_3).

Boc-Tyr(cHex)-Ile-Val-Asp(cHex)-Asp(cHex)-Val-OH

This peptide was synthesised on a $BrCH_2$-Nbb-resin (7.6 g, 2.8 mmol) according to protocols previously described (14, 16). The first amino acid (Boc-Val-OH, 2 excess) was incorporated using the cesium salt procedure (32) at room temperature for 4 hr in an essentially quantitative yield. In order to avoid the formation of diketopiperazines, the third amino acid was incorporated using the method described by Suzuki (33). At the end of the synthesis, the photolysis was carried out in batches of 1 g up a total of 5 g (1.10 mmol) of peptide-resin to provide 0.52 mmol (47 %) of crude peptide. This material was purified by MPLC (ca. 110 µmol were injected at each run, convex gradient formed from 400 mL each of 3:3:4 and 3:5:2 of DMF, H_2O, and CH_3CN containing 0.5% of propionic acid, and a flow rate of 180 mL/h). After lyophilisation the residue was dissolved in 3 mL of DMA and the peptide was precipitated by the addition of 9 mL of H_2O. After 3 hr at 4°C the solid was filtered (Millipore GSPW, 0.22 µm) to provide 0.20 mmol (38 %) of protected peptide (98% pure by HPLC). Amino acid analysis: Asp, 2.02; Val, 1.98; Ile, 0.83; Tyr, 0.92. FAB-MS (thioglycerol/NaCl matrix), m/z 1091 $(M + Na)^+$. The most significant signals in the ^{1}H-NMR spectrum of the peptide (DMSO-d_6, 200 MHz) were: δ 8.6-8.5 (m, 2xNH), 8.5-8.3 (m, 2xNH), 8.33 (d, NH), 8.23 (d, NH), 8.12 (d, NH), 7.9-7.1 (m, Ar Fmoc), 7.53 (d, NH), 7.3-7.1 (m, Ar Phe), 7.23 (s, Ar Bzl), 6.9 (bs, Asn γ-NH_2), 6.82 (dd, Ar Tyr), 4.7-4.0 (m, Asn α-CH, Cys α-CH, Tyr α-CH, Phe α-CH, Thr α-CH, β-CH, CH_2 Bzl), 1.82 (s, Acm CH_3), 1.79 (s, Acm CH_3), 1.4-1.2 (m, Thr γ-CH_3).

Boc-Pro-[2-^{3}H]Gly-Arg(Tos)-Cys(Acm)-His-pMeBHA-resin

The different amino acids were attached to a pMeBHA-resin (1.0 g, 0.26 mmol) following the same protocol used for the Nbb-resin. After the incorporation of histine, the peptide-resin was treated with HOBt (160 mg, 1.05 mmol, 4 equiv) in DMF (5 mL) for 4 h. After washings with DMF (3x1 min) the synthesis was continued. Once the peptide was assembled onto the resin, the amino acid analysis gave 0.18 mmol of peptide/g resin and Pro, 0.91; Gly, 0.99; His, 1.04; Arg, 1.06. Likewise, a portion (100 mg) of this peptide--resin was treated with 4.5 mL of anhydrous HF in the presence of 0.5 mL of p-cresol for 1 h at 0°C to provide the crude peptide, which was more than 96% pure by analytical HPLC and with a specific activity of $1.7x10^5$ cpm/µmol.

Procedure for the coupling of protected segments

Couplings were first tested on a small amount of peptide resin (2-3 mol) in a 1 mL polypropylene syringe fitted with a polyethylene disc before carrying out the large scale experiment. These were performed using a mechanical shaker in a silanized screw-cap tube reaction vessel (1.0 x 6.0 cm) with a Teflon-lined cap, a sintered glass frit and a stopcock, with the following procedures:

i) On Boc-peptide-resin: 1) CH_2Cl_2, 4 x 1 min; 2) 40% TFA/CH_2Cl_2, 2 x 1 min + 1 x 30 min; 3) CH_2Cl_2, 5 x 1 min; 4) 5% DIEA/CH_2Cl_2, 3 x 2 min; 5) CH_2Cl_2, 4 x 1 min; 6) Protected peptide and an equimolar amount of HOBt in DMF were added to the resin at 0°C, after 5 min DCC (1 equiv respect to peptide) in DMF was added and stirred 1 hr at 0°C and then at room temperature; 6) DMF, 4 x 1 min; 7) DMF, 3 x 1 min; 8) CH_2Cl_2, 4 x 1 min. From the incorporation of the segment 35-43 2% of β-mercaptoethanol was added to step 2) in order to avoid oxidation of Trp. For the last three segments 1 equiv of NMM was added to step 6.

ii) On Fmoc-peptide-resin: Steps 2, 3, and 4 of the Boc-procedure were substituted by 50% piperidine/CH_2Cl_2, 3 x 2 min. Filtrates corresponding to treatment with piperidine and subsequent washings with CH_2Cl_2 were pooled and the 9-fluorenylmethylpiperidine formed was quantified spectrophotometrically at 301 nm.

Boc-(53-64)-pMeBHA-resin. Steps 1-5 of the protocol described above were applied to Boc-(60-64)-toxin resin (417 mg, 75 μmol). Boc-(53-59)-toxin (160 μmol, 2.1 equiv.) dissolved in 1.55 mL of DMF and HOBt (25 mg, 160 μmol) dissolved in 0.2 mL were added to the peptide-resin at 0°C. After 5 min of shaking, DCC (33 mg, 160 μmol) in 0.2 mL of DMF was added and the mixture was stirred 1 hr at 0°C and then 20 hr at room temperature. After this time the amino acid analysis showed a coupling yield of 96%. Chloranil test was negative.

Boc-(44-64)-pMeBHA-resin. The first coupling was carried out with 96 μmol (1.3 equiv.) of Boc-(44-59)-toxin dissolved in 2 mL of DMF and 72 μmol of (53-64)-pMeBHA-resin. After 40 h the yield was 91%. The second coupling was performed with 55 μmol (0.7 equiv.) of protected peptide dissolved in 1.6 mL of DMF and the yield after 24 h was essentially quantitative.

Fmoc-(35-64)-pMeBHA-resin. The first coupling was carried out with 112 μmol (1.6 equiv.) of Fmoc-(35-43)-toxin dissolved in 1.25 mL of DMF and 70 μmol of (40-64)-pMeBHA-resin. After 16 hr the yield was 90%. The second coupling was carried out with 44 μmol (0.6 equiv.) of peptide dissolved in 1 mL of DMF, with a yield essentially quantitative after 16 hr.

Boc-(32-64)-pMeBHA-resin. Quantification of 9-fluorenylmethylpiperidine after deprotection of the N-terminus of Fmoc-35-64-resin was in accord with the expected value. Two couplings were performed on 64 μmol of peptide--resin. For the first, 115 μmol (1.8 equiv.) of protected peptide were dissolved in 1 mL of DMF and the yield after 28 hr was 94%. The second coupling was carried out with 100 μmol of peptide in 1 mL of DMF and the yield was essentially quantitative after 16 hr.

Fmoc-(18-64)-pMeBHA-resin. A single coupling was carried out with 156μmol of protected peptide in 3 mL of DMF and 56 μmol of peptide-resin. After 48 hr, several amino acid hydrolyses gave a ranged yield of about 80%. Ninhydrin test was in this case clearly negative.

Boc-(17-64)-pMeBHA-resin. After piperidine treatment and washings, the 9-fluorenylmethylpiperidine formed was quantified spectrophotometrically and 93% of the expected amount (if the coupling yield of segment 18-31 had been quantitative) was found. Boc-Gly-OH (31 mg, 180 μmol, 10 equiv.) in 1 mL of DMF was coupled on 18 μmol of peptide-resin in presence of DCC and HOBt. After 90 min, the aminoacid analysis showed a quantitative yield.

Fmoc-(11-64)-pMeBHA-resin. A first coupling was carried out with 85 µmol (5 equiv.) of protected peptide dissolved in 1 mL of DMF, DCC (18 mg, 85 µmol), HOBt (13 mg, 85 µmol), NMM (9.5 µL, 85 µmol), and 17 µmol of peptide-resin (90% yield). The coupling was repeated in the same conditions with 28 µmol (1.6 equiv.) of peptide in 1 mL of DMF and the yield was quantitative.

Boc-(5-10)-pMeBHA-resin. Again, the amount of 9-fluorenylmethylpiperidine found was in accord with the theoretical value. Two couplings were performed in the same conditions and excesses as before (17 µmol of peptide-resin) with a 91% yield for the first coupling and a quantitative yield after the second coupling.

Boc-(1-64)-pMeBHA-resin. After one single coupling with 85 µmol (5 equiv.) of protected peptide in 0.2 mL of DMF, in the presence of HOBt, DCC, and NMM, the ninhydrin test was negative. Evaluation of "one segment preview" by Edman degradation demonstrated, on the basis of the ratio Asp/Val (residues 3 and 6) in the third cycle of the sequencing, that the coupling yield was 99% (see Figure I).

Amino acid analyses to quantify coupling yields

Peptide-resins (ca. 0.5 mg) were first treated with 50% TFA/CH_2Cl_2 (1 x 5 min), CH_2Cl_2 (4 x 1 min), 50% piperidine/CH_2Cl_2 (1 x 5 min), and CH_2Cl_2 (5 x 1 min) in order to remove protected peptides covalently bonded to the imidazole of histidine. Hydrolyses were carried out at 150°C with 12N HCl/propionic acid (1:1) and a crystal of phenol over 45 min (see Table IV for the amino acid analysis of different peptide-resins).

Table IV

Amino acid analysis of the different peptide-resins[a]

	53-64	44-64	35-64	32-64	18-64	11-64	5-64	1-64
Asp	0.99 (+1)	2.21 (1+1)	2.00 (2)	2.16 (2)	3.65 (2+2)	5.07 (4+1)	6.84 (5+2)	7.21 (7+1)
Glu	-	-	0.93 (+1)	1.82 (1+1)	3.47 (2+2)	3.74 (4)	3.83 (4)	4.13 (4)
Gly	2.17 (1+1)	1.91 (2)	3.04 (2+1)	3.96 (3+1)	4.95 (4+1)	6.37 (5+1)	6.14 (6)	7.28 (6+1)
Ala	-	1.28 (+1)	2.14 (1+1)	1.91 (2)	2.91 (2+1)	3.06 (3)	3.00 (3)	3.06 (3)
Val	1.11 (+1)	0.86 (1)	0.87 (1)	1.10 (1)	1.21 (1)	n.d (1)	3.24 (1+2)	3.61 (3+1)
Ile	-	-	-	-	-	-	0.86 (+1)	0.92 (1)
Leu	-	1.18 (+1)	0.93 (1)	0.81 (1)	1.90 (1+1)	1.78 (2)	1.91 (2)	2.24 (2)
Tyr	-	2.23 (+2)	3.72 (2+2)	3.85 (4)	5.03 (4+1)	n.d (6)	n.d (6+1)	n.d (7)
His	1.69 (1+1)	1.78 (2)	2.23 (2)	2.20 (2)	2.65 (2)	n.d (2)	n.d (2)	n.d (2)
Lys	1.09 (+1)	2.06 (1+1)	1.97 (2)	1.94 (2)	4.25 (2+2)	3.98 (4)	4.18 (4)	5.63 (4+1)
Arg	1.95 (1+1)	1.50 (2)	2.17 (2)	2.09 (2)	n.d (2+1)	n.d (3)	n.d (3)	3.02 (3)

[a]In (m+n) m corresponds to the number of residues contained in the peptide-resin before the coupling, and n the number of residues in the protected peptide.

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