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New Strategy for the Synthesis of Large Peptides as Applied to the C-terminal Cysteine-Rich 41 Amino Acid Fragment of the Mouse Agouti Protein

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Abstract: In this new convergent peptide synthetic approach the advantages of the Fmoc and Boc techniques are combined. Boc-protected peptide fragments prepared on 2-chlorotrityl resin by Fmoc-technique were coupled sequentially in solution. As an example, the C-terminal (91-131) part of the agouti protein comprising all the 5 disulfide bridges in the original molecule has been synthesized by this method. © 1997 Elsevier Science Ltd.

The agouti gene, expressed in mice bearing the lethal yellow mutation, encodes the 131 amino acid agouti protein. The agouti protein inhibits eumelanin production in the skin, causing the yellow coat color of the animals. Beside this effect, agouti induces various diseases, among others insulin-resistant diabetes, sensitivity to development of tumors etc[1]. The C-terminal cysteine-rich fragment of the protein has been shown to possess the α-MSH antagonist activity of the whole molecule[2].

We report here the synthesis of the C-terminal agouti (91-131) fragment

P-C-V-A-T-R-D-S-C-K-P-P-A-P-A-C-C-D-P-C-A-S-C-Q-C-R-F-F-G-S-A-C-T-C-R-V-L-N-P-N-C,

using a new strategy developed for the synthesis of large peptides. This approach combines the advantages of well known and routinely utilized peptide synthetic techniques[3,4], reducing

i) the difficulties caused by the lower solubility of the growing protected peptide chain;

ii) the amount of contaminants brought about by the incompleteness of the deprotection and coupling cycles during the solid phase elongation of the longer peptides.

The sequence of agouti(91-131) was split into five smaller fragments (PCVATRDSCKPP, APACCDP, CASCQCRFFG, SACTCRVLNP and NC) containing C-terminal glycine or proline residues. The functional groups of the amino acid side chains were protected with benzyl type protecting groups which are stable under the reaction conditions applied both in the Boc- and in the Fmoc-techniques possessing the following advantages:

i.) The solubility of the Boc/benzyl protected peptides in organic solvents (e.g., DMF, NMP, DMSO, etc.) has been shown to be higher than that of the *t*-butyl type side chain protected peptides[5] giving rise to the minimization of the problems due to the low solubility of the peptides during fragment condensation;

ii.) The removal of N^{α}-Boc-protecting group from large peptides in neat trifluoroacetic acid (TFA) is more advantageous than that of N^{α}-Fmoc in piperidine-dimethyl formamide[6], since the acidolytic removal of the Boc group is less sensitive to steric factors. An additional advantage is that neat TFA is an excellent solvent for peptides. If necessary, scavengers can be added to capture the *t*-butyl cations. As a result, the formation of the deletion peptides caused by the incomplete removal of the N^{α}–protecting group is minimized.

In the synthesis of protected peptides the side chain protecting groups were as follows: Asn: trityl (Asn(Trt)), Asp: cyclohexyl (Asp(cHex)), Arg: *p*-toluenesulphonyl (Arg(Tos)), Cys: acetamido-methyl (Cys(Acm)), Gln:trityl (Gln(Trt)), Lys: 2-chloro-benzyloxycarbonyl (Lys(ClZ)), Ser: benzyl (Ser(Bzl)), Thr: benzyl (Thr(Bzl)).

The C-terminal dipeptide fragment was prepared in solution, while the N^{α}-Boc-protected fragments were synthesized on 2-chlorotrityl resin[7] by solid phase method using Fmoc-technique. The synthesis of the fragments was carried out using the standard Fmoc protocol on 2-chlorotrityl resin (nominal capacity: 1.2 mmol/g) prepared in our laboratory. The loading of the resin was adjusted to obtain 0.4-0.6 mmol/g initial capacity as determined by the Fmoc assay. The couplings were carried out by using 2 equivalents of amino acid derivative, 1.9 equivalents of O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) in 1-methyl-2-pyrrolidinone (NMP) or dimethylformamide (DMF), in the presence of N,N-diisopropylethylamine (DIEA). In cases when the Kaiser test was positive after 30 minutes, the resin was washed and coupled again until no free amino groups were detected. The Fmoc groups were cleaved by 30% piperidine in DMF. The use of 2-chlorotityl resin is advantegous in this synthetic approach since the loss of the peptide chain on this support at dipeptide phase is negligible even with C-terminal glycine or proline. The N-terminal amino acid of the fragments was incorporated in its N^{α}-Boc-protected form. The peptides were cleaved from the resin by using a mixture of dichloromethane-methanol-acetic acid 8:1:1, or dichloromethane-trifluoroethanol-acetic acid 8:1:1. The cleavage of the peptide-resin bond was complete within 2h. The fragments were purified by repeated precipitation from different solvent mixtures. Their purity was checked by RP-HPLC and TLC. The yield of the crude products was generally above 80%, with the yield of the purified peptides over 50% as calculated from the starting resin loadings. The purity of the fragments was higher than 95%, based on RP-HPLC trace. The protected fragments were characterized by amino acid analyses and FAB-MS measurements.

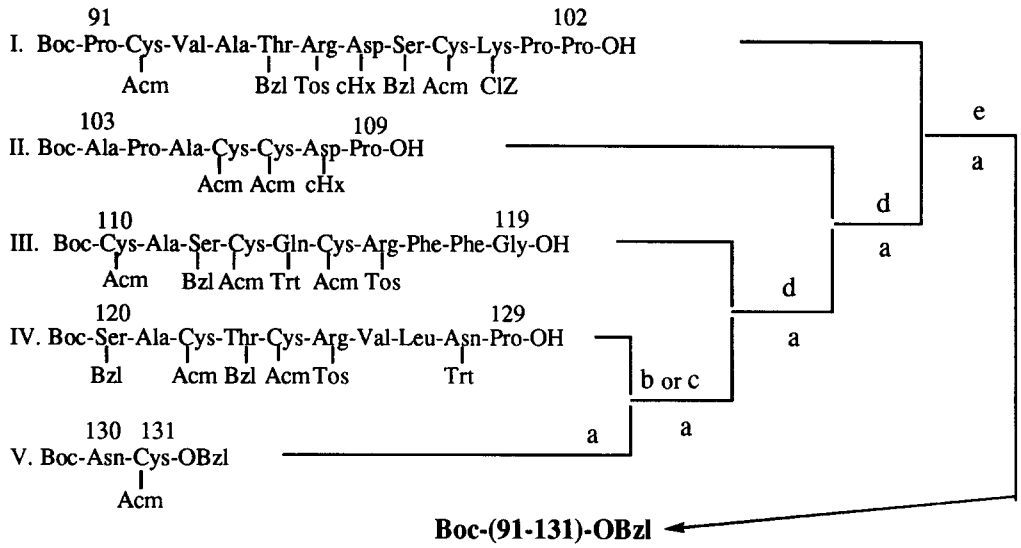
To find optimal conditions for the fragment condensations, the rate of the reaction and the purity of the product obtained from the coupling of fragment IV with fragment V were compared using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) as condensing agents, respectively. The yield and the purity of the products were indistinguishable, thus further on only the EDC-based, highly optimized method developed by Sakakibara and coworkers[8,9] was applied. The synthesis started from Fragment V and after the removal of the Boc group the fragments were coupled sequentially as illustrated in Scheme 1.

The cleavage of the N-terminal Boc group was performed in neat trifluoroacetic acid followed by the usual workup including the replacement of TFA with HCl in dioxane. In the couplings the carboxyl component was in 20% molar excess. The completeness of the couplings was monitored by fluorescamine test. If unreacted amino groups were detected after 1 day of coupling, another 20% molar excess of the N-terminal fragment and coupling reagents were added to drive the reaction to completion. Surprisingly, in the synthesis of Boc-agouti(103-131)-OBzl the reaction mixture contained unreacted amino groups even after adding 40% excess of the acylating component. In this case the reaction mixture was worked up and coupled again with 0.5 equivalents of the acylating component.

The excess of acylating carboxyl component and condensing reagents was removed by reprecipitations from appropriate solvent mixtures. The yields of fragment condensations were over 80% in each case as calculated on the amount of starting amino component used. The overall yield of the fragment condensations leading to the fully protected Boc-agouti(91-131)-OBzl was 43%.

All the protecting groups, except the cysteine S-acetamido groups (Acm), were removed by liquid HF (10% anisole, -5°C, 1h) allowing the purification of the 10-Acm-protected peptide without the danger of oxidation of the mercapto groups to disulfide bridge(s). The crude product was purified by Sephadex G-25 gel filtration followed by preparative RP-HPLC. The yield of the HF cleavage and purification was 50%.

The cystein Acm groups were removed by mercury(II) acetate in 50% acetic acid, followed by 2-mercaptoethanol treatment[10]. The crude SH-peptide was separated from the mercury(II) complexes by Sephadex G-25 column chromatography using 5% acetic acid eluent. The product was further purified on



Scheme I.

The synthesis scheme of the fully protected agouti protein (91-131) fragment. a: TFA; b: EDC/HOBt/DIEA, DMF; c: BOP/DIEA, DMF; d: EDC/HOBt/DIEA, DMF/DMSO; e: EDC/HOBt/DIEA, DMSO.

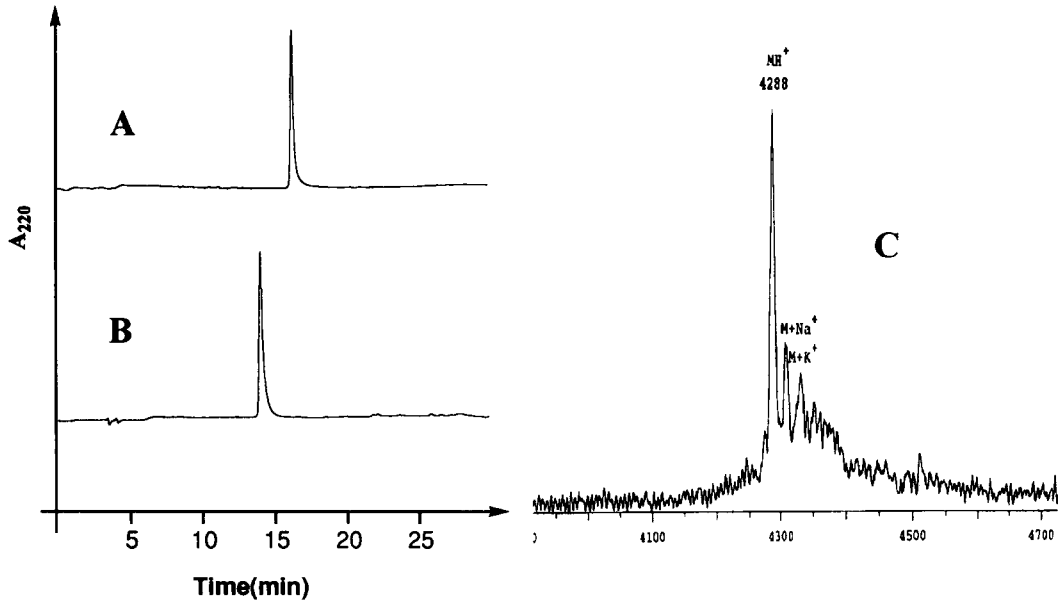


Figure 1.

***A and B**:HPLC profile of the cyclized peptide on Vydac 250 x 4.6 mm C-18, 300 Å column. A: linear gradient (components: 0.1% trifuoroacetic acid in water and 0.07% trifluoroacetic acid in acetonitrile) from 5 to 50% in 25 min. B: linear gradient (components: 1/15M pH=7.0 phosphate buffer in water:acetonitrile 9:1 and 4:6) from 0 to 80% in 25 min.*
C: The MALDI-TOF mass spectrum of the peptide (linear mode, matrix: sinapic acid).

preparative RP-HPLC after 2-mercaptoethanol reduction in pH 8.6 tris(hydroxymethyl)aminomethane hydrochloride (TRIS) buffer containing 5M guanidine hydrochloride to yield 52% chromatographically homogeneous reduced peptide.

The oxidative folding reaction of the agouti(91-131) was investigated in model experiments. Under more than 30 different reaction conditions, the effects of temperature, pH, organic solvents, different buffers, inorganic salts, redox (oxidized/reduced glutathion) and denaturating reagents were tested[11]. In experiments yielding a main component, the same main component was identified as characterized by its RP-HPLC behavior. The parameters of the optimal condition for constructing the five disulfide bonds to give the highest yield of the folded peptide were as follows: peptide concentration $1.2x10^{-5}$ M; 0.1M TRIS buffer pH 8.5; peptide/oxidized glutathion/reduced glutathion 1:10:100; 1M guanidine hydrochloride; 4°C; 2 days. After preparative RP-HPLC purification 16 mg (32%) of chromatographically homogeneous agouti(91-131) (Fig. 1 A,B) was obtained from 50 mg SH-peptide giving the expected molecular weight in MALDI-TOF (Fig. 1 C).

This peptide synthetic approach combines the speed of the solid phase peptide synthesis with the advantage of the classical solution phase peptide synthesis in purification of the intemediates. It is generally applicable for the synthesis of peptides containing amino acid side chain protecting groups stable under the conditions of both Fmoc and Boc methodology. With the recent wide selection of protection groups this criterion can be easily fulfilled and can be further optimized for different purposes.

This synthetic strategy can be extended to fragment condensation on solid support as well. In a preliminary experiment we have compared the solid phase and solution techniques using in both cases the same peptide fragments and methodology. The yield and the homogenity of the crude 10-Acm peptide amide prepared by the solid phase fragment condensation method was comparable to those of the linear peptide prepared in solution.

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