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SYNTHETIC PEPTIDE AMIDES BY DIRECT AMMONOLYSIS FROM THE POLYSTYRENE-DIVINYLBENZENE RESIN

W. Parr, C. Yang and G. Holzer

Department of Chemistry
University of Houston
Houston, Texas 77004

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Frequently, one desires to cleave a peptide as an amide from the Merrifield resin since many peptide hormones are C-terminal amidated. Two principle ways exist presently to do so. These involve:

(a) direct ammonolysis with metanolic ammonia, which has been applied for oxytocin and vasopressin and their analogs where glycine is the C-terminal amino acid (1-7).

(b) removal of the peptide by transesterification with an alcohol in the presence of trimethylamine and subsequent treatment of the ester with ammonia where more steric hindered amino acids such as valine, phenylalanine are present (8,9). The drawback of these two techniques is that neither method can be applied when the usual protecting esters (o-benzyl and o-tert.-butyl) for the side chain of the carboxyl groups of aspartic and glutamic acid are used. Under those conditions both will be partially converted directly into the amide when method (a) is employed and first transesterified and then converted into the amide when method (b) is used.

Bayer, Breitmaier, Jung and Parr (10) reported the synthesis of a new solid support which readily undergoes ammonolysis in dimethylformamide (DMF) with liquid ammonia even if amino acids are present, which might cause steric hindrance.

We varied this technique and applied it to the Merrifield resin and found a simple method for cleaving C-terminal amino acids from the chloromethylated resin as amides. In a general experiment, 1 g of the Boc-amino acid polymer, suspended in 50 ml DMF, was transferred into a pressure bottle, cooled down in one acetone-dry ice bath and 50 ml of dried liquid ammonia

(cooled to -70°C) were added. The bottle was immediately closed, transferred into a Parr-Medium-Pressure Hydrogenation Apparatus and shaken at room temperature for 5 days. The ammonia was allowed to escape through the relief-valve and last traces of ammonia were removed in vacuo. The crude product was purified by liquid chromatography over Al_2O_3 (neutral). The obtained yields are given in Table I.

TABLE I

Direct Ammonolysis With Various Boc-Amino Acids Polymers

Polymers	Cleavage Product[a]	Amount Cleaved[c]
Boc-L-Phe-Polymer	Boc-L-Phe-NH_2	70-75%
Boc-L-Pro-Polymer	Boc-L-Pro-NH_2	60-70%
Boc-L-Val-Polymer	Boc-L-Val-NH_2	60-70%
Boc-Gly-Polymer	Boc-Gly-NH_2	80-90%
Boc-O-Benzyl-L-Tyr-Polymer	Boc-O-Benzyl-L-Tyr-NH_2[b]	>90%

[a]Identified by TLC on silica gel (F 254 Merck; Solvents: Pyridine 70, methyl ethyl ketone 30, water 20, acetic acid 10) after removal of the Boc-group with trifluoroacetic acid by comparison with authentic samples.

[b]L-Tyr was identified as L-Tyr-NH_2 after removal of the Boc-and O-Benzyl-groups with HBr/CH_3-COOH.

[c]Average yield, obtained from three independent experiments.

Table I illustrates clearly that under the conditions specified the C-terminal amino acids of certain peptide hormones, e.g., secretin (valine), gastrin (phenylalanine), and thyrocalcitonin (proline) can be removed from the resin in high yields.

To test the applicability of this procedure for longer peptides the C-terminal hexapeptide amide of secretin L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine amide was selected because this peptide amide is very difficult to remove from the resin (11). Furthermore, this peptide has been characterized very well (10,11).

The synthesis was carried out with three grams of (Boc)-L-valine resin ester (0.39 m moles of valine/g.). The following steps were used to introduce each new amino acid: Cleavage of Boc-group with 1 N HCl in glacial acetic acid for 30 min., neutralization of hydrochloride with

10% triethylamine in DMF for 10 min., coupling of new Boc-amino acids (5 fold excess, reaction time 2 hrs.), using dicyclohexylcarbodiimide as condensing agent. L-glutamine was introduced as p-nitrophenyl ester of the Boc-amino acid (reaction time eight hours). After each step, excess reagents were removed by washing successively with methylene chloride, DMF, ethanol, glacial acetic acid, and DMF.

The fully protected hexapeptide resin was then transferred into a pressure bottle, and the cleavage in liquid NH_3 and DMF was carried out as described above. After shaking 5 days at room temperature, and after removal of ammonia, the resin was filtered and washed twice with DMF. DMF was evaporated in vacuo at 40°C. The Boc-group was cleaved by treating the crude product with trifluoroacetic acid for 30 min. Trifluoroacetic acid was then removed in vacuo and the residue was twice recrystallized from DMF-ether. Yield 482 mg, (64%); m.p. 250° (decomp.); Ref. 255-260°. The amino acid analysis after hydrolysis with 6 N HCl, 24 hrs. at 100°C gave the following molar ratios: Leu: 2.92; Glu: 1.04; Gly: 1.00; Val: 0.96; NH_3: 2.10; (Gly being taken as 1.00). The amide showed two spots (R_f = 0.68 and a trace at R_f = 0.42) by thin layer chromatography of the N-trifluoroacetate on silica gel (F 254 Merck, solvents pyridine 70: methyl ethyl ketone 30: water 20: acetic acid 10). The major spot with R_f = 0.68 was identified as secretin C-terminal hexapeptide amide by comparing R_f values with an authentic sample.

More important, however, is the synthesis of a C-terminal amidated peptide with a free carboxyl group in the side chain of aspartic and glutamic acid. Under the conditions specified, benzyl esters of glutamic and aspartic acid are partially converted into amides as shown by thin layer chromatography (12). Even free carboxyl groups which in general cannot readily be converted into amides show traces of the corresponding glutamine and asparagine (12). In both cases one obtains mixtures of peptides and peptide amides which cannot easily be separated and the final product shows impurities.

However, tert.-butyl ester provides ammonia resistant protection for aspartic and glutamic acid and does not undergo ammonolysis under the conditions specified. The tert.-butyl esters can readily be removed with trifluoroacetic acid after ammonolysis.

The disadvantage of this technique is that very labile protecting groups for the NH_2-groups have to be used. As an example, we have synthesized a scotophobin analog:

Ser-Asp-Asn-Asn-Gln-Gln-Gly-Lys-Ser-Ala-Gln-Gln-Gly-Gly-Tyr-NH_2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Using Boc-amino acids, the chain 15-3 was built up in the usual manner. Aspartic acid (position 2 in the above sequence) was coupled to the peptide chain as N-2-diphenyl isopropyloxycarbonyl-β-O-tert. butyl aspartate. The N-protecting group can be cleaved in the presence of tert. butyl ester group with acetic acid (13). The N-terminal amino acid serine was attached as Boc-o-benzyl-serine in the known manner. After cleavage of the peptide by direct ammonolysis as described above, a crude product was obtained, which after deprotection with HBr and purification by liquid and thin layer chromatography gave an overall yield of this about 50% pure pentadecaamide, which is identical to the natural product obtained from rat brain. Details about the synthesis and the bioassay will be published soon (14,15).

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