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Cleavage and Deprotection of Peptides from MBHA-resin with Bromotrimethylsilane

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Abstract: *A procedure was developed for the cleavage of thymosin α_1 from MBHA-resin with bromotrimethylsilane that would be pratctical for large scale manufacture of commercial quantities of the peptide.*

Since the introduction of solid phase peptide synthesis (SPPS) by Merrifield in 1963,[1] the technique has been used in the production of a large number of peptides in small and large quantities. The first stage of the technique consists of peptide chain assembly with protected amino acid derivatives on a polymeric support. The second stage of the technique is the cleavage of the peptide from the resin support with the concurrent cleavage of all side chain protecting groups to give the crude free peptide. In some cases, strategy is chosen that cleaves side chain protecting groups prior to or after the removal of the peptide from the solid support. The third stage of the process is purification of the crude cleaved product.

In Merrifield's first report, he employed a beaded form (200 to 400 mesh) of a nitrated chloromethylated divinylbenzene-styrene co-polymer as a solid phase support and N-carbobenzyloxy (Z) groups for alpha-amino acid protection. Z groups were removed with 10 % hydrogen bromide in acetic acid and the peptide was cleaved from the support by saponification. In subsequent reports by Merrifield and others following his approach,[2-5] divinylbenzene-styrene co-polymers were used that were chemically stable to chain assembly but labile to anhydrous mineral acid cleavage conditions. He employed for amino protection the N-t-butyloxycarbonyl (Boc) group and benzyl ester or ether protection for aspartic and glutamic acids or for serine and threonine. This approach is commonly termed Boc / benzyl chemistry as opposed to the Sheppard approach.[6] Sheppard's modifications employed 9-fluorenylmethyloxycarbonyl (FMOC) amino acid derivatives and peptide to support linkages cleavable with relatively mild acid reagents (trifluoroacetic acid) for peptide cleavage. The cleavage method that has predominated Boc / benzyl chemistry since introduction by Sakakibara in 1965[7] employs anhydrous liquid hydrogen fluoride (HF) as a cleavage reagent. This HF method gave products of higher purity and in greater yield. This method introduced an extremely hazardous operation into the peptide process, but received wide acceptance because of significant improvements in yield and purity. The HF method and other strong acidolytic cleavage procedures suffer from a common drawback that limits the process scale. This is decomposition of the cleaved peptide by the strongly acidic reaction media before the peptide product can be isolated from it. This problem can be adequately managed at small scale when small amounts of reagents are used and can be quickly removed, but for large scale processing the longer residence times that usually occur lead to reduced yields and less pure products. We present here the

results of an investigation into the use of an alternate reagent for the cleavage of peptide resin that is applicable to large scale processing.

The need to develop a process for the manufacture thymosin α_1 as a therapeutic agent prompted a review of literature for a cleavage strategy for SPPS technology. This review revealed a recent report by H. Yajima and co-workers[8] on the use of hard acid cleavage reagents. This report discussed the use of trifluoromethanesulfonic acid (TFMSA), its trimethylsilyl ester (TMSOTF) and bromotrimethylsilane (TMSBr) with thioanisole as a cation acceptor in trifluoroacetic acid (TFA) solution. After a careful review of this report, we decided to investigate the work with TMSBr because it appeared that this reagent was well suited for the thymosin α_1 peptide resin and of these three reagents TMSBr was reported to give the least rearrangement product of aspartic acid to the isomeric beta-derivative. This is an important consideration for thymosin α_1 synthesis since it contains three aspartic acid residues as well as a carboxyl terminal asparagine residue.

The thymosin α_1 peptide resin used in our experiments was prepared as described previously[9] through side chain attachment strategy[10] at the carboxyl terminal asparagine residue to a para-methylbenzhydrylamine resin with a substitution level of 0.6 milliequivalents per gram of the starting resin.[11] Protecting groups on the peptide resin for the hydroxyl functions were benzyl ethers (Bzl), the carboxylic acid functions were benzyl esters (OBzl) and the epsilon amino groups of lysine were 2-chlorobenzyloxycarbonyl (ClZ) groups. Its structure is Ac-Ser(Bzl)-Asp(OBzl)-Ala-Ala-Val-Asp(OBzl)-Thr(Bzl)-Ser(Bzl)-Ser(Bzl)-Glu(OBzl)-Ile-Thr(Bzl)-Thr(Bzl)-Lys(ClZ)-Asp(OBzl)-Leu-Lys(ClZ)-Glu(OBzl)-Lys(ClZ)-Lys(ClZ)-Glu(OBzl)-Val-Val-Glu(OBzl)-Glu(OBzl)-Ala-Glu(OBzl)-Asp(MBHA-Resin)-OBzl. All the protecting groups of this peptide resin could be easily cleaved by the TMSBr / thioanisole / TFA reagent. The cleavage of peptide from a MBHA resin with TMSBr had not been reported previously.

Our first experiment was designed to duplicate the reaction conditions given previously for TMSBr[8] using 200 mg of peptide resin in 10 mL of TFA at 0° C and treating with a solution of 4.7 mL of thioanisole and 5.3 mL of TMSBr in TFA. After 1 hr at 0°C, 100 mL of ethyl ether was added. The resultant solids were filtered after 1 hr and treated with a 1 M NH_4F solution adjusting the pH to 8.0 with 1 M NH_4OH. After 30 minutes at 0° C, the mixture was filtered. Analysis of the filtrate by reverse phase HPLC showed only trace amounts of product. From this result it was apparent that very little cleavage had occured. Reaction conditions and the procedure were modified with the goals of obtaining more complete cleavage and a more efficient work up procedure that could be adapted to large scale processing. These modifications were as follows. Neat TMSBr was added to a stirred mixture of the peptide resin in thioanisole and TFA at -10° C, followed by vacuum rotary evaporation of volatile reactants at 30° C, addition of ethyl acetate, filtration and extraction of the solids with 0.05 M NH_4OAc. The filtrate was analyzed by HPLC. The results are shown in figure 1 (A). On a larger scale, 25 gm of peptide resin and 82 mL of dry thioanisole in 312 mL TFA at -15 to -10° C were stirred during the dropwise addition of 62 mL of TMSBr followed by an additional 90 minutes stirring at ambient temperature. The solvents and reactants were evaporated rapidly at 30° C and the residue stirred with 1.8 L of ethyl acetate for 1.5 hours. The suspension was filtered through celite and the filter cake

stirred with 0.05 M ammonium acetate while adjusting to pH 7 with dilute ammonium hydroxide. After filtration, the filtrate was evaporated on a rotary evaporator to remove volatile organic material from the crude product solution. This solution was purified by ion-exchange on DEAE cellulose media (Matrex Cellufine A-500, Amicon) and preparative HPLC RP silica C18 (YMC AQ 10 micron). The fractions from the preparative HPLC were desalted by a Hamilton PRP-1 media and isolated by freeze-drying to yield 3.7 g of purified thymosin α_1 peptide, representing a yield of 30 % based on the titer of the starting MBHA resin. Amino acid analysis(110° C, 24 hr, 6 N HCl) Amino acid, found (theory): Asp 4.0 (4), Thr 2.80 (3), Ser 2.82 (3), Glu 6.04 (6), Ala 3.04 (3), Val 2.23 (3), Ile 0.98 (1), Leu 1.03 (1), Lys 3.97 (4). HPLC results are shown in Figure 1 (B). The mass spectra indicated the major component to be 3109.1(M+H)+. This result indicates a molecular weight of 3108.1 (theory 3108.3). These results are consistent for thymosin α_1 with a purity of 99 %.

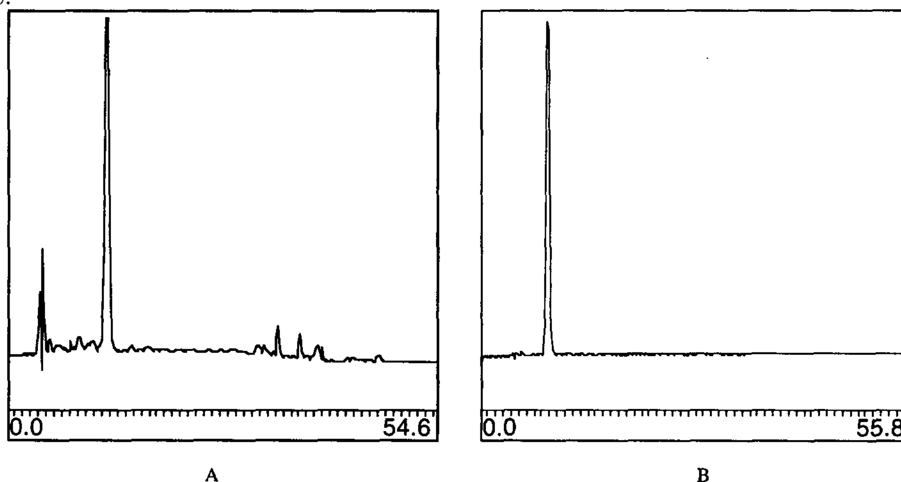

Fig. 1. HPLC chromatograms of crude peptide (A) and HPLC purified thymosin α_1 (B). Detection at 214 nm. Solvent A: 0.035 M potassium phosphate, pH 5.7. Solvent B: 40 % acetonitrile in 0.035 M potassium phosphate. Chromatogram A: isocratic 30 % B, 20 min, linear gradient 30 % B to 40 % B in 13 min, isocratic 40 % B. Chromatogram B: isocratic 30 % B. Column: YMC-Pack ODS-AMQ, 5 µm, 200 Å, 250 x 4.6 mm I. D.

TMSBr / thioanisole / TFA is an excellent reagent for the cleavage of thymosin α_1 peptide MBHA resin. The reagent does not appear to be scale limited since extending the residence time of the cleaved peptide product in the reaction mixture from 1 to 3 hours did not affect its quality.

We believe this reagent will be widely applicable in SPPS as well as solution phase technology. A side chain protection strategy, however, must be selected for the peptide chain assembly that is TMSBr / thioanisole / TFA cleavable (for example, mesitylenesulfonyl for protection for the guanidine side chain of arginine) and scavengers selected for the cleavage based on the amino acid residue content of the peptide

product. We will continue our work with this reagent to test its applicability to other peptide resins.

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