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Affinity Purification of Synthetic Peptides and Proteins on Porous Graphitised Carbon

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Abstract: The affinity of the Tbfmoc group for porous graphitised carbon has been exploited for the purification of synthetic peptides containing up to 85 amino acids. The hydrophobicity of the group has also been used to simplify peptide purification by HPLC.

Over the last three decades great progress has been made in solid phase peptide synthesis. This, combined with the widespread use of HPLC for peptide purification has resulted in the synthesis of peptides containing up to 20-30 amino acid residues becoming almost routine. In contrast, only a few proteins, e.g. HIV-1 protease[1] (99 residues) and ubiquitin[2] (76 residues), have succumbed to chemical synthesis and and crystallisation hence this area still represents a considerable challenge.

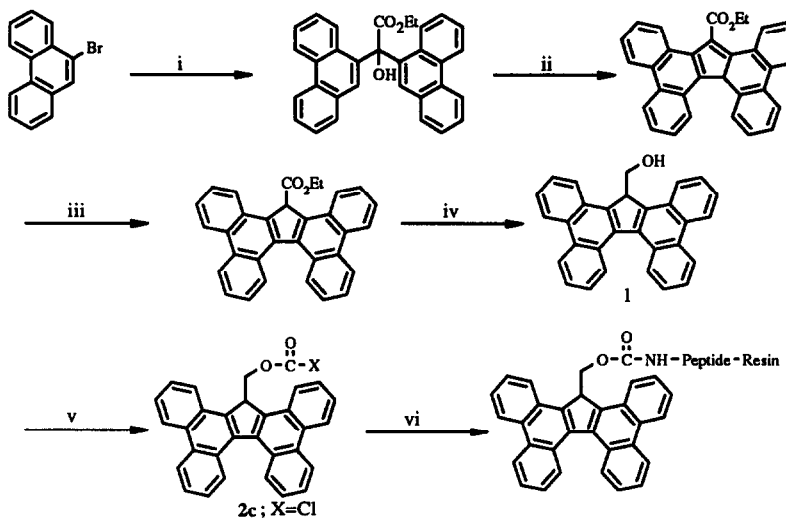
One of the main obstacles to the stepwise chemical synthesis of proteins is the difficulty in purification of the final product, due to the accumulation of truncated peptides on the resin. Truncated peptides are formed when the coupling of an amino acid fails to go to completion and the N^{α}-termini of these truncations are routinely capped with acetic anhydride to ensure they play no further part in synthesis. Several methods have been suggested for separating the desired peptide from the acetylated truncated peptides by derivatising the peptide N-terminus with a group capable of an affinity-type binding to a solid support. Examples include the binding of N^{α}–biotinylated peptides to immobilised avidin[3], N^{α}-dinitrophenyl (DNP) peptides to immobilised DNP antibody[4] and the reaction of an N^{α}-thiol group with an iodoacetamide resin[5].

Recently[6], we reported the potential of the base labile N^{α}-protecting group, tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxycarbonyl (Tbfmoc) for affinity purification of peptides on porous graphitised carbon (PGC)[7]. We now report an improved synthesis of the Tbfmoc precursor, tetrabenzo[*a,c,g,i*]fluorenyl-17-methanol (1) (Scheme 1), without recourse to chromatographic purification of intermediates, and the application of this methodology to the purification of peptides containing up to 85 amino acids

Previously, the Tbfmoc group had been introduced using Tbfmoc-Gly-OH as the N-terminal amino acid in solid phase peptide synthesis. Although a number of N^{α}-Tbfmoc amino acids have been prepared, it was thought to be more efficient if a single Tbfmoc reagent could be used to introduce the group directly onto the N-termini of resin-bound peptides. Model studies of the reaction of a resin-bound pentapeptide, Leu.Ile.Phe.Ala.Gly-Resin, with two tetrabenzo[*a,c,g,i*]fluorenyl-17-methyl mixed

carbonates (2a; X=O-nitrophenyl, 2b; X=O-pentafluorophenyl) were carried out. The extent of reaction was monitored by HPLC of the crude cleaved product and showed inefficient incorporation of the Tbfmoc group. However, better results were obtained using chloroformate (2c; X=Cl) in CH_2Cl_2 when virtually quantitative incorporation of the Tbfmoc group was observed by sonicating in the presence of diisopropylethylamine. A range of peptides of varying length (Table 1) were then synthesised by the Fmoc/t-Bu method of solid phase synthesis[8]. After reaction of the resin-bound peptides with chloroformate 2c, the Tbfmoc peptides were cleaved from the resin with TFA + scavengers (peptides 3, 4 and 6) or TFA/TMSBr[9] (peptide 5).

Scheme 1



i. Mg, THF; $(CO_2Et)_2$; ii. TFA/CH_2Cl_2; iii. Et_3N/CH_2Cl_2; iv. Diisobutylaluminium hydride/CH_2Cl_2; v. triphosgene/ N,N-dimethylaniline/CH_2Cl_2; vi. Peptide-Resin/diisopropylethylamine/CH_2Cl_2

Table 1: Peptides purified using Tbfmoc group

	Peptide	No. of Amino Acids
3	Hepatitis B surface antigen PreS1 (1-23) ayw[10]	23
4	Gastrin releasing peptide[11]	27
5	Bacteriophage λ Ral $(Acm)_4$[12]	66
6	MeCP2 methylated DNA binding domain (78-162)[13]	85

Table 2: Tbfmoc 3 deprotection on PGC in 70% CH_3CN

Deprotection Solution	Deprotection Time
1% piperidine	60 min
10% piperidine	10 min
10% triethylamine	>3 h
10% pyrrolidine	10 min

Initial trials were carried out on peptide 3, when a solution of the crude Tbfmoc peptide in 70% aqueous acetonitrile was applied to a short column of PGC (50-100 μm). HPLC of the eluent showed complete retention of the Tbfmoc peptide, whilst the acetylated truncated peptide impurities eluted. Deprotection of the Tbfmoc group and release of the purified peptide (Figure 1) was then effected by washing the column with 1% piperidine/70% aqueous acetonitrile until no further material eluted. A similar purification could also be achieved by simply adding PGC (either 7 μm or 50-100 μm) to a solution of the crude peptide in 70%

aqueous acetonitrile, whereby the Tbfmoc peptide is adsorbed onto the surface of the PGC and the truncated impurities remained in solution. Adsorption of the Tbfmoc peptide onto PGC was monitored by the uv absorbance of the Tbfmoc group at 364 nm. The supernatant (containing the impurities) was then removed and the purified peptide released from the PGC by suitable base treatment. Using this procedure, the base-deprotection of the adsorbed Tbfmoc peptide was studied more closely. Several deprotection mixtures were tried (Table 2) and the release of the peptide from the carbon was monitored by HPLC. Surprisingly, deprotection of the adsorbed Tbfmoc peptide was much slower than the same reaction in homogeneous solution which is complete within 1 minute in 1% piperidine. This is presumably due to steric hindrance of the acidic 17-H of the Tbfmoc group by the surface of the PGC. Consequently a piperidine concentration of at least 10% (v/v) is recommended for rapid deprotection of Tbfmoc peptides on PGC.

Having established the criteria for efficient deprotection of the Tbfmoc group, purification of larger peptides 4 and 6 on PGC was attempted. In the case of peptide 4, while a reasonably good purification of the peptide was achieved (Figure 2), variable recoveries of product were obtained. This situation became even worse for peptide 6, when, although preferential adsorption of the Tbfmoc peptide 6 occurred, subsequent treatment with piperidine/aqueous acetonitrile failed to release the purified peptide from the PGC. These problems were traced to poor solubility of peptides 4 and 6 in piperidine/aqueous acetonitrile mixtures. Alternative solvent systems were tried and some success was achieved using aqueous acetonitrile solutions containing urea or guanidine hydrochloride. However, best results were obtained with 10%piperidine/50% aqueous isopropanol which gave good recoveries of purified products. These results indicate that it is crucial to the success of this method to maintain conditions in which the peptide is soluble and recent experience with other synthetic proteins has shown 1:1 6M guanidine

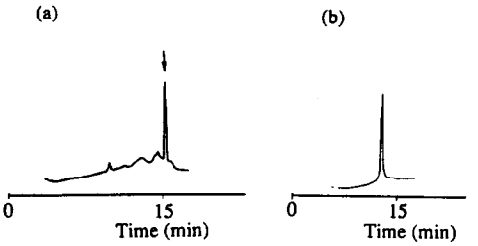


Figure 1: HPLC chromatograms[14] of 3: (a) crude Tbfmoc peptide; (b) after purification on PGC

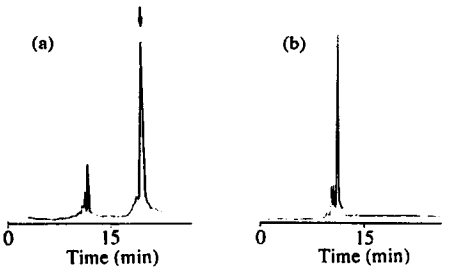


Figure 2: HPLC chromatograms[15] of 4: (a) crude Tbfmoc peptide; (b) after purification on PGC

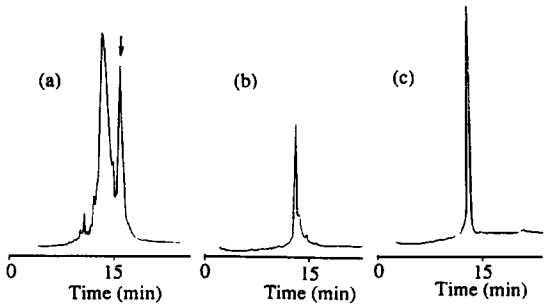


Figure 3: HPLC chromatograms[15] of 6: (a) crude Tbfmoc peptide; (b) after purification on PGC; (c) after purification by preparative HPLC

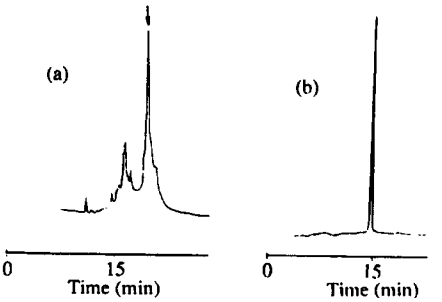


Figure 4: HPLC chromatograms[16] of 5: (a)crude Tbfmoc peptide; (b) after purification by preparative HPLC

hydrochloride/isopropanol to be highly effective in this respect.

The Tbfmoc group also allows the simplification of peptide purification by RP-HPLC. As illustrated in Figures 1-4, the highly hydrophobic Tbfmoc group caused the Tbfmoc peptides (marked with an arrow) to elute significantly later than the truncations. Unambiguous identification of the Tbfmoc peptides was further facilitated by the the specific uv absorbance of the Tbfmoc group at 364 nm and its fluorescence at 424 nm. The ease of purification by HPLC was exemplified by the purification of peptides **5** and **6**, when trivial separation of the Tbfmoc peptides from the impurities was possible. The Tbfmoc group was then removed by treatment with piperidine and after neutralisation of the solutions (acetic acid) and further preparative HPLC, peptides **5** and **6** were obtained in pure form (Figures 3(c) and 4).

In conclusion, introduction of the Tbfmoc group onto the N-termini of resin-bound peptides offers a simple, quick and highly effective method for purification of peptides and small proteins by HPLC or an affinity-type binding to PGC. No problems have been encountered due to the presence of difficult residues (e.g. Cys, Met), other than the requirement for Cys residues to remain protected during purification and air is excluded during purification of Met-containing peptides. Recent experience in our laboratory suggests that this methodology will be valuable for the initial stage in the purification of synthetic proteins *ca.*10 kDa molecular weight.

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14. Aquapore C_{18} (7 μm) column (4.6 x 100 mm), A = 0.1% aqueous TFA, B = 0.1% TFA/CH_3CN, flow rate 1 ml/min, linear gradient 10-90% B over 20 min, monitoring at 214 nm.
15. HPLC conditions as ref 13 with linear gradient 10-90% B over 30 min. The minor satellite peaks proved to be the two sulphoxides formed by aerial oxidation of **4**.
16. Vydac C_{18} (5 μm) column (4.6 x 250 mm), A = 0.1% aqueous TFA, B = 0.1% TFA/CH_3CN, flow rate 1 ml/min, linear gradient 10-90% B over 30 min, monitoring at 214 nm.

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