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A Novel Purification Protocol for Soluble Combinatorial Peptide Libraries

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Abstract: Four soluble peptide libraries, synthesised *via* a simple Multiple Peptide Synthesis method using automated SPPS, have been purified using tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl attached to the N^{α} terminus of the completed sequences The moiety imparts a number of useful properties on the library peptides which have been exploited to enable their separation from chemically similar impurities *via* an affinity chromatography technique.

An important application of combinatorial chemistry is in the production of soluble peptide libraries. Major impurities accumulated during Solid Phase Peptide Synthesis (SPPS)[1] are truncated peptide fragments (truncates) with acetylated N^{α} termini. These species, being of similar chemical nature to the product, can be difficult to eliminate during purification, however it is important to remove them from the libraries as they may interfere during biological screening.

The tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl (Tbfmoc) was designed to facilitate the separation of truncates, and other impurities accumulated during chemical peptide synthesis, from the desired product[2]. After removal of the Fmoc group, the Tbfmoc moiety is attached, *via* the chloroformate, to the free N^{α} of the completed peptide sequence, *see Fig. 1*. The truncates are not affected due to their acetylated N^{α} terminus. Purification is achieved by exploiting a number of properties, described in *Table 1*, which the Tbfmoc moiety imparts on the peptide.

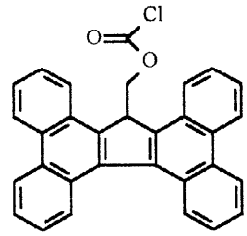

Figure 1. Tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl chloride.

Table 1. Properties imparted on the peptide by the Tbfmoc.

Property	Purification Advantage
Extremely high affinity for carbon.	Tbfmoc-peptides adsorb, truncates can washed out.
UV absorbance maximum at 364 nm	Can easily differentiate between Tbfmoc and non-Tbfmoc material.
Imparts different solubility characteristics on peptide.	Tbfmoc peptides can elute later on RP HPLC than non-Tbfmoc impurities.

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This methodology has been applied to the purification of single peptides/proteins and the following work describes its application to the purification of four randomly selected, soluble peptide libraries.

Library 1 (3 members)

H-DKEUV **Y** FUHLDIIW-OH
F
W

Library 2 (27 members)

H-D **P** NAY **S** G **E** LFDA-OH
G **T** **D**
D **Y** **F**

Library 3 (9 members)

H-DPN **A** YSGE **L** FDA-OH
G **I**
Abu **V**

Library 4 (9 members)

H-DAEFRHDSGYEV **H** HNKLVFFAEDVGSN **K** GAIIGLMVGGVV-OH
R **R**
S **A**

Figure 2. Four peptide libraries synthesised.

Diversity was achieved using automated Solid Phase Peptide Synthesis, employing the most simple method of Multiple Peptide Synthesis[3], whereby the synthesiser cartridges corresponding to the positions of diversity were loaded with equimolar quantities of the three appropriate amino acids.

Each library was synthesised on an ABI 430A automated synthesiser on an 0.25 mmol scale using the Fmoc orthogonal protecting strategy with Wang resin. Non-optimum conditions were applied to each synthesis using 1:1 DIC/HOBT with single coupling throughout in order to demonstrate the effectiveness of the Tbfmoc application to pure library synthesis.

This method could result in a non-equimolar concentration of each library member, but was selected due to its rapid, facile nature. The quantitative representation of each product was not important for demonstration of the efficiency of the Tbfmoc method, however the third library was re-synthesised using a modified split/pool technique[3] in order to achieve equimolar representation, hence affording a library suitable for screening. This was accomplished by dividing the resin into three equal portions, at the positions of diversity, and manually coupling each amino acid separately. Each library was cleaved using the standard technique of 95% TFA + scavengers.

Consideration of *fig. 3* shows the level of purification attainable using the Tbfmoc methodology by comparing the HPLC trace of crude library 3 (straight from cleavage, containing Tbfmoc), with that of the library after being subjected to Tbfmoc purification. The library peptides in the crude sample are clearly distinguishable from the truncates and other impurities due to the 364 nm absorbance of the Tbfmoc group (see hatched area in *fig. 3*).

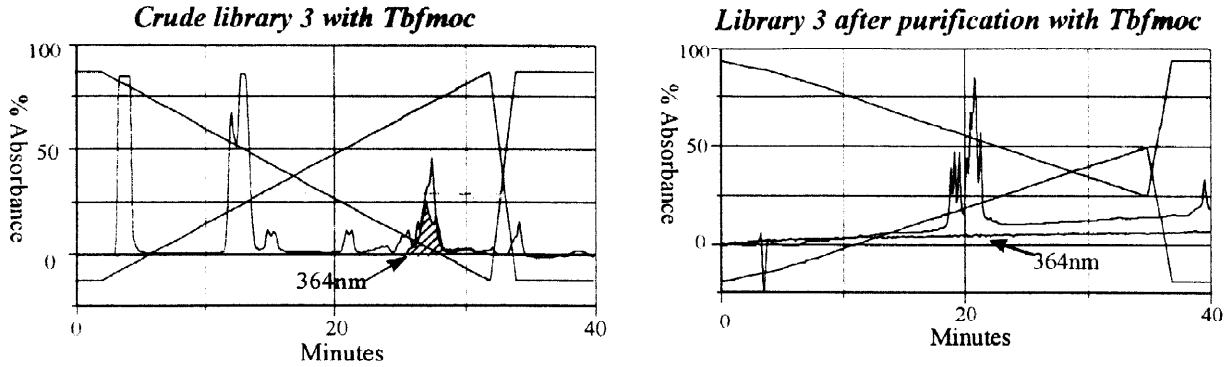

Figure 3. HPLC traces of Library 3 before and after purification *via* the Tbfmoc moiety. *With dual wavelength monitoring at 214nm and 364nm.*

The affinity of Tbfmoc for carbon was exploited to achieve purification of each of the libraries. Porous graphitised carbon (PGC)[4,5] was used to effect the purification of libraries 1 and 2.

The crude material, isolated from resin cleavage, was adsorbed onto PGC from a 1:1 6M guanidine hydrochloride/isopropanol solution by vortexing, leaving all non-Tbfmoc material, including truncates, in solution. Complete adsorption of 364 nm material was confirmed by HPLC, then the suspension centrifuged and the supernatant poured off. The PGC was washed repeatedly with the solvent system, until a flat baseline was attained on HPLC indicating all the impurities and truncates had been washed away. The libraries were then cleaved from the Tbfmoc moiety using 10% piperidine in the solvent system, leaving the remnant of the Tbfmoc group still adsorbed on the carbon. The piperidine supernatant, containing the library, was neutralised with glacial acetic acid and desalted by passing it down a G15 Sephadex gel column.

Each of the libraries discussed was analysed by mass spectrometry and amino acid analysis which confirmed and identified the presence of each library member. The MALDI ToF mass spectrometry of library 2 illustrates the diversity within the library is shown in *fig. 4*. Of the 27 members, 10 were of degenerate mass giving 17 mass variants (given in the table).

TABLE OF MASS DISTRIBUTION

Predicted Distribution	**Peak**/Value	Predicted distribution	**Peak**/Value
1285	**A**/1287.9	1361	**E**/1360.9
1299		1363	**F**/1364.3
1303	**B**/1303	1375	
1313		1379	**G**/1379.6
1317	**C**/1319.4	1393	**H**/1398.3
1331		1407	
1335	**D**/1334.3	1411	**I**/1413.4
1345		1425	**J**/1425.4
1349			

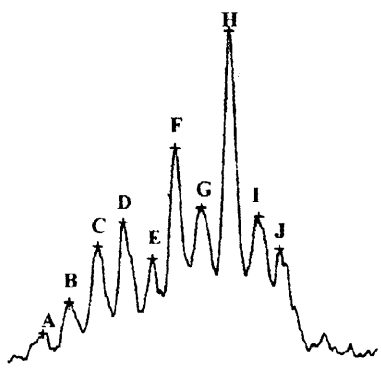

Figure 4. MALDI ToF mass spectrum of library 2.

An alternative form of carbon, chromatographic charcoal, was employed in the purification of libraries 3 and 4. The guanidine solvent system, used previously, was not ideal as it was difficult to eliminate. The purification was therefore modified to avoid its use by adsorbing the library onto the carbon directly from the cleavage solution.

The vortex/centrifuge technique was retained. A polar solvent system, suitable to wash the impurities from the carbon and cleave the library from the Tbfmoc, was selected by determining the solubility of the crude peptide material without Tbfmoc.

The charcoal required pre-treatment to remove undesirable material, by pre-washing with a 10% piperidine solution of the chosen solvent system until the supernatant remained clear and colourless. Finally it was rinsed with the system before use.

PGC was directly compared with charcoal, both using the revised procedure and charcoal was found to afford the better overall yield, 83.8% compared with 53.7% for PGC.

The synthesis of library 4, nine analogues of the biologically active 40 amino acid β-amyloid peptide including the natural sequence, was undertaken in order to demonstrate the ability of the Tbfmoc methodology to achieve facile purification of libraries of larger peptides. A more stringent coupling method, using a novel coupling agent[6], was employed in the synthesis since the overall coupling was poor (<30%). However Tbfmoc successfully separated the library members from the undesirable material rapidly and in good yield *via* charcoal purification. The HPLC, amino acid analysis and electrospray mass spectroscopy results confirmed that only the desired library members had been isolated.

In conclusion, Tbfmoc has been successfully used to facilitate the purification of four soluble peptide libraries. Purification was achieved by exploiting the affinity of Tbfmoc for carbon. The methodology affords rapid, facile separation of the desired peptides from their chemically similar impurities resulting in libraries containing only the requisite members.

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