Resin Effects in Solid-phase Peptide Synthesis. Enhanced Purity of Tryptophan-containing Peptides through Two-step Cleavage of Side Chain Protecting Groups and Peptide–Resin Linkage

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Alkylation of tryptophan residues during acidic cleavage of *tert*-butyl side chain protecting groups is reduced in the absence of thiol-scavenging reagents if the peptide remains attached to the solid support throughout the deprotection reaction.

In solid-phase peptide synthesis, it has been near universal practice to cleave the majority of side chain protecting groups and to detach the peptide from the resin support in a single step. Thus the Boc-benzyl system of Merrifield[1] utilises benzyl-based protecting groups for amino acid side chains and a similarly acid-labile benzyl ester attachment to the (polystyrene) resin. In the milder Fmoc-*tert*-butyl-polyamide system[2] and similarly conceived polystyrene-based variants, the more labile *tert*-butyl-based side chain protecting groups are combined with an equally reactive *p*-alkoxybenzyl ester peptide–resin linkage. We now show that this experimentally convenient practice may not always be advantageous in terms of product purity, and that the presence of the resin support may significantly affect the outcome of the side chain deprotection reaction.

Glp-Gly-Pro-Trp-Leu-Glu_5-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-X

1a; X = NH_2

b; X = Gly-OH

Y-Phe-OCH_2-C_6H_4-CO-Nle-Resin

2

Y-Gly-OCH_2-C_6H_4-OCH_2CO-Nle-Resin

3

Y-Gly-OCH_2-CH=CH-CO-β-Ala-Nle-Resin

4

Fmoc-Glu-Glu-Trp-Gly-OH

5

The 17 residue gastrin analogue **1a** contains a tyrosine and two tryptophan residues susceptible to attack by carbocations,

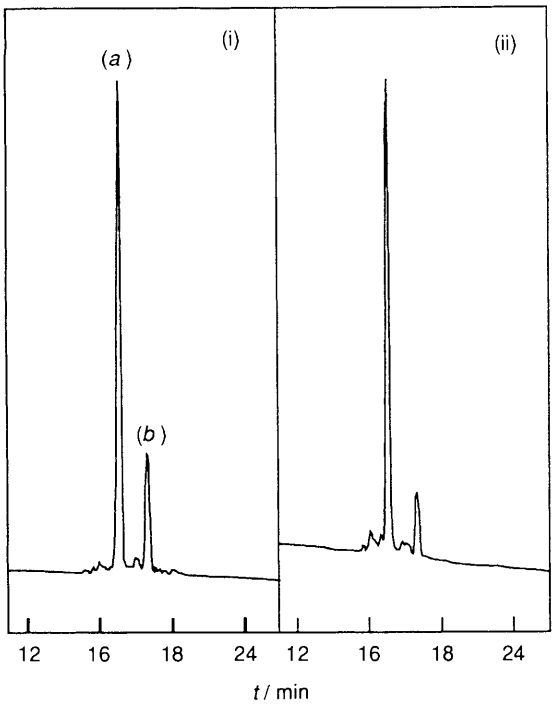

Fig. 1 HPLC profiles of crude gastrin octadecapeptide **1b** prepared (*i*) using the hydroxymethylphenoxyacetyl resin **3**, and (*ii*) using the hydroxycrotonyl resin **4**. Side chain protecting *tert*-butyl groups were cleaved using a dilute suspension of resin in 95% trifluoroacetic acid–phenol (see text). HPLC conditions: Aquapore RP 300 column. Buffer *A*, 0.1% aq. trifluoroacetic acid; *B*, 90% acetonitrile, 10% *A*; linear gradient 10–90% *B* over 25 min. In other experiments, peaks (*a*) and (*b*) were collected and identified by amino acid analysis and FAB-MS as the expected octadecapeptide **1b** [*m/z* 2138 (M + H), 2160 (M + Na^+)] and mono *tert*-butyl [*m/z* 2194 (M + H^+), 2216 (M + Na^+)] derivatives respectively.

Table 1 % of Mono-*tert*-butylated peptide in the trifluoroacetic acid deprotection of the hepta-*tert*-butyl peptide sequence **1b** linked to Kieselguhr-supported polydimethylacrylamide resin through esters of the allyl alcohol **4** or alkoxybenzyl alcohol **3** linkage agents[a]

	(*a*) 'Dilute'		(*b*) 'Concentrated'	
Scavenger (%)	Allyl ester **4**	Alkoxybenzyl ester **3**	Allyl ester **4**	Alkoxybenzyl ester **3**
1 None	17	24	26	40
2 Water (5)	9	17	24	40
3 Phenol (5)	13	20	18	30
4 Phenol (20)	9	13	—	—
5 EDT (5)	9	—	10	10
6 EDT (2.5), anisole (2.5)	11	10	12	10
7 EDT (10), anisole (10)	8	13	—	—

[a] All reactions were carried out for 60 min at room temp. EDT = ethanedithiol. Proportions of scavenger are v/v except for phenol (w/v). 'Dilute' experiments contained *ca.* 10 mg of peptide resin suspended in 1 ml of cleavage reagent. 'Concentrated' experiments were carried out in the minimum of reagent (*ca.* 60 μl) required to cover the same quantity of resin. Other experiments using multicomponent scavenger mixtures including ethanedithiol and thioanisole were also carried out but gave more complex product mixtures.[7]

Table 2 Preparation of sequence **5** on polydimethylacrylamide and polystyrene supports[a]

Resin	Linker	% Crude[b]	% Purified[c]
Polydimethylacrylamide	Alkoxybenyl ester **3**	78	83.5
Polydimethylacrylamide	Allyl ester **4**	94	92
Polystyrene	Allyl ester **4**	95	90

[a] Side chain *tert*-butyl groups were cleaved by treatment for 1 h with the minimum of 95% aq. trifluoroacetic acid to cover the resin. Values given are the percentage of total product in the main peak [b] before, and [c] after partial purification by anion exchange chromatography on Aquapore AX3000 resin.

and seven residues normally protected by *tert*-butyl groups during Fmoc–*tert*-butyl–polyamide solid-phase synthesis.[2] In syntheses of **1a** and other gastrin-related peptides described earlier,[3] we noted that remarkably little *tert*-butylation of the tryptophan indole rings occurred during acid-catalysed cleavage of the *tert*-butyl derivatives, even in the absence of good scavenging agents for *tert*-butyl cations. The peptide chain was attached to the insoluble polydimethylacrylamide resin through the acid-stable linkage agent shown in **2** and was later released by ammonolysis to generate the required heptadecapeptide *amide*.† In contrast, in a later unpublished synthesis of the octadecapeptide *acid* **1b** (an analogue of the presumed biogenetic precursor of human gastrin), substantial *tert*-butylation of indole residues occurred, and was a function of the 'concentration' of the insoluble peptide–resin suspended in the cleaving trifluoroacetic acid. This last synthesis utilised the normal acid-labile hydroxymethylphenoxyacetic acid linkage agent shown in **3**, so that in contrast to the foregoing, cleavage of side chain *tert*-butyl groups was accompanied by release of the free peptide into solution.

This partial 'protection' of the tryptophan residues by attached resin seemed likely to be generally important in solid-phase syntheses of tryptophan-containing peptides and has now been investigated in more detail.

The allyl ester peptide resin linkage[4] depicted in **4** provides a system allowing independent cleavage of side chain *tert*-butyl derivatives and the peptide–resin linkage in the preparation of peptide acids, analogous to that provided by **2** for peptide amides. It was formed by acylation of H-Nle-polydimethylacrylamide resin[2] (itself prepared from commercial Pepsyn K) first with Fmoc-β-alanine and then, after Fmoc-group cleavage, with the symmetrical anhydride of 4-bromocrotonic acid.[5] Reaction with the caesium salt of Fmoc-glycine gave the Fmoc-glycyl derivative **4** (Y = Fmoc), which was extended through 17 cycles‡ of continuous-flow peptide synthesis[2] to the gastrin sequence **1b**. Fmoc-glycyl polydimethylacrylamide resin **3** (Y = Fmoc) contained in a second column reactor attached to the same automatic synthesiser (LKB Biolynx) was extended simultaneously in the same reagent stream. The final peptide resins had amino acid compositions: (*a*) allyl ester linkage **4**: Asp, 1.01; Glu, 6.18; Gly, 2.92; Ala, 1.00; Leu, 1.97; Tyr, 0.98; Phe, 1.00; Pro, 0.94; Nle, 1.32; (*b*) hydroxymethylphenoxyacetyl ester linkage **3**: Asp, 1.00; Glu, 6.15; Gly, 2.95; Ala, 0.99; Leu, 1.98; Tyr, 0.98; Phe, 1.00; Pro, 0.94; Nle, 1.03.

The washed and dried peptide–resins were treated separ-

† In studies presented at a satellite meeting held during the 21st European Peptide Symposium in 1990, similar results using the same linkage agent were described by R. Cotton (ICI Pharmaceuticals Division, UK).

‡ Fmoc-amino acid pentafluorophenyl ester derivatives were used for the first 16 residues. Terminal pyroglutamic (Glp) residue was unprotected and added using (*O*-benzotriazol-1-yl)-tetramethyluronium tetrafluoroborate (TBTU).

ately with trifluoroacetic acid in the presence or absence of *tert*-butyl cation scavengers. HPLC analysis (Table 1) was performed directly on the crude peptide from resin **3** after evaporation of the trifluoroacetic acid and washing of the residue with ether; peptide resin from **4** was cleaved with tetrakistriphenylphosphinepalladium(0) in the presence of hydroxybenzotriazole.[6] Palladium derivatives and low molecular weight impurities were removed by chromatography on Sephadex G25 of the ether-washed aqueous extract. This procedure was shown not to remove any peptide-related constituents. HPLC profiles for crude octadecapeptide **1b** from the acid-labile alkoxybenzyl ester linkage **3** and the acid-stable allyl ester linkage **4** are illustrated in Fig. 1.

The protecting effect of the resin is clearly shown in entries 1–4 in Table 1 which cover experiments in which no or relatively weak scavengers (water, phenol) were used. The degree of *tert*-butylation is in all cases significant and becomes very substantial (40%) when the minimum volume of deprotecting reagent is used. Peptide linked to the resin through the acid-stable allyl ester **4** gave uniformly better results than that linked through the acid-labile alkoxybenzyl ester **3**. We ascribe these results to reduced concentration of reactive *tert*-butyl cations in the gel phase relative to free solution. In polydimethylacrylamide resin, the multiple tertiary amide groups will be partially protonated when permeated by trifluoroacetic acid, providing an energetically unfavourable environment for other positively charged species. Alternatively, following Lundt *et al.*[8] who postulate that *tert*-butylation occurs largely through intermediate *tert*-butyl trifluoroacetate, the acid-catalysed equilibration of *tert*-butyl trifluoroacetate and reactive *tert*-butyl cation will be displaced in favour of the former within the solvated gel phase.

The situation is markedly different when the deprotection is carried out in the presence of a powerful scavenger such as ethanedithiol (experiments 5–7). Formation of the *tert*-butylated peptide now occurs to a lower and essentially uniform degree regardless of peptide-resin linkage or resin 'concentration'. In this case, rapid and *irreversible* capture of *tert*-butyl cations presumably occurs.[8] The base level of about 10% *tert*-butylation (which is also observed in the earlier experiments) may conceivably arise by an intramolecular or similar direct transfer process, unaffected by the presence of scavengers or by solution or gel phase environment.

The foregoing results were confirmed and extended to the polystyrene series in a second series of experiments using the Fmoc-sequence **5**. More complex initial HPLC profiles were frequently observed with this simpler peptide although the degree of *tert*-butylation was apparently lower. For the allyl ester linked peptides, this is in part due to the difficulty in separating palladium-containing and other low molecular weight species from short peptides. Results from reversed phase HPLC analysis of crude and anion-exchange purified§ peptides are presented in Table 2. Higher yields of purer products were obtained using both polydimethylacrylamide and polystyrene resins linked to peptide through the allyl ester **4** rather than the alkoxybenzyl ester linkage **3**. In the polystyrene series, the beneficial effect of the resin environment during acidic de-*tert*-butylation was again observed. It may be due to the apolar environment provided by the neutral resin, or again to partial protonation.

We conclude that the conventional practice of choosing similarly labile side chain protecting groups and peptide–resin linkages is not always beneficial in solid-phase peptide synthesis. When such systems are used in the presence of susceptible amino acids (*e.g.* tryptophan, methionine, cysteine and tyrosine), scavengers which react rapidly and irreversibly with carbocations, *e.g.* ethanedithiol, are strongly indicated. On the other hand, prolonged treatment of tryptophan-containing peptides with ethanedithiol–trifluoroacetic acid mixtures can lead to substantial substitution of the mercaptan into the indole nucleus.[8] Remarkably, the incidence of this side reaction is, in our experience, *reduced* when the proportion of ethanedithiol in the cleavage mixture is *increased*; we presently favour a concentration of 20% EDT in trifluoroacetic acid. Complex mixtures of scavengers (*cf.* ref. 7) do not necessarily lead to purer products than ethanedithiol alone.

Received, 22nd August 1991; Com. 1/04412E

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§ Preliminary experiments showed that no separation of equally charged peptide derivatives occurred in this partial purification.