Comparative Investigation of the Cleavage Step in the Synthesis of Model Peptide Resins: Implications for N^{α}-9-Fluorenylmethyloxycarbonyl-Solid Phase Peptide Synthesis

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Based on our studies of the stability of model peptide-resin linkage in acid media, we previously proposed a rule for resin selection and a final cleavage protocol applicable to the N^{α}-*tert*-butyloxycarbonyl (Boc)-peptide synthesis strategy. We found that incorrect choices resulted in decreases in the final synthesis yield, which is highly dependent on the peptide sequence, of as high as 30%. The present paper continues along this line of research but examines the N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc)-synthesis strategy. The vasoactive peptide angiotensin II (AII, DRVYIHPF) and its [Gly^8]-AII analogue were selected as model peptide resins. Variations in parameters such as the type of spacer group (linker) between the peptide backbone and the resin, as well as in the final acid cleavage protocol, were evaluated. The same methodology employed for the Boc strategy was used in order to establish rules for selection of the most appropriate linker-resin conjugate or of the peptide cleavage method, depending on the sequence to be assembled. The results obtained after treatment with four cleavage solutions and with four types of linker groups indicate that, irrespective of the circumstance, it is not possible to achieve complete removal of the peptide chains from the resin. Moreover, the Phe-attaching peptide at the C-terminal yielded far less cleavage (50—60%) than that observed with the Gly-bearing sequences at the same position (70—90%). Lastly, the fastest cleavage occurred with reagent K acid treatment and when the peptide was attached to the Wang resin.

Key words peptide synthesis; peptidyl resin; cleavage; linker group

Since its inception,[1] the solid-phase peptide synthesis (SPPS) methodology has been systematically improved as the result of a wide variety of experimental investigations. These efforts have ranged from optimizing the coupling reaction itself (through the use of efficient acylating reagents, microwave irradiation and variations in temperature)[2—5] to broadening our knowledge of the complex peptide-resin solvation process.[6—9] Predictably, methods such as nuclear magnetic resonance[10,11] and Fourier transform infrared spectroscopy[12,13] have also been tested in attempts to further improve SPPS. In our case,[14—17] we pioneered the application of the electron paramagnetic resonance (EPR) technique, which is based on the use of a previously developed amino acid-type marker.[18,19]

All of these efforts have led to ongoing improvement of the SPPS method. Intriguingly, little attention has yet been given to the possibility that incomplete cleavage occurs or that there is premature removal of peptide chains from the solid support. Within this context, we previously proposed some rules for the selection of resins used in the N^{α}-*tert*-butyloxycarbonyl (Boc) chemistry. Our proposal was based on the stability of model peptide-resin linkages toward existing acid cleavage procedures,[20,21] as well as on the degree to which premature peptide chains are removed during trifluoroacetic acid (TFA)/Boc removal of peptide-resins. The results of other studies[22,23] have indicated the need for caution in selecting the type of resin to be used in the synthesis of peptide sequences containing C-terminal residue in either the α-carboxamide or the α-carboxyl function. In addition, appropriate final cleavage experiments, which are typically carried out either in anhydrous hydrogen fluoride[24] or in a trifluoromethanesulfonic acid/TFA/thioanisole cocktail,[25] should be performed, since a significant decrease in the amount of cleaved peptide can occur in this step. The type of the C-terminal residue and the length of the peptide sequence seem to affect the overall cleavage yield (which, surprisingly, can be as high as 30%) as a consequence of incomplete final chain removal accompanied by premature loss from the resin during TFA removal of peptide-resins.

The results of a comparison between benzhydrylamine-resin (BHAR)[26] and methylbenzhydrylamine-resin (MBHAR),[27] both used for the synthesis of α-carboxamide peptides in the Boc-chemistry, led us to conclude that the latter is the resin of choice mainly when the resin-bound amino acid is of the hydrophobic type. However, in the presence of a hydrophilic residue at the C-terminal position, the difference between the two aminated resins in terms of their efficiency depends on the length of the peptide. When the peptide sequence is longer, BHAR produces higher yields than does MBHAR. This is a consequence of the greater stability of the peptide-resin linkage of the former toward successive TFA treatments in each synthesis cycle.[20,21]

In the present study, we aimed to take a similar approach to establishing rules for the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc)-protecting group synthesis strategy.[28] Since it is impossible to occur premature peptide chain cleavage from the resin during the Fmoc group removal in piperidine/dimethylformamide (DMF) solution, other parameters were varied. The first was the cleavage capacity of different acid cocktails used routinely for the final peptide cleavage in

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the Fmoc-synthesis methodology. The second was the type of linker group used for separating the peptide chain from the resin matrix. These variations were therefore tested using two types of peptide sequences that differed in the hydrophobicity of their C-terminal amino acids. The vasoactive angiotensin II (AII, DRVYIHPF) and its [Gly^8]-AII analogue were deliberately synthesized using different linker groups attached to the solid support and designed for the synthesis of peptides containing carboxamide or carboxyl groups at their C-terminal position. Besides the Wang resin[29] that attaches a *p*-benzyloxybenzyl alcohol spacer to a polystyrene-type solid support and is used routinely for the synthesis of peptide acid, the other three tested resins were all characterized by containing different linker groups coupled to MBHAR support. Amongst these, the HMPA resin[30] uses the 4-hydroxymethylphenoxyacetic acid linker (also for the synthesis of peptide acids) whereas the Knorr[31] and Rink[32] resins attach 4-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)-methoxy-formamido]-2,4-dimethoxybenzyl-phenoxyacetic acid and 4-[(2′,4′-dimethoxyphenyl) Fmoc-aminomethyl] phenoxyacetamido groups, respectively. Among the known acid cocktails used for final cleavage, the following solutions were selected[28,33]: (**1**): TFA/water (9.5 : 0.5); (**2**): TFA/*p*-cresol/water (9 : 0.5 : 0.5); (**3**): TFA/ethanedithiol (EDT)/*p*-cresol/water (7.5 : 1.5 : 0.5 : 0.5); and (**4**): TFA/EDT/phenol/thioanisole/water (8.25 : 0.25 : 0.5 : 0.5 : 0.5, reagent K).

Table 1 compares the cleavage yields of AII and [Gly^8]-AII, each submitted to four types of linker resins and to the TFA cleavage solutions mentioned above. Based on the results of these experiments, several conclusions were drawn. First, according to previous results applied to Boc protocol,[20,21] or even to those obtained in the acid hydrolysis-related investigation of the peptide resins necessary for further amino acid analysis,[34] greater resistance to acid cleavage was observed with peptides containing Phe at their C-terminal extremities than with those containing Gly at the same location. The use of the former resulted in 50—60% peptide removal, compared with 80—90% for the latter. In addition, a higher yield was observed when solution 4 (reagent K) was used. Furthermore, faster removal of peptide chains occurred when those chains were bound to Wang-resin. Finally, even after 2 h of acid treatment, none of peptide resins presented complete cleavage of peptide chains from their respective solid supports. The mean purity of the cleaved peptides ranged from 70 to 80%, with molecular weights and amino acid compositions that were consistent with the theoretical values.

To determine the time course of the process of peptide chain cleavage in the most stable AII-resins, the acid treatment (with reagent K) was extended for up to 6 h at 25 °C (Fig. 1). Even after this long cleavage time, only the peptide-Wang support presented near total peptide removal from the resin. The cleavage values for the peptides attached to other supports (Rink or HMPA resins) did not surpass 70%. These findings suggest that considerable caution should be taken in the planning of cleavage procedures. A significant (20—30%) loss in the overall yield can occur during this step, depending on the type of peptide-resin pair and cleavage procedure. Finally, the AII-HMPA-resin was tested in order to evaluate the known high stability of the Arg 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) side-chain group in reagent K. Even after 1 h of treatment, approximately 12% of this protecting group remained attached to the peptide backbone, and was only completely removed after 2 h.

Table 1. Percentage of Peptide Cleavage from the Resin in Different Acid Solution (at 25 °C, for 2 h)

Peptidyl-resin	Yield of cleavage (%)			
	1[a)]	**2**[b)]	**3**[c)]	**4**[d)]
[Gly^8]AII-Wang-R	86	83	74	93
[Gly^8]AII-Rink-R	79	79	77	80
[Gly^8]AII-HMPA-R	79	70	68	84
[Gly^8]AII-Knorr-R	81	77	75	88
[Phe^8]AII-Wang-R	64	65	64	76
[Phe^8]AII-Rink-R	58	45	48	67
[Phe^8]AII-HMPA-R	49	46	47	60
[Phe^8]AII-Knorr-R	59	50	49	69

a) TFA/water (9.5 : 0.5); *b*) TFA/*p*-cresol/water (9 : 0.5 : 0.5); *c*) TFA/EDT/*p*-cresol/water (7.5 : 1.5 : 0.5 : 0.5) and *d*) TFA/EDT/phenol/thioanisole/water (8.25 : 0.25 : 0.5 : 0.5 : 0.5, reagent K).

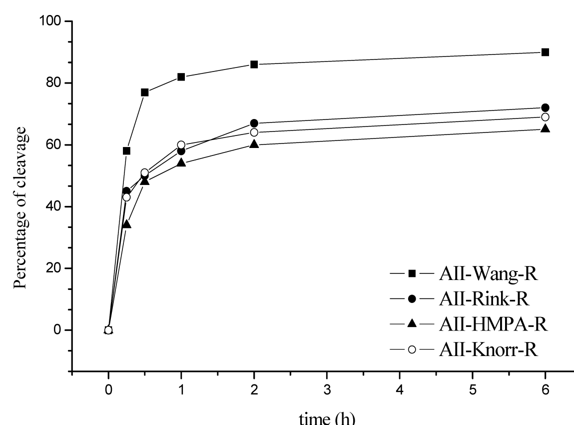

Fig. 1. Time-Course Study of AII Cleavage from Wang, Rink, Knorr and HMPA-Resins Using Reagent K, at 25 °C

Concerning the low cleavage yield of peptide sequences attaching hydrophobic residues at the C-terminal position, some recent experiments (not shown) has indicated hat the use of anhydrous HF or TFMSA/TFA/thioanisole treatments allow, regardless of the type of resin and linker group, a cleavage yield of about 95% for this type of peptide sequences. These findings thus suggest that these cleavage treatments, routinely applied only in Boc chemistry would be also applicable to overcome the mentioned chain removal shortcoming in the Fmoc synthesis strategy.

In conclusion, the present study revealed significant variation in the degree to which peptide chains were cleaved from the solid support in the Fmoc-peptide chemistry. Similarly to what had been observed for the Boc-synthesis strategy, the amount of peptide removed from the resin is strongly dependent upon the type of linker group, the cleavage solution and the type of C-terminal residue in the peptide sequence. Previous studies have demonstrated that crude peptide purity is dependent on the type of cleavage cocktail used.[28,35] As a complement, the present study demonstrated the critical influence of various factors affecting the overall synthesis yield, especially in terms of incomplete removal of peptide from the solid support, which has been ignored by some authors. These losses can be much greater than predicted but

can be avoided by establishing the appropriate combination of resin type, linker group and cleavage protocol used.

Experimental

All Fmoc amino acids were purchased from Advanced Chemtech (Louisville, KY, U.S.A.) or Bachem Inc. (Torrance, CA, U.S.A.). Solvents and reagents were acquired from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland).

Peptide Synthesis The peptides were synthesized manually according to Fmoc chemistry. The following side-chain protecting groups were used: *t*-butyl for Asp and Tyr residue; Pmc for Arg residue; and trityl for His residue. In each synthetic cycle, the N^{α}-Fmoc deprotection step was carried out in 20% piperidine/DMF for 20 min, followed by washings with dichloromethane (DCM) and DMF. The coupling reactions were performed with a three-fold excess of the acylating component diisopropylcarbodiimide/*N*-hydroxybenzotriazole in DMF/DCM (1 : 1). After approximately 2 h of coupling, the ninhydrin test was performed to estimate the completeness of the reaction. Cleavage from the resin and removal of the side-chain protecting groups were simultaneously with different acid cocktails as detailed below. After the cleavage procedure had been completed, the crude peptides were precipitated with anhydrous ethyl ether, separated from the soluble nonpeptide materials by centrifugation, extracted into 5% acetic acid in water and lyophilized.

Time-Course Cleavage Study In several small syringes, each equipped with a polypropylene filter, the cleavage solution was added to isolated portions (approximately 50 mg each) of protected peptide resins, stirred for 2 h at 25 °C and cleaved using solvents 1 through 4 (see text). After the cleavage reaction was complete, the resin was submitted to exhaustive washings with, consecutively, ethyl ether, DCM, methanol (MeOH), 10% acetic acid (AcOH)/water, water and MeOH to guarantee the removal of all cleaved peptides and other by-products of the reaction. After this treatment, small aliquots of each dried resin were hydrolyzed as previously reported[34] for further amino acid analysis. The calculated peptide content of the cleaved resin was compared to the value obtained for the initial peptide-resin pair, taken as 100%, and checked against the amount of cleaved peptide. To evaluate the purity of removed peptide, the cleaved peptide was isolated by precipitation with cold ethyl ether in the resin, further extracted with 10% AcOH/water and lyophilized.

Amino Acid Analysis As recently proposed,[34] prior to cleavage, all peptide-resin pairs were hydrolyzed with a mixture of 12 N HCl/propionic acid for 100 h at 130 °C to guarantee quantitative removal of peptide chains from the resin. Pyrex tubes with plastic Teflon-coated screw caps (13×1 cm) were used for the hydrolyses, and the amino acid analyses were performed in a Biochrom 20 plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) to determine the amount of peptide attached to the resin.

Analytical RP-HPLC The RP-HPLC analyses were carried out in TFA/acetonitrile gradient using a Waters Associates HPLC system consisting of two 510 HPLC pumps, automated gradient controller, Rheodyne manual injector, 486 UV detector and 746 data module (Waters, Eschborn, Germany). We used Solvent A (0.1% TFA/H_2O) and Solvent B (60% acetonitrile/0.1% TFA/H_2O with a gradient of 5—95% in 30 min) at a flow rate of 1.5 ml/min. A C_{18} column (0.46×25 cm, 5 μm particle size and 300 Å pore size; Vydac, Hesperia, CA, U.S.A.) was employed. Detection was at λ= 210 nm.

Liquid Chromatography/Mass Spectrometry The crude lyophilized peptides were analyzed on a system composed of a Micromass Platform LCZ Mass Spectrometer (Micromass, Manchester, U.K.), a Waters Alliance HPLC, a Waters 996 Photodiode Array detector, and a Compaq Workstation. The peptides were loaded onto a Waters Nova-Pak C_{18} reverse-phase HPLC column (2.1×150 mm, 3.5 μm particle size and 60 Å pore size), using Solvents A (0.1% TFA/H_2O) and B (0.1% TFA in CH_3CN/H_2O) at a flow rate of 0.4 ml/min, detection at 210 nm and a mass range of 500—3930 Daltons.

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