Resin Selection Based on the Lability of Peptidyl-Resin Linkage towards HF and TFA Steps: Dependence on the C-Terminal Amino Acid and Peptide Length[1)]

Guita N. JUBILUT,[a] Maria Terêsa MIRANDA,[b] Mineko TOMINAGA,[a] Yoshio OKADA,[c] Antonio MIRANDA,[a] and Clóvis R. NAKAIE*,[a]

Department of Biophysics, Universidade Federal de São Paulo,[a] Rua 3 de Maio 100, CEP 04044–020, SP, Brazil, Department of Biochemistry, Chemistry Institute, Universidade de São Paulo,[b] Av. Prof. Lineu Prestes 748, CEP 05508–900, SP, Brazil, and Faculty of Pharmaceutical Sciences, Kobe Gakuin University,[c] Nishi-ku, Kobe 651–2180, Japan. Received May 26, 1999; accepted July 22, 1999

Ideally, the solid support used for *tert*-butyloxycarbonyl (Boc)-peptide synthesis method must allow sufficient stability of the peptide linkage towards TFA-α-amino deprotection but adequate lability to final HF cleavage. Due to these conflicting characteristics, the choice of the correct resin for peptide synthesis is complex and dependent upon many factors. Aiming to clarify this issue, a time-course study of the trifluoroacetic acid (TFA) and HF steps using model peptidyl-resins was developed. The peptidyl-resin bond stability was strongly dependent upon the resin and the carboxy-terminus residue. The decreasing order of acid stability for resins was: benzhydrylamine-resin (BHAR)>*p*-methylbenzhydrylamine-resin (MBHAR)≅4-(oxymethyl)-phenylacetamidomethyl-resin (PAMR)>chloromethyl-resin (CMR) and Phe>Gly≅His≅Asp for C-terminal amino acids. HF-cleavage times of near 6 h (BHAR) and 2—3 h (MBHAR and PAMR) were necessary for quantitative cleavage of hydrophobic Phe residue-containing sequence at its C-terminal portion. When premature chain loss in TFA and incomplete cleavage in HF values were both quantitatively considered, a significant decrease in the overall yield (up to 35%) was observed in some resins. Moreover, MBHAR was more suitable than BHAR only when the peptide C-terminal residue is hydrophobic. The data also allow the prediction that due to more significant chain loss in TFA when MBHAR is used, BHAR will be the resin of choice for much longer than 40-mer peptide sequences containing C-terminal hydrophilic residues. Otherwise PAMR is the best resin for the synthesis of free carboxyl peptides but significantly low HF cleavage was observed when the C-terminal amino acid is of the hydrophobic-type.

Key words peptide synthesis; peptidyl-resin cleavage; benzhydrylamine-resin; methylbenzhydrylamine-resin

It is well known that, despite almost four decades having passed since its inception, Merrifield's solid phase peptide synthesis technique[2—5)] still presents limitations that affect the overall yield of synthesis. In addition to various side reactions which may occur in different steps of the synthesis cycle there are, for instance, problems of incomplete α-amine deprotection[6)] and couplings.[7)] In this regard, more attention has been given to the latter where an intense search for more efficient acylating reagents[8)] as well as conditions for improved resin solvation[9,10)] has been observed. In this context, we have examined, either by microscopic measurement of bead sizes[11)] or by electron paramagnetic resonance spectroscopy and using an amino acid-type spin probe,[12)] the motion of peptide chains of model peptidyl-resins within the solvated polymer matrix.[13)] The importance of the knowledge on resin solvation has been recently confirmed by improving difficult coupling reactions during the synthesis of a long and hydrophobic transmembrane receptor segment.[14)]

Otherwise, although routinely neglected, a significant decrease in the overall yield also may occur in some circumstances, when incomplete HF cleavage and premature detachment of the peptide from the resin in the *tert*-butyloxycarbonyl (Boc)-chemistry strategy in the trifluoroacetic acid (TFA)-α-amine deprotection reaction step are considered. These effects depend basically upon the lability of the peptide-resin linkage which in turn, is affected by the resin itself, the C-terminal amino acid and how often this chemical bond is submitted to TFA treatment during peptide growth. In short, a clearer rule for the choice of the best resin must exist dependent upon the type of the peptide fragment to be synthesized.

The use of methylbenzhydrylamine-resin (MBHAR)[15)] instead of benzhydrylamine-resin (BHAR),[16)] both designed for the synthesis of α-carboxamide peptides, has already been advocated to overcome incomplete HF cleavage with the former resin, mainly when hydrophobic amino acids such as Phe or Val are at the peptide C-terminal position. On the other hand, as emphasized in the same study, care should be taken regarding the use of MBHAR as the higher lability of peptide link to this amine resin may induce premature detachment of peptide chain during TFA deprotection. Obviously, this shortcoming in the use of MBHAR will be much more significant if a long sequence containing hydrophilic C-terminal amino acids is to be synthesized.

Similarly, to avoid peptide chain loss during the TFA steps during free α-carboxy terminal peptide synthesis, the 4-(oxymethyl)-phenylacetamidomethyl spacer containing-resin (Pam-resin or PAMR) was introduced[17,18)] as a replacement for the classical chloromethyl-resin (CMR).[2,3)] The ester linking to the latter is reported to be slightly labile to TFA deprotection and the comparatively higher stability of the peptide-PAMR linkage is due to the electron withdrawing effect of the acetamido group in the *para* position of the phenyl ring to which the peptide is attached. However, the significant acid stability of the peptide-PAMR linkage may otherwise induce incomplete HF cleavage as observed for BHAR, mainly when a bulky and hydrophobic residue is attached to the resin.

* To whom correspondence should be addressed.

In summary, despite all this previous knowledge, there are still questions left open regarding the proposition of a more clear resin selection rule for Boc-based peptide synthesis. This paper reports data found in the time-course study of HF and TFA treatments of peptidyl-BHAR, -MBHAR, -PAMR and -CMR. For this, the vasoactive peptide angiotensin II (DRVYIHPF, AII) bearing different types of amino acids at its C-terminal position (Phe, Gly, His and Asp) was assembled in these resins and further taken as models for quantitative acid stability evaluation.

Table 1 shows the results obtained in the time-course cleavage of selected peptidyl-resins towards a standard HF cleavage protocol.[19] Owing to the possibility in presenting undesirable side reactions when synthesized in CMR,[2,3] the His- and Asp-containing AII at the C-terminus were not used to compare this resin with the PAMR. The comparison between these resins was therefore based solely on Gly^8- and Phe^8-containing peptides. As expected,[15,17,18] the nature of the polymeric support affected the rate of cleavage of the peptidyl-resin linkage. The observed decreasing order of stability towards HF was BHAR>MBHAR≅PAMR>CMR. These data were consistent with those found in our previous study[20] that investigated the stability of model aminoacyl-resin bonds towards the classical 12 N HCl/propionic acid hydrolysis procedure (1 : 1, v/v, at 130 °C). In this study we have demonstrated that in contrast to the proposed standard protocol,[21] a much longer hydrolysis time (around 70 h) was necessary if complete acidolysis was desired, regardless of the resin-bound group. Therefore, this hydrolysis protocol followed by amino acid analysis was applied in the present study to quantify the exact amount of peptide still attached to the polymer after HF or TFA treatments. The data shown in Table 1 also confirm the dependence of cleavage yield on the nature of the AII C-terminal residue.[15,22] The decreasing order of stability in HF of resin-bound amino acids was Phe>Gly≅His≅Asp.

Table 1. Percentage of HF Cleavage (at 0 °C) of Model Peptidyl-Resins

Peptidyl-resin	Time (h)					
	0.5	1	2	3	4	6
[Phe^8]AII-BHAR	—	67	—	88	94	99
[Phe^8]AII-MBHAR	—	86	—	98	—	—
[Phe^8]AII-PAMR	—	85	—	98	—	—
[Phe^8]AII-CMR	—	96	—	100	—	—
[Gly^8]AII-BHAR	84	90	93	99		—
[Gly^8]AII-MBHAR	91	95	99	—	—	—
[Gly^8]AII-PAMR	89	95	98	—	—	—
[Gly^8]AII-CMR	93	98	100	—	—	—
[His(N^{im}-Tos)^8]AII-BHAR	88	94	99	—	—	—
[His(N^{im}-Tos)^8]AII-MBHAR	94	98	—	—	—	—
[Asp(β-OcHex)^8]AII-BHAR	88	95	99	—	—	—
[Asp(β-OcHex)^8]AII-MBHAR	95	98	—	—	—	—

Interestingly, an almost 6 h HF treatment was necessary to attain complete [Phe]^8-AII cleavage from BHAR (Table 1). When MBHAR or PAMR were used, 2—3 h HF cleavage is needed to allow complete [Phe]^8-AII recovery from the resin. In contrast, for the more hydrophilic C-terminal Gly, His or Asp-containing AII analogues, the HF time-course results showed that, except for BHAR, the other resins allowed quantitative peptide detachment in close to 1—2 h HF reaction. It should be noted that, although a HF reaction time not longer than 1—2 h has been recommended in order to minimize some side reactions,[2,3] this problem was not observed with the AII-type sequence. Figure 1 shows the analytical HPLC profiles of crude [Gly]^8-AII sequence after 1, 4 and 6 h HF cleavage at 0 °C revealing that no relevant decrease in the purity of peptide was observed as a consequence of longer HF treatments.

Table 2 shows the 1 to 20 h TFA time-course results for the four peptidyl-resins thus simulating TFA-exposure of up to 40 amino acid long sequences. Exactly the same order of peptidyl-resin bond lability related either to the type of resin or to the C-terminal amino acid measured in HF (Table 1) or during peptidyl-resin hydrolysis,[20] was observed in this TFA treatment. The most substantial peptide loss was observed for [Gly]^8-AII after 20 h TFA-exposure when bound to CMR (22%), whereas only 5% chain removal was quantified for the more stable [Phe]^8-AII-BHAR.

Surprisingly, the resin acidolysis data shown in Table 2 regarding PAMR and CMR seemed not to agree completely with the already known almost 100-fold increase in acid stability of the peptide-resin bond comparing PAMR with the

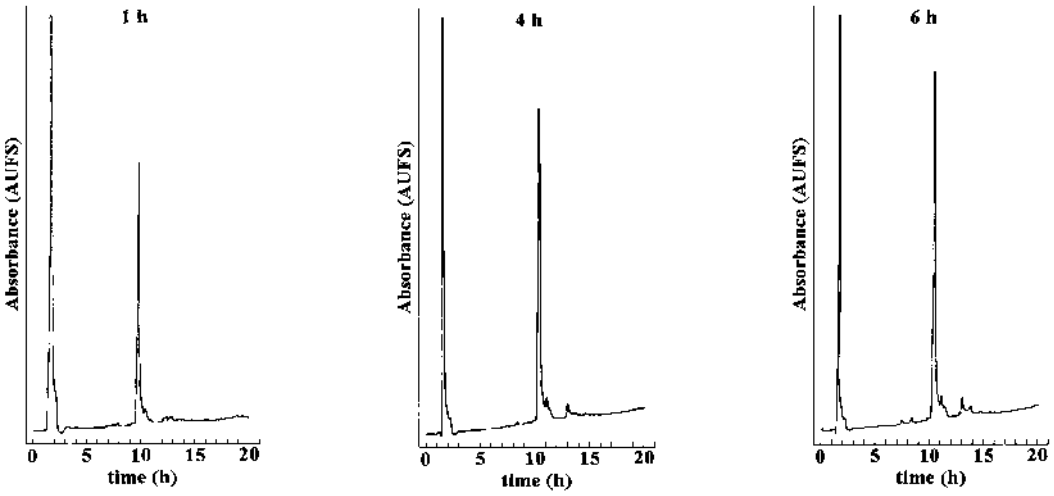

Fig 1. HPLC Profiles of the [Gly^8]AII-MBHAR after 1, 4, 6 h HF Treatment at 0 °C

CMR resin.[17,18] There are no clear explanations yet for this discrepancy although some differences in experimental protocols between these early reports and the present study must be emphasized. Besides the use of 30% instead of 50% TFA/dichloromethane (DCM) solution for deprotection, the peptidyl-resins in our TFA treatment investigation, contrary to the earlier studies, were washed and rinsed as much as possible with newly prepared TFA solution in order to better resemble mixing-washing processes that the resin beads are submitted to during each TFA step in the synthesis cycle. Moreover, batches of both PAMR and CMR acquired from different commercial sources were deliberately used in this study in order to avoid extraneous interference in this investigation which might occur depending upon the synthesis protocol of each manufacturer. Although clearly more stable when bound to PAMR than to CMR, an additional indication of a not so drastic difference in the peptide-resin linkage stability between these two resins was also noticed in the HF time-course study already discussed and shown in Table 1.

In summary, to clarify the selection rule for resin depending upon the nature and length of peptide sequence to be synthesized, the yields of both HF and TFA treatments data shown in Tables 1 and 2 were simultaneously considered. This was done by multiplying the yield of 1 h HF standard reaction of each peptidyl-resin with the yields, for instance, of 8 and 20 h TFA-exposures. This strategy allows therefore the prediction of the final yield simulating the synthesis of 16 and 40 amino acid long peptides, respectively. The corresponding theoretical yields found for these syntheses are summarized in Table 3 and allow the following comments: a) due to strong stability towards HF cleavage, the lowest yield was observed for peptidyl-BHAR bearing the hydrophobic Phe residue at C-terminus. In this case, the low final yield calculated after considering the loss in both acid steps was near 65%, regardless of the peptide chain length; b) to compare correctly the resins employed for the synthesis of α-free-carboxyl-peptides (PAMR and CMR), we have to be aware of the well-known problems which arise when CMR is used.[2,3,23] Peptides with C-terminal amino acids containing functional groups which undergo alkylation easily such as His, Cys, Met or side chains prone to ester interchange such as Asp or Glu can not be synthesized using CMR. Thus the use of PAMR is clearly indicated for the synthesis of these sequences. However care should be taken in the case of sequences containing C-terminal hydrophobic residues when assembled in PAMR. In this case, almost 20% of the peptide chains still remain attached to the resin after 1 h standard HF cleavage protocol (Table 3); c) by comparing the two amino polymers used for α-carboxamide peptide synthesis, MBHAR seemed to be more suitable than BHAR only when the C-terminal residue is of the hydrophobic-type. Contrariwise, when a hydrophilic amino acid is attached to the resin, they are equivalent. Moreover, the comparative yields shown in Table 3 also allow the prediction that BHAR might be more appropriate than MBHAR for the synthesis of peptide sequences containing hydrophilic amino acids at the carboxy-terminus portion and with a peptide length much longer than 40-residues.

Taken together, we believe that the present findings based on a quantitative acid stability study of model peptidyl-resins in HF and TFA may guide the correct selection of resin. In addition, these data reveal that in contrast to general belief, a significant decrease in the overall synthesis yield may occur during these often neglected acid steps. Alternative polymers characterized by more appropriate acid lability properties seem to be still awaiting their introduction into the Boc-chemistry peptide synthesis field.

Table 2. Percentage of Chain Loss of Model Peptidyl-Resins in 30% TFA/DCM

Peptidyl-resin	Time (h)					
	1	4	8	12	16	20
[Phe^{8}]AII-BHAR	0	1	3	4	5	5
[Phe^{8}]AII-MBHAR	0	1	3	5	7	9
[Phe^{8}]AII-PAMR	0	1	4	5	7	9
[Phe^{8}]AII-CMR	0	1	4	6	10	15
[Gly^{8}]AII-BHAR	0	0	3	4	5	8
[Gly^{8}]AII-MBHAR	0	0	4	6	9	14
[Gly^{8}]AII-PAMR	0	0	4	7	10	12
[Gly^{8}]AII-CMR	0	2	6	9	14	22
[His(N^{im}-Tos)^{8}]AII-BHAR	0	1	3	5	6	8
[His(N^{im}-Tos)^{8}]AII-MBHAR	0	2	5	8	11	16
[Asp(β-OcHex)^{8}]AII-BHAR	0	1	4	5	7	9
[Asp(β-OcHex)^{8}]AII-MBHAR	0	2	5	7	11	17

Experimental

Most of the solvents and reagents were purchased from Fluka or Aldrich and all met ACS standards. Trifluoroacetic acid was acquired from Fluka and the anhydrous hydrogen fluoride (5 liters capacity cylinder) was from Merck Co.

Peptide Synthesis Stepwise build-up of the peptides was done manually by Boc-chemistry solid phase methodology. BHAR, MBHAR, PAMR and Merrifield's CMR with substitution degree ranging from 0.2 to 0.6 mmol/g were used. These resins were acquired from different companies (Advanced

Table 3. Theoretical Synthesis Yield of Model Peptidyl-Resins Considering the TFA and HF Treatments[a]

	Theoretical yield (%)							
	BHAR[b]		MBHAR[b]		PAMR[b]		CMR[b]	
	16[c]	40[c]	16	40	16	40	16	40
C-Terminal residue								
Phe	65	64	83	78	82	77	92	82
Gly	87	85	91	82	91	84	92	76
His	91	86	93	82	—	—	—	—
Asp	92	87	94	81	—	—	—	—

a) No other source of error or side reactions were considered in this study, other than derived from partial HF or TFA peptide chain cleavage. *b*) Resins. *c*) Number of residues.

Chemtech, Novabiochem, Peninsula and Bachem). The N^{α}-*tert*-butyloxycarbonyl (Boc) protecting group was removed with 30% TFA in DCM in the presence of 2% anisole for 30 min. The following side chain protecting groups were used: *p*-toluenesulfonyl (Tos) for Arg and His (in the form of the dicyclohexylamine salt), 2-bromobenzyloxycarbonyl (2-BrZ) for Tyr and cyclohexyl group (cHex) for Asp. Amine group neutralization was performed for 1×1 min and 1×10 min with 10% triethylamine (TEA). Coupling reactions were done using 2.5 excess of Boc-amino acid/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/diisopropylethyl-amine (DIEA) (1 : 1 : 2) in DCM or in 1 : 1 (v/v) DCM/dimethylformamide (DMF) mixture. All couplings were monitored by qualitative ninhydrin test. Before HF and TFA time-course investigation, the integrity of the synthesized peptidyl-resins was confirmed by examining the purity of the synthesized peptide. For this, a small amount of samples was cleaved and the purity of the crude peptide was assessed by analytical reverse-phase high performance liquid chromatography (RP-HPLC), mass spectrometry and amino acid analysis.

Amino Acid Analysis Before the HF and TFA time-course studies, all peptidyl-resins were hydrolyzed at 130 °C with 12 N HCl/propionic acid mixture for 70 h to guarantee quantitative removal of peptide chains from the resin as recently proposed.[19] Pyrex tubes with plastic Teflon-coated screw caps (13 cm×1 cm) were used for the hydrolyses and the amino acid analyses were performed in a Beckman System 6300 amino acid analyzer to determine the amount of peptide attached to the resin.

TFA Time-Course Treatment Study Approximately 500 mg of each peptidyl-resin was added to a 20 ml reaction vessel and treated with 30% TFA/DCM solution containing 2% anisol for up to 20 h reaction time with shaking. At a given time, the resin was filtered and washed with TFA/DCM (1 : 3, v/v, 5×1 min) followed by DCM washings. The resin was again suspended in freshly prepared TFA/DCM mixture and shaking was continued. The monitoring of chain loss was determined at 1, 4, 8, 12, 16 and 20 h. The percentage of peptide lost in each treatment time was quantified by amino acid analysis: initial and final peptide contents of the resin were compared using the already mentioned hydrolysis procedure. All TFA time-course monitoring data were carried out in duplicate and the average value for each reaction time was calculated.

HF Time-Course Cleavage Study Portions of protected peptide-resins (approximately 0.05 mmol each) were treated with anhydrous HF in the presence of *o*-cresol and dimethylsulfide (5% each) at 0 °C for up to 6 h, with mixing. Excess HF was eliminated under vacuum and the cleaved peptide-resin was washed initially with ethyl acetate to remove scavengers. After drying, the peptide extraction from the resin was carried out with 10% and 90% aqueous AcOH (5×5 ml, each). The resulting extracted peptide solution was lyophilized and the amount of peptide cleaved was checked against the remaining amount of peptide still bound to the extracted resin. The determination of the yield of HF cleavage was quantified through resin hydrolysis followed by amino acid analysis as aforementioned.

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

Mass Spectrometry MALDI-MS The crude peptides obtained were analyzed on a Micromass Spectrometer, model TofSpec SE, using α-cyano-4-hydroxycinnamic acid as the solid matrix.

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References and Notes

1) Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—369 (1967); **11**, 1726—1732 (1972). The symbols represent the L-isomer unless otherwise specified. The following additional abbreviation are used: AcOH, acetic acid; 2-Br-Z, 2-bromobenzyloxycarbonyl; BHAR, benzhydrylamine-resin; Boc, *tert*-butyloxycarbonyl; CMR, chloromethyl-resin; cHex, cyclohexyl; C_{18}, octadecyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; RP-HPLC, reverse phase high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; PAMR, 4-(oxymethyl)-phenylacetamidomethyl-linker-containing resin; RP-HPLC, reversed phase high performance liquid chromatography; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl.
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