Solid Phase Peptide Synthesis. A Study on the Effect of Trifluoroacetic Acid Concentration on the Removal of the *tert*-Butyloxycarbonyl Protecting Group[1]

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Received July 21, 1975

An acid concentration of 20% (v/v) TFA in CH_2Cl_2 was used to remove the α-amino protecting group during the solid phase synthesis of the tri- and tetrapeptides corresponding to the carboxy terminus of bovine pancreatic RNase A. Thin layer chromatography of the cleaved and desalted tripeptide showed significant amounts of the deletion peptides alanylvaline and serylvaline in addition to the expected alanylserylvaline. Similar analysis of the tetrapeptide showed five major ninhydrin positive by-products. Inadequate removal of the α-amino protecting group was determined to be the chief cause of these impurities. A study on the synthesis of the tri- and tetrapeptides using a variety of acid concentrations between 20 and 50% (v/v) TFA in CH_2Cl_2 indicated that 40–45% TFA was the weakest possible acid concentration that could be employed to remove the α-amino protecting group quantitatively and produce a reasonably pure tri- and tetrapeptide on the solid support used.

One of the main advantages of the solid phase synthesis of peptides[2] is the potential for automation. The ideal automated procedure would require a set of tactics which could be repeated for a large number of amino acids in a variety of sequences.[3]

The Boc[4] group has found wide acceptance as the α-amino protecting group in solid phase synthesis.[5] Removal of the Boc group from the α-amino function has been accomplished by 4 N HCl–dioxane,[6] 1 N HCl–glacial acetic acid,[7] TFA,[8–12] 50% (v/v) TFA–CH_2Cl_2,[13] 20% (v/v) TFA–CH_2Cl_2,[14,15] and a variety of TFA concentrations in CH_2Cl_2 between 20 and 50%.

In spite of the desire to have one concentration of acid which can be used to remove the Boc group throughout the entire synthesis of a peptide, there have been instances[16] where quantitative removal of the Boc group has required a variety of acid concentrations throughout the synthesis.

The ease of preparation of various concentrations of anhydrous TFA as opposed to the preparation of various anhydrous HCl concentrations has given this acid widespread use for the removal of the Boc group. A 50% concentration of TFA in CH_2Cl_2, the most widely used concentration of TFA for Boc group removal, has been shown to cause branching at lysine residues where the side chain has been protected by the benzyloxycarbonyl group.[17] It is also known that a 50% TFA solution results in a small amount of cleavage of the ester bond linking the peptide to the resin with a concomitant loss of the growing peptide chain.[13]

Despite the availability of a more stable protecting group for the side chain of lysine[17] as well as the observation that the reduction of the concentration of TFA from 50 to a 20% (v/v) solution in CH_2Cl_2 had only a minor effect on the stability of the ester bond linking the peptide to the resin,[15] the tendency has been to use lower acid concentrations for the removal of the Boc group.

In order to study the effectiveness of various concentrations of TFA in CH_2Cl_2 on the removal of the Boc group, the tripeptide and tetrapeptide from the carboxy terminus of RNase A were chosen as the model peptides. The reasons for selecting these peptides were twofold. Firstly, several studies have been carried out on the use of synthetic analogues of the carboxy terminus of RNase A to regenerate activity from pepsin and carboxypeptidase inactivated RNase A.[18–22] If the solid phase synthesis of a model peptide corresponding to the carboxy terminus of RNase A could be studied step by step to determine the ideal conditions for coupling each individual amino acid, then a series of analogues could be prepared in a reasonably pure state without the necessity of analyzing every step for complete Boc group removal and coupling. Secondly, it is possible that the difficulty in removing the Boc groups or achieving complete coupling of the amino acids may be caused in part by steric hindrance from the bulky protected side chains of the amino acid derivatives. Since the carboxy terminal tripeptide contains the bulky valine and benzyl protected serine as well as one alanine which has relatively little hindrance and the tetrapeptide contains, in addition, the bulky aspartic acid β-benzyl ester, a study of these peptides may give some indication as to whether large and/or protected side chains have a major effect on the deprotection of the α-amino group and subsequent coupling reaction during solid phase peptide synthesis.

The tripeptide was synthesized by coupling Boc-alanine and Boc-*O*-benzylserine to Boc-valine resin using DCC after removing the Boc group with 20% TFA in CH_2Cl_2. After cleavage of the peptide from the resin using HBr in TFA[23] and desalting on Sephadex G-10, the product obtained showed one neutral spot by electrophoresis on cellulose thin layer plates using 0.1 M pyridine acetate at pH 6.0. However, thin layer chromatography on silica gel in the solvent system 1-butanol–glacial acetic acid–water (4:1:1) resulted in three major ninhydrin positive spots at R_f 0.38, 0.31, and 0.27 and four weak ninhydrin positive spots at R_f 0.53, 0.44, 0.21, and 0.15.

Preparative TLC of the impure product resulted in seven samples (Table I) which, after desalting, produced partial purification of the three major spots as shown by rechromatographing the samples.

Rechromatography of sample 3 (Table I) showed one major spot at the expected R_f value and a very weak spot at R_f 0.31. Amino acid analysis following acid hydrolysis showed nearly equimolar quantities of valine and alanine with a trace amount of serine.

Rechromatography of sample 4 (Table I) showed that the major component of this sample had an R_f of 0.38 with a minor component at R_f 0.31 and a very weak spot at R_f 0.27. Comparison of the amino acid analyses of samples 3 and 4 showed that the latter had considerably more serine and less alanine than the former.

Sample 5 (Table I) showed a major spot at R_f 0.31 with a spot of minor intensity at R_f 0.27 when examined by TLC. A second preparative TLC of sample 5 resulted in a product which gave a single spot of R_f 0.31 on TLC and amino acid analysis showed a trace of alanine and near equal quantities of serine and valine.

Table I. Preparative TLC of Ala-Ser-Val Synthesized Using 20% (v/v) TFA–CH_2Cl_2

Sample no.	R_f value in original chromatogram	R_f values of ninhydrin positive spots in TLC of sample after preparative TLC			Amino acid content of sample (nM of amino acid)		
		Major	Minor	Weak[a]	Ala[b]	Ser[b,c]	Val
1[d,e]	0.53		0.53 and 0.44		4.0 (0.82)	7.6 (1.56)	4.9 (1)
2[d,e]	0.44		0.53 and 0.44		6.5 (1.07)	8.6 (1.42)	6.1 (1)
3	0.38	0.38		0.31	81.3 (0.98)	3.1 (0.04)	83.1 (1)
4	0.31	0.38	0.31	0.27	54.4 (0.68)	21.2 (0.27)	79.6 (1)
5	0.27	0.31	0.27		84.6 (0.72)	98.0 (0.83)	118.4 (1)
	f	0.31			Trace	68.4 (0.92)	74.6 (1)
6	0.21	0.27			133.0 (0.90)	119.0 (0.80)	148.4 (1)
7[d]	0.15		0.21 and 0.15		12.6 (1)	6.7 (0.53)[g]	

[a] An estimation of the relative intensity of the spots. [b] The values in parentheses are relative to valine. [c] Uncorrected for serine destruction during acid hydrolysis. [d] Not separated by preparative TLC. [e] Difficult to detect by ninhydrin as the spots faded rapidly. [f] Sample no. 5 was again subjected to preparative TLC and material with R_f 0.31 was isolated. [g] This value in parentheses is relative to alanine.

Rechromatography of sample 6 (Table I) resulted in a single spot of R_f 0.27 and amino acid analysis showed equal quantities of alanine, serine, and valine.

Preparative TLC could therefore show that the ninhydrin positive spot at R_f 0.27 was the required alanylserylvaline tripeptide while the spot of R_f 0.31 was the serylvaline dipeptide. The spot of R_f 0.38, although very slightly impure after preparative TLC, was concluded to be the alanylvaline dipeptide.

The two spots of highest R_f values (0.53 and 0.44) faded rapidly after spraying with ninhydrin and were not completely separated by preparative TLC. Amino acid analysis (Table I) of the two samples from preparative TLC showed that some separation may have taken place since sample 1 had a lower alanine content than sample 2. Both samples contained both spots as shown by TLC; however, the relative intensities could not be determined. When a sample was taken from the synthesis vessel at the serylvaline resin stage, the spot of R_f 0.53 was found, whereas the spot of R_f 0.44 did not occur until after alanine had been coupled. Therefore it was concluded that the material with R_f 0.53 was probably an impurity of the serylvaline dipeptide, while the spot of R_f 0.44 was perhaps due to an impurity of the alanylserylvaline peptide. However, a definite identification of these impurities had not been made.

The spots of R_f 0.21 and 0.15 were not separated by preparative TLC, but the amino acid analysis of sample 7 containing both spots (Table I) showed the presence of serine and alanine. Again, a sample of the peptide resin taken at the serylvaline resin stage showed only the spot of R_f 0.15 while that with R_f 0.21 appeared after coupling alanine. When these impurities were compared on TLC with a sample of free serine and free alanine, the alanine was shown to have an R_f value of 0.21 and serine an R_f value of 0.15. Therefore, these two impurities were concluded to be alanine and serine, respectively. Although the presence of serine in the alanylserylvaline samples suggested inefficient washing, other experiments which involved three 20-min acid treatments for deprotection also resulted in the presence of detectable amounts of serine in the product. Since the serine could not be removed after three acid treatments, inefficient washing would not explain its presence in the product. Free valine had an R_f of 0.37 and could not be separated from the alanylvaline dipeptide if it was present at all.

The impurities in the tripeptide product could have resulted from either incomplete removal of the α-amino protecting groups or incomplete coupling of the amino acid derivatives. If incomplete coupling was responsible, then a change in the acid concentration for α-amino deprotection would not be expected to alter the number and amount of impurities to any extent. However, if incomplete Boc group removal was responsible then alteration of the acid concentration used for α-amino group deprotection should result in a change in impurities.

Table II. A Study of the Ala/Val Ratios after Coupling Boc-Ala to Boc-*O*-benzylserylvaline Resin[a] Deprotected with 20 and 50% TFA[b]

Acid concn,[b] %	Sample no.[c]	Ala/Val ratio[d]
20	1	0.92
	2	0.87
	3	0.90
50	1	1.09
	2	0.99
	3	1.08

[a] Synthesized by deprotecting Boc-valine resin with 50% (v/v) TFA in CH_2Cl_2 prior to coupling Boc-*O*-benzylserine. [b] By volume in CH_2Cl_2. [c] Three couplings performed, taking a sample after each coupling. [d] From amino acid analysis after HBr–TFA cleavage from resin, desalting, and acid hydrolysis.

To discover which of the above possibilities was responsible for the impurities found in the product, Boc-valine resin was deprotected with 20% TFA in CH_2Cl_2 and Boc-*O*-benzylserine was coupled. The Boc-*O*-benzylserylvaline resin was then divided into two fractions. One fraction was deprotected with 20% TFA in CH_2Cl_2 and coupled with Boc-alanine three times taking a small sample of the resin peptide after each coupling. The second fraction was deprotected using 50% TFA in CH_2Cl_2 and coupled with Boc-alanine three times taking samples after each coupling. The alanine/valine ratios from amino acid analysis of the samples after cleavage from the resin, desalting, and acid hydrolysis are shown in Table II. There was no major difference in the alanine/valine ratios within the group of three samples from each of the experiments; however, the alanine/valine ratios of the three samples from the experiment using 50% TFA were approximately 17% higher than those of the three samples from the experiment using 20% TFA, indicating a better incorporation of alanine after deprotection of the Boc-*O*-benzylserylvaline resin by 50% TFA. The TLC of the six samples showed no major difference in the spot intensities within each group; however, the 50% samples showed a great decrease in the intensity of the spot attributed to the serylvaline dipeptide as well as an increase in the intensity of the spot attributed to the alanylvaline dipeptide when compared with the 20% samples. These results indicated that the majority of the impurities was due to incomplete removal of the α-amino protecting group during the acid treatment. However, it cannot be ruled out

that a small amount of the impurities, a change in which cannot be detected by TLC, was due to incomplete coupling.

The tripeptide was synthesized seven times by the solid phase method using acid concentrations from 20 to 50% TFA in CH_2Cl_2 at 5% intervals in order to obtain an acid concentration which gave a maximum of product with a minimum of impurities. The TLC of the products obtained are shown in Figure 1. There was an indication of a decrease in the impurities proceeding from 20 to 25% TFA and a slight further decrease from 25 to 30%; however, there was no further visible decrease in impurities between 30 and 50% TFA. The amino acid analyses of these products (Table III) showed only a slightly low alanine content of the product prepared using 20% TFA.

In order to quantitate the impurities and have a closer look at the effect of acid concentrations on impurities between 30 and 50% TFA, the peptide was synthesized using different acid concentrations and employing the Dorman titration[24] to examine Boc removal and amino acid coupling. Table IV shows the results obtained.

Examination of the effect of different acid concentrations on deprotection of the Boc-valine resin indicated that an acid concentration of 45% gives maximum removal of the Boc group. Boc removal from the Boc-*O*-benzylserylvaline resin showed an increase (18%) on proceeding from 20 to 25% TFA reaching a maximum at 40%. It was also observed that, in general, deprotection of the Boc-valine resin was better than deprotection of the Boc-*O*-benzylserylvaline resin, although not to any significant extent except in the peptide prepared with 20% TFA. The erratic behavior of the Boc-alanyl-*O*-benzylserylvaline resin during titration after the deprotection step cannot be explained at the present time.

Table III. Amino Acid Composition of Tripeptides Prepared Using Different Acid Concentrations for Boc Group Removal

Acid concn[a]	Amino acid ratio of cleaved and desalted peptide[b]		
	Ala	Ser[c]	Val
20	0.90	0.89	1
25	0.99	0.91	1
30	0.97	0.91	1
35	1.01	0.95	1
40	0.96	0.86	1
45	1.02	0.98	1
50	1.12	1.01	1

[a] Percent by volume TFA in CH_2Cl_2. [b] Values relative to valine. [c] No correction for serine destruction during acid hydrolysis.

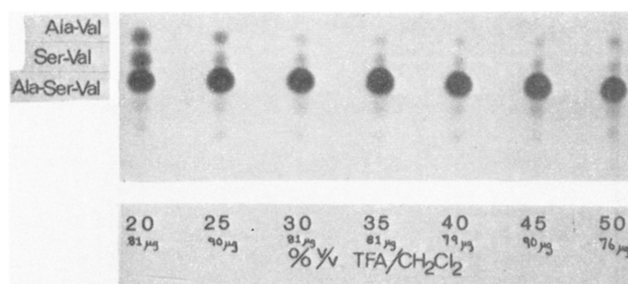

Figure 1. Thin layer chromatogram of crude tripeptide products synthesized using different TFA concentrations to remove the Boc group. The amount of sample applied to the plate is listed under the acid concentration.

In order to calculate the percentage of impurities in each of the samples (Table V), coupling was assumed to be 100% and the calculations were made from the percent deprotection in Table IV. Using the 20% TFA in CH_2Cl_2 experiment as an example, the calculations were performed as follows: Boc-valine resin was 87% deprotected (Table IV) thus leaving 13% as Boc-valine resin. A 100% coupling of Boc-*O*-benzylserine would result in a product consisting of 87% Boc-*O*-benzylserylvaline resin and 13% Boc-valine resin. Deprotection of this mixture of products was 73% complete (Table IV), assuming that the deprotection is equally distributed between the Boc-*O*-benzylserylvaline resin and Boc-valine resin; after 100% coupling of Boc-alanine, the mixture would consist of 73% of 87% or 63.5% Boc-alanine-*O*-benzylserylvaline resin, 27% of 87% or 23.5% Boc-*O*-benzylserylvaline resin, 73% of 13% or 9.5% Boc-alanylvaline resin, and 27% of 13% or 3.5% Boc-valine resin.

These calculations showed that the valine and alanylvaline impurities, which were due to incomplete deprotection of Boc-valine resin, reached a minimum at 45% TFA, while the serylvaline impurity due to incomplete deprotection of the Boc-*O*-benzylserylvaline resin reached a minimum at 40% TFA. The maximum yield of the alanylserylvaline tripeptide occurred at about 45% TFA. Figure 2 was a graph of these results.

Both Figure 1 and Table V indicated an unusually high content of alanylvaline and serylvaline in the tripeptide product obtained from the synthesis using 50% TFA in CH_2Cl_2. This was a surprising result and since it was expected that 50% TFA would be as efficient as, or better than, 40 or 45% TFA in removing the Boc group, the impurities probably resulted from incomplete coupling of Boc-*O*-benzylserine and Boc-alanine.

It was important to remember that the values of the free amino groups (Table I) and hence the percentage impurities (Table V) were relative values only since the highest

Table IV. Preparation of Ala-Ser-Val Using Different Concentrations of Acid and Monitoring the Synthesis by the Dorman Procedure[a,b]

Acid concn[c]	Boc-Val resin deprotection, µequiv Cl⁻/g[d]	Boc-Ser(Bzl) coupling, µequiv Cl⁻/g	Boc-Ser(Bzl)-Val resin deprotection, µequiv Cl⁻/g	Boc-Ala coupling, µequiv Cl⁻/g	Boc-Ala-Ser(Bzl)-Val resin deprotection, µequiv Cl⁻/g
20	157 (87)[e]	6 (3)	132 (73)	5 (3)	168 (93)
25	164 (91)	5 (3)	164 (91)	7 (4)	179 (99)
30	170 (94)	6 (3)	166 (92)	8 (4)	163 (91)
35	173 (96)	8 (4)	170 (94)	7 (4)	170 (94)
40	172 (96)	8 (4)	173 (96)	7 (4)	180 (100)
45	176 (98)	7 (4)	172 (96)	7 (4)	167 (93)
50	170 (94)	5 (3)	169 (94)	10 (6)	172 (96)

[a] Amino acid analysis of Boc-Val resin indicated 163 µM Val/g Boc-Val resin. [b] The accuracy and precision of the Dorman monitoring of total amino groups after deprotection were determined by six chloride determinations on a 500-mg sample of 40% TFA deprotected Boc-Val resin and found to be ±6 and ±1.6%, respectively. [c] Percent by volume of TFA in CH_2Cl_2. [d] µequiv Cl^- is assumed to be equivalent to µM free amino groups. [e] Numbers in parentheses indicate the percent free amino groups relative to the 40% TFA–CH_2Cl_2 deprotection of Boc-Ala-Ser(Bzl)-Val resin.

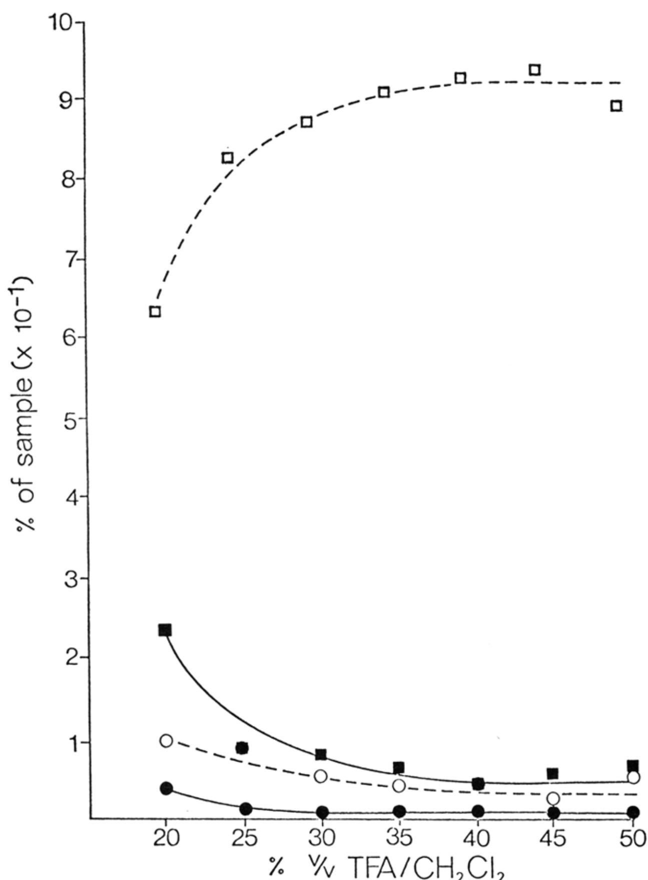

Figure 2. Comparison of decreasing formation of impurities (●—●, valine; ■—■, serylvaline; O- - - -O, alanylvaline) and increasing yield of product (□- - - -□, alanylserylvaline) with increasing TFA concentrations during synthesis of the alanylserylvaline tripeptide.

Table V. Percentage of Impurities in Tripeptides Prepared Using Various Acid Concentrations[a,b]

Acid concn[c]	% Val	% Ala-Val	% Ser-Val	% Ala-Ser-Val[d]
20	3.5	9.5	23.5	63.5
25	0.8	8.2	8.2	82.8
30	0.5	5.5	7.5	86.5
35	0.2	3.8	5.8	90.2
40	0.2	3.8	3.8	92.2
45	0.1	1.9	3.9	94.1
50	0.4	5.6	5.6	88.4

[a] Assuming 100% coupling. [b] Calculated from percent deprotection in Table IV (see text). [c] Percent by volume TFA in CH_2Cl_2. [d] This column shows the increasing yield of tripeptide product with increasing acid concentration.

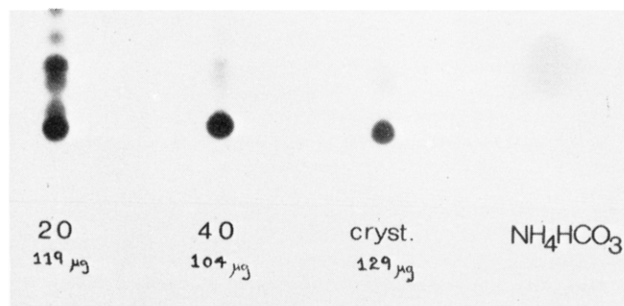

Figure 3. Comparison of the crude tetrapeptide synthesized with 20% (v/v) TFA in CH_2Cl_2 (20) with the crude tetrapeptide synthesized with 40% (v/v) TFA in CH_2Cl_2 (40) and the crystallized tetrapeptide (cryst). Residual NH_4HCO_3 in the samples after desalting and lyophilization migrates to the same position as the alanlyserylvaline tripeptide thus making the estimation of purity of the tetrapeptide difficult. The amount of the sample applied to the plate is listed under the appropriate label.

value for the chloride determination was arbitrarily taken as 100% removal of the Boc group.

The tripeptide resin prepared using 20% TFA to remove the α-amino protecting group was further deprotected with 20% TFA and Boc-aspartic acid β-benzyl ester was coupled to form the tetrapeptide resin. After HBr–TFA cleavage from the resin and desalting on Sephadex G-10, electrophoresis on cellulose thin layer plates in 0.1 M pyridine acetate at pH 6 and 600 V showed one neutral ninhydrin positive spot and one large, diffuse spot of net negative charge. Thin layer chromatography of this peptide resulted in six ninhydrin positive spots of major intensity at R_f values of 0.38, 0.31, 0.27, 0.25, 0.20, and 0.15. The first three spots of R_f 0.38, 0.31, and 0.27 could be identified (by analogy to the tripeptide studies) as alanylvaline, serylvaline, and alanylserylvaline, respectively. The other three spots were the result of coupling aspartic acid and therefore were likely to be aspartylalanylvaline, aspartylserylvaline, and aspartylalanylserylvaline.

Since the TLC and Dorman titration studies on the tripeptide indicated that 40% TFA was the weakest acid concentration that could be used to remove the Boc group, the tetrapeptide was resynthesized using 40% TFA to deprotect the α-amino function.

The cleaved, desalted peptide was run on TLC and compared to the tetrapeptide synthesized using 20% TFA (Figure 3). A small amount of the serylvaline dipeptide and the alanylserylvaline tripeptide was still present in this crude tetrapeptide; however, when compared with the tetrapeptide synthesized using 20% TFA the purity was considerably improved.

The tetrapeptide synthesized using the 40% TFA could be further purified by ion exchange chromatography on DEAE cellulose using an ionic gradient. After ion exchange chromatography, desalting, and treatment of the lyophilized product with aqueous HCl to form the hydrochloride salt, the tetrapeptide could be crystallized from an aqueous methanol solution by addition of dry ether. The hydrochloride salt of the tetrapeptide synthesized using 20% TFA and purified in the same manner could not be crystallized.

In conclusion, these studies indicated that a minimum of 40% TFA in CH_2Cl_2 should be employed to remove the Boc group during the solid phase synthesis of the tetrapeptide corresponding to the carboxy terminus of RNase A using the solid support described in the Experimental Section.

No convincing evidence could be found to show that steric hindrance by the amino acid side chains contributed significantly to the formation of impurities in the tripeptide or tetrapeptide by hindering α-amino group deprotection or the subsequent coupling reaction.

The mechanical properties of different supports may differ considerably[3] and therefore certain supports may require higher acid concentrations for Boc removal, while others may give the same result at lower acid concentrations; hence the need for careful study of the mechanical properties of the solid support before commencing a synthesis.

Experimental Section

Boc-alanine, Boc-valine, Boc-O-benzylserine, and Boc-aspartic acid β-benzyl ester were purchased from Fluke AG, Buchs, Switzerland, and Serva Feinbiochemica, Heidelberg, Germany. Melting

points and R_f values on precoated silica gel thin layer plates were determined for all derivatives. Chloromethylated copolystyrene–1% divinylbenzene resin (Bio-Beads SX-1, 200–400 mesh, 1.25 mequiv of chlorine per gram, control no. 10948) was obtained from Bio Rad. All solvents and reagents were of analytical grade except for *N,N*-dimethylformamide, which was spectroscopic grade.

Thin layer chromatography was carried out on precoated silica gel plates purchased from Merck in the solvent system 1-butanol–glacial acetic acid–water (4:1:1). Electrophoresis was carried out on precoated cellulose plates purchased from Merck and samples were run in 0.1 M pyridine acetate at pH 6.0 for 40 min at 600 V (~4 mA) in a Camag electrophoresis unit. After thin layer chromatography or electrophoresis, ninhydrin positive material was detected by thoroughly drying the plates and spraying with a 5–10% solution of ninhydrin in a buffer consisting of glacial acetic acid–pyridine–acetone (2:1:64).

Peptide resins and free peptides were prepared for amino acid analysis as described elsewhere[13] and analyzed on a Beckmann Unichrome Amino Acid Analyzer.

Solid Phase Synthetic Procedure. The synthetic procedure was initially carried out in a mechanical shaker of the type described in ref 5. The synthesis of peptides incorporating the Dorman titration procedure was carried out using the Peptider purchased from Penninsula Laboratories, San Carlos, Calif. The amount of valine esterified to the resin varied between 0.139 and 0.269 mM/g Boc-valine resin. α-Amino group deprotection was carried out with redistilled TFA in CH_2Cl_2, and neutralization was performed using a 10% solution of triethylamine in CH_2Cl_2. Couplings were performed using *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2 (250 mg/ml). Initially, two 1-h couplings were performed but this was later reduced to one 1-h coupling step when the Dorman titration was employed because TLC studies indicated that there was no visible change in impurities after the first coupling. A threefold excess of Boc-amino acid and DCC was used for each coupling.

One cycle of the synthesis including the Dorman titration was performed on 500 mg of amino acid resin as follows: 1, 2 × 3 min, CH_2Cl_2, 10 ml/wash; 2, 1 × 3 min, TFA–CH_2Cl_2, 10 ml; 3, 2 × 10 min, TFA–CH_2Cl_2, 10 ml; 4, 6 × 3 min, CH_2Cl_2, 10 ml/wash; 5, 1 × 1 min, 10% $(CH_3CH_2)_3N$–CH_2Cl_2, 10 ml; 6, 1 × 6 min, 10% $(CH_3CH_2)_3N$–CH_2Cl_2, 10 ml; 7, 6 × 3 min, CH_2Cl_2, 10 ml/wash; 8, 1 × 3 min, 0.3 M pyridine hydrochloride–CH_2Cl_2, 10 ml; 9, 1 × 10 min, 0.3 M pyridine hydrochloride–CH_2Cl_2, 10 ml; 10, 4 × 3 min, CH_2Cl_2, 20 ml/wash; 11, 3 × 3 min, dimethylformamide, 20 ml/wash; 12, 1 × 1 min, 10% $(CH_3CH_2)_3N$–dimethylformamide, 10 ml; 13, 1 × 6 min, 10% $(CH_3CH_2)_3N$–dimethylformamide, 10 ml; 14, 4 × 3 min, dimethylformamide, 10 ml/wash; 15, 6 × 3 min, CH_2Cl_2, 10 ml/wash; 16, 1 × 10 min, Boc-amino acid–CH_2Cl_2, 10 ml; 17, 1 × 60 min, DCC; 18, 3 × 3 min, CH_2Cl_2, 10 ml/wash; 19, 3 × 3 min, CH_3CH_2OH (absolute), 10 ml/wash; 20, 3 × 3 min, CH_2Cl_2, 10 ml/wash. The washings in steps 12–14 were collected for chloride determination.

The peptide samples were cleaved from the resin by bubbling HBr through a suspension of the resin in 50% TFA–CH_2Cl_2 for 80 min. The suspension was filtered and the resin washed with concentrated TFA (2 × 5 ml) and then the combined filtrate and washings were evaporated to dryness. The residue was dissolved in 0.05 M NH_4HCO_3, adjusted to pH 8.2 with 1 N NaOH, and stirred for 1 h at room temperature to reverse any N to O acyl shift which may have occurred during the HBr cleavage. After lyophilization, the sample was dissolved in 0.05 M NH_4HCO_3 and desalted by application to Sephadex G-10 and elution with the bicarbonate buffer. The peptide was detected in the eluate by reading the fractions at 230 nm.

Estimation of Free Amino Groups on the Resin. Free amino groups were determined according to the method described by Dorman.[24] Washes 12–14 above were collected, acidified with 20 ml of 1 N HNO_3, and titrated with 0.1 N silver nitrate. Titrations were performed automatically using the Autoburette ABU 12, Titragraph SBR 2c, and Titrator TTT 1c purchased from Radiometer Copenhagen.

Preparative Thin Layer Chromatography. Approximately 20 mg of crude peptide was dissolved in 2 ml of deionized water and 0.2 ml of this solution was applied to each of ten precoated silica gel plates (20 × 20 cm, 0.25 mm in thickness). The plates were developed to a height of approximately 13 cm (4–5 h) in 1-butanol–glacial acetic acid–water (4:1:1). Then they were thoroughly dried and the center covered with tinfoil leaving 2 cm exposed on both sides. The exposed sides were sprayed with ninhydrin and the corresponding spots on both sides were joined by drawing straight lines across the plates above and below the spots. The centers of the plates were thus divided into seven sections which were separately scraped from the plates. The corresponding sections of the plates were combined and extracted three times with 30% (v/v) glacial acetic acid–water. Centrifugation for 15 min at 3000 rpm in a Heraeus Christ centrifuge was required to remove completely silica gel from the samples which were then lyophilized and desalted on Sephadex G-10 using 0.05 M NH_4HCO_3 as eluent. The samples so isolated were then rechromatographed to determine purity.

Ion Exchange Chromatography and Crystallization of the Tetrapeptide. After the tetrapeptide prepared using 40% TFA was cleaved from the resin, desalted, and lyophilized, it was dissolved (30 mg) in 2 ml of 0.05 M pyridine acetate at pH 6.0 and applied to a DEAE cellulose column (2.5 × 11.5 cm). After 50 ml of 0.05 M pyridine acetate buffer had passed through the column a linear ionic gradient was started at 0.05 M pyridine acetate, pH 6.0 (100 ml), to 0.5 M pyridine acetate, pH 6.0 (100 ml). The eluted peptide was detected by alkaline hydrolysis and the ninhydrin reaction on 0.1-ml aliquots of the fractions. The tubes containing the major fraction eluting at 160 ml were combined and lyophilized. The lyophilized product was desalted on Sephadex G-10 using the bicarbonate buffer mentioned above.

A sample of the tetrapeptide (13 mg) was dissolved in 0.2 ml of aqueous HCl, pH 1.7, and centrifuged to remove suspended particles. Approximately 1 ml of methanol was added to the solution followed by ether until the solution became cloudy. The cloudy solution was then refrigerated for 3 weeks. This resulted in the recovery of 7.25 mg of crystalline tetrapeptide.

Acknowledgments. I would like to thank Dr. B. Gutte for his many suggestions and continued interest in this work. I am indebted to Mrs. M. Hoffman for her excellent technical assistance and Mr. K. Bärwald and Mr. W. Kullmann for many helpful discussions. I would also like to thank Mr. G. Leipertz for his excellent photographic work.

Registry No.—Boc-alanine, 15761-38-3; Boc-valine, 13734-41-3; Boc-*O*-benzylserine, 23680-31-1; Boc-aspartic acid β-benzyl ester, 7536-58-5; aspartylalanylserylvaline, 57739-05-6; alanylserylvaline, 57694-90-3; TFA, 76-05-1.

References and Notes

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(12) Abbreviations used are: TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; TLC, thin layer chromatography.
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