ANALYTICAL BIOCHEMISTRY 174, 399–414 (1988)

Mass Spectrometric Evaluation of Synthetic Peptides for Deletions and Insertions[1,2]

R. B. MERRIFIELD, JAMES SINGER, AND BRIAN T. CHAIT

The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received March 30, 1988

A new technique to evaluate methods for the synthesis of peptides was developed. It is based on the identification and quantitation of peptide by-products by mass spectrometry. Model oligopeptides containing 10 or 20 alanine residues were synthesized by automated solid phase methods using a variety of protocols, and the levels of deletion and insertion peptides were measured by the ^{252}Cf fission fragment ionization time-of-flight spectrometric technique in which the total, unfractionated, synthetic product was deposited on a film of nitrocellulose and analyzed. The introduction of D-alanine at every third residue of the model eliminated peptide conformation problems that led to incomplete reactions in the all L model. Couplings with preformed symmetrical anhydrides in dimethylformamide gave rise to significant levels of both deletion peptides and insertion peptides. The best of the protocols examined was a double coupling of *tert*-butyloxycarbonyl-alanine by *in situ* activation with dicyclohexylcarbodiimide in dichloromethane. [D-Ala^{3,6,9,12,15,18}]Ala$_{20}$–Val was synthesized with an average deletion of only 0.036% per step and an average insertion of only 0.029% per step, which is equivalent to a stepwise yield of 99.93% for the target peptide.

KEY WORDS: solid phase peptide synthesis; ^{252}Cf fission fragment ionization mass spectrometry; coupling efficiency; deletion peptides; insertion peptides; oligopeptide models; Ala$_{10}$–Val; Ala$_{20}$–Val.

From the first publication on solid phase peptide synthesis (1) to the present there has been interest and concern about the completeness of the deprotection and coupling reactions. To achieve fully satisfactory synthetic results it is necessary to have rapid and quantitative reactions and to avoid peptide by-products, which arise from termination, deletion, insertion, branching, and modification reactions. The numerous methods that have been devised to monitor the occurrence of such side reactions and to measure the kinetics of solid phase reactions have been reviewed (2–5). The most useful ones have involved (I) the spectrophotometric measurement of uptake of reagents (6–7) or formation of by-products (8,9); (II) quantitation of unreacted amino component by $HClO_4$ titration (10), by uptake of Cl^- (11,12), picrate$^-$ (13), or $^{35}SO_4^=$ (14), by ninhydrin analysis (15,16), by displacement of a colored aldehyde from its Schiff base (17) or of other chromophoric adducts (18,19); (III) measurement of deletion peptides by preview sequencing of the peptide-resin (20) by mass spectrometric analysis of small peptides after cleavage and partial hydrolysis (21) or by direct mass spectrometry of final peptide product (22); (IV) ion exchange (23,24), reverse phase (25), or HPLC separation of the products after cleavage of a model test peptide from the support. Some of these are real-time methods while others give an answer after the fact. Those in

[1] This work was supported in part by Grant AM01260 from the U.S. Public Health Service and by Grant RR00862 for the Rockefeller University Mass Spectrometric Biotechnology Research Resource. The spectropolarimeter was purchased by the Rockefeller University with funds from National Science Foundation Grant PCM 84-00268.

[2] We dedicate this paper to Professor Hiruaki Yajima on the occasion of his retirement from Kyoto University.

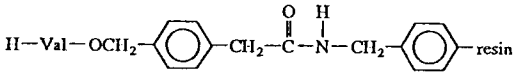
0003-2697/88 $3.00

group I are convenient and rapid, but not very sensitive (1–3%). Those in group II depend on chemical reactions, which may reduce their accuracy, but often they are sensitive to 0.1 to 0.3%. Method III can also be good to 0.1 to 0.3%, but it is subject to several problems and gives only an average measure of the extent of the reactions. Method IV can be quite sensitive with small model peptides, but is slow and has certain limitations. Taken together these monitoring methods have been very helpful in following the course of the reactions, in evaluating synthetic products, and in developing synthetic methodology, but they clearly need to be supplemented and improved.

We report here a new, quantitative method based on mass spectrometry, which has certain advantages in sensitivity and in the identification of the reaction by-products. Recent improvements in sample preparation (26,27) for fission fragment mass spectrometry (28–30) now allow highly sensitive, quantitative detection of the parent ion of peptides up to about 10,000 Da and, therefore, large model peptides designed specifically to reflect the by-products just described can be synthesized and analyzed with precision. The use of homopolymers has been introduced to provide a large amplification factor for quantitation of by-products, and detection limits for insertion and deletion peptides of <0.02% per step have been demonstrated. This has led to an improved analysis of reaction conditions for solid phase synthesis.

THE TEST SYSTEM

The model peptides L-Ala_{10}-Val-OCH_2-Pam-resin[3] and L-Ala_{20}-Val-OCH_2-Pam-resin were selected for the initial studies. The support was copoly(styrene-1%-divinylbenzene) resin beads.



(Val-OCH_2-Pam-R) was selected for the attachment of the peptide to the support because the resulting substituted benzyl ester bond is very stable to acidolysis, and losses of peptide chain during synthesis were negligible (31). Valine served also as a marker for chemical analysis of the synthetic product. The 10 or 20 alanine residues provided the necessary amplification factor because a deletion of an alanine residue at any position in the chain would yield the same Ala_9-Val or Ala_{19}-Val product, and this follows also for multiple deletions. Similar reasoning applies to potential insertion peptides where more than one alanine residue is added at a single synthetic cycle. The use of alanine, which lacks a third functionality, essentially eliminated the production of branched chains or of modification peptides in which the peptide chain was altered by various chemical side reactions during the synthesis and workup. N^{α}-trifluoroacetyl-Ala_n-Val and other N^{α}-blocked termination peptides are also detected by the technique, although with no amplification factor. With this test system several variables in the synthesis were studied.

It was soon found that these all L model peptides were not the best choice because they were subject to conformational constraints leading to incomplete reactions that were not a function of the resin or the peptide chemistry. That problem was overcome by introduction of D-Ala residues along the chain. The model peptides now recommended are

1. [D-$Ala^{2,5,8}$]Ala_{10}ValOCH_2-Pam- R : (L-Ala-D-Ala-L-Ala-L-Ala-D-Ala-L-

[3] Abbreviations used: The peptide nomenclature follows the general rules recommended by the IUPAC-IUB Commission on Biochemical Nomenclature; see *J. Biol. Chem.* **247**, 977 (1972). Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIEA, diisopropyl ethyl amine; DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; Pam, phenylacetamidomethyl; NMM, *N*-methylmorpholine; SA, symmetric anhydride; NC, nitrocellulose; DVB, divinylbenzene; TFA, trifluoroacetic acid.

Ala–L-Ala–D-Ala–L-Ala–L-Ala–L-Val–OCH_2Pam–resin)

2. [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val–OCH_2–Pam– R : L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–D-Ala–L-Ala–L-Ala–Val–OCH_2Pam–resin.

MATERIALS AND METHODS

Peptide Synthesis

The model peptides were all synthesized on the Applied Biosystems Model 430A synthesizer in order to maintain uniform and reproducible results. The starting material was Boc–Val–OCH_2–Pam–copoly(styrene-1%-divinylbenzene) resin beads (32,33), 0.7 mmol/g. The initial conditions were those of the standard ABI program for a double coupling with preformed symmetrical anhydrides in dimethylformamide (DMF). Thus, the chemistry was essentially the same as that developed and described previously (34) for solid phase synthesis, but with a number of changes in detail that were introduced by the manufacturer in order to adapt the technique to this automated synthesizer. The reagents, conditions, and programs were then varied.

Synthetic Protocols

I. Preformed symmetrical anhydride coupling in dimethylformamide. The Boc-amino acid (2 mmol, 4 eq relative to the amino component) is dissolved in 2 ml CH_2Cl_2 and transferred to the activator vessel. Dicyclohexylcarbodiimide (DCC) (1 mmol, 2 eq) in CH_2Cl_2 is added and, after activation for 10 min, the resulting anhydride is transferred, with filtration and rinsing, to the concentrator vessel. The solvent is evaporated with N_2 gas and warmed to maintain a temperature near 25°C, while the CH_2Cl_2 is exchanged with DMF. After 16 min in the concentration vessel, the solution is transferred to the reaction vessel containing the deprotected peptide-resin (0.5 mmol, ~0.7 g). The volume of solvent during coupling is 4 ml. Coupling is continued for 22 min with vortex mixing and then filtered and washed repeatedly with DMF. For a double coupling, the Boc-peptide-resin is washed with 5% DIEA in DMF before introduction of the second preparation of activated amino acid. In preparation for the next synthetic cycle the peptide-resin is washed with CH_2Cl_2, deprotected with 70% TFA in CH_2Cl_2, once for 2 min and once for 18 min, then neutralized with 5% DIEA in CH_2Cl_2 three times for 2 min each, and finally washed with CH_2Cl_2 and DMF.

II. Preformed symmetrical anhydride coupling in dichloromethane. This procedure is the same as protocol I, except the CH_2Cl_2 is not exchanged with DMF and the DMF washes are replaced with CH_2Cl_2 washes.

III. In situ symmetrical anhydride coupling. The Boc-amino acid (4 eq) in CH_2Cl_2 is transferred to the activator vessel and, without delay, to the concentrator vessel and then to the reaction vessel containing 1 eq of amino component. DCC (2 eq) in CH_2Cl_2 is introduced into the rinsed activating vessel and immediately transferred, with rinsing, to the concentrator and on to the reaction vessel. The activation and coupling reactions are continued for 60 min.

IV. Preformed hydroxybenzotriazole coupling. The Boc-amino acid (4 eq) in DMF is added to the activator vessel containing HOBt (4 eq) in DMF. Then DCC (4 eq) in an equal volume of CH_2Cl_2 is added. After 24 min, the solution is transferred, with filtration and rinsing, to the concentrator and then to the reaction vessel where coupling is carried out for 40 min.

V. In situ dicyclohexylcarbodiimide coupling. This is basically the standard solid phase DCC method. The Boc-amino acid (4 eq) in CH_2Cl_2 is transferred to the activator and, without delay, to the concentrator vessel and, without concentrating, on to the reaction vessel containing 1 eq of amino component. DCC (4 eq) in CH_2Cl_2 is introduced into the rinsed activator vessel and immedi-

ately transferred, with rinsing, to the concentrator and on to the reaction vessel. The activation and coupling are continued for 40 min. To avoid clogging of the filters by precipitated dicyclohexylurea, an equal volume of methanol is added to the reaction vessel before draining. After 2 min of vortexing the peptide-resin is filtered and washed with $MeOH/CH_2Cl_2$ (1:1) and then with CH_2Cl_2.

Preparation of peptides for analysis. The synthetic peptide-resins were cleaved with HF:anisole (9:1) for 1 h, 0°C. After evaporation of HF at 0°C and extraction with ether to remove scavenger, the peptides were extracted into trifluoroacetic acid (~0.4 mg/ml) or trifluoroethanol (~0.2 mg/ml) and used directly for chemical characterization and for mass spectrometric analysis.

Further characterization of the synthetic peptides. Aliquots of the crude, unfractionated, cleaved peptides were hydrolyzed in 6 N HCl in sealed, evacuated tubes for 48 h, 110°C, and the amino acid ratios were determined on a Beckman 6300 amino acid analyzer.

Other aliquots were analyzed for homogeneity on a Shimadzu LC6A HPLC instrument at 210 nm on an analytical C_{18} column (Vydac No. 218TP, Visalia, CA) using a 30-min linear gradient from 0 to 100% solvent B into solvent A:

Solvent A: Acetonitrile, 10%

+ 0.05% trifluoroacetic acid in water, 90%

Solvent B: Acetonitrile, 60%

+ 0.05% trifluoroacetic acid in water, 40%.

Circular dichroism spectra were recorded on an Aviv 60DS CD spectropolarimeter at 22°C in 1-mm cells. The spectra were measured from 190 to 300 nm at 1-nm intervals and three scans were averaged. The estimated percentages of α helix, β sheet and random coil structures in the peptides were calculated by the Aviv Prosec program, which is based on the conformational standards of Chang *et al.* (35).

Mass Spectrometric Analysis

Sample preparation. Samples were prepared for mass spectrometric analysis by adsorption of peptide from solution onto a thin nitrocellulose (NC) film (27). The NC film was produced by electrospraying 50 μg of NC (1 mg/ml in acetone) onto a flat, thin (2 μm) aluminized polyester support with a surface area of 1 cm^2. One nanomole of peptide dissolved in 2–10 μl of trifluoroacetic acid or trifluoroethanol was spread on the NC layer. Following adsorption of the peptide to the NC surface and evaporation of the solvent, the sample foil was inserted into the vacuum lock of the mass spectrometer where the film was thoroughly dried by evacuation. The resulting bare layer of peptide molecules bound to the surface of the NC was then inserted into the mass spectrometer for analysis.

^{252}Cf fission fragment ionization mass spectrometry. The mass spectra were obtained with the Rockefeller University ^{252}Cf fission fragment ionization time-of-flight mass spectrometer (29,30). In this instrument, the sample of NC-bound peptide adhering to the aluminized polyester support is placed in front of a ^{252}Cf source. The spontaneous fission of ^{252}Cf ($t_{1/2}$ = 2.6 years) results in the emmission of two highly energetic (~100 MeV) fission fragments traveling in opposite directions. The passage of one of these fission fragments through the sample causes desorption of sample ions. These sample ions are accelerated and are then allowed to drift through a 3-m-long flight tube, at the end of which they are detected. When a ^{252}Cf fission fragment ionizes the sample, its complementary fragment strikes a closely proximate detector to provide a time reference from which the flight times of the sample ions are measured. The time of flight of the ions provides a direct measure of their mass-to-charge ratio. In the present configuration the ion flux through the sample foil is 2000 fission fragments/s. Sample ions are accelerated by a 10.0-kV potential and are postaccelerated just prior to detection by a further 8.5 kV.

To accurately determine the amount of deletion and insertion peptides in a given sample, it is necessary that the spectral peaks corresponding to these species be clearly discerned from the noise and also that these peaks contain a sufficiently high number of ion counts. The spectral accumulation time required to satisfy these conditions depends on the amounts of deletion and insertion peptides relative to the amount of target peptide present in the sample. For large relative amounts of deletion and insertion peptides (>2%), accumulation times between 10 and 60 min were found to be adequate. For the smallest relative amounts of these materials (<1%) it proved necessary to accumulate spectra for as long as 24 h. It should be noticed that the fission fragment flux through the sample is sufficiently low to cause negligible damage to the total sample over the time scale of these measurements. Thus the effect of increasing the spectrum accumulation time is simply to increase the counting statistics and the signal-to-noise ratio rather than to alter the relative contributions to the spectrum of the various components present in the sample.

Each ion species of a given atomic composition is observed in the spectrum as a cluster of closely related isotopic component peaks. These clusters arise because the naturally occurring elements that compose the ion are not isotopically pure. The fission fragment mass spectrometer has sufficient resolution to resolve these isotopic components below mass-to-charge ratio (m/z) 1000. In this case the mass of the most abundant isotopic component was determined. Above m/z 1000 the fission fragment mass spectrometer has insufficient resolution to resolve the isotopic components. In this case the average mass of the various isotopic components was determined. In both of the above cases the accuracy of the mass determinations was in general better than 300 ppm (0.03%).

RESULTS

The initial experiments with the model peptides began with the synthesis of all L-Ala_{10}–Val–OCH_2–Pam–resin by protocols that included a double coupling with preformed symmetrical anhydride. For run 1 both couplings were in DMF, for run 2 the first was in DMF and the second was in CH_2Cl_2, and for run 3 the first was in CH_2Cl_2 and the second was in DMF. A typical mass spectrum of the parent ion region from 600 to 1000 mass units of the crude, unpurified peptide from run 3 is shown in Fig. 1. In this fission fragment time-of-flight analysis each peptide present in the preparation gives rise to a set of positive ions composed primarily of $(M + H)^+$, $(M + Na)^+$, and $(M + 2Na–H)^+$. An analysis of these data showed a main set of peaks corresponding to M_{10} (i.e., Ala_{10}–Val) and less abundant sets for M_9 and M_8, which include all of the species of single deletion and double deletion peptides that were produced during the synthesis. In addition a set of ions was found corresponding to M_{11}, which is composed of the single insertion peptides formed during this synthesis. In addition there are low levels of species derived from both deletions and insertions occurring in the same molecule. Those species corresponding to $(M + H)^+$ are designated by unprimed numbers, 8, 9, 10, 11; those corresponding to $(M + Na)^+$ ions are designated 8′, 9′, 10′, 11′; and the $(M + 2Na–H)^+$ ions are labeled 8″, 9″, 10″, and 11″.

Calculation of the Levels of Deletion and Insertion Peptides

The relative amounts of the various ions from run 3 are listed in Table 1 together with their observed and calculated masses. The agreement between theory and observation was within 0.4 mass unit for every ion and provided good evidence for the identity of each species. Note that the same mass ion is produced no matter in which position the L-Ala residue is deleted or inserted and this provides the amplification of this method. The average deletion and insertion per synthetic step were deduced from the observed distribution of final products after 10 cycles of the

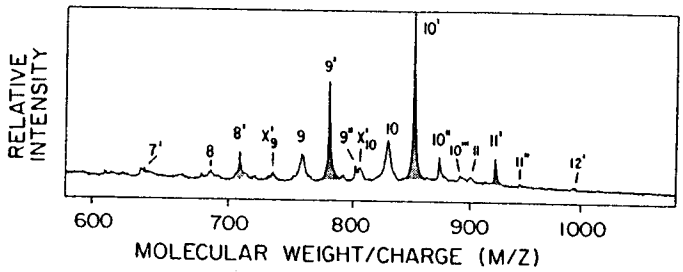


FIG. 1. Mass spectrum of L-Ala_{10}–Val, run 3. The first coupling was by preformed symmetrical anhydride in dichloromethane and the second coupling by preformed symmetrical anhydride in dimethylformamide. The sample was dissolved in trifluoroethanol (0.2 mg/ml) and applied to nitrocellulose film without washing. The numbers over each peak are the number of alanine residues. The nonprimed numbers are for $(M + H)^+$ species, the single primed numbers are for $(M + Na)^+$ species, and the double primed numbers are for $(M + 2Na-H)^+$ species. In addition, peak $10''' = (Ala_{10}–Val + Cu)^+$, $X'_{10} = (Ala_{10}–Val + Na–HCOOH)^+$ and $(Ala_{10}–Val + Na–NH_2CH_2CH_3)^+$, and $X'_9 = (Ala_9–Val + Na–HCOOH)^+$ and $(Ala_9–Val + Na–NH_2.CH_2CH_3)^+$.

synthesis by fitting the data to a trinomial distribution in which the general probability term (36) is given by

$$P(x_1, x_2, x_3) = \frac{n!}{x_1!x_2!x_3!} P_1^{x_1} P_2^{x_2} P_3^{x_3}, \quad [1]$$

where n is the number of cycles in the synthesis, x_1 is the number of deletions in n cycles, x_2 is the number of insertions in n cycles, $x_3 = n - (x_1 + x_2)$, P_1 is the probability of a deletion, P_2 is the probability of an insertion, and $P_3 = 1 - (P_1 + P_2)$. The abundance of a given final product is calculated by adding together all the significantly contributing terms given by Eq. [1]. Thus, for example, the abundance of the final product containing 10 alanine residues is

TABLE 1

COMPARISON OF MEASURED AND CALCULATED MASS FOR THE COMPONENTS OF UNFRACTIONATED SYNTHETIC L-Ala_{10}–Val, RUN 3, AND THEIR RELATIVE INTENSITIES FROM THE MASS SPECTRUM SHOWN IN FIG. 1

Peak number	Peak identity	Mass-to-charge ratio		Relative intensity[b]
		Measured[a]	Calculated[a]	
7′	$(Ala_7–Val + Na)^+$	637.4	637.3	2.4
8	$(Ala_8–Val + H)^+$	686.6	686.4	5.2
8′	$(Ala_8–Val + Na)^+$	708.5	708.4	19.2
9	$(Ala_9–Val + H)^+$	757.6	757.4	18.2
9′	$(Ala_9–Val + Na)^+$	779.5	779.4	64.7
9″	$(Ala_9–Val + 2Na–H)^+$	801.5	801.4	10.5
10	$(Ala_{10}–Val + H)^+$	828.9	828.5	26.2
10′	$(Ala_{10}–Val + Na)^+$	850.6	850.4	100.0
10″	$(Ala_{10}–Val + 2Na–H)^+$	872.5	872.4	15.7
11	$(Ala_{11}–Val + H)^+$	899.5	899.5	4.0
11′	$(Ala_{11}–Val + Na)^+$	921.6	921.5	14.7
11″	$(Ala_{11}–Val + 2Na–H)^+$	943.5	943.5	1.7
12′	$(Ala_{12}–Val + Na)^+$	992.7	992.5	1.2

[a] Mass-to-charge ratio of most abundant isotopic component.
[b] Determined from the heights of the peaks in Fig. 1.

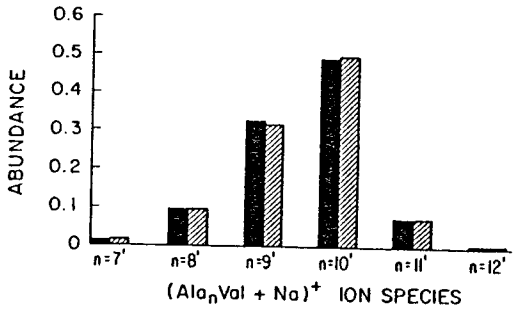


FIG. 2. Observed and calculated abundances of deletion and insertion peptides for the model peptide Ala_{10}–Val, run 3. The solid bars are the experimentally observed abundances of the $(M_n + Na)^+$ ions produced from the various products of the synthesis, $M_n = Ala_n$–Val where $n = 7$–12. The hatched bars are the abundances calculated by fitting the data to a trinomial probability distribution as described under Materials and Methods. For the products of run 3 a deletion probability of 6.2% per step and an insertion probability of 1.45% per step gave the best fit.

$$P(0 \text{ deletions, } 0 \text{ insertions, } 10)$$
$$+ P(1 \text{ deletion, } 1 \text{ insertion, } 8)$$
$$+ P(2 \text{ deletions, } 2 \text{ insertions, } 6)$$
$$+ \ldots$$

and the abundance of the final product containing nine alanine residues is

$$P(1 \text{ deletion, } 0 \text{ insertions, } 9)$$
$$+ P(2 \text{ deletions, } 1 \text{ insertion, } 7)$$
$$+ \ldots$$

and so on.

The fittings were made under the assumption that the probability of deletion or insertion is the same for each cycle of the synthesis. The results of the above-described fitting procedure for run 3 are given in Fig. 2 where a deletion probability of 6.2% per step and an insertion probability of 1.45% per step gave the best fit to the data. The deletion and insertion probabilities per step deduced by this procedure for all 10 of the L-Ala_{10}–Val synthetic runs are summarized in Table 2 and those for the additional synthetic runs are in Table 3.

For samples in which total by-products were small, adequate average values for the percentages of deletions and insertions per step could be estimated from the ratio of the observed height of the appropriate $(M + Na)^+$ peak to the sum of all observed $(M + Na)^+$ peak heights.

The Effects of Solvent and Coupling Protocol

The results of varying the solvent, and activation and coupling protocols for the synthesis of L-Ala_{10}–Val, are shown in Table 2. In runs 1–3 the couplings were with preformed symmetrical anhydrides, with the solvent for the double couplings being DMF/DMF, DMF/CH_2Cl_2, or CH_2Cl_2/DMF. Both deletions and insertions were large and little affected by the reaction solvent or the order in which they were used. *In situ* activation and coupling with symmetrical anhydrides, run 5, did not affect deletions (extent of coupling) significantly but it did reduce the amount of insertion peptides by a factor of 6 or 7, as expected. However, when a base such as diisopropyl ethyl amine or *N*-methylmorpholine was present during the second coupling, runs 6, 7, and 8, the level of insertions was increased markedly. The slight increase in the coupling reaction (lower deletions) was overshadowed by the deleterious effect on insertions.

Coupling with preformed HOBt esters in DMF:CH_2Cl_2, 1:1, run 9, also kept the insertion peptides relatively low (0.2% per step) but gave slow and incomplete coupling, with an average of 7.4% deletions per step. *In situ* activation and coupling with DCC, run 10, also reduced the insertion level, but not as much as expected and the reactions were not complete. The average yield of target peptide was 95.3%.

A single experiment with a polyacrylamide resin and with preactivated HOBt esters in DMF (data not shown) gave 4.3% deletions per step, which is also high and comparable to the value obtained on the polystyrene support suggesting that the poor coupling is not

TABLE 2

L-Ala_{10}-Val-OCH_2Pam-*S*-DVB SYNTHESES: EFFECTS OF ACTIVATION METHOD AND SOLVENT ON YIELDS OF DELETION AND INSERTION PEPTIDES

Run No.	Activation and solvent		Total $n-1$ peptide[a] (%)	Average deletions per cycle (%)	Total n + 1 peptide[a] (%)	Average insertions per cycle (%)	Average stepwise yield of target peptide[b] (%)
	First coupling	Second coupling					
1	Pre SA/DMF[c]	Pre SA/DMF	25.8	4.3	9.6	1.70	94.0
2	Pre SA/DMF	Pre Sa/CH_2Cl_2[d]	29.2	5.6	6.8	1.30	93.1
3	Pre SA/CH_2Cl_2	Pre SA/DMF	32.2	6.2	7.3	1.45	92.3
4	Pre SA/DMF	Situ SA/CH_2Cl_2[e]	27.7	4.4	5.5	0.90	94.7
5	Situ SA/CH_2Cl_2	Situ SA/CH_2Cl_2	28.5	4.4	1.3	0.20	95.4
6	Situ SA/CH_2Cl_2	Situ SA/CH_2Cl_2 +20% DIEA after 20 min	16.2	2.2	6.2	1.00	96.8
7	Situ SA/CH_2Cl_2	Situ SA/CH_2Cl_2 +1% NMM after 20 min	13.2	1.9	6.1	0.80	97.3
8	Situ SA/CH_2Cl_2	Situ SA/CH_2Cl_2 20% NMM after 20 min	18.7	2.2	4.1	0.50	97.3
9	Pre HOBt/DMF/CH_2Cl_2[f]	Pre HOBt/DMF/CH_2Cl_2	34.3	7.4	1.1	0.22	92.4
10	Situ DCC/CH_2Cl_2[g]	Situ DDC/CH_2Cl_2	28.8	4.4	1.7	0.26	95.3

[a] Calculated as 9′/(8′ + 9′ + 10′ + 11′) and 11′(8′ + 9′ + 10′ + 11′). The percentages of the peptides containing one less or one more alanine residue are shown only to indicate the approximate magnitudes of the total deletion and insertion events. Each of these peaks will be a mixture of the single deletion or insertion peptides plus much lower amounts of peptides derived from various combinations of deletions plus insertions occurring in the same peptide. The levels of components containing a net decrease or increase of two and three residues were also measured and used for the analysis but are not shown here.

[b] 100% − (% deletions per step + % insertions per step).

[c] Pre SA means preformed symmetrical anhydride. This synthesis was carried out in dimethylformamide according to protocol I (Materials and Methods).

[d] Preformed symmetrical anhydride coupled in CH_2Cl_2 by protocol II.

[e] Situ SA means *in situ* symmetrical anhydride. The anhydride was formed in the presence of the amino component and coupled simultaneously by protocol III.

[f] Pre HOBt means preformed *N*′-hydroxybenzotriazole ester. The synthesis was carried out according to protocol IV.

[g] Situ DCC means *in situ* dicyclohexylcarbodiimide. The Boc-amino acid was activated and coupled in the presence of the amino component by protocol V.

a consequence of the type of resin support, but more likely a result of conformational constraints of the model poly-L-alanine peptide.

The average stepwise yield of the target peptide, L-Ala_{10}-Val, in this series of experiments ranged from 92 to 97% and that was clearly not satisfactory.

The Effect of the Composition of the Model Polypeptide

Since the model L-Ala_{10}-Val did not give fully satisfactory results due, presumably, to conformational constrains imposed by the all L structures, we decided to disrupt the helix-producing chain by insertion of several D-Ala residues. The model selected was L-Ala-D-Ala-L-Ala-L-Ala-D-Ala-L-Ala-L-Ala-D-Ala-L-Ala-L-Ala-L-Val. Table 3 summarizes the results on this new model, and it will be seen that in all cases the results are much improved.

Comparison of preformed symmetrical anhydride syntheses in which the first coupling was either in DMF or CH_2Cl_2, runs 11 and

TABLE 3

SYNTHESES OF [D-Ala^{2,5,8}]Ala$_{10}$–Val–OCH$_2$Pam–*S*–DVB AND [D-Ala^{3,6,9,12,15,18}]Ala$_{20}$–Val–OCH$_2$Pam-*S*-DVB: EFFECTS OF THE ACTIVATION METHOD AND SOLVENT ON YIELDS OF DELETION AND INSERTION PEPTIDES

Run No.	Activation and solvent[a]		Total $n-1$ peptide (%)	Average deletions per cycle (%)	Total $n+1$ peptide (%)	Average insertions per cycle (%)	Average stepwise yield of target peptides (%)
	First coupling	Second coupling					
	[D-Ala^{2,5,8}]Ala$_{10}$–Val–OCH$_2$Pam-*S*-DVB						
11	Pre SA/DMF	Pre SA/CH_2Cl_2	4.7	0.50	4.9	0.52	98.80
12	Pre SA/CH_2Cl_2	Pre SA/DMF	3.1	0.31	1.0	0.10	99.59
13	Pre SA/CH_2Cl_2 Stand 1 h	Pre SA/DMF	2.0	0.20	2.9	0.30	99.50
14	Pre HOBt/DMF/CH_2Cl_2	Pre HOBt/DMF/CH_2Cl_2	3.1	0.31	0.20	0.02	99.67
15	Situ SA/CH_2Cl_2	Pre SA/DMF	4.3	0.45	2.8	0.30	99.25
16	Situ DCC/CH_2Cl_2	None	2.6	0.27	0.20	0.02	99.71
17	Situ DCC/CH_2Cl_2	Pre SA/DMF	1.3	0.13	0.94	0.10	99.77
18	Situ DCC/CH_2Cl_2	Situ DCC/CH_2Cl_2	1.6	0.15	0.32	0.03	99.82
	[D-Ala^{3,6,9}]Ala$_{11}$–Val–OCH$_2$Pam-S-DVB						
19[b]	Situ DCC/CH_2Cl_2	Situ DCC/CH_2Cl_2	0.39	0.036	0.38	0.035	99.93
	[D-Ala^{3,6,9,12,15,18}]Ala$_{20}$–Val–OCH$_2$Pam-*S*-DVB						
20[c]	Situ DCC/CH_2Cl_2	Situ DCC/CH_2Cl_2	0.72	0.036	0.56	0.029	99.93

[a] The activation and coupling procedures are the same as described in Table 2.
[b] This is a repeat of run 18, except it was carried to Ala$_{11}$–Val.
[c] This is a continuation of run 19.

12, again showed little difference in yields of deletion peptides, but a fivefold decrease in insertion peptides when the solvent was CH_2Cl_2. However, if the anhydride was allowed to stand in CH_2Cl_2 for an additional 1 h (run 13), the level of insertions increased by a factor of 3. The coupling with preformed HOBt esters in DMF:CH_2Cl_2 1:1, run 14, was markedly improved with this model peptide over the all L model. The result was somewhat better than that with the preformed symmetrical anhydride in DMF and the occurrence of insertion peptides was very small (<0.02%/cycle). *In situ* symmetrical anhydrides also gave a comparable extent of coupling and a decreased insertion reaction, run 15.

Single *in situ* DCC couplings in CH_2Cl_2, run 16, were comparable to the double couplings with symmetrical anhydrides or HOBt esters and produced very little insertion product. These results were further improved by a first coupling with *in situ* DCC in CH_2Cl_2 and a second coupling with preformed symmetrical anhydrides in DMF, run 17, where the average stepwise yield was 99.77%. Two syntheses were carried out by a double *in situ* DCC coupling protocol, runs 18 and 19. In run 18 the target peptide was Ala$_{10}$–Val and in run 19 it was Ala$_{11}$–Val. The results were remarkably good, with average stepwise yields of 99.82 and 99.93%. The slight differences may represent small differences in variables that were not controlled adequately during the

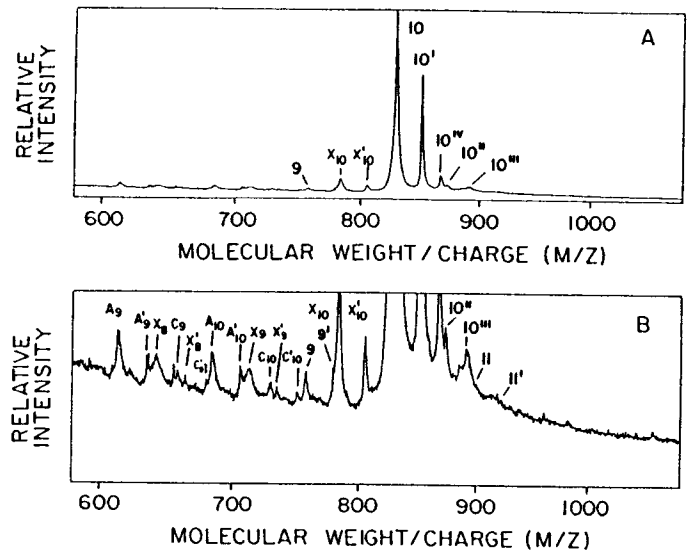


FIG. 3. (A) Mass spectrum of [D-$Ala^{2,5,8}$]Ala_{10}–Val, run 18. Double coupling by *in situ* DCC in dichloromethane. The mass spectrometer data were collected for 16 h to increase the sensitivity of detection of deletion and insertion peptides. The peaks are numbered as in Fig. 1. In addition, peak X'_{10} = (Ala_{10}–Val + Na–HCOOH)$^+$ and (Ala_{10}–Val + Na–$NH_2CH_2CH_3$)$^+$, peak X_{10} = (Ala_{10}–Val + H–HCOOH)$^+$ and (Ala_{10}–Val + H–$NH_2CH_2CH_3$)$^+$, and peak 10^{IV} = (Ala_{10}–Val + K)$^+$. (B) Mass spectrum of [D-$Ala^{2,5,8}$]-Ala_{10}–Val, run 18. The data from (A) are amplified 10-fold to show the deletion and insertion peptides more clearly. Six different series of fragment ions are present, denoted X'_i, X_i, C'_i, C_i, A'_i, and A_i. These labels refer to the fragment notation Scheme I given in the text. For simplicity we have not explicitly indicated the hydrogen transfers. The primed labels refer to fragments originating from the (Ala_{10}–Val + Na)$^+$ parent ion while the unprimed labels refer to fragments originating from the (Ala_{10}–Val + H)$^+$ ion species.

synthesis. In these latter two analyses the precision was enhanced by collecting data for 16 h. The limits of detection of the peaks are estimated to be <0.2%, which for the deca-alanine model gives us the ability to see as little as 0.02% deletions or insertions per synthetic cycle and, therefore, an average stepwise yield of target peptide of greater than 99.96%.

The partial mass spectum between 600 and 1000 mass units of the crude, unpurified peptide from run 18 is shown in Fig. 3A. Figure 3A is plotted to show the largest ion, 10, at full scale. The major Ala_{10}–Val ionic species observed in run 3 (Fig. 1) are all clearly present. Comparison with Fig. 1 immediately demonstrates that run 18 yielded much lower levels of deletion and insertion peptides than did run 3. The peak for the deletion peptide 9 is so small as to be hardly discernable in Fig. 3A, and the insertion peptide peaks 11 and 11′ cannot be seen at all. The large dynamic range of the measurements allows the mass spectrum to be expanded 10-fold in the intensity scale, Fig. 3B. It was then possible to estimate peak 9 as 1.57% of all (M + H)$^+$ peaks and peak 11 as 0.3% of all (M + H)$^+$ peaks. The resulting average stepwise yield of deletion peptides was then calculated to be 0.15%, and for insertion peptides the stepwise yield was 0.03%, giving a stepwise yield of target peptide of 99.82%. The results of the fit to the data are shown in Fig. 4. Again comparison with Fig. 2 graphically demonstrates that run 18 yielded much lower levels of deletion and insertion peptides than did run 3.

In the expanded spectrum, Fig. 3B, a number of additional ion peaks are observed at masses below the 10 and 10′ peaks. With the exception of the 9 and 9′ peaks, these result from products of unimolecular fragmenta-

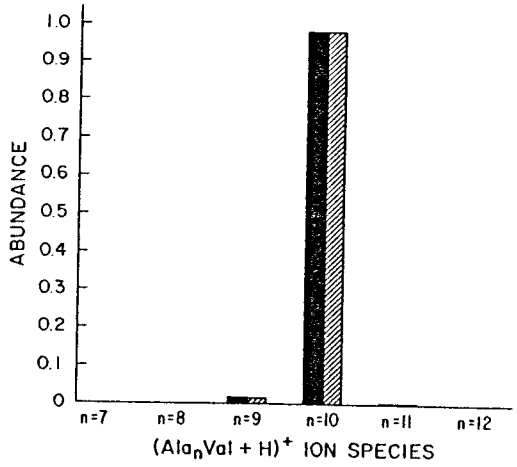


FIG. 4. Observed and calculated abundances of deletion and insertion peptides for the model peptide Ala_{10}–Val, run 18. The solid bars are the experimentally observed abundances of the $(M_n + H)^+$ ions produced from the various products of the synthesis, M_n = Ala_n–Val where n = 9–11. The hatched bars are the abundances calculated by fitting the data to a trinomial probability distribution as described under Materials and Methods. For the products of run 18 a deletion probability of 0.15% per step and an insertion probability of 0.03% per step gave the best fit.

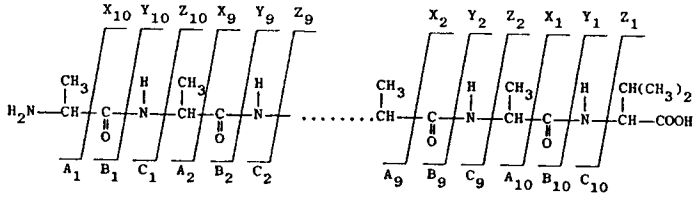
tion of the 10 and 10′ ions which occurs during the ^{252}Cf fission fragment ionization process. The identities of these fragment ions are indicated by the peak labels which refer to the notation in Scheme 1. For simplicity we have not explicitly indicated the hydrogen transfers. The unprimed labels refer to fragments originating from the $(Ala_{10}–Val + H)^+$ parent ion while the primed labels refer to fragments originating from the $(Ala_{10}–Val + Na)^+$ ion species. Note that peaks originating from deletion peptides can be differentiated from these fragment ion peaks.

The Effect of Extending the Model Peptide to 20 Alanine Residues

A single synthesis has been carried out in which the model peptide chain was lengthened in order to increase the amplification of by-product detection and to examine the effect of chain length on synthetic efficiency. Thus, [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val–OCH_2–Pam–S–DVB was assembled by the *in situ* DCC in CH_2Cl_2 double coupling protocol. The synthesis was a continuation of run 19. The results are shown in Fig. 5 and Table 3. Figure 5A is plotted to show the largest ion, 20′, at full scale. In it the seven Ala_{20}–Val ionic species corresponding to those detected previously for the Ala_{10}–Val series (Fig. 3) were clearly present. Peaks for the deletion peptide 19 and insertion peptides 21 and 21′ were so small that they cannot be discerned in Fig. 5A. However, when the spectrum was expanded 10-fold, Fig. 5B, it became possible to estimate peak 19 as 0.72% of all $(M + H)^+$ peaks and peak 21′ as 0.56% of all $(M + Na)^+$ peaks. The resulting average stepwise yield of deletion peptides was then calculated to be 0.036%, and for insertion peptides the stepwise yield was 0.029%, giving a stepwise yield of target peptide of 99.93%. These average stepwise yields after 20 steps were very close to those measured after 11 steps, indicating that the two side reactions were not a function of peptide chain length and that our assumption that deletions and insertions occurred uniformly throughout the synthesis was reasonable.

Analytical HPLC Evaluation of the Model Peptides

The presence of deletion and insertion peptides in run 3 could also be detected by high-performance liquid chromatography. However, the resolution and sensitivity was not as great as that with mass spectrometry and, furthermore, the technique does not directly identify the peptides. The peptides from the highly efficient runs 17–20 appeared to be homogeneous by this technique. However, these peptides contained three or six D amino acid residues, and peptides with deletions and insertions at different residues are chemically different and will not behave identically by this technique. Therefore, they will give rise to broader, multicomponent peaks that are more difficult to detect, and thus much of the amplification present in the MS procedure is lost.



SCHEME I

Circular Dichroism of the Model Peptides

The conformation of the peptides was examined by measuring circular dichroism as a function of solvent. The all L-Ala_{10}–Val peptide in dilute (0.1 mg/ml) solution was highly helical (92%) in 0.01 N HCl in the presence of only 10% trifluoroethanol, TFE (needed for solubility). In 20 and 80% TFE the peptide was essentially 100% helical. In sharp contrast, neither [D-$Ala^{2,5,8}$]Ala_{10}–Val nor [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val showed any significant amount of helicity by CD measurements, even in the presence of 80% TFE. The latter two peptides were mixtures of β-sheet and random coil in a ratio of approximately 2 or 3 to 1.

Amino Acid Analysis of the Model Peptides

Data on amino acid analyses of acid hydrolysates of three of the HF-cleaved peptides (before any fractionation) are summarized in Table 4. If the synthesis proceeded in high yield, the ratio of Ala/Val should coincide with the number of alanine residues in the model and the deviation from the expected ratio should be a rough measure of the frac-

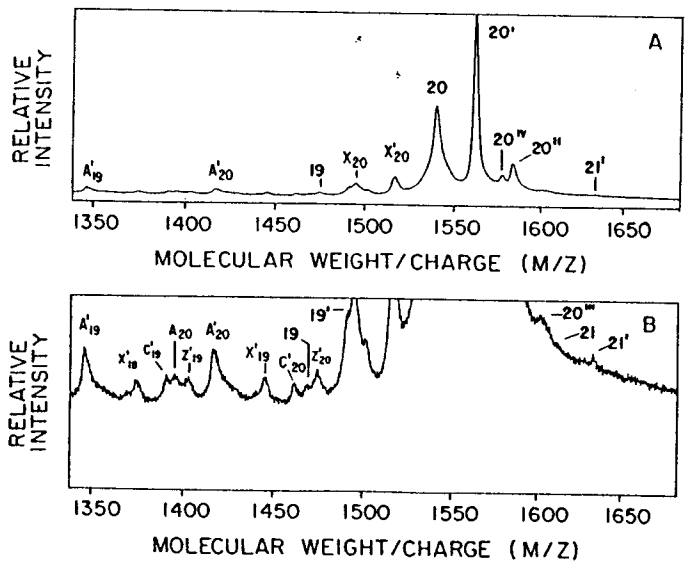


FIG. 5. (A) Mass spectrum of [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val, run 20. Double coupling by *in situ* DCC in dichloromethane. This is a 24-h spectrum accumulation. The lines indicate the expected molecular weight positions of the Ala_{19}–Val single deletion peak and the Ala_{21}–Val insertion peak. The peaks are identified as in Figs. 1 and 3. (B) Mass spectrum of [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val, run 20. The data from Fig. 5A are amplified 10-fold to show the deletion and insertion peptides more clearly. The notation for the fragment ions is described in the caption to Fig. 3B.

TABLE 4

AMINO ACID ANALYSES OF SYNTHETIC MODEL PEPTIDES[a]

Run No.	Peptide	Amino acid ratio	
		Found	Expected
3	L-Ala_{10}–Val	9.03	10
17	[D-$Ala^{2,5,8}$]Ala_{10}–Val	9.73	10
20	[D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val	20.7	20

[a] The unfractionated peptides obtained after HF cleavage from the resin support were hydrolyzed 48 h, 110°C, in 6 N HCl and analyzed directly on the Beckman 6300 analyzer.

tion of deletion minus insertion peptides produced, as seen for run 3. For the high-yield runs the agreement was good. The Ala/Val ratio in run 17 was 9.73 vs 10 and for run 20 it was 20.7 vs 20. The MS data suggest that these small deviations (±3%) from theory result from experimental errors in hydrolysis and analysis.

DISCUSSION

The fission fragment time-of-flight mass spectrometric technique has proven to be of great use in the analysis of synthetic peptides. It has recently been much improved by the development of the thin-film nitrocellulose method for sample introduction, which has markedly increased the sensitivity of technique and has increased the useful mass range. The model peptides described here extend to molecular masses of 1800 Da and were easily measured. This mass spectrometric method gives rise to several different cationic species of each peptide including the $(M + H)^+$, $(M + Na)^+$, and $(M + 2Na–H)^+$ ions. The sodium which contributes to the latter two ionic species is nearly always present in peptide samples as a trace impurity. Traces of potassium and copper impurities are also sometimes present and give rise to weak $(M + K)^+$ and $(M + Cu)^+$ ions. The typical pattern that this set of ions presents is not a detriment but, instead, is often helpful in identifying parent ions and differentiating them from fragment ions produced during the ^{252}Cf fission fragment ionization process. The observed masses of the peptide ions are typically within 0.3 mass unit of the calculated value.

The purpose of this study was to provide a sensitive way to estimate the synthetic efficiency of solid phase peptide synthesis, as reflected in the levels of deletion and insertion peptides that are produced. Such a method then allows us to study some of the synthetic variables and to define the most effective synthetic procedure. We selected two homo-oligomers as model peptides, Ala_{10}–Val and Ala_{20}–Val. The omission of a single Ala during the synthesis will thus produce the single-deletion peptides Ala_9–Val or Ala_{19}–Val, which can be detected and quantitated by the MS analysis. Since deletion of a residue at any step will produce the same Ala_9 or Ala_{19} peptide the size of the observed deletion peptide peak will be amplified by a factor of 10 or 20 over the size that would be observed if each deletion gave a different product. This reasoning also applies to multiple deletions or to insertion peptides in which more than one residue is added at a single step.

The first studies were with the all L model peptide. As described under Results, preformed symmetrical anhydride activation and coupling gave high levels of deletion and insertion peptides and they were not greatly different when the two couplings were both carried out in dimethylformamide or first in DMF and second in CH_2Cl_2 or first in CH_2Cl_2 and second in DMF. The formation of the anhydride *in situ,* in the presence of the amino component, did not give better coupling but did reduce the level of insertion peptides. *In situ* DCC coupling gave similar results. The naming of these two procedures is based on previous use of the terms, although in each a mixed mechanism may exist. *In situ* symmetrical anhydride means that two equivalents of Boc-amino acid and one equivalent of DCC are used, in which case the Boc-aminoacylisourea is formed first and

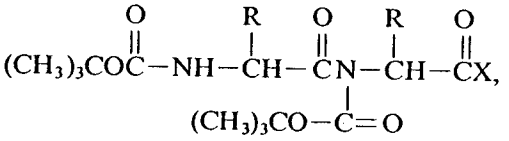
presumably converted to the anhydride by excess carboxylate before aminolysis to give the peptide bond. *In situ* DCC means that equivalent amounts of Boc amino acid and DCC are used. In solid phase peptide synthesis, the Boc-aminoacylisourea probably reacts directly with the peptide chain, although it has also been suggested that it proceeds to the anhydride before coupling.

The deletion peptides arise from incomplete deprotection, neutralization, or coupling reactions of a peptide chain. For single deletions the peptide chain resumes growth at the next cycle of synthesis. For multiple deletions, this lack of growth occurs more than once in a single chain, either consecutively or at later stages of the synthesis. We believe the main cause in the case of L-Ala_{10}–Val to be some form of conformational restriction of the peptide chains which causes them to become less solvated and less accessible to the incoming activated amino acid. Both prediction and circular dichroism measurements suggest that the problem is due to the helicity of the peptide. Based on these and previous studies (37), we do not believe that peptide–polymer interactions are responsible. The experiment with a polyacrylamide resin reported here supports this view since the extent of coupling was comparable to that found with a polystyrene resin of different polarity.

No detectable levels (>0.4%) of trifluoroacetyl peptides could be found for the Ala_n–Val peptides, with n = 9–11. Large amounts were not expected because the Pam-resin support was shown previously (38) to avoid the main side reaction leading to chain termination by trifluoroacetylation.

The extent of coupling of the anhydride was increased in the presence of a base. This effect may be due to the removal of a proton or other temporary blocking group from the amino component or possibly to an effect on peptide conformation. However, the presence of base during the formation and coupling of symmetrical anhydrides is not recommended because it catalyzes the rearrangement leading to the insertion reaction.

The insertion peptides arise by a mechanism that we have called intramolecular urethane acylation (23). This reaction, which has been known for many years (39,40), results from an attack of the urethane nitrogen of one component of the anhydride on the carbonyl carbon of the other component to give a dipeptide derivative II,



II

which becomes activated by anhydride interchange and then couples to the growing peptide chain. The net effect is the introduction of two residues of amino acid, i.e., the insertion of an extra residue into the chain. This reaction is very facile with glycine, but the present data show that it can occur to a significant extent with Boc-alanine. The reaction is minimal at −10°C, but is known to be accelerated at room temperature and also by the presence of tertiary amines or by prolonged standing of the anhydride before coupling (23), and these findings have been confirmed here. The level of insertion peptides is reduced by using *in situ* anhydride coupling, where the time of standing after activation is much shorter, or with hydroxybenzotriazole esters, which have little tendency to form intermediate II.

The extent of coupling was greatly increased by introduction of D-alanine into the peptide chain of the model and the recommended model peptides now contain several D-alanine residues. This was expected to reduce the tendency of the polyalanine to assume a rigid helical conformation, and the prediction was confirmed by the circular dichroism measurements. The measurements on the free peptide were made in mixtures of 0.01 N HCl and trifluoroethanol, and we do not know the extent of helicity of the resin-bound peptide in CH_2Cl_2 although for the all L peptide it is assumed to be high. The ran-

dom chains containing D residues were then more accessible to the activated amino acid and the deletion peptides could be reduced to very low levels, averaging as little as 0.04% per step. In such a peptide the deletion of a D residue or an L residue will give diastereoisomers that are not chemically identical, but which will behave identically in the mass spectrometer. Thus, the amplification feature is not lost as it would be in chromatographic or other methods of analysis that depend on the shape and chemical properties of the peptides.

The best procedure that we have found for the synthesis of these model peptides is the original DCC coupling in CH_2Cl_2 (1). The activated intermediate has very little tendency to form the dipeptide derivative by intermolecular reaction, which would lead to insertion peptides, and the coupling reaction in most instances proceeds rapidly and in high yield. In some cases, however, peptide conformation problems can reduce coupling rates and yields (41). In those instances we recommend a first coupling with DCC in CH_2Cl_2, followed by a second coupling with a symmetrical anhydride in DMF or with an HOBt ester in DMF. The solvent effect can usually drive the reaction to near completion and the insertion reaction will be minimal because only a few amino groups will be present at the beginning of the second coupling.

In an attempt to study the effect of chain length on the efficiency of the coupling reaction, the length of the model peptide was extended to 20 alanine residues. Thus, [D-$Ala^{3,6,9}$]Ala_{11}–Val-resin, run 19, was extended to [D-$Ala^{3,6,9,12,15,18}$]Ala_{20}–Val-resin, run 20, (Table 3). The data show that the peptide chain can be extended to 20 alanine residues with quite high efficiency. The average deletions per cycle were 0.036% at 11 alanines and remained at 0.036% after 20 alanines, showing that the coupling efficiency did not decline over this range of chain length. There was also no significant difference in the average level (0.035% vs 0.029%) of insertion peptides per step on going to 11 or to 20 alanine residues.

The other important conclusion from these findings is that the standard symmetrical anhydride coupling in DMF recommended for the ABI instrument is not safe and is likely to lead to significant levels of insertion peptides, especially when long peptides are synthesized. The danger would be reduced, however, if lower temperatures and shorter standing times could be arranged.

The molecular weight of Ala_{20}–Val is far from the limits of the mass spectrometric analysis. Therefore, an extension of these experiments to much longer peptides and to models containing other amino acids should be feasible and should help establish more general limits to the efficiency of solid phase peptide synthesis.

ACKNOWLEDGMENTS

We thank Mrs. Tanuja Chaudhary and Mr. Louis Grace for assistance in obtaining the mass spectra and Mr. Barry Cunningham for the amino acid analyses and for his assistance with some of the synthetic work.

REFERENCES

1. Merrifield, R. B. (1963) *J. Amer. Chem. Soc.* **85**, 2149–2154.
2. Marshall, G. R., and Merrifield, R. B. (1971) *in* Biochemical Aspects of Reactions on Solid Supports (Stark, G. R., Ed.), pp. 111–169, Academic Press, New York.
3. Hirt, J., de Leer, E. W. B., and Beyerman, H. C. (1973) *in* The Chemistry of Polypeptides (Katsoyannis, P. G., Ed.), pp. 363–387, Plenum, New York.
4. Erickson, B. W., and Merrifield, R. B. (1976) *in* The Proteins (Neurath, H., and Hill, R. L., Eds.), Vol. 2, 3rd ed., pp. 255–527, Academic Press, New York.
5. Barany, G., and Merrifield, R. B. (1979) *in* The Peptides (Gross, E., and Meienhofer, J., Eds.), Vol. 2, Part A, pp. 1–284, Academic Press, New York.
6. Gut, V., and Rudinger, J. (1987) *in* Peptides 1968 (Bricas, E., Ed.), pp. 185–186, North-Holland, Amsterdam.
7. Wang, S. S., and Merrifield, R. B. (1969) *Int. J. Peptide Protein Res.* **1**, 235–244.
8. Bodanszky, M., and Sheehan, J. T. (1966) *Chem. Ind. (London)*, 1597.

9. Rudinger, J., and Gut, V. (1967) *in* Peptides (Beyerman, H. C., van de Linde, A., and Maassen van den Brink, W., Eds.), pp. 89–90, North-Holland, Amsterdam.
10. Brunfeldt, K., Roepstorff, P., and Thomsen, J. (1969) *Acta Chem. Scand.* **23**, 2906–2907.
11. Dorman, L. C. (1969) *Tetrahedron Lett.* 2319–2321.
12. Hancock, W. S., Prescott, D. J., Vagelos, P. R., and Marshall, G. R. (1973) *J. Org. Chem.* **38**, 774–781.
13. Gisin, B. F. (1972) *Anal. Chim. Acta* **58**, 248–249.
14. Beyerman, H. C., van der Kamp, P. R. M., de Leer, E. W. B., Maassen van den Brink, W., Parmentier, J. H., and Westerling, J. (1973) *in* Peptides 1971 (Nesvadba, H., Ed.), pp. 162–164, North-Holland, Amsterdam.
15. Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) *Anal. Biochem.* **34**, 595–598.
16. Sarin, V. K., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) *Anal. Biochem.* **117**, 147–157.
17. Esko, K., Karlsson, S., and Porath, J. (1968) *Acta Chem. Scand.* **22**, 3342–3343.
18. Losse, G., and Ulbrich, R. (1973) East German Patent No. 83,529; *Chem. Abstr.* **78**, 84822.
19. Cameron, L., Meldal, M., and Sheppard, R. C. (1987) *J. Chem. Soc. Chem. Commun.*, 270.
20. Niall, H. D., Tregear, G. W., and Jacobs, J. (1972) *in* Chemistry and Biology of Peptides (Meienhofer, J., Ed.), pp. 695–699, Ann Arbor Science Pub., Ann Arbor, MI.
21. Bayer, E., Eckstein, H., Hägele, K., König, W. A., Brüning, W., Hagenmaier, H., and Parr, W. (1970) *J. Amer. Chem. Soc.* **92**, 1735–1738.
22. Biemann, K., and Scoble, H. A. (1987) *Science* **237**, 992–998.
23. Merrifield, R. B., Mitchell, A. R., and Clarke, J. E. (1974) *J. Org. Chem.* **39**, 660–668.
24. Erickson, B. W., and Merrifield, R. B. (1973) *J. Amer. Chem. Soc.* **95**, 3757–3763.
25. Yamashiro, D. (1979) *Int. J. Pept. Protein Res.* **13**, 5–11.
26. Johnsson, G. P., Hedin, A. B., Hakansson, P. L., Sundquist, B. U. R., Save, B. G. S., Nielsen, P. F., Roenstorff, P., Johansson, K. E., Kamensky, I., and Lindberg, M. S. L. (1986) *Anal. Chem.* **58**, 1084–1087.
27. Chait, B. T., and Field, F. H. (1986) *Biochem. Biophys. Res. Commun.* **134**, 420–426.
28. Macfarlane, R. D., and Torgerson, D. F. (1976) *Science* **191**, 920–925.
29. Chait, B. T., Agosta, W. C., and Field, F. H. (1981) *Int. J. Mass Spectrom. Ion Phys.* **39**, 339–366.
30. Chait, B. T., Gisin, B. F., and Field, F. H. (1982) *J. Amer. Chem. Soc.* **104**, 5157–5162.
31. Mitchell, A. R., Kent, S. B. H., Engelhard, M., and Merrifield, R. B. (1978) *J. Org. Chem.* **43**, 2845–2852.
32. Tam, J. P., Kent, S. B. H., Wong, T. W., and Merrifield, R. B. (1979) *Synthesis*, 955–957.
33. Hagenmaier, H., and Frank, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1973–1976.
34. Merrifield, R. B., Vizioli, L. D., and Boman, H. G. (1982) *Biochemistry* **21**, 5020–5031.
35. Chang, C. T., Wu, C.-S. C., and Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–31.
36. Hoel, P. G. (1966) Introduction to Mathematical Statistics, 3rd ed., p. 117, Wiley, New York.
37. Sarin, V. K., Kent, S. B. H., and Merrifield, R. B. (1980) *J. Amer. Chem. Soc.* **102**, 5463–5470.
38. Kent, S. B. H., Mitchell, A. R., Engelhard, M., and Merrifield, R. B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2180–2184.
39. Wieland, T., and Heinke, B. (1956) *Justus Liebigs Ann. Chem.* **599**, 70.
40. Kotake, H., and Saito, T. (1966) *Bull. Chem. Soc. Japan* **39**, 853.
41. Kent, S. B. H., and Merrifield, R. B. (1981) *in* Peptides 1980 (Brunfeldt, K., Ed.), pp. 328–333, Scriptor, Copenhagen.