Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 2791–2795, May 1981
Biochemistry

Solid-phase peptide synthesis under continuous-flow conditions

(polystyrene supports/high-pressure liquid chromatography/peptide analysis/model tetrapeptide/ovalbumin)

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Communicated by Bruce Merrifield, January 30, 1981

ABSTRACT **A system is described for solid-phase synthesis of peptides under continuous-flow conditions with liquid chromatographic equipment, conventional polystyrene supports, and well-defined chemistry. The model tetrapeptide Leu-Ala-Gly-Val was assembled in 99.3% purity in about 4 hr on microporous copoly(styrene-1% divinylbenzene). During coupling, the preformed symmetric anhydrides were conserved by being recycled. Relative yields of the peptide products were determined quantitatively in 20 min by reverse-phase high-pressure liquid chromatography. This rapid assay system was used to examine the influence on product yields of (*i*) the time and number of couplings per cycle, (*ii*) microporous versus macroporous polystyrene, and (*iii*) *tert*-butoxycarbonyl (Boc) group versus 9-fluorenylmethoxycarbonyl for amine protection. Use of microporous polystyrene and two 30-min couplings of Boc-amino acids per cycle gave the best results. This continuous-flow system provides a rapid and efficient approach to solid-phase peptide synthesis. A 17-residue peptide from chicken ovalbumin was obtained in similar purity and yield from a discontinuous synthesis and from a continuous-flow synthesis.**

Solid-phase peptide synthesis (1, 2) has played a major role in defining the structural features important for the biologic activities of numerous polypeptides, including hormones, neuropeptides, and even enzymes. The discontinuous mode of operation described by Merrifield (3) in 1963 involves the addition of each reagent solution and wash solvent to the solid support in several discrete portions. In 1970–1972, Bayer *et al.* (4) and Scott *et al.* (5, 6) showed that solid-phase synthesis can also be performed by passing a continuous flow of each solution or solvent through a column containing the solid support. A simple, rapid, readily available, and easily automated system for solid-phase peptide synthesis under continuous-flow conditions would be useful for the synthesis of large polypeptides. This paper describes a continuous-flow system based on commercially available modular equipment (7–9) for high-pressure liquid chromatography (HPLC), conventional microporous polystyrene supports, and well-established chemistry (1, 2).

MATERIALS AND METHODS

Continuous-Flow Synthesis. The model tetrapeptide Leu-Ala-Gly-Val (3, 10, 11) was assembled under continuous-flow conditions with a commercial HPLC system (Waters Associates) consisting of a model 6000A pump, model U6K manual sample injector, model 440 dual-wavelength UV monitor, and a stainless steel column (0.39-cm inner diameter by 6-cm length) bearing 10-μm inlet and outlet filters (Fig. 1). Two manual three-way valves present on the pump were used to control the flow of liquids through the solid support in the column. Valve 1 was used to select the source of new liquid entering the pump and

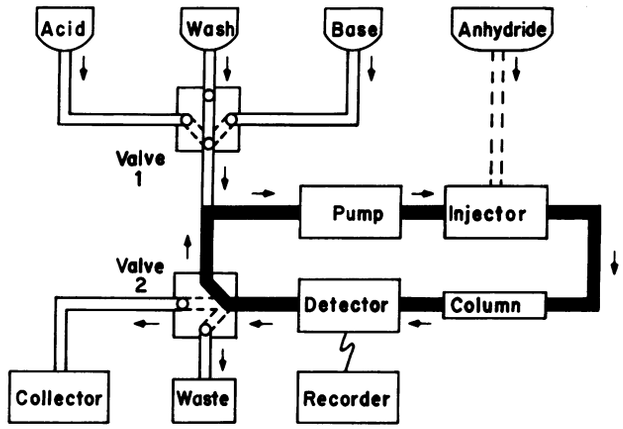

FIG. 1. Flow chart for a continuous-flow solid-phase synthesizer. The dark lines indicate the flow pattern for recycling during coupling.

valve 2 was employed to direct the liquid leaving the detector. Four pump-head microfilters were replaced by large-bore washers to prevent their clogging with particulate matter, which increases the back pressure and can cause extrusion of the polystyrene beads through the column outlet filter (7). The resulting column back pressure ranged from 500 to 1000 pounds per square inch gauge (psig). In addition, cavitation and degassing of CH_2Cl_2 solutions as they were drawn into the pump heads was minimized by maintaining the acid, wash, and base reservoirs under slight nitrogen pressure (4 psig).

A typical synthetic cycle used dichloromethane (freshly distilled from anhydrous Na_2CO_3) as solvent, *tert*-butoxycarbonyl (Boc) amino acids, and phenylacetamidomethyl (PAM)-modified polystyrene. A column containing Boc-Val-OCH_2-PAM-polystyrene (10) beads (200–400 mesh; 0.08–0.1 g) was pumped with various solutions at 4.0 ml/min.

Step 1. The Boc-amino groups were deprotected by pumping for 5 min a solution‡ containing 0.01 M methanesulfonic acid and 0.10 M trifluoroacetic acid from the acid reservoir through valve 1, pump, injector, column, detector, and valve 2 to waste.

Step 2. Valve 1 was turned to the wash reservoir and solvent was pumped for 4 min through the column to waste.

Step 3. The resulting protonated amino groups were neutralized by turning valve 1 to the base reservoir and pumping

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Abbreviations: Boc, *tert*-butoxycarbonyl; DMF, *N,N'*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-pressure liquid chromatography; PAM, phenylacetamidomethyl; TLC, thin-layer chromatography; equiv., equivalent(s); psig, pounds per square inch gauge.

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‡ Erickson, B. W. & Wang, C. Y. (1975) 170th National Meeting of the American Chemical Society, ORGN 081 (abstr.).

for 1 min a solution of 0.35 M triethylamine through the column to waste.

Step 4. Step 2 was repeated.

Step 5. A solution of Boc-amino acid symmetric anhydride was prepared in advance by reaction of the thin-layer chromatography (TLC)-pure Boc-amino acid (2.0 equiv.) and *N,N'*-dicyclohexylcarbodiimide (1.0 equiv.) in CH_2Cl_2 for 10 min at 20–25°C. The resulting mixture was microfiltered twice (Millipore filter FHLP 013) to remove the precipitated dicyclohexylurea. The anhydride solution was adjusted to 0.175 M and placed in the 2-ml sample loop of the manual sample injector. As soon as this solution was injected, valve 2 was turned so that the liquid leaving the detector was recycled (Fig. 1) through the pump, injector, column, and detector for 30 min to conserve the valuable, slowly reacting anhydride solution. The 3.5-ml recycled volume consisted of 2 ml in the sample injection loop, 0.6 ml in the column, and 0.9 ml in the tubing. Thus, the concentration of symmetric anhydride in the column was 0.175 M during the first pass through the column (0.5 min) and was diluted to about 0.10 M during subsequent passes.

Step 6. Valve 2 was turned to waste and step 2 was repeated.

Steps 7–10. Steps 3–6 were repeated. The elapsed time per synthetic cycle was 83 min.

This synthetic cycle was carried out three times to couple successively Boc-Gly, Boc-Ala, and Boc-Leu to the support. Boc-Leu-Ala-Gly-Val-OCH_2-PAM-polystyrene was removed from the column and treated with anisole/liquid HF, 1:9 (vol/vol), for 1.0 hr at 0°C to obtain the free tetrapeptide and any deletion peptide by-products. The reaction mixture was washed with ether and extracted with 1% aqueous acetic acid. The extract was lyophylized and redissolved in microfiltered water (Millipore Milli-Q system) for analysis.

Peptide Analysis. Authentic samples of Leu-Ala-Gly-Val and its single-deletion and double-deletion peptides were synthesized by the solid-phase method, purified by reverse-phase HPLC, and shown to be homogeneous by amino acid analysis and by TLC in two systems (Table 1). Concentrations of stock solutions of the tetrapeptide and each deletion peptide were determined by duplicate amino acid analysis of hydrolyzed aliquots. Fresh standard mixtures of these solutions were analyzed just before analyzing each set of reaction mixtures.

Peptide standards and reaction mixtures containing these peptides were analyzed with an HPLC system (Waters Associates) consisting of two model 6000A high-pressure pumping systems, model 660 solvent programmer, model 710A automatic sample injector, and a stainless steel column (0.39 × 30 cm) containing μBondapak C_{18} reverse-phase support. Peptides were eluted for 25 min at 2.0 ml/min with a linear gradient of 0–16% (vol/vol) 2-propanol (Burdick and Jackson) in 5 mM triethylammonium phosphate (pH 6.3). The 220-nm absorbance of the eluted peptides was measured with a Hitachi model 635M dual-wavelength UV monitor set at 0.08 and 0.64 absorbance units full scale (Fig. 2).

Table 1. Analysis of Leu-Ala-Gly-Val and deletion peptides

Peptide sequence*	TLC†, (R_f)		HPLC‡	
	System A	System B	Retention time, min	Rel. molar absorbance
LAGV	0.16	0.41	14.4 ± 0.2	[1.00]
LGV	0.19	0.54	14.0 ± 0.2	0.80
LAV	0.20	0.52	13.7 ± 0.2	0.89
LV	0.39	0.60	12.1 ± 0.2	0.76
AGV	0.60	0.32	3.7 ± 0.1	0.67
AV, GV	0.66	0.36	——	——

* L, leucine; A, alanine; G, glycine; V, valine.

† System A was reverse-phase silica gel plates (Analtech) developed with 5% (vol/vol) methanol in 0.01 M sodium phosphate buffer (pH 6.3). System B was silica gel plates (GF, Analtech) developed with 1-butanol/acetic acid/water, 4:1:1 (vol/vol).

‡ Relative (Rel.) peak areas per mol of peptide at 220 nm, at which 1 nmol of LAGV corresponded to a peak area of 2.25 mm^2 at a detector scale of 0.64 absorbance units per 10-cm scale and a chart speed of 1 cm/min.

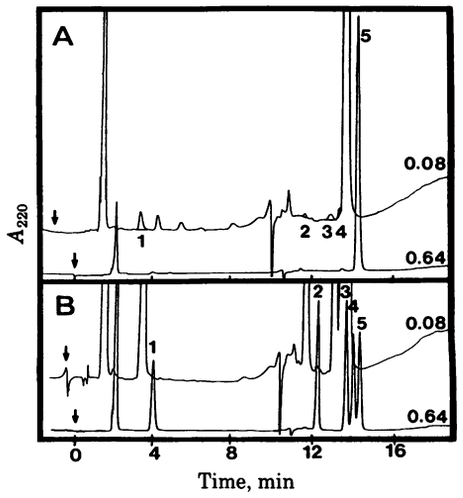

FIG. 2. Reverse-phase HPLC analysis of the model peptide Leu-Ala-Gly-Val (5) and four deletion peptides: Ala-Gly-Val (1), Leu-Val (2), Leu-Ala-Val (3), and Leu-Gly-Val (4). (*A*) Crude peptide mixture from synthesis S-8 of Table 3. (*B*) Mixture of synthetic peptide standards (50–70 nmol).

RESULTS AND DISCUSSION

Peptide Chemistry. Solid-phase peptide synthesis can be carried out in the continuous-flow mode with the same reagents and reactions normally used in the discontinuous mode. Specifically, protonated CF_3COOH is used for deprotection of the Boc-amino group, triethylamine is employed for neutralization of the resulting protonated amino group, and a Boc-amino acid symmetric anhydride is used to couple the next amino acid.

Although 6.5 M CF_3COOH in CH_2Cl_2 is used for deprotection in the discontinuous mode (1, 2), 0.01 M CH_3SO_2OH/0.10 M CF_3COOH in CH_2Cl_2‡ was used in the continuous-flow mode to minimize abrupt changes in resin swelling and potential corrosion of the pump during extended use. This solution contains the same deprotecting species present in 6.5 M CF_3COOH, which is not neutral CF_3COOH but its conjugate acid, $CF_3C(OH)_2^+$ (Fig. 3). A minimum of 5 equiv. of $CF_3C(OH)_2^+$ per Boc-amino site was pumped through the solid support during the deprotection step.

The 6.5 M CF_3COOH and CH_3SO_2OH/CF_3COOH solutions are comparable in their ability to remove the temporary

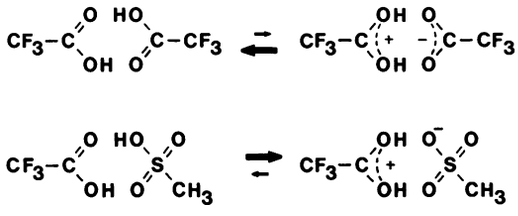

FIG. 3. Formation of protonated CF_3COOH (pK_a, *ca.* −3.5) by equilibrium self-protonation of CF_3COOH (pK_a 0.3) or by stoichiometric protonation with a small amount of CH_3SO_2OH (pK_a −6).

Boc protection group relative to removal of benzylic sidechain protecting groups, such as the benzyloxycarbonyl group on the ε-amino group of lysine.‡ The benzyl ester linkage between the peptide carboxyl group and hydroxymethyl-PAM-polystyrene is relatively resistant to cleavage by the CH_3SO_2OH/CF_3COOH solution. Only 0.34% of the tetrapeptide was released from Leu-Ala-Gly-Val-OCH_2-PAM-polystyrene during treatment with the CH_3SO_2OH/CF_3COOH solution for 8 hr.

Peptide Analysis. The success of a synthesis of Leu-Ala-Gly-Val depends mainly on the efficiency of the deprotection, neutralization, and coupling steps and on the chemical inertness of the solid support. Because very efficient synthesis of this model peptide (>98% yield) can be achieved in the discontinuous mode when microporous $HOCH_2$-PAM-polystyrene is used as the support (10, 12), it was used to explore the efficiency of solid-phase synthesis in the continuous-flow mode.

Relative molar yields of Leu-Ala-Gly-Val and its deletion by-products have been determined by low-pressure ion-exchange chromatographic separation and ninhydrin detection (13, 14). We have developed an alternate method that uses reverse-phase HPLC separation and UV detection (9). Injection of 100 nmol of a reaction mixture onto the μBondapak C_{18} column and measurement of the 220-nm peptide absorbance simultaneously at sensitivities of 0.08 and 0.64 absorbance units (full-scale) allows detection of as little as 0.2% of a peptide by-product. This reverse-phase HPLC assay can analyze about 5% of the peptide sample needed for the ion-exchange assay in about 5% of the time. One drawback is that the double-deletion peptides Ala-Val and Gly-Val are not sufficiently hydrophobic to be retarded by the column and elute with the solvent front. But separation of the third double-deletion peptide (Leu-Val) and all three single-deletion peptides (Table 1) is adequate to assess the efficiency of each cycle of a synthesis. One advantage is that UV monitoring also allows detection of by-products with a blocked terminal amino group. For example, CF_3CO-Gly-Val elutes at 13.2 min under the conditions described in Table 1.

Coupling Conditions. Several small-scale syntheses of Leu-Ala-Gly-Val (10–80 μmol, 4–34 mg) were performed with the manual continuous-flow synthesizer to explore the influence of coupling conditions on yield of the desired tetrapeptide (Table 2). Use of one 15-min coupling step per cycle was more efficient when the anhydride concentration was 0.10 M (Table 2, synthesis S-2) than when it was 0.045 M (synthesis S-1). As in the discontinuous mode, the important parameter for coupling efficiency is the concentration of the Boc-amino acid anhydride. With only one coupling step per cycle, the observed yields were not very reproducible (compare syntheses S-2 and S-3) because a single chemical or equipment fault could lower the yield of the desired product. For example, the final coupling of Boc-Leu to Ala-Gly-Val-OCH_2-PAM-polystyrene is generally less complete than the other coupling steps. The percentage of by-products decreased by about 50% when this coupling step was repeated (compare syntheses S-3 and S-4). Using two coupling steps per cycle (synthesis S-5) and increasing the coupling period from 15 to 30 min (synthesis S-6) gave higher, more reproducible yields of the desired tetrapeptide.

By-Product Distribution. Incomplete coupling during the first synthetic cycle gives the deletion peptide Leu-Ala-Val, during the second cycle produces Leu-Gly-Val, and during the last cycle yields Ala-Gly-Val, whereas Leu-Val arises through incomplete coupling during both the first and second cycles. The yields of each of these by-products were measured quantitatively by reverse-phase HPLC (Table 3).

The use of microporous $HOCH_2$-PAM-polystyrene as the solid support and just one 30-min coupling per cycle (Table 3, synthesis S-7) gave only 89% of the desired tetrapeptide and substantial amounts of each of the three single-deletion peptides. With two 30-min couplings per cycle (Table 3, synthesis S-8), the yield of Leu-Ala-Gly-Val was 98.3%.

Table 2. Continuous-flow synthesis of Leu-Ala-Gly-Val

Synthesis code*	Couplings per cycle, no.	Coupling time, min	Symmetric anhydride†		Tetrapeptide,‡ mol %
			M	Equiv.	
S-1	1	15	0.045	4	80.0
S-2	1	15	0.100	31	95.1
S-3	1	30	0.100	31	89.5
S-4	1§	15	0.170	15	94.3
S-5	2	15	0.100	31	96.4 ± 0.1
S-6	2	30	0.100	31	97.7 ± 0.6

* Microporous Boc-Val-OCH_2-PAM-polystyrene [0.13 mmol of valine per g of resin; 80–100 mg = 10–13 μmol of valine; prepared from copoly(styrene-1% divinylbenzene) (Bio-Beads SX-1, Bio-Rad)] was placed in an 0.39 × 6.0 cm column, except that in syntheses S-1 and S-4 a 0.78 × 10 cm column was used.

† Calculated values during recycling.

‡ Amount of Leu-Ala-Gly-Val present in the synthetic product mixture relative to the combined amounts of this peptide and four deletion peptides (Table 1); both S-5 and S-6 are an average of two syntheses.

§ Two couplings of Boc-Leu symmetric anhydride were used.

Two syntheses were also conducted on reaction scales (0.1 and 0.45 mmol) comparable to those used in peptide research. The concentration of Boc-amino acid symmetric anhydride and the times for each synthetic operation were maintained, but the amount of resin was increased 7-fold (Table 3, synthesis S-9) and, in addition, the amino acid loading was increased 5-fold (synthesis S-10). These syntheses used 10 and 2.2 molar equiv. of each symmetric anhydride, respectively. Since >99% of both product mixtures was the desired tetrapeptide, product purity was unaffected by a 34-fold increase in the scale of the synthesis. Synthesis S-10 gave 150 mg of the tetrapeptide. Thus, solid-phase synthesis under continuous-flow conditions has furnished this model peptide in the same high purity obtained by addition of each solvent and solution in several discrete portions (10, 12, 13).

Macroporous Support. A macroporous polystyrene support, which consisted of a rigid, highly crosslinked matrix containing large connected pores, was aminomethylated (14) and coupled with Boc-Val-oxymethylphenylacetic acid (10). This support, which did not swell in CH_2Cl_2, gave only 87% of the desired peptide and substantial amounts of two deletion peptides with one coupling step per cycle (Table 3, synthesis S-11). When two couplings were used per cycle, one by-product was nearly eliminated but the other was increased (synthesis S-12). In both syntheses, the coupling of Boc-Leu symmetric anhydride was very inefficient, which suggests that the rigid support tends to exclude the anhydride after only a few synthetic cycles. Substantial improvements in coupling efficiency would be needed before such rigid macroporous supports become as useful as the swellable microporous supports.

Deprotection with Base. The preceding syntheses used the Boc group for temporary protection of the α-amino groups and a 4-(acetamidomethyl)benzyl ester link to the polystyrene matrix. The Boc group was removed under moderately acidic conditions [$CF_3C(OH)_2^+$], and the ester link was cleaved under strongly acidic conditions (liquid HF). Also examined was a chemically milder procedure consisting of the 9-fluorenylmethoxycarbonyl (Fmoc) group (15) for α-amino protection and a 4-alkoxybenzyl ester link (16) to the microporous matrix. This orthogonal strategy (2) allowed removal of the Fmoc group under basic conditions (piperidine) and cleavage of the ester link

Table 3. Distribution of Leu-Ala-Gly-Val and deletion peptides after continuous-flow synthesis

Synthesis code	Amino protection	Solid support*	Valine loading, mmol/g	30-min couplings per cycle	Peptide distribution† relative mol %				
					LAGV	AGV	LAV	LGV	LV
S-7	Boc	A	0.13	1	89.1	4.2	5.2	1.1	0.2
S-8	Boc	A	0.13	2	98.3	1.3	0.4	<0.1	0.1
S-9	Boc	A	0.13	2	99.3	0.3	0.2	0.2	<0.1
S-10	Boc	A	0.64	2	99.1	0.5	0.4	<0.1	<0.1
S-11	Boc	B	0.27	1	86.6	9.7	0.6	2.9	0.2
S-12	Boc	B	0.27	2	83.3	16.1	0.5	0.3	0.4
S-13	Fmoc‡	C	0.80	2	96.1	2.3	1.6	<0.1	<0.1
S-14	Fmoc‡	C	0.44	2	96.3	0.2	3.5	<0.1	<0.1
S-15	Fmoc§	C	0.44	2	98.6	<0.1	1.4	<0.1	<0.1

* Support A, microporous hydroxymethyl-PAM-polystyrene prepared from copoly(styrene-1% divinylbenzene) (Bio-Beads SX-1, Bio-Rad); support B, hydroxymethyl-PAM-polystyrene prepared from macroporous polystyrene (Dionex, Sunnyvale, CA); support C, microporous 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) (*p*-alkoxybenzylalcohol resin, Chemical Dynamics, South Plainfield, NJ). Each synthesis used 0.08–0.10 g of Boc-Val-resin in a 0.39 × 6.0 cm column, except S-9 (0.76 g, 0.10 mmol) and S-10 (0.70 g, 0.45 mmol) in an 0.78 × 12 cm column pumped at 6 ml/min.

† L, leucine; A, alanine; G, glycine; V, valine.

‡ Soluble Fmoc-peptides were deprotected with 1 M piperidine in dioxane/water, 1:1 (vol/vol).

§ Soluble Fmoc-peptides were deprotected with 2 M piperidine in DMF, and the resulting free peptides were precipitated by addition of ether.

under moderately acidic conditions [$CF_3C(OH)_2^+$]. Separate neutralization with a basic solution was not needed.

4-(Hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) was esterified by using Fmoc-Val, *N,N'*-dicyclohexylcarbodiimide, and 4-(dimethylamino)pyridine (17). Leu-Ala-Gly-Val was assembled by successive addition of Fmoc-amino acid symmetric anhydrides (17, 18). The Fmoc group was removed by treatment for 5 min with 2.0 M piperidine in dimethylformamide (DMF) (18). In order to use this solution, a second model 6000A pump and a model 660 solvent programmer were added to the manual synthesizer. The wash step before deprotection consisted of a 5-min treatment with CH_2Cl_2, a 4-min linear gradient from CH_2Cl_2 to DMF, and a 1-min treatment with DMF. Conversely, the wash step after deprotection consisted of a 2-min treatment with DMF, a 5-min linear gradient from DMF to CH_2Cl_2, and a 5-min treatment with CH_2Cl_2. Use of these gradients avoided abrupt changes in the solvent composition and the volume of the solid support.

After removal of the resin from the synthesizer, the peptide was cleaved by treatment for 1.0 hr with 6.5 M CF_3COOH in CH_2Cl_2 (17). However, cleavage by the CH_3SO_2OH/CF_3COOH solution in the continuous-flow synthesizer was inefficient. Treatment of Fmoc-Val-resin (0.10 g, 44 μmol of valine) with 0.01 M CH_3SO_2OH/0.10 M CF_3COOH at 4.0 ml/min for 30 min [120 equiv. of $CF_3C(OH)_2^+$] released only 15% of the Fmoc-Val. The phenolic ring of the covalent link evidently competes with the α-carboxyl group of the ester for the fixed amount of $CF_3C(OH)_2^+$ available. Unlike a concentrated CF_3COOH solution, which continuously generates $CF_3C(OH)_2^+$, the dilute CH_3SO_2OH/CF_3COOH solution contains only 0.01 M $CF_3C(OH)_2^+$ (Fig. 3).

The mixture of Fmoc-peptides was deprotected with 1.0 M piperidine in dioxane/water, 1:1 (vol/vol), to give a mixture of free peptides. HPLC analysis showed that with both high and moderate loadings of valine (Table 3, syntheses S-13 and S-14) 96% of the mixture was the desired tetrapeptide. The Fmoc-Gly couplings were incomplete for both loadings and the Fmoc-Leu coupling was incomplete for the higher loading. These results are nearly as good as those obtained using Boc protection and the PAM link. Part of the mixture of Fmoc-peptides obtained in synthesis S-14 was deprotected with 2 M piperidine in DMF, and the resulting free peptides were precipitated by addition of anhydrous ether (17). The resulting peptide mixture (synthesis S-15) contained only about 40% of the deletion peptide Leu-Ala-Val present when the precipitation step was not used (synthesis S-14). This precipitation step should be omitted when attempting to measure the actual amounts of by-products produced during synthesis.

Ovalbumin Heptadecapeptide. Application of solid-phase peptide synthesis under continuous-flow conditions to a specific biologic problem is illustrated by synthesis of chicken ovalbumin-(238–254)-heptadecapeptide, which contains 10 of the 20 genetically coded amino acids. Studies with an ovalbumin fragment (19) and by sequence comparison (20) suggest that during ovalbumin biosynthesis, this 17-residue ovalbumin segment may be the molecular signal that permits translocation of the nascent polypeptide chain across the endoplasmic reticulum membrane. Boc-Thr(Bzl)-Met(O)-Ser(Bzl)-Met(O)-Leu-Val-Leu-Leu-Pro-Asp(OBzl)-Glu(OBzl)-Val-Ser(Bzl)-Gly-Leu-Glu(OBzl)-Gln-OCH_2-PAM-polystyrene was assembled from Boc-amino acids.

Two syntheses of the ovalbumin peptide were carried out in parallel on a 50-μmol scale (Table 4). The discontinuous synthesis used a 21-min deprotection with 6.5 M CF_3COOH and two 120-min couplings with Boc-amino acid activated *in situ* (10), whereas the continuous-flow synthesis employed a 6-min deprotection with the CH_3SO_2OH/CF_3COOH solution and two 45-min couplings with preformed Boc-amino acid symmetric anhydride. The coupling times used in both syntheses were probably longer than necessary. With these procedures, the total elapsed time for each synthetic cycle was about 390 min for the discontinuous synthesis and 132 min for the continuous-flow synthesis. Even shorter cycle times have been used on occasion (21). Diisopropylethylamine was used for neutralization to minimize cyclization of the O^{β}-benzyl-aspartyl residue to an aspartimide residue during peptide assembly (22).

The peptide mixtures obtained by cleavage with anisole/HF were analyzed by reverse-phase HPLC with a linear gradient of 18–48% acetonitrile in 10 mM ammonium acetate (pH 5.2) and detection at 220 nm. The discontinuous- and continuous-flow products contained 74% and 72% of the desired 17-residue peptide, respectively. The major side reaction in each synthesis was aspartimide formation (1, 2, 22). The discontinuous synthesis produced 16% of the aspartimide-247 analogue, whereas the continuous-flow synthesis gave 5.5% of this analogue and 15.5% of its major hydrolysis product, the β-aspartyl-247 analogue. These by-products were isolated by preparative HPLC and identified by digestion with carboxypeptidase Y fol-

Table 4. Synthetic cycles for assembly of ovalbumin-(238–254)

Cycle step	Discontinuous: Reagent	Discontinuous: Shaking time, min	Continuous flow: Reagent	Continuous flow: Pumping time, min
Deprotection	6.5 M CF_3COOH	1 + 20	0.01 M CH_3SO_2OH 0.10 M CF_3COOH	6
Wash	None	6 × 1	None	6
Neutralization	0.30 M DIEA	2 × 2	0.30 M DIEA	3
Wash	None	6 × 1	None	6
Coupling	0.08 M Boc-aa 0.08 M DCC (12 equiv. each)	120	0.10 M Boc-aa anhydride (16 mol equiv.)	45
Wash	None	8 × 1	None	6
Repeat last four steps				

The solvent was dichloromethane. Abbreviations: aa, amino acid; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine. For both syntheses, 0.50 g of Boc-Gln-OCH_2-PAM-copoly(styrene-1% divinylbenzene) (0.10 mmol of glutamine per g of resin; 50 μmol) gave 82 mg (86% yield) of crude peptide.

lowed by amino acid analysis and by conversion of the aspartimide analogue into the β-aspartyl analogue. The presence of these by-products, which are often formed during HF cleavage of O^{β}-benzyl-aspartyl-containing peptides (22), is independent of the physical mode of peptide assembly. Their presence would be minimized by protecting the aspartyl residue as the cyclohexyl ester and conducting the HF cleavage at −20°C (22).

The relative molar percentage of deletion peptides produced is a useful measure of the efficiency of the two modes of peptide assembly. The discontinuous synthesis gave a mixture containing 9.8% of other by-products assumed to be single-deletion peptides, which corresponds to formation of an average of 0.61% deletion peptides per cycle. The corresponding values for the continuous-flow synthesis were 7.0% and 0.44%. These results are close to the 0.3% of deletion peptides formed per cycle during large-scale synthesis S-10 of Leu-Ala-Gly-Val and during discontinuous syntheses of this tetrapeptide (14) and thymosin α-1 (23). Thus, these discontinuous and continuous-flow syntheses provided the ovalbumin-(238–254) segment in comparable yield and purity.

Features of the Continuous-Flow System. The continuous-flow system offers several practical features for stepwise synthesis of peptides. Because the entire system is closed, filling of the reservoir vessels and loading of the activated amino acids are the only reagent handling steps. Subsequent addition of each liquid to the column requires only the turning of a valve. The efficiency of washing the solid support with a continuous flow of solvent is inherently greater than that with a discontinuous washing procedure. The times for deprotection, neutralization, and washing are short because addition and removal of the acid, base, and wash solutions occur at the same time as the solid support is being treated with these solutions. The amounts of the Boc-amino acid symmetric anhydrides used are minimized by recycling them through the column. The scale of synthesis is conveniently varied by changing the size of the column and the flow rate.

Individual operations could be monitored by continuously measuring the UV absorbance of the liquid leaving the column. This approach has been used by Birr (24) to monitor the progress of the reactions and the washes performed by his centrifugal solid-phase synthesizer. In principle, such monitoring data could serve as the information needed for real-time feedback control of an automated continuous-flow synthesizer (25).

Dr. Lukas was a Fellow of the Damon Runyon–Walter Winchell Cancer Fund and Dr. Prystowsky was a Fellow of the Cancer Research Institute. We thank Soliman Bakr, Margot Rubinstein, and Daniel Bloomfield for technical assistance and Patricia M. Bihn for skillful editorial assistance. This work was supported in part by U.S. Public Health Service Grants HL 19795, AI 15301, and CA 24435, and National Science Foundation Grant PCM 77-25761.

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