Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2771–2775, July 1977
Biochemistry

Concept of internal structural controls for evaluation of inactive synthetic peptide analogs: Synthesis of [$Orn^{13,14}$]apamin and its guanidination to an apamin derivative with full neurotoxic activity

(solid phase peptide synthesis/[4-homoarginine]apamin/bee venom toxin)

WESLEY L. COSAND*† AND R. B. MERRIFIELD

Rockefeller University, New York, New York 10021

Contributed by R. B. Merrifield, April 25, 1977

ABSTRACT The importance of arginine residues 13 and 14 in the bee venom neurotoxin, apamin, was tested by the synthesis of replacement analogs. [13,14-di-N^{δ}-trifluoroacetylornithine]Apamin was synthesized by the solid phase method on a benzhydrylamine resin. It was deprotected to [13,14-diornithine]apamin, which was then guanidinated to produce the 4-homoarginine-13,14-diarginine analog, [Har^4]apamin. Neither the trifluoroacetylornithine analog nor the ornithine analog produced any detectable symptoms when injected intravenously into mice. However, the synthetic [Har^4]apamin exhibited the full neurotoxic activity of native apamin and of [Har^4]apamin derived from the natural toxin. This provided an *internal structural control* for the correctness of the primary structure of the inactive synthetic analogs and strengthened the conclusion that one, or both, of the arginine residues plays an important role in the action of apamin.

Caution must be exercised in interpreting results from synthetic peptide analogs that are inactive or of low activity (1–3). The lack of activity may be a consequence of the new structure itself or of an unrecognized difficulty in the synthesis that resulted in an incorrect sequence or in a structural modification. We think this uncertainty can be overcome in selected cases, and would like to emphasize the concept of an *internal structural control* for inactive synthetic peptide analogs. If the analog can be converted by a simple reaction into a fully active peptide that is identical with the naturally occurring parent molecule, we have provided an internal control for the correctness of the structure of the inactive peptide.

Several examples of such conversions can be found, although we have not located a reference to the idea of using them as internal structural controls for the purpose just outlined. The most common examples are those involving the conversion of inactive protected peptides into active free peptides by chemical deprotection steps (4, 5) or the enzymic formation of active peptides from synthetic precursors of low activity (6–8). For structure-function studies the most useful interconversion would involve the change of one amino acid residue into another. A pertinent transformation of this type is the conversion of an ornithine residue to an arginine residue by guanidination. This approach to the synthesis of arginine-containing peptides was suggested by Fruton (9) and has been applied several times (10–14).

We have undertaken to test the value of an internal structural control in a study of the role of arginine in the neurotoxin, apamin. This octadecapeptide from bee venom has been isolated and purified (15), its structure determined (16–18), and its synthesis described (19). Chemical modification suggested that the two arginines at positions 13 and 14 might be required for activity (20). Therefore, a synthetic approach to the question was designed in which positions 13 and 14 would contain N^{δ}-trifluoroacetylornithine in place of arginine. The trifluoroacetyl groups could be removed to give the diornithine derivative and it in turn could be guanidinated to the diarginine derivative. Although the latter would also contain homoarginine (Har) at position 4, it was an acceptable target molecule because it was already known (20) that [Har^4]apamin, the 4-homoarginine-13,14-diarginine analog derived from native apamin, is fully active. Thus, if either [$Orn(Tfa)^{13,14}$]apamin or [$Orn^{13,14}$]apamin was inactive and could be converted into [Har^4]apamin that was fully active, we would have the desired internal structural control from which reliable conclusions‡ about these replacements of arginine could be drawn.

MATERIALS AND METHODS

Reagents. Dichloromethane (Eastman) was distilled from Na_2CO_3 and diisopropylethylamine (Aldrich) was distilled from ninhydrin. Commercial N^{α}-Boc-amino acids were checked for homogeneity by thin-layer chromatography. N^{α}-Boc-N^{im}-Dnp-L-histidine was recrystallized in 70% yield from isopropanol (21).

S-(4-Methylbenzyl)-L-cysteine was prepared in 82% yield by the method of Erickson and Merrifield (22) and was converted to the *tert*-butyloxycarbonyl derivative with *t*-butylazidoformate by the Schnabel method (23) under an inert atmosphere to prevent formation of traces of the sulfoxide (S. Kent and D. Live, unpublished data). Yield 58%; mp 74°; thin-layer chromatography, R_F 0.91, in chloroform/methanol/acetic acid (85:10:5). *Anal.* Calcd. for $C_{16}H_{23}NO_2S$: C, 59.08; H, 7.08; N, 4.31. Found: C, 59.14; H, 7.03; N, 4.17.

N^{δ}-Trifluoroacetyl-L-ornithine [Orn(Tfa)] was prepared by treatment of ornithine with *S*-ethyl thioltrifluoroacetate (24), purified on a silica gel column in butanol/acetic acid/water (4:1:1), and recrystallized from 50% ethanol. Yield, 40%; mp 228–231°; thin-layer chromatography, R_F 0.45, in butanol/acetic acid/water (4:1:1). *Anal.* Calcd for $C_7H_{11}F_3NO_3$: C, 36.80; H, 4.86; N, 12.13. Found: C, 36.80; H, 4.84; N, 12.20.

Boc-Orn(Tfa) was synthesized by an anhydrous reaction of N^{δ}-Tfa-Orn with *tert*-butylazidoformate and triethylamine in dimethyl sulfoxide based on a method of Stewart and Young (25).

[*U*-^{14}C]Glutamine (0.1 mCi/mmol) and [*G*-^{3}H]asparagine (1 mCi/mmol), Schwartz-Mann, were converted to the N^{α}-*tert*-butyloxycarbonyl derivatives with *tert*-butylazidoformate

Abbreviations: Har, homoarginine; Tfa, trifluoroacetyl; iPrOH, isopropanol; LD_{50}, dose at which 50% of the animals died.

* In partial fulfillment of the degree of Doctor of Philosophy, Rockefeller University.

† Present address: Department of Chemistry, Lewis and Clark College, Portland, OR 97219.

‡ Subject to the reasonable assumption that an unrecognized modification that is without effect in the parent structure will not become of major importance in the analog.

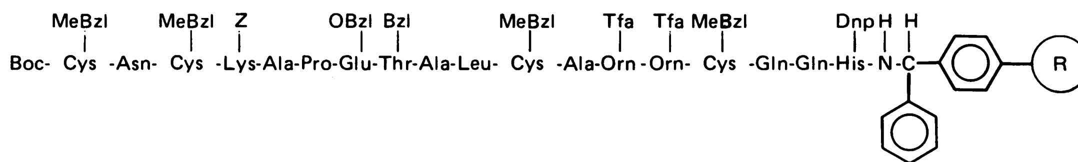

FIG. 1. Protected [Orn^{13,14}]apamin-benzhydrylamine-resin.

in aqueous dioxane containing magnesium oxide according to Schwyzer *et al.* (26).

The Instrument. The peptide was synthesized on the automated Beckman peptide synthesizer model 990 which had been modified to perform the functions in the order: meter, drain, transfer, mix. This allowed the instrument to meter the next reagent while still mixing the previous one and resulted in a substantial saving in time. Contamination of the reaction vessel was minimized by positioning three rinse ports between the stirring-drive magnets. The ports connected directly (without passing through a metering vessel) to a dichloromethane reservoir, which was programmed to be pressurized concurrently with another metered reservoir.

Synthesis of the Peptide. The peptide was synthesized by the solid-phase method (2, 27, 28) using the protecting groups shown in Fig. 1. The synthesis consisted of a stepwise double coupling with dicyclohexylcarbodiimide-activated Boc-amino acids according to the following automated protocol: (*1*) 50% trifluoroacetic acid/CH_2Cl_2 for 2 min, (*2*) 50% trifluoroacetic acid/CH_2Cl_2 for 30 min, (*3*) CH_2Cl_2 seven times for 2 min, (*4*) isopropanol (iPrOH) twice for 2 min, (*5*) CH_2Cl_2 five times for 2 min, (*6*) iPrOH twice for 2 min, (*7*) CH_2Cl_2 five times for 2 min, (*8*) 5% diisopropylethylamine/CH_2Cl_2 three times for 2 min, (*9*) CH_2Cl_2 five times for 2 min, (*10*) iPrOH twice for 2 min, (*11*) CH_2Cl_2 five times for 2 min, (*12*) 3 equivalents of Boc-amino acid/CH_2Cl_2 for 5 min, then 3 equivalents of dicyclohexylcarbodiimide/CH_2Cl_2 for 120 min, (*13*) CH_2Cl_2 five times for 2 min, (*14*) iPrOH twice for 2 min, (*15*) CH_2Cl_2 five times for 2 min, (*16*) iPrOH twice for 2 min, (*17*) CH_2Cl_2 five times for 2 min, and (*18*) repeat steps *8–17*. Since Boc-Asn and Boc-Gln were coupled as the hydroxybenzotriazole esters (29), the automated procedure was interupted before those coupling steps. The dicyclohexylcarbodiimide 3 equivalents and hydroxybenzotriazole 3 equivalents in dimethylformamide were mixed at 0°, the Boc-amino acid 3 equivalents in dimethylformamide were added, and the resulting solution was immediately added manually to the reaction vessel. The automated mode was then resumed.

Determination of Toxicity. The toxicity of the peptides was determined by the lethality after intracaudal injection in mice. The samples were dissolved in sterile saline so that 100 μl would contain approximately one LD_{50}. (LD_{50} is the dose at which 50% of the animals died.) Then six groups of six mice each (male, 20 ± 2 g, Rockefeller Institute strain Swiss white mice) were injected with 50–160 μl. The animals became quiet for a dose-dependent period of a few minutes, then hyperactive, and finally died of respiratory failure. The numbers of deaths after 24 hr were counted and the data were evaluated by the method of Litchfield and Wilcoxon (30).

RESULTS

Synthesis of Boc-Cys(MeBzl)-[^{3}H]Asn-Cys(MeBzl)-Lys(Z)-Ala-Pro-Gly(OBzl)-Thr(Bzl)-Ala-Leu-Cys(MeBzl)-Ala-Orn(Tfa)-Orn(Tfa)-Cys(MeBzl)-Gln-[^{14}C]Gln-His-(Dnp)-benzhydrylamine-resin

The synthesis began with 5.00 g (0.48 mmol of amine per g) of benzhydrylamine resin (31). Boc-His(Dnp) (2.4 mmol) was coupled with dicyclohexylcarbodiimide-activation to give a resin containing 0.27 mmol of His per g of resin. The remaining sites were acetylated with acetylimidazole. The protected peptide was then assembled in a stepwise manner on the peptide synthesizer by the protocol given under *Materials and Methods*. Each nonlabeled residue was coupled twice with 3 equivalents of Boc-amino acid and 3 equivalents of dicyclohexylcarbodiimide relative to the histidine content in a minimal volume of CH_2Cl_2. Boc-Gln^{17} was introduced with a ^{14}C label, and Boc-Asn with an ^{3}H label. In the first coupling only 0.5 equivalent of radioactive Boc-amino acid was used, and this was followed with two additional couplings with 3 equivalents of nonlabeled amino acid. The specific activity of the peptide chain was 55 μCi of ^{14}C per mmol and 590 μCi of ^{3}H per mmol.

A sample was removed for analysis of unreacted amino groups by the fluorescamine method (32) after the third coupling of Asn^2 and Gln^{17} and the second coupling of the other residues. All of these tests were negative. Based on the published sensitivity (32), less than 0.3% of a deletion could be expected at any residue of this synthesis. The synthesis was also monitored by amino acid analysis. The observed amino acid ratios indicated good coupling at each step, and there was no significant loss of peptide chains from the resin support during the synthesis (0.26/mmol of peptide per g of resin after the last residue). At the end of the synthesis the Boc group was removed in trifluoroacetic acid and His(Dnp) was deprotected with 2.5% thiophenol in dimethylformamide for 1 hr. The peptide-resin was removed from the reaction vessel and dried under reduced pressure at 25°. Found, 9.02 g; calculated, 8.84 g.

Preparation of [Orn(Tfa)^{13,14}]apamin

A 2.00-g sample (303 μmol) of the partially protected peptide-resin was treated with HF containing 10% anisole for 30 min at 0°. This cleaved the peptide and removed the remaining protecting groups, except Tfa on the ornithines. The peptide was extracted with 5% acetic acid and lyophilized; the yield was 234 μmol (77%).

Reduction and Chromatographic Purification. The cleavage product was reduced with dithiothreitol under nitrogen in pH 9 Tris for 2 hr. It was desalted on Sephadex G-25 in 5% acetic acid containing 1 mM EDTA and 2 mM dithiothreitol. The main peak of radioactivity had a nearly constant ^{3}H/^{14}C cpm ratio of 5.5 ± 0.2. It was rechromatographed on Whatman CM-52 carboxymethylcellulose with a linear gradient of ammonium acetate, pH 4.65, 0.025–1.00 M, containing 1 mM EDTA and 2 mM dithiothreitol. The main peak of radioactivity (72 μmol) was poured into 3 liters of 0.1 M Tris·HCl, pH 8. To promote disulfide exchange, 1.3 mmol of 2-mercaptoethanol was added and the solution was stirred in an open beaker for 5 days, after which no sulfhydryl remained. The lyophilized material was desalted on Sephadex G-25 in 5% acetic acid. The radioactive peak yielded 39 μmol of peptide.

Preparation of [Orn^{13,14}]apamin

A 1.00-g sample (152 μmol) of the synthetic protected peptide-resin, from which the N^{α}-Boc and N^{im}-Dnp groups had

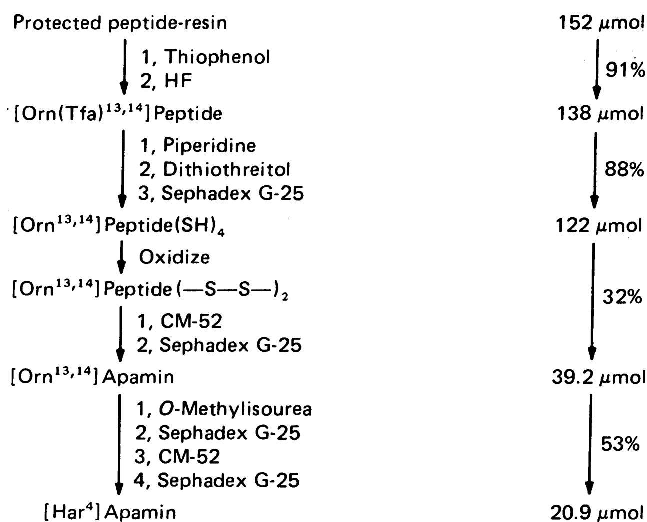

FIG. 2. Preparation of synthetic apamin analogs.

been removed, was cleaved with 9 ml of HF containing 1 ml of anisole for 1 hr at 0°. The yield was 138 μmol (91%). The procedure is summarized in Fig. 2.

Removal of Tfa Groups, Reduction, and Desalting. The cleavage product was treated with 60 ml of 1 M aqueous piperidine at 0° for 2 hr. Solid Tris (1.45 g) was added and the pH was adjusted to 8 with HOAc. Nitrogen was bubbled through for 15 min, dithiothreitol (0.6 g) was added, the solution was flushed again with N_2, and the flask was stoppered. After 10 hr, 10 ml of HOAc was added and the solution was desalted on a Sephadex G-25 column in 5% acetic acid at 4° under N_2.

Oxidation and Ion-Exchange Chromatography. The main peak of radioactivity (122 μmol) was pooled and poured into 3.5 liters of 0.1 M Tris-HCl and adjusted to pH 8. After the solution had been stirred in an open beaker for 48 hr no free sulfhydryl could be detected with 5,5′-dithiobis(2-nitrobenzoic acid). The oxidized peptide was chromatographed on a 2.8 × 11-cm column of CM-52 at pH 5.1 in 0.1 M ammonium acetate. The fractions between 330 and 434 ml were desalted on Sephadex G-25 in 0.05 M acetic acid. Radioactivity showed a single peak, which was pooled and lyophilized. The yield was 39.2 μmol.

The location of the S—S bonds has not been established with certainty, but there is evidence that it is the same as in native (18) and previously synthesized (19) apamin.

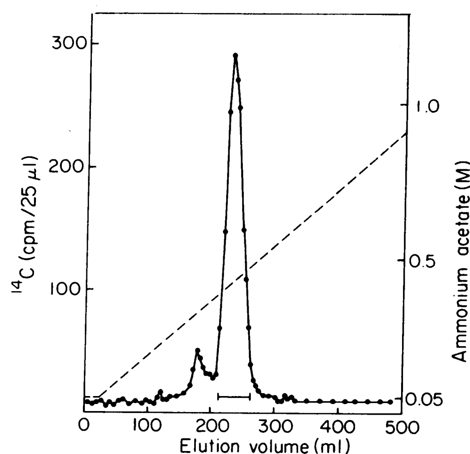

FIG. 3. Chromatographic purification of [Har⁴]apamin on a carboxymethylcellulose column. Solid line, ^{14}C counts; broken line, ammonium acetate concentration.

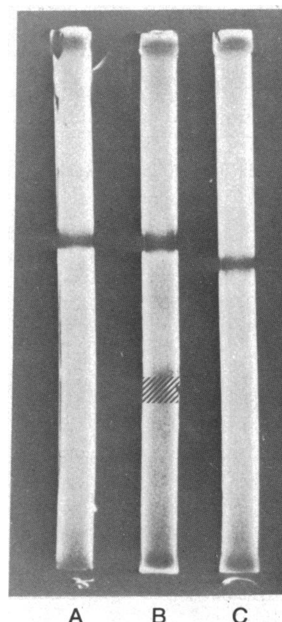

FIG. 4. Gel electrophoresis of apamin analogs. Gels were 30% polyacrylamide/0.3% *N,N′*-methylenebisacrylamide, run in 0.9 M acetic acid for 5 hr at 150 v. Stained with amido black and only slightly destained before photographing. Samples (40 μg in 10% glycerol) are: *A*, natural apamin; *B*, synthetic [Har⁴]apamin; *C*, synthetic [Orn^{13,14}]apamin. The stippled area on gel *B* shows the position of the running dye, methyl green.

Preparation of [Har⁴]apamin

Guanidination (33). Lyophilized [Orn^{13,14}]apamin (29.4 μmol) was dissolved in 5 ml of water and mixed with 5 ml of 1 M *O*-methylisourea that had been adjusted to pH 11 with NaOH. The α-amino group does not guanidinate under these conditions (34). After 7 days the solution was acidified with 5 ml of HOAc and desalted on Sephadex G-25. The main component was separated from a retarded peak that contained about 5% of the total ^3H but no ^{14}C.

Chromatographic Purification. The desalted, guanidinated peptide was chromatographed on a 2.1 × 5.5-cm column of CM-52 with a 600-ml linear gradient from 0.05 to 1.00 M ammonium acetate, pH 5.1 (Fig. 3). ^{14}C counts showed a small by-product at 189 ml and the main peptide at 239 ml. Material from this peak was pooled, desalted on Sephadex G-25 in 0.05 M HOAc, and lyophilized. The yield was 15.6 μmol.

Characterization of [Orn^{13,14}]Apamin and [Har⁴]Apamin. The synthetic peptides and native apamin were examined by gel electrophoresis (35) (Fig. 4). The bands of the two synthetic peptides and the native peptide were comparably sharp and homogeneous by this technique.

Table 1 presents amino acid analyses for native apamin and the synthetic apamin analogs. They show quite acceptable ratios of amino acids and demonstrate clearly the disappearance of the three equivalents of lysine plus ornithine upon guanidination and the appearance of one equivalent of homoarginine and two equivalents of arginine.

Neurotoxic Activity. The apamin derivatives were assayed by injection into the caudal vein of mice. The LD_{50} of native apamin for 20-g mice has been reported to be 40–50 nmol (36);

Table 1. Amino acid molar ratios in acid hydrolyzates of apamin and synthetic apamin analogs

Amino acid	[Orn(Tfa)^{13,14}]-Apamin	[Orn^{13,14}]-Apamin	[Har^4]-Apamin*	Native apamin†
Lys + Orn	2.98	3.04	<0.01	1.06
Arg	0	0	2.01	2.06
Har	0	0	1.00	0
His	1.07	1.12	1.00	0.97
Asp	0.93	0.95	0.97	1.03
Thr	0.90	0.81	1.07‡	0.88
Glu	3.01	3.01	2.91	3.09
Pro	1.20	1.02	1.01	N.D.
Ala	3.01	2.99	2.97	2.95
Cys	N.D.§	3.68¶	3.78‖	N.D.
Leu	1.01	0.88	1.06	1.05

* Average of nine analyses, three each at 24, 48, and 72 hr.
† Natural apamin was a gift of Dr. T. P. King.
‡ Extrapolated to zero hydrolysis time.
§ Not determined.
¶ Determined as ½ cystine.
‖ Determined as cysteic acid.

Table 2. Neurotoxic activity of natural apamin and synthetic apamin analogs

Compound	LD_{50}* (nmol)	Relative activity
Natural apamin	47	1.00
Natural [Har^4]apamin		1.00†
Synthetic [Har^4]apamin	44	1.07
Synthetic [Orn^{13,14}]apamin	>1200‡	No activity (<1%)
Synthetic [Orn(Tfa)^{13,14}]apamin	>280‡	No activity (<5%)

* Calculated from data at six concentrations on groups of six mice each by the method of Litchfield and Wilcoxon (30).
† Taken from Vincent *et al.* (20).
‡ No observable symptoms at this dose.

we found a value of 47 nmol (Table 2). Guanidinated apamin is reported to have an activity of 1.0 relative to the native apamin (20). The synthetic guanidinated apamin, [Har^4]apamin, was found to have an LD_{50} of 44 nmol, that is, within experimental error it was as toxic as both the natural peptide and the [Har^4]apamin prepared from natural material. [Orn^{13,14}]-Apamin, the replacement analog containing ornithine residues at positions 13 and 14 instead of arginines and retaining the positive charge, elicited no toxic symptoms whatever at a dose of 1200 nmol/20-g mouse. It was impractical to inject larger amounts. If the analog had been 4% as active as natural apamin, one half of the animals should have died; even if it had been only 1% as active, very characteristic symptoms would have appeared. [Orn(Tfa)^{13,14}]Apamin showed no symptoms at a dose of 280 nmol/mouse. Higher levels could not be tested.

DISCUSSION

The solid phase synthesis of fully protected resin-bound [Orn^{13,14}]apamin proceeded in a straightforward manner. Fluorescamine monitoring indicated that the coupling reactions were at least 99.7% complete; this finding is in line with the quantitative preview data of Van Rietschoten *et al.* (19) on their original solid phase synthesis of apamin itself, in which they estimated an average yield of 99.3% for the coupling reactions. The yields in the two HF cleavages were 77% and 91%, and the deprotections of Dnp-His and Tfa-Orn as well as the guanidination reaction went in high yields. The main losses occurred at the oxidation to the disulfide monomer and during the several chromatographic steps, giving an overall yield of only 14%. The synthetic [Orn^{13,14}]apamin and [Har^4]apamin were homogeneous by ion exchange chromatography and polyacrylamide gel electrophoresis and gave good amino acid analyses. There was no chemical reason to suppose that the peptides were not pure and of the intended amino acid sequence.

The purpose of this work was to test the concept of an *internal structural control* for inactive synthetic peptide analogs. Neither the [Orn(Tfa)^{13,14}]apamin nor the [Orn^{13,14}]apamin produced any symptoms of neurotoxicity whatever in mice at the highest levels that could be tested. As expected, the guanidinated derivative containing arginines at positions 13 and 14 (as well as homoarginine at position 4) was fully as active as natural apamin and [Har^4]apamin derived from the native toxin (20). These results provided the desired internal control and strengthen the conclusion that analogs of the correct amino acid sequence were synthesized. We can then conclude that the observed inactivity of the ornithine derivatives was due to the intended structure and not to an unrecognized error in sequence. In addition, we can infer that at least one of the arginines at positions 13 and 14 plays an important role in the activity of apamin, although the level at which it functions has not been established. Arginine may provide electrostatic, steric, or conformational properties that are necessary for proper folding, stability, transport, or action at the receptor site.

This work was supported in part by Grant AM 01260 from the U.S. Public Health Service and by funds from the Hoffmann-La Roche Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Merrifield, R. B. (1969) *Adv. Enzymol.* **32,** 221–296, see also p. 277.
2. Rudinger, J. (1971) in "Drug design," in *Medicinal Chemistry Monograph Series*, ed. Ariens, E. J. (Academic Press, New York), Vol. II, pp. 319–419.
3. Meienhofer, J. (1973) in *Hormonal Proteins and Peptides*, ed. Li, C. H. (Academic Press, New York), Vol. 2, pp. 45–267.
4. Hofmann, K. (1962) *Annu. Rev. Biochem.* **31,** 213–246.
5. Bodanszky, M., Ondetti, M. A., Rubin, B., Piala, J. J., Fried, J., Sheehan, J. T. & Birkhimer, C. A. (1962) *Nature* **194,** 485–486.
6. Skeggs, L. T., Jr., Lentz, K. E., Kahn, J. R. & Schumway, N. P. (1958) *J. Exp. Med.* **108,** 283–297.
7. Najjar, V. A. & Merrifield, R. B. (1966) *Biochemistry* **5,** 3765–3770.
8. Oparil, S., Tregear, G. W., Koerner, T., Barnes, B. A. & Haber, E. (1971) *Circ. Res.* **29,** 682–690.
9. Fruton, J. S. (1949) *Adv. Protein Chem.* **5,** 1–82.
10. Katchalski, E. & Spitnik, P. (1951) *J. Am. Chem. Soc.* **73,** 3992–3995.
11. Barrass, B. C. & Elmore, D. T. (1957) *J. Chem. Soc.*, 3134–3139.
12. Bodanszky, M., Ondetti, M. A., Birkhimer, C. A. & Thomas, P. L. (1964) *J. Am. Chem. Soc.* **86,** 4452–4459.

13. Scoffone, E., Marchiori, F., Rocchi, R., Vidali, G., Tamburro, A., Scatturin, A. & Marzotto, A. (1966) *Tetrahedron Lett.*, 943–949.
14. Borin, G., Toniolo, C., Moroder, L., Marchiori, F., Rocchi, R. & Scoffone, E. (1972) *Int. J. Protein Res.* **4**, 37–45.
15. Habermann, E. & Reiz, K. G. (1965) *Biochem. Z.* **341**, 451–466.
16. Haux, P., Sawerthal, H. & Habermann, E. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 737–738.
17. Shipolini, R., Bradbury, A. F., Callewaert, G. L. & Vernon, C. (1967) *Chem. Commun.*, 679–680.
18. Callewaert, G. L., Shipolini, R. A. & Vernon, C. A. (1968) *FEBS Lett.* **1**, 111–113.
19. Van Rietschoten, J., Granier, C., Rochat, H., Lissitzky, S. & Miranda, F. (1975) *Eur. J. Biochem.* **56**, 35–40.
20. Vincent, J. P., Schweitz, H. & Lazdunski, M. (1975) *Biochemistry* **14**, 2521–2525.
21. Buis, J. (1973) Doctoral Dissertation, Catholic University, Nijmegen, The Netherlands, p. 127.
22. Erickson, B. W. & Merrifield, R. B. (1974) *Isr. J. Chem.* **12**, 79–85.
23. Schnabel, E. (1967) *Ann. Chem.* **702**, 188–196.
24. Schallenburg, E. E. & Calvin, M. (1955) *J. Am. Chem. Soc.* **77**, 2779–2783.
25. Stewart, J. M. & Young, J. D. (1969) *Solid Phase Peptide Synthesis* (Freeman, San Francisco), p. 29.
26. Schwyzer, R., Sieber, P. & Kappeler, H. (1959) *Helv. Chim. Acta* **42**, 2622–2624.
27. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154.
28. Erickson, B. W. & Merrifield, R. B. (1976) in *The Proteins*, eds. Neurath, H. & Hill, R. L. (Academic Press, New York), 3rd ed., Vol. 2, pp. 257–527.
29. Hruby, V. J., Muscio, F., Groginsky, C. M., Gitu, P. M., Saba, D. & Chan, W. Y. (1973) *J. Med. Chem.* **16**, 624–629.
30. Litchfield, J. T., Jr. & Wilcoxon, F. (1949) *J. Pharmacol. Exp. Ther.* **96**, 99–113.
31. Pietta, P. G., Cavallo, P. F., Takahashi, K. & Marshall, G. (1974) *J. Org. Chem.* **39**, 44–48.
32. Felix, A. M. & Jimenez, M. H. (1973) *Anal. Biochem.* **52**, 377–381.
33. Chauvet, J. & Acher, R. (1967) *Biochem. Biophys. Res. Commun.* **27**, 230–235.
34. Chervenka, C. H. & Wilcox, P. E. (1956) *J. Biol. Chem.* **222**, 635–647.
35. Krieger, D. (1976) Ph.D. Dissertation, Rockefeller University.
36. Reiz, K. G. (1965) Doctoral Dissertation, University of Würzburg, as quoted in Spoerri, P. E., Jentsch, J. & Glees, P. (1973) *Neurobiology* **3**, 207–214.