compounds include 4,5-dehydromuscarine (III) and -muscarone, (IV) and 2-methyl-5-trimethylammoniummethylfuran (II). This antiplanar conformation is, however, nearly that found in acetyl-β-methylcholine[10] and in lactoylcholine[6] (V). In the furan and in 2-methyl-4-trimethylammoniummethyl-1,3-oxazoline (VI), the Me group is in the plane of the ring, as it is in acetylcholine or acetyl-β-methylcholine; τ C7–C6–O1–C5 is always 180° in esters due to the partial double bond character of the C6–O1 bond.[5,6,10,15]

Absolute Configuration.—Much information is available on the absolute configuration of potent muscarinic agonists. In addition to this 1,3-dioxolan[1,3] and muscarine,[16] evidence of potency and absolute configuration are available for muscarone,[16] acetyl-α-methylcholine,[10,17] acetyl-β-methylcholine,[10,17] acetoxycyclopropyltrimethylammonium,[18] and 3-acetoxyquinuclidine.[19] With the exception of muscarone (for which we can offer at this stage no explanation of the more or less equal activities of the two enantiomers), all the muscarinically more active enantiomers of these compounds have an absolute configuration consistent with those of this 1,3-dioxolan and muscarine. In Table III are listed three observed torsion angles of the more potent enantiomers of these substances. It is seen that τ O1–C5–C4–N varies from +73 to +137°, τ C6–O1–C5–C4 = 180 ± 37°, and τ C7–C6–O1–C5 equals either 180 or −137°.

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Solid Phase Synthesis and Antibacterial Activity of N-Terminal Sequences of Melittin

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N-Terminal peptides representing the 1–8, 7–17, 1–17, and 14–17 sequences of melittin were synthesized by the solid phase method and shown to be homogeneous by elemental and amino acid analyses and by tlc and tl electrophoresis. The yields were 40–66% starting from BOC-amino acid resin esters prepared from dimethyl(arylmethylene)sulfonium bicarbonate resins. Some interesting observations were made during the deblocking of BOC-Ile-O-resin reflecting steric and environmental considerations. Whereas melittin exhibited general antibacterial activity, none of the synthetic peptides showed any significant activity, thus demonstrating that the antibacterial activity of melittin does not reside in the N-terminal portion of the molecule.

The basic polypeptide melittin (1) is the main com-

H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Try-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

1

ponent of the venom of the honey bee, *Apis mellifica.*[1] Crude, dry bee venom contains 40–50% (by weight) **1** and about 10% of **1** is formylated at the N-terminus.[2]

The primary structure of **1** is rather unique. The N-terminal end, *i.e.*, 1–20 sequence, consists mainly of amino acids having hydrophobic side chains, exceptions being 7-lysine and 10,11-threonine.[3] The C-terminal end, on the other hand, consists mainly of amino acids containing basic and/or hydrophilic side chains. Hence, the molecule is a natural surfactant and many of its toxic reactions have been attributed to this property. For example, the hemolytic and inflammatory properties of **1** may be explained by its ability to disrupt lipid biomembrane structures.[4] The interaction of **1** with artificial lipid membranes has also been demonstrated.[5] Not all of the biological properties of **1** are untoward. Jentsch[6] reported that **1** at 2.5×10^{-6} *M* suppressed the uptake of thymidine, uridine, and leucine in DNA, RNA, and protein biosynthesis of cancer (ascities) cells. Melittin has been shown to protect mice against lethal doses of X radiation when administered sc.[7] Finally, Fennell, *et al.*,[8] showed that **1** was the antibacterial component of bee venom. These workers found that **1** was more effective against Gram-positive than Gram-negative bacteria. They raised the point of whether the antibacterial property of **1** was associated with the entire peptide molecule. In other words, might a smaller peptide sequence of **1** also exhibit the observed antibacterial activity, but without having the toxicity of **1**?

This question has been a principal concern of ours. In this connection we wish to report herein the synthesis and antibacterial activity of peptide sequences of the N-terminal portion of **1**, namely the 1–8, 1–17, 7–17, and 14–17 sequences. These peptides were synthesized by the Merrifield[9] solid phase method. As an adjunct

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(1) J. Jentsch and E. Habermann in "Peptides," H. C. Beyerman, A. van de Linde, and W. M. van den Brink, Ed., North-Holland Publishing Co., Amsterdam, 1967, p 263.

(2) G. Kreil and G. Kreil-Kiss, *Biochem. Biophys Res. Commun.*, **27**, 2751 (1967).

(3) E. Habermann and J. Jentsch, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 37 (1967).

(4) G. Weissmann, R. Hirschorn, and K. Krakauer, *Biochem. Pharmacol.*, **18**, 1771 (1969), and ref referred to therein.

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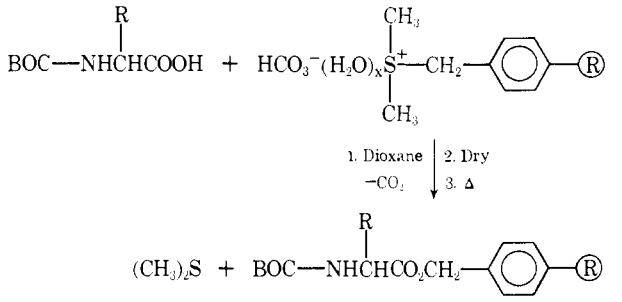
(9) (a) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); (b) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964); (c) R. B. Merrifield, *Advan. Enzymol.*, **32**, 221 (1969).

to this work we also wish to demonstrate the synthetic applicability of using N-protected amino acid resin esters that were prepared from basic dimethyl(arylmethylene)sulfonium resins.[10,11]

Results and Discussion

Peptide Synthesis.—Syntheses were initiated with *N*-BOC[12] amino acid resin esters, 0.48–1.0 mmole of BOC amino acid/g of resin ester. The resin esters were prepared by the method of Dorman and Love.[10] Peptide chains were built up in the usual stepwise



manner using N^{α}-BOC-L-amino acids.[9] The ϵ-NH_2 of lysine and the β-OH of threonine were protected by carbobenzoxy and benzyl groups, respectively. The general procedure for introducing each new N-terminal amino acid residue involved, (a) deblocking of resin-bound N-terminal BOC protective group; (b) neutralization with NEt_3 in $CHCl_3$ or DMF; and (c) DCI coupling of the requisite BOC-amino acid in CH_2Cl_2. The finished peptide was decoupled from its resin support with HBr in F_3CCO_2H.

Initially the progress of each amino acid addition was determined qualitatively by (1) removing a small sample of resin at the end of the coupling cycle and decoupling the peptide from it with F_3CCO_2H saturated with HBr gas, (2) evaporating the mixture, (3) triturating the residue with buffer, and (4) tlc of the resulting solution. Homogeneity of the chromatograms, particularly with respect to the previous cycle, was indicative of coupling success during that cycle. This technique had the obvious drawback of requiring too much time (*ca.* 3 hr) and was of dubious sensitivity. Nevertheless, a number of incomplete coupling reactions were detected by this method which allowed us to remedy the situation by repeating the coupling step or starting over again. Near the end of this work the pyridine·HCl method for determining completeness of coupling reactions was developed and used thereafter.[13]

In several syntheses the quantity of available N-terminal amine after each deblocking step was determined by Cl^- titration of the Et_3N–DMF neutralization filtrate.[14] This procedure enabled us to make a peculiar, but interesting observation during deblocking, probably reflecting both steric and resin composition phenomena. Data in Table I show the

TABLE I

DEBLOCKING DATA FOR MELITTIN 14–17 SYNTHESIS

Synthesis Cycle			3 2 1	
Amino acid sequence			BOC-Pro-Ala-Leu-Ile-O-Resin	
			14 15 16 17	
Run	Initial resin BOC-Ile-O-resin, mmole/g	Cycle	H_2N resin, mmole/g	Deblocking conditions
1	0.52	1	0.32	1 *N* HCl–AcOH, 30 min
		2	0.50	1 *N* HCl–AcOH, 30 min
		3	0.50	1 *N* HCl–AcOH, 30 min
2	0.52	1	0.23	3 *N* HCl–dioxane, 40 min
		1	0.42	1 *N* HCl–AcOH, 30 min
		2	0.53	1 *N* HCl–AcOH 40 min
		3	0.53	1 *N* HCl—AcOH 40 min
3	0.48	1	0.40	1 *N* HCl–AcOH, 30 min
		1	0.48	1 *N* HCl–AcOH, 30 min
		2	0.48	1 *N* HCl–AcOH, 30 min
		3	0.48	1 *N* HCl–AcOH, 30 min

amount of N-terminal resin amine liberated after the deblocking steps during the synthesis of the 14–17 sequence of **1**. In the first run normal deblocking conditions, *i.e.*, treatment of the resin with 1 *N* HCl in AcOH for 30 min, produced only 0.32 mmole (62%) of the resin amine in cycle 1 (deblocking of BOC-Ile-O-resin). This value increased to 0.50 mmole in cycles 2 and 3. In the second run more vigorous deblocking conditions, *i.e.*, successive treatment with 3 *N* HCl in dioxane for 40 min and 1 *N* HCl in AcOH for 30 min, in cycle 1 failed to effect complete deblocking, 0.42 mmole (80%) of resin amine being liberated. Again, complete deblocking was achieved in cycles 2 and 3. The BOC-Ile-O-resin used in the third run was prepared from an entirely different batch of resin, *viz*, starting from unsubstituted polymer. This different resin ester was prepared to help determine if the deblocking pattern observed in the first two runs was due to some intrinsic property of the resin support, the amino acid sequence involved, or some inherent property of this particular resin ester. A 30-min treatment of this resin with 1 *N* HCl in AcOH produced about 83% deblocking in cycle 1, an improvement over corresponding values of the first two runs, but still considerably incomplete. Extension of the acid treatment for another 30 min, however, did bring about complete deblocking. As previously described, complete deblocking in cycles 2 and 3 was achieved in the shorter, normal deblocking time. (Nonetheless, as a precaution against incomplete deblocking, 60-min deblocking times were used in each cycle of the synthesis of the 1–17 melittin sequence).

Interestingly, no apparent difficulties were encountered with deblocking in the synthesis of the 7–17 melittin sequence.[15] This synthesis, however, was initiated with a BOC-Ile-O-resin of higher amino acid capacity, *i.e.*, 0.87 mmole of BOC-Ile/g of resin ester. These observations, taken together, seem to indicate that there may be some factor(s) in addition to steric hindrance—*cf.* steric hindrance of amino acid alkyl side

(10) L. C. Dorman and J. Love, *J. Org. Chem.*, **34**, 158 (1969).

(11) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Co., San Francisco, Calif., 1969, p 8.

(12) Abbreviations: BOC = *tert*-butyloxycarbonyl; Ⓡ = styrene–2% divinylbenzene copolymer resin, 200–400 mesh; DCI = dicyclohexylcarbodiimide; Z = carbobenzoxy.

(13) L. C. Dorman, *Tetrahedron Lett.*, 2319 (1969).

(14) (a) R. B. Merrifield, *J. Amer. Chem. Soc.*, **86**, 304 (1964); (b) E. Bayer and H Hagenmaier, *Tetrahedron Lett.*, 2037 (1968).

(15) C. B. Anfinsen, D. Ontjes, M. Ohno, L. Corley, and A. Eastlake [*Proc. Natl. Acad. Sci.*, **58**, 1806 (1967)] also had no apparent difficulties with BOC-Ile-O-resin in synthesizing the 132–139 sequence of *Staphyloccal nuclease*. Since each cycle was not monitored, it is possible that a small deviation in deblocking may have gone unnoticed.

chains, Ile > Leu > Ala—affecting the rate and extent of deblocking of BOC-Ile-O-resin. One such factor may be attributed to changes in resin composition or environmental considerations. As the relative composition of the resin increases in amino acid or peptide content, *e.g.*, BOC-Ile-O- or (BOC-Leu-Ile-O- + BOC-Ile-O-), deblocking of the BOC group of C-terminal isoleucine is facilitated,[16] presumably as the result of increased swelling of the resin. Klostermeyer, *et al.*,[17] observed that swelling of the resin diminished markedly after substitution with amino acids *without* side chains. In retrospect, this experience with deblocking—as with coupling—points up the value of monitoring solid phase reactions,[18] particularly when working in unfamiliar systems.

The 1–8 sequence of **1** was synthesized starting from BOC-Val-O-resin ester, 1.04 mmoles of BOC-Val/g of resin ester. The yield of octapeptide was 49% when deblocking of BOC groups (after incorporation of lysine) was effected by a 40-min treatment with F_3CCO_2H. The yield was raised to 64% when deblocking was done with 30-min treatments with 1 *N* HCl in AcOH. Most probably, the lower yield was the result of a higher incidence of premature benzyl ester cleavage during the 40-min F_3CCO_2H treatment.[19]

The synthesis of the N-terminal peptides of **1** demonstrates that N-protected amino acid resin esters prepared from basic sulfonium resins are suitable for solid-phase peptide synthesis. This work also demonstrates that resin esters of comparatively high capacity, *e.g.*, 0.87–1.0 mmole of amino acid/g, can also be used although we do not, *a priori*, advocate this practice. Peptide yields, calculated on the basis of starting resin esters, ranged from 40% for **1** (1–17) to 64% for **1** (1–8). While these yields are not extraordinary compared with some (59–81%) reported in the literature,[9b,20] when the esterification step[21] is included in the calculations these yields may fare somewhat better. We will cite examples of the use of trifunctional amino acid resin esters, prepared from sulfonium resins, in subsequent reports.

Biological Results.—An *in vitro* agar petri dish dilution test was used for determining the bacteriocidal activity of peptides. Droplets of the test bacteria were applied to the surface of an agar plate containing the peptide at a fixed concentration (ppm). Growth of the bacteria was measured after incubation of the agar plate. Compounds causing no reduction in growth at 2000 ppm were considered inactive. Melittin, isolated from crude dry bee venom (see Experimental Section), killed *Staphylococcus aureus*,[22] *Escherichia coli*, *Salmonella typhosa*, *Mycobacterium phlei*, *Candida albicans*, and *Trichophyton mentagrophytes* at 2000 ppm. *Staph. epidermis* and *Bacillus subtilis* were killed by **1** at 500 ppm. None of the synthetic peptides exhibited any bacterial activity demonstrating that the antibacterial activity of **1** does not reside in the N-terminal portion of the molecule.

Experimental Section[23]

N-BOC-L-amino acids were prepd by the procedures of Schwyzer, *et al.*,[24] and Schnabel.[25] BOC-*O*-benzyl-L-threonine was prepd by the procedure of Mizoguchi, *et al.*[26] Ascending tlc was conducted on silica gel supported either on glass plates (F-254, Merck AG) or plastic sheets (type 6061, Eastman). Solvent systems for tlc: 1, *n*-BuOH–AcOH–H_2O, 3:1:1 (v/v); 2, *n*-BuOH–Pyr–AcOH–H_2O, 30:20:6:24 (v/v). Tl electrophoresis was performed on silica gel supported on glass plates (250 μ, Merck AG). Buffer systems for tle: 3,[27a] H_2O–Pyr–AcOH, 225:25:1 (v/v), pH 6.5; 4, 0.05 *M* phosphate, pH 8.0. Arginine was used as a reference for tle and migration of a peptide is expressed as the ratio of its migration to that of arginine in a given buffer, *i.e.*, E_{Arg} 3, etc. Electropherograms and chromatograms were developed by either spraying with ninhydrin in *n*-BuOH and/or with *tert*-butyl hypochlorite soln [2% in AcOH and $(ClCH_2)_2$, 1.8:0.2, v/v], followed, after air drying, with a spray of equal vols of *p*-tolidine (satd 2% AcOH) and 1% KI.[27b] Optical rotations were obtained with a Perkin-Elmer polarimeter, Model 141. Amino acid anal. were performed on peptide hydrolysates (6 *N* HCl at 110° for 24 hr) using a Phoenix automatic recording amino acid analyzer.

Resin Esters.—*N*-BOC-amino acid resin esters were prepd according to the general procedures of Dorman and Love[10] as described here for BOC-isoleucine-O-resin. Chloromethylated styrene–2% divinylbenzene resin (0.78 mmole of CH_2Cl/g, 200–400 mesh), 20 g (15.6 mmoles of CH_2Cl), was stirred in a closed mixture of 50 ml of MeOH, 25 ml of CH_2Cl_2, and 5 g (81 mmoles) of Me_2S for 5 days at room temp. The resin was collected on a filter and washed successively with MeOH, dioxane, and MeOH. It was not sucked dry, but stored damp, under refrigeration (45 g). It was found to contain 0.289 and 0.068 mequiv/g of ionic ($S^+Me_2Cl^-$) and covalent (CH_2Cl) Cl, respectively.[10] This resin (39.7 g, 11.5 mequiv of Cl^-) was suspended in 50 ml (50 mequiv) of 1 *N* $KHCO_3$ and poured into a glass 2.5-cm column, (i.d.). After standing several hours the column was drained slowly during 4.5 hr and rinsed slowly with 50 ml of H_2O. Titration of the eluent and rinse showed it to contain 7.0 mequiv (61% exchange) of Cl^-. Further elution of the column with 50-ml portions of aq $NaHCO_3$ (and H_2O) exchanged another 3.9 mequiv of Cl^- making the total extent of exchange 95%.[28] The dimethylsulfonium bicarbonate resin was collected on sintered glass and washed well (H_2O) to remove residual HCO_3^-. Potentiometric titration of resin samples (*ca.*

(16) This factor, if valid, would probably hold only to the point wherein very high capacities would involve a high proportion of buried resin sites. At this point steric factors would probably dominate over all others. [M. Bodanszky and R. J. Bath, *Chem. Commun.*, 1259 (1969).]

(17) H. Klostermeyer, J. Halstrom, P. Kusch, J. Föhlen, and W. Lunkenheimer in "Peptides," H. C. Beyerman, A. van de Linde and W. M. van den Brink, Ed., North Holland Publishing Co, Amsterdam, 1967, p 113.

(18) (a) H. Hagenmaier, *Tetrahedron Lett.*, 283 (1970); (b) K. Braunfeldt, R. Roepstorff and J. Thomsen, *Acta Chem. Scand.*, **23**, 2906 (1969); (c) E. Bayer, H. Eckstein, K. Hägele, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, *J. Amer. Chem. Soc.*, **92**, 1735 (1970).

(19) We later learned that F_3CCO_2H alone at 25% can cleave a significant amount of peptide from the resin in 1 hr (*cf.* ref 9c). Undoubtedly, a shorter deblocking time would have been sufficient.

(20) (a) M. Manning, *J. Amer. Chem. Soc.*, **90**, 1348 (1968); (b) R. B. Merrifield, *J. Org. Chem.*, **29**, 3100 (1964); (c) S. Visser, J. Roeloffs, K. E. T. Kerling, and E. Havinga, *Recl. Trav. Chim. Pays-Bas*, **87**, 559 (1968).

(21) Resin esterification yields from basic sulfonium resins are generally 86–96% while those from the chlormethylated resin are *ca.* 14–50% (*cf.* ref 22 of ref 10).

(22) Jentsch[5] reported melittin as having no antibacterial activity against *Staph. aureus* contrary to Fennell, *et al.*,[4] and this study. Jentsch gave no details of the test method in his paper. The discrepancy is most probably due to interpretation of the degree of activity and possibly, to differences in strains used in the tests.

(23) Where analyses are indicated only by symbols of the elements, analytical results obtained for the elements are within ±0.4% of theoretical values. Peptide yields are calcd on the basis of mmoles of peptide isolated after intermediate and/or final purification relative to the total mmoles of starting BOC-amino acid, *viz.*, as resin ester. When a portion of crude peptide is purified, the amt so isolated is normalized for the entire crude batch and the yield is calcd on this normalized amount relative to the amt of starting BOC-amino acid.

(24) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

(25) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

(26) T. Mizoguchi, G. Levin, D. W. Woolley, and J. M. Stewart, *J. Org. Chem.*, **33**, 903 (1968).

(27) (a) J. R. Sargent and B. P. Vadlamudi, *Anal. Biochem.*, **25**, 583 (1968); (b) R. H. Mazur, B. W. Ellis, and P. S. Canmarata, *J. Biol. Chem.*, **237**, 1619 (1962).

(28) Note that low capacity sulfonium chloride resins require a larger quantity of $NaHCO_3$ to effect exchange of Cl^- by HCO_3^- (*cf.* Experimental Section of ref 10). Any residual sulfonium chloride groups are converted back into chloromethyl groups during the heating phase of the esterification.

1 g) suspended in 20 ml of *M* Na_2SO_4[29] with 0.1 *N* H_2SO_4 showed the resin to contain 0.390 mequiv/g of HCO_3^-.

The remainder of the esterification was carried out as described in ref 10. Esterification yields 86%, 0.48 mmole of *N*-BOC-L-isoleucine/g of resin.

Synthesis Apparatus.—Solid phase peptide syntheses were carried out in a cylindrical reaction vessel (2 × 16 cm) which was fitted with a ground glass female joint (29/25) at the top and a fritted glass plate of medium porosity at the bottom. The tube was tapered sharply below the plate to a 3-way stopcock with openings to the tube, a N_2 source, and to a suction funnel. This arrangement allowed either filtration of the tubes' contents by applied vacuum or agitation ("percolation") of the tubes' contents by gentle N_2 upward flow through the fritted plate. The tube could be closed, when desired, with cold fingers of various lengths (protrusion of these into the reactor tube helped reduce the vol of the reactor tube, when needed, to assure complete contact of the resin and reactants in soln) fitted at the top with a male joint (29/25). The N_2 source and filtration flask were disconnected when the tube was closed and rocked mechanically. In the peptide syntheses, agitation was done by percolation with N_2 bubbling in all steps with the exception of the coupling steps of 1 (1–17 and 14–17 sequences) syntheses in which mechanical rocking was used.

H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-OH (Melittin 1–8 Sequence).—The synthesis was commenced with BOC-valine resin ester, 0.91 g (0.95 mmole of BOC-L-valine). The resin was homogenized by brief swelling in CH_2Cl_2 followed by filtration. The following steps were used to introduce each new amino acid residue, (1) in the initial cycle the BOC group was removed by a 30-min treatment with 25 ml of 1 *N* HCl in AcOH. In the remaining cycles BOC removal was achieved by a 40-min treatment with 25 ml of F_3CCO_2H; (2) wash with AcOH (3 × 25 ml); (3) wash with EtOH (3 × 25 ml); (4) wash with $CHCl_3$ (3 × 25 ml); (5) neutralize the HCl salt with 25 ml of a soln of NEt_3 in $CHCl_3$ (1:6, v/v) for 10 min; (6) wash with $CHCl_3$ (3 × 25 ml); (7) wash with CH_2Cl_2 (1 × 25 ml); (8) add 2.0 mmoles of the appropriate BOC-amino acid (the carbobenzoxy group was used to protect the ϵ-NH_2 of lysine) dissolved in 12–15 ml of CH_2Cl_2 (5 ml of solvent used for rinsing in this step and the next) and mix for 10 min; (9) add 2.0 mmoles of DCI dissolved in 5 ml of CH_2Cl_2 and agitate the mixture for 3–5 hr; (10) wash with CH_2Cl_2 (3 × 25 ml); (11) wash with EtOH (3 × 25 ml); (12) wash with glacial AcOH (3 × 25 ml); and (13) remove a small sample, *ca.* 5 mg, of resin peptide from the reaction mixture and suspend it in *ca.* 1 ml of F_3CCO_2H satd with HBr. After 1 hr the mixture was freed of acids *in vacuo* in a desiccator; the residue was triturated with a small drop of pH 7, 0.4 *M* pyridine acetate buffer and this soln was examined by tlc. Homogeneity of the chromatogram and lack of detection (ninhydrin) of a spot corresponding to the previous cycle were indicative of complete coupling during that cycle. After the last coupling cycle and a final CH_2Cl_2 wash, the resin peptide was dried *in vacuo* (1.34 g). The peptide was decoupled from its support by suspending the resin in 35 ml of F_3CCO_2H and passing a slow, steady stream of dry HBr through the mixture for 90 min. The system was protected from atmospheric moisture with a $CaCl_2$ drying tube. The polymer was removed by filtration and rinsed several times with F_3CCO_2H. The combined filtrate and washings were immediately freed of the bulk of acids *in vacuo* at room temp; the residue of crude peptide further dried *in vacuo* over solid KOH; the resulting semisolid was dissolved in AcOH and evapd and dried as before to yield 0.600 g of crude octapeptide.

A 0.287-g sample of crude octapeptide was dissolved in 30 ml of pH 8.6 buffer (collidine–Pyr–AcOH, 40:40:0.3 ml to 4 l. with H_2O)[30] and filtered to remove some insol material (dry wt 11 mg). The filtrate was applied to a column (1.25 × 98 cm) of Dowex 1-X2 resin (200–400 mesh) that was preequilibrated with pH 8.6 buffer. The column was eluted with an increasing gradient of AcOH starting with 20 ml of H_2O on the resin bed, 200 ml of H_2O in a mixing flask stirred magnetically, and 280 ml of 1 *N* AcOH in the reservoir component. The reservoir was recharged with 2 *N* AcOH when emptied. A flow rate of *ca.* 36 ml/hr was maintained and fractionation was monitored by automatic drop counting. Fractions were analyzed by the ninhydrin method using 0.2-ml aliquots. The desired octapeptide was located between fractions 93–108 (317–369 ml). These fractions were pooled and freeze-dried yielding 0.182 g (49%)[31] of octapeptide as white fluffy solid: $[\alpha]^{25}D$ −91.3° (*c* 0.69, 1 *N* AcOH); tlc (silica gel/plastic support) $R_{f\,1}$ 0.65, $R_{f\,2}$ 0.71; tle $E_{Arg\,3}$ 0.84, $E_{Arg\,4}$ 0.30. Amino acid ratios found were: Ile, 0.96; Leu, 0.96; Val, 1.99; Ala, 1.05; Gly, 2.18; Lys, 1.00. *Anal.* ($C_{35}H_{65}N_9O_9 \cdot CH_3CO_2H \cdot 3H_2O$) C,H,N.

In another synthesis of this octapeptide in which deblocking (step 1) was achieved in each cycle with 1 *N* HCl in AcOH, the yield was raised to 64%. All anal. were in accord with those of the preceding synthesis.

H-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-OH (Melittin (7–17 Sequence).—Synthesis of this undecapeptide was achieved in a stepwise manner as described in the preceding experiment starting from BOC-isoleucine resin ester (1.0 g, 0.87 mmole of BOC-L-isoleucine). Deblocking of BOC groups throughout was effected with 1 *N* HCl in AcOH for 30 min. Two millimoles of BOC-amino acids and DCI was used in each cycle. The OH of threonine was protected with a benzyl group. Tlc examination of intermediate peptides, as previously, showed these to be essentially homogeneous. Half of the peptide resin was decoupled with HBr in CF_3CO_2H as before and there was obtained 0.456 g of cream white solid.

This undecapeptide was purified by ion-exchange on a column (1.25 × 105 cm) of Dowex 1-X2 using an AcOH gradient as before. Chromatography of a 0.314-g portion yielded 0.210 g (59%) of the desired peptide appearing in fractions 11–29 (3.8 ml each). This undecapeptide was quite homogeneous by tlc; on rechromatography of 0.172 g on the Dowex 1-X2 column, the peptide eluted in a sharp band (Figure 1) without the appearance of any other peaks. There was recovered 0.150 g (87% recovery) of peptide as a white fluffy solid: $[\alpha]^{25}D$ −107° (*c* 0.375, 1 *N* AcOH); tlc (silica gel/plastic support) $R_{f\,1}$ 0.48; $R_{f\,2}$

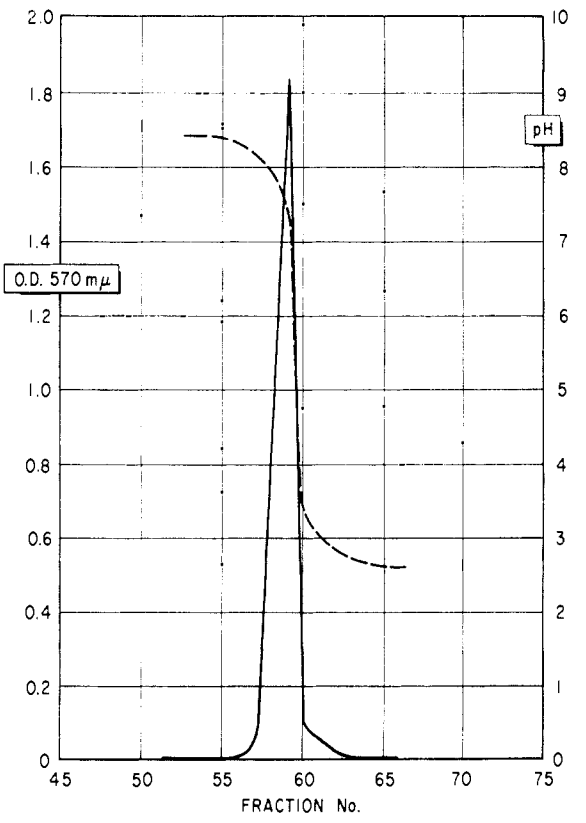


Figure 1.—Chromatography of melittin (7–17 sequence) on Dowex 1-X2 resin using an increasing concentration gradient of acetic acid. Solid line, OD of ninhydrin–peptide color formation at 570 mμ; broken line, pH.

(29) The SO_4^{2-} ions in solution exchange with resin-bound HCO_3^- ions and thereby facilitate the titration. The 20-ml portion of 1 *M* sulfate solution required a 0.05-ml blank of 0.1 *N* H_2SO_4 acid.

(30) G. Funatsu, A. Tsugita, and H. Fraenkel-Conrat, *Arch. Biochem. Biophys.*, **105**, 25 (1964).

(31) Corrected for volume of fractions used for ninhydrin analyses.

0.75. Tle $E_{Arg\ 3}$ 0.58, $E_{Arg\ 4}$ 0.12. Amino acid ratios found were: Thr, 2.02; Pro, 1.11; Gly, 1.12; Ala, 1.15; Val, 1.00; Ile, 1.05; Leu, 2.94; Lys, 0.96. *Anal.* ($C_{53}H_{96}N_{12}O_{14}\cdot 3\ H_2O$) C,H,N,$H_2O$.

H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-OH (Melittin 1–17 Sequence).—The starting resin ester was BOC-Ile-O-resin, 0.92 g (0.49 mmole of BOC-L-isoleucine). This synthesis was carried out in a same stepwise manner as the two previous syntheses with these modifications. The deblocking 1 *N* HCl in AcOH) time in step 1 was extended to 1 hr. Preliminary experiments with this resin ester showed deblocking to be incomplete, particularly in the first cycle, after the usual 30-min period (*cf.* to data in Table I). DMF was substituted for $CHCl_3$ in steps 4–6. The neutralization filtrate of step 5 and the first DMF wash in step 6 were combined, chilled, acidified with dil HNO_3, and titrated with 0.1 *N* $AgNO_3$ for Cl^- to determine the quantity of available N-terminal amine resulting from deblocking. This value ranged from 0.49 mmole initially to 0.37 mmole in the last deblocking step. One millimole of BOC-amino acid and DCI was used in all cycles except the lysine cycle for which 1.5 mmoles of reagents were used. As a precaution against possible incomplete coupling of N^{α}-BOC-N^{ϵ}-Z-lysine, the resin was treated with a soln of Ac_2O (5 ml) and NEt_3 (1.5 ml)[9a] in CH_2Cl_2 (25 ml) for 0.5 hr at the end of this cycle.

The yield of crude tan solid heptadecapeptide, after decoupling, evapg, and drying was 0.717 g. A 0.102-g sample of crude heptadecapeptide was shaken (mechanically) in 40 ml of 0.1 *M* AcOH for *ca.* 20 min and the mixture centrifuged after the resulting foam had cleared. The supernatent soln (insol material, *ca.* 5 mg) was applied to a column (1.5 × 27 cm) of Cellex-SE which had been equilibrated with 0.1 *M* AcOH. The column was eluted with 135 ml of 0.1 *M* AcOH. Thereafter gradient elution was applied starting with 5 and 80 ml of 0.1 *M* AcOH on the column bed and mixing chamber, respectively, and 150 ml of pH 4.5, 0.2 *M* NH_4OAc in the adjoining reservoir. Fractions of 3 ml were collected at a flow rate of *ca.* 15 ml/hr. The peptide was located in fractions 55–69 (homogenous by tlc) and isolated by pooling of these fractions and freeze-drying, yield 0.049 g (40%) of white powder. Amino acid ratios found were: Thr, 1.95; Pro, 1.02; Gly, 3.18; Ala, 2.11; Val, 1.82; Ile, 1.89; Leu, 3.8; Lys, 1.05. Further chromatography on a column (1.5 × 90 cm) containing Sephadex G-15 beads produced white, light flakes and fibers; $[\alpha]^{25}D$ −97.1° (*c* 0.53, 1 *N* AcOH); tlc (silica gel/glass plates) $R_{f\ 1}$ 0.55; $R_{f\ 2}$ 0.61; tle $E_{Arg\ 3}$ 0.50; $E_{Arg\ 4}$ 0.00. Amino acid ratios found were: Thr, 1.97; Pro, 1.05; Gly, 3.35; Ala, 2.12; Val, 1.67; Ile, 1.83; Leu, 3.94; Lys, 1.00. *Anal.* ($C_{17}H_{138}N_{18}O_{20}\cdot CH_3CO_2H\cdot 3H_2O$) C,H,N,O,$H_2O$.

H-Pro-Ala-Leu-Ile-OH (Melittin 14–17 Sequence).—This synthesis was carried out as described in the preceding experiment with exceptions as noted; starting resin ester BOC-Ile-O-resin (1.00 g, 0.48 mmole of BOC-Ile). The deblocking time in cycle 1 was 1 hr (two 0.5-hr intervals, 83% deblocking achieved after first interval, complete after second interval). In the two remaining cycles, 0.5-hr deblocking times were sufficient for complete deblocking. In step 13 resin samples were no longer removed, instead, the nondestructive Py·HCl procedure[13] was employed to determine the completeness of coupling reactions. Coupling was ≥99% in all three cycles.

The crude peptide (0.270 g) obtd after decoupling, evapns of acids, and drying, was purified by ion exchange on a column (1.25 × 32 cm) of Dowex 50-X4 preequilibrated with pH 4, 0.1 *M* of Py acetate buffer. The peptide was dissolved in 7 ml of buffer and applied to the column. The column was eluted with the same buffer and fractions of 3.3 ml were collected at a rate of *ca.* 20 ml/hr. The desired tetrapeptide eluted in fractions 140–203; these were pooled and freeze-dried to yield 0.124 g (63%) of fluffy, white solid, homogeneous by tlc. A 85-mg sample was dissolved in dil MeOH, filtered, and pptd with dioxane. After several days of refrigeration, the mixture was centrifuged, the peptide was washed 3 times with MeOH–dioxane (1:2) by centrifugation and dried, yielding 76 mg (89% recovery): $[\alpha]^{25}D$ −106° (*c* 0.68, 1 *N* AcOH); tlc (silica gel/plastic sheets) $R_{f\ 1}$ 0.62; $R_{f\ 2}$ 0.70; tle $E_{Arg\ 3}$ 0.57; $E_{Arg\ 4}$ 0.48. Amino acid ratios found were: Pro, 2.14; Ala, 2.09; Ile, 1.84; Leu, 1.84. *Anal.* ($C_{20}H_{36}N_4O_5$) C,H,N.

Melittin.—A soln of 121 mg of crude dry bee venom (Nutritional Biochemical Corp.) in 1 ml of pH 4.5, 0.1 *M* NH_4OAc buffer was fractionated on a column (2.5 × 92 cm) of Sephadex G-50 with the ^-OAc buffer as eluent. Fractions were monitored with uv at 280 mμ. The melittin fractions were pooled and lyophilized yielding 71 mg of cream white solid. Amino acid analysis of this polypeptide showed it to contain *ca.* 10 mole % of histidine. Repetition of the chromatography on Sephadex G-50 with 68 mg of this crude 1 produced 29 mg of melittin: Tlc (silica gel/glass plates) $R_{f\ 2}$ 0.27 (homogeneous by uv and chlorination). Amino acid ratios found were (theory): Thr, 1.84 (2); Ser, 0.94 (1); Glu, 2.07 (2); Pro, 0.91 (1); Gly, 3.13 (3); Ala, 2.06 (2); Val, 2.00 (2); Ile, 2.94 (3); Leu, 3.88 (4); Lys, 2.91 (3); Arg, 1.87 (2); NH_3, 4.9 (3). There were no histidine peaks in the chromatogram indicating reasonably pure 1.[2]

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