3.14) has completely disappeared in **21** and the signal of the C-2 olefinic proton which in **2** appears as a broad singlet[1] (or as a triplet,[22] $J = 1.7$ Hz) in **21** is a singlet.

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Synthesis of a Hydrophobic Potassium Binding Peptide[1]

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Abstract: Based on the known structures of alkali ion complexing agents the design of a homodetic cyclopeptide that would be able to bind the potassium ion was undertaken. A molecular model of the peptide *cyclo*-[-L-Val-D-Pro-D-Val-L-Pro-]$_3$ exhibited that property as judged by the oxygen-lined cavity it could provide in one of its most probable conformations. The linear peptide was synthesized by the solid-phase method starting with L-proline at the C terminus. After cleavage from the resin and cyclization the neutral cyclododecapeptide was found to form a crystalline, hydrophobic 1:1 complex with potassium picrate.

Among the ligands for metal ions the so-called "ion carriers" constitute a group of compounds which are able to complex with alkali ions and make them soluble in nonpolar media. This quality has generated great interest mainly for three reasons. First, some of the ion carriers exhibit antibiotic activity[2-9] and they show dramatic effects on the ionic balance in mitochondria[10,11] and in red blood cells.[12,13] Second, they have been found to produce similar effects in lipid membranes[13,14] (lipid bilayers) and in other artificial systems related to membranes.[3,4,11,15-18] All of these properties appear to be a consequence of the ion complexing ability which is thought to be a necessary though not sufficient condition for activity in natural membranes.[4] Third, spectroscopic investigation of some of these compounds has contributed significantly to the understanding of conformational principles in molecules of biological origin.[3-5,15,19,20]

Alkali ion carriers of known structure from natural sources are depsipeptides (valinomycin,[21] the en-

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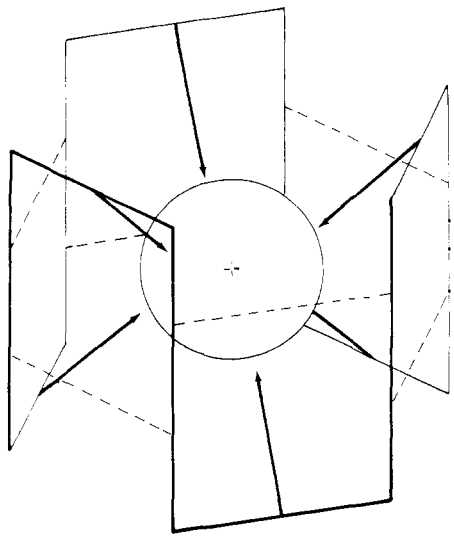

Figure 1. General architecture of a cyclododecapeptide folded in a way to provide a cavity lined with six amide carbonyl dipoles (arrows) to accommodate a potassium ion. The remaining amide groups form hydrogen bonds (broken lines) to stabilize the six U-shaped loops of the backbone (*cf.* Figure 2).

niatins,[22] beauvericin,[23] and monamycin[24]), peptides (alamethicin[25] and antamanide[26]), polyenes (amphotericin B[6]), and polyethers (the actins,[27] monensin,[7] nigericin,[8] and X-537A[9]). Some synthetic carriers have also become known, among them several depsipeptides,[3,4] a peptide,[28] and cyclic polyethers ("crown" compounds) or polythioethers.[17,29]

The inertness of most of the ion carriers which does not allow much room for chemical modification is a disadvantage. If one is to attempt to shed some light on the unanswered questions about the requirements of a molecule to exhibit alkali ion complexing one is therefore forced to resort to the total synthesis of compounds. The present study describes the design and synthesis of a neutral cyclododecapeptide which solubilizes alkali salts in an organic phase through complexation with the cation.

Despite their diversity in origin and chemical composition there remain a few structural features that are common to all of the alkali ion ligands and which are believed to be the roots of their activity. They all contain polar as well as nonpolar groups which are generally arranged in a cyclic structure. The folding of these molecules in their complexing conformation is such that the polar groups point toward the center to provide a polar cavity for the cation. The nonpolar groups, on the other hand, point outward, forming a lipophilic exterior. The result is a charged lipophilic complex which is large enough to allow its penetration into nonpolar media that normally exclude small charged particles. These general features of molecular architecture have been found to be realized in several instances by X-ray crystallography.[7-9,16,30]

Additional clues for the design of a carrier that would consist of α-amino acids exclusively are found in the well-investigated structure of a valinomycin–potassium complex.[16,19] This molecule is folded in a way that all of the six ester carbonyl oxygens point toward the center where they form the corners of an octahedron that encompasses the potassium ion (Figure 1). The backbone of the cyclododecadepsipeptide consisting of alternating hydroxy and amino acid residues encircles the cation in three complete sine waves, thus forming six loops. Each peptide carbonyl is engaged in a hydrogen bond with the closest peptide N–H in the sequence to form a bridge across the loop. These hydrogen bonds are thought to be essential in stabilizing this particular folding of the backbone. All of the side chains of the hydroxy and amino acid residues (methyl and isopropyl groups) point outward, thus shielding the potassium ion and the hydrogen bonds from the solvent.

From studies with space-filling models it becomes clear that the choice of changes in the nature or chirality of the hydroxy and amino acid residues that would not distort the symmetrical arrangement of the coordinating atoms is very limited.[4,31] There are two rules in particular that should not be violated. First, every other residue should provide an N–H which can, through hydrogen bonding, contribute to a stabilization of the "active conformation." Second, the orientation of the side chains, which is dictated by the optical configuration of the individual hydroxy or amino acid residues, should be so chosen that they could not prevent the formation of these hydrogen bonds.[31] However, there is no indication whatsoever that would require the carbonyl oxygens that coordinate to the potassium ion to be part of an ester group. In space-filling molecular models, substituting the ester bonds by amide bonds does not significantly alter the over-all geometry of the molecule. Nevertheless, in order not to introduce additional N–H groups that could conceivably stabilize other conformations or decrease the lipophilic character of the compound, these amide bonds should be part of an imino acid, such as proline.

Based on these considerations we chose to synthesize the cyclododecapeptide *cyclo*-[-L-Val-D-Pro-D-Val-L-Pro-]$_3$.[32] It may be considered an analog of valinomycin in that the L-lactic acid residues were replaced by L-proline and the D-α-hydroxyisovaleric acid residues by D-proline. In this molecule, should it assume a conformation analogous to valinomycin (Figure 2), the six carbonyl oxygens coordinating to the potassium ion still belong to the valine residues but they are now part of an amide rather than an ester bond.

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Results and Discussion

The synthesis was performed essentially according to the established procedures[33] of solid-phase peptide synthesis[34] with the aid of a Beckman peptide synthesizer Model 990. Polystyrene-*co*-1% divinylbenzene resin was chloromethylated with chloromethyl methyl ether and stannic chloride[34,35] which was converted, first, to acetoxymethyl resin[36,37] and then aminolyzed with diethylamine to yield hydroxymethyl resin. Boc-L-proline was coupled to the resin with *N,N'*-carbonyldiimidazole[36,38] and the remaining hydroxymethyl groups were acetylated with acetic anhydride. Proline was chosen as the C-terminal residue in order to minimize the chance of racemization in the ultimate cyclization step, for imino acids are known not to undergo this side reaction upon activation of the carboxyl group.[39] During the synthesis of the dodecapeptide-resin[40] 50% trifluoroacetic acid in methylene chloride was used for deprotection and 5% diisopropylethylamine in methylene chloride for neutralization. Coupling was with 2 equiv of *tert*-butyloxycarbonyl amino acids and *N,N'*-dicyclohexylcarbodiimide (DCC)[41] in methylene chloride for 2 hr followed by another 2 hr with 1 additional equiv of each reagent.

The unusual tendency of the peptide ester, H-D-Val-L-Pro-resin, to cyclize to give D-valyl-L-proline diketopiperazine called for a modification of the coupling procedure with DCC.[42] In methylene chloride the intramolecular aminolysis was found to be catalyzed by carboxylic acids, *e.g.*, by Boc-D-proline which was added to the dipeptide-resin prior to the addition of DCC. In this "regular" DCC coupling the loss of dipeptide amounted to approximately 70% while, using a "reversed" coupling (adding DCC prior to Boc-D-Pro-OH), the loss was only 10–20%. Furthermore, the sequence D-valyl-L-prolyl- has recently been shown to be cleaved from a tripeptide (H-D-Val-L-Pro-Sar-OH) to give D-valyl-L-proline diketopiperazine and sarcosine.[43] Since our peptide contains this sequence and its chemically equivalent optical antipode (-L-Val-D-Pro-) exclusively, taking measures to suppress diketopiperazine formation seemed advisable. For that reason the reversed DCC coupling procedure[42] was used throughout the synthesis.

Nevertheless, the loss of peptide chains in the first stages of the synthesis was not prevented completely as determined with the picric acid method.[44] The amine content of the resin was 72% of its original value at the tripeptide and 62% at the pentapeptide stage. There was no significant further decrease during the synthesis and the amount of amine found in the dodecapeptide-resin was 60% of the value for the proline-resin at the beginning of the synthesis. Thus, the yield of peptide decreased more rapidly during the first five to six coupling cycles than in the later ones, an observation that has also been made during the synthesis of peptide sequences unrelated to this one.[45]

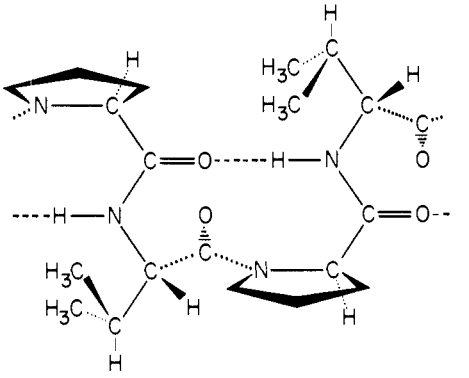

Figure 2. Probable conformation of one of the hydrogen bonded loops in the potassium complex of *cyclo*-[-L-Val-D-Pro-D-Val-L-Pro-]$_3$. The sequence shown represents one-third of the molecule.

The peptide was cleaved from the resin with hydrobromic acid in acetic acid or TFA and purified by Sephadex LH-20 chromatography. Cyclization was with Woodward's reagent K.[46] The crystalline cyclododecapeptide gave the expected elemental analysis and, after hydrolysis, amino acid analysis by the method of Manning and Moore[47] indicated equimolar amounts of L-Val, D-Pro, D-Val, and L-Pro. The compound showed the calculated molecular weight of 1176 by mass spectrometry.[48]

The cyclic dodecapeptide was demonstrated to bind potassium ions in the following experiments. A known amount of the peptide was dissolved in methylene chloride and solid potassium picrate (which is insoluble in this solvent) was added. The yellow color of the picrate was immediately taken up by the solvent and spectrophotometric determination of the solubilized picrate indicated a 1:1 complex with the peptide. Upon evaporation of the solvent the compound was obtained in crystalline form. Judging from the two-phase dissociation constants (K_{D2})[49] in the system methylene chloride–water the peptide ($K_{D2} = 7 \times 10^{-6}$ *M*) showed a sevenfold higher affinity for potassium picrate than valinomycin ($K_{D2} = 5 \times 10^{-5}$ *M*).

The ir spectra of the cyclododecapeptide and its potassium complex are shown in Figure 3. The two N–H stretch bands of the uncomplexed peptide indicate the presence of both free (3397 cm^{-1}) and hydrogen-bonded (3317 cm^{-1}) amide hydrogens. Upon complexation these bands merge and undergo a bathochromic shift to form a single band at a frequency of 3280 cm^{-1}. The amide I band which is broad and structured in the free peptide (1630–1670 cm^{-1}) is considerably narrower in the complex. Except for a sharp ab-

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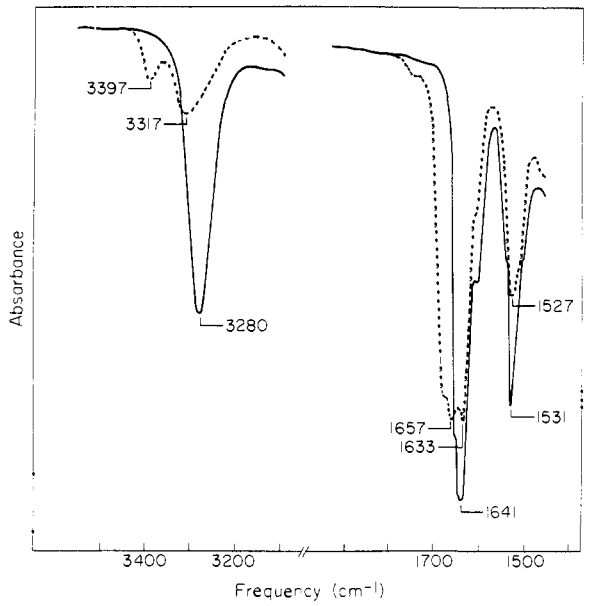

Figure 3. Partial ir spectra of the free peptide (broken line) and of its potassium picrate complex (solid line) in methylene chloride (concn, 2.5×10^{-3} *M*).

sorption band at a frequency of 2060 cm^{-1} due to the thiocyanate ion the potassium thiocyanate complex gave the same spectrum as the potassium picrate complex of Figure 3. The data for the free peptide are consistent either with a backbone structure indicated in Figure 1 in which most of the intramolecular hydrogen bonds are intact or with a mixture of conformers with a varying degree of intramolecular hydrogen bonding. Complexation appears to eliminate non-hydrogen-bonded amide hydrogens in favor of one single type of hydrogen-bonded N–H (Figure 2) as would be required for a symmetrical, compact complex of the type shown in Figure 1. The narrowing of the amide I band upon complexation is thought to be due to the superposition of the hydrogen-bonded proline carbonyl band by the band of the valine carbonyls.

The present work is an example of the possibility to program[50] the sequence of a polypeptide in a way that it contains all the information necessary to express a simple predetermined function, in this case to bind an alkali ion and form a hydrophobic complex.

Experimental Section

Amino acid analyses (Beckman Spinco amino acid analyzer Model 121) were performed by Miss L. Apacible and elemental analyses by Mr. T. Bella of Rockefeller University. Infrared spectra were taken on a Perkin-Elmer 237B or 621 ir spectrophotometer which were made available to us through the courtesy of Professor L. C. Craig of Rockefeller University. The melting points were determined in open capillaries and are not corrected. A Schmidt & Haensch polarimeter was used to measure the optical rotations. Solid-phase reactions were carried out on an automatic Beckman peptide synthesizer Model 990 or on a mechanical shaker[35] in screw cap vessels equipped with a fritted disk and a stopcock.[42] All chemicals and solvents were reagent grade. Trifluoroacetic acid, acetic acid, diisopropylethylamine, triethylamine, pyridine, acetic anhydride, and methylene chloride were redistilled prior to use.

Substitutions of resins are expressed in μequivalents per gram of benzyl polymer ($\cdot CH_2-C_6H_4-$resin) according to the formula,[51] $s = a/(1 - ae)$, where s = substitution in μequivalents per gram; a = analytical concentration of substituent on the resin in μequivalents per gram, and e = equivalent weight of the substituent bound to the benzyl polymer in gram per μequivalent. Peptide-resins were hydrolyzed with propionic acid–concentrated HCl (1:1, v/v),[52] and peptides with 6 *N* HCl, at 140° for 3–6 hr in sealed vessels.

Chloromethyl Resin. Polystyrene-*co*-1% divinylbenzene resin (100 g, Bio-Rad SX-1)[53a] was gently stirred at 90–100° in each of the following solvents for 30 min: benzene, methanol, dimethylformamide, dioxane–2 *N* aqueous NaOH (1:1, v/v), and dioxane–2 *N* aqueous HCl (1:1, v/v).[54] After each heating period the resin was filtered and washed thoroughly with the subsequent solvent. The last washes before air drying were with hot methanol, hot benzene, methanol, and methylene chloride. The resin was stirred in 600 ml of freshly distilled chloromethyl methyl ether at room temperature for 30 min and then cooled to −2 to 0° (ice–acetone bath). While, under exclusion of moisture, over a period of approximately 10 min a solution of 12 ml of stannic chloride in 100 ml of hexane was added from a dropping funnel to the stirred solution, the temperature remained within the range of −2 to +2°. After another 30 min at 0° 1000 ml of ice cold chloroform was added. The resin was filtered, washed thoroughly (dioxane–water (3:1, v/v), dioxane–2 *N* aqueous hydrochloric acid (3:1), dioxane–water (3:1), dioxane, water, methanol, and water), allowed to stand in methanol for 0.5 day, and floated in CH_2Cl_2 to remove small particles. The chloride content was 1950 μequiv/g (by combustion).

Hydroxymethyl Resin. Chloromethyl resin (20 g, substitution 1950 μequiv/g) was stirred and kept at 100–110° in 150 ml of methyl Cellosolve–potassium acetate (9:1, v/w) for 16 hr, filtered, and washed with methyl Cellosolve and CH_2Cl_2. This treatment was repeated for two more 16-hr periods. Washings included methyl Cellosolve, methyl Cellosolve–water (2:1, v/v), methyl Cellosolve, CH_2Cl_2, and methanol. Chloride was below the detectable limit (<30 μequiv/g by combustion). The resin was refluxed in 100 ml of diethylamine overnight, washed with CH_2Cl_2 and methanol, and dried. This procedure for converting acetoxymethyl resin to hydroxymethyl resin was adopted in order to convert, at the same time, any remaining traces of chloromethyl groups into diethylaminomethyl groups, thus eliminating the chance of the formation of quaternary ammonium sites later on. There was no ester band detectable at 1750 cm^{-1} indicating that essentially all of the acetoxy groups had been converted into hydroxy groups.

***tert*-Butyloxycarbonyl-L-prolyl-Resin.** To a cold (−5°, acetone–ice bath) suspension of 5.85 g (36 mmol) of carbonyldiimidazole in 100 ml of CH_2Cl_2 Boc-L-Pro-OH[53b] (7.75 g, 36 mmol) was added. After 30 min of stirring at −5° all of the components had gone into solution. Hydroxymethyl resin (30 g, 54 mequiv) was added and more solvent to make a volume of 250 ml. The suspension was stirred at room temperature for 3 days, filtered, and thoroughly washed with CH_2Cl_2, dimethylformamide, and methanol. The remaining hydroxymethyl groups were esterified by a treatment of the resin with acetic anhydride–pyridine (1:1, v/v) at room temperature for 30 min. Washings were with benzene, CH_2Cl_2, and methanol. Hydrolysis of the resin indicated a substitution of 630 μmol of proline per gram of resin. The substitution of a sample withdrawn after 18 hr reaction time was 265 μmol/g.

***tert*-Butyloxycarbonyl-D-proline.** D-Proline[53c] was N-protected with the Boc group using a procedure of Schnabel:[55] mp 133–134°; $[\alpha]^{26}D$ +63.6 (*c* 1, acetic acid) [lit.[55] (L isomer) mp 134–136°, $[\alpha]^{18-25}D$ −68.5° (*c* 1, acetic acid)].

Anal. Calcd for $C_{10}H_{17}NO_4$: C, 55.80; H, 7.96; N, 6.51. Found: C, 56.02; H, 7.95; N, 6.32.

Trifluoroacetate of L-valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-prolyl-Resin. First Run. The starting material was 4.52 g of *tert*-butyloxycarbonyl-L-prolyl-resin (substitution, 590 μequiv/g by picrate determination[44] and 595 μequiv/g by amino acid analysis). The peptide chain was built up using the following cycle: (a) deprotection with 50% (v/v) TFA in CH_2Cl_2, 2 × 15 min; (b) neutralization with 5% (v/v) diisopropylamine in CH_2Cl_2; (c) coupling with a twofold

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Table I. Analytical Data of Boc-Peptide-Resins (Second Run)

		Peptide					
		Tri	Penta	Hepta	Nona	Undeca	Dodeca
Pro:Val	Calcd	2:1	3:2	4:3	5:4	6:5	6:6
	Found	1.88:1	2.90:2	3.88:2	5.08:4	6.14:5	5.88:6
Free amine[a]		0.3	0.5	0.6	0.7	0.6	0.7

[a] As determined with picric acid,[44] expressed in per cent of the amount of peptide found by amino acid analysis. These values have not been corrected for the blank value of Boc-Pro-resin (0.4% or 2.6 μmol/g) and may therefore in fact be too high.

excess of DCC and acyl component (Boc-D-Val-OH,[56] Boc-D-Pro-OH, Boc-L-Val-OH,[53c] or Boc-L-Pro-OH,[53b] respectively) and a second coupling with 1 equiv each for 2 hr. The resin was washed thoroughly with CH_2Cl_2 after each step and with dimethylformamide and CH_2Cl_2 after each cycle, and was deprotected at the end of the synthesis. Because of the loss of peptide through intramolecular aminolysis that was observed when the dipeptide-resin was exposed to the acyl component a reversed DCC coupling[42] was used throughout: the DCC was added first followed by the Boc-amino acid in several batches. In order to inactivate unreacted amino groups and also hydroxy groups that may have formed upon loss of peptide from the resin, acetylation steps (acetic anhydride–pyridine, 1:1, 20 min) were added after the tri-, tetra-, penta-, and nonapeptide stages. The total amount of amine found in the deprotected peptide resins as determined with picrate was as follows: H-L-Pro-resin, 2.36 mequiv (100%); tripeptide-resin, 1.70 mequiv (72%); pentapeptide-resin, 1.47 mequiv (62%); heptapeptide-resin, 1.48 mequiv (62%); nonapeptide-resin, 1.39 mequiv (59%); undecapeptide-resin, 1.37 mequiv (58%); dodecapeptide-resin, 1.43 mequiv (60%). The dried dodecapeptide-resin trifluoroacetate had a substitution of 370 μequiv/g by picrate determination, thus indicating that 63% of the peptide chains were retained on the resin; amino acid analysis, Pro:Val = 6.6:6.0 (calcd 6:6).

Second Run. Starting with 5.0 g (2.8 mmol) of *tert*-butyloxycarbonyl-L-prolyl-resin this batch of peptide was synthesized on an automatic Beckman peptide synthesizer Model 990 which had been programmed to perform the same operations as described for the first run, with the following exceptions. The first couplings to the di- and tripeptide were done manually to allow addition of the Boc-amino acid in small portions and the acetylation steps were omitted. Samples of Boc-peptide-resins were taken and analyzed for free amino groups and amino acid content (Table I). These results indicate a better than 99.3% coupling yield at the stages examined. The yield of dodecapeptide-resin was 58% based on the substitution (340 μequiv/g by amino acid analysis).

L-Valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-proline Hydrochloride. Dodecapeptide-resin trifluoroacetate of the first run (2.4 g, 0.60 mmol) was allowed to swell in CH_2Cl_2 (10 min) and was treated with 30% HBr in acetic acid for 90 min at room temperature. The resin was filtered and washed with glacial acetic acid and the filtrates were combined, evaporated, and lyophilized from glacial acetic acid (470 mg). This crude product was chromatographed on a 2.6 × 90 cm column of Sephadex LH-20 in 10^{-3} *N* HCl and 100 5-ml fractions were collected. The main component emerged as a symmetrical peak (by ninhydrin reaction with an aliquot of the fractions[57]) in the fractions 50 through 60. Evaporation and lyophilization from water yielded 335 mg (46%). The corresponding yield for material from the second run of the previous paragraph was 43%. Titration of 4.77 mg of this lyophilized product with 0.01 *M* NaOH in 0.1 *M* NaCl showed it to be a mixture of 2.10 μmol of peptide hydrochloride and 1.96 μmol of free peptide. These data indicate a molecular weight of 1154 for the peptide (calcd 1194). The apparent pK_a of 7.6 was within the expected range for the N-terminal amino group of a peptide. Hydrolysis followed by quantitative amino acid analysis gave a ratio of Pro:Val = 6.0:5.6 (calcd 6:6).

***cyclo*-[-L-Valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-prolyl-L-valyl-D-prolyl-D-valyl-L-prolyl]. HCl·H-[-L-Val-D-Pro-D-Val-L-Pro-]$_3$-OH** (1.0 g, 0.8 mmol) was neutralized in aqueous solution with triethylamine to pH 5.5 and lyophilized. According to a procedure by Blaha and Rudinger[46] it was activated in 50 ml o dimethylformamide with Woodward's reagent K[53d] (270 mg, 1.0 mmol) at −10° to +10° for 120 min. The solution was diluted with 800 ml of CH_2Cl_2 and cyclization was effected by addition of triethylamine (210 μl, 1.5 mmol). After 120 hr at room temperature the solvent was evaporated at reduced pressure and the oily residue was taken up in 75% methanol and passed through a 200-ml column of mixed bed ion exchange resin (AG 501-X8 (D)[53a]). The effluent (150 ml) was collected and evaporated at reduced pressure with gradual replacement of the solvent by glacial acetic acid. This procedure was used because the peptide tended to precipitate from aqueous or alcoholic solutions in an insoluble form. After lyophilization from acetic acid the product was homogeneous by tlc (silica gel G plates[53c]) in three systems (1-butanol–acetic acid–pyridine–water, 15:10:3:2, R_f = 0.73; 1-butanol–acetic acid–water, 4:1:1, R_f = 0.47; 1-butanol, R_f = 0.18), ninhydrin-negative, and gave a yellow-white spot with the iodine–tolidine reaction.[58] Crystallization by slow evaporation of a solution of the peptide in $MeCl_2$–benzene (1:1) gave 138 mg (16%) of needles: mp >330° dec (with browning at 300°); $[\alpha]^{29}D$ −2.2 ± 1.2° (*c* 0.2, TFA). The peptide retained 1 equiv of acetic acid.

Anal. Calcd for $C_{60}H_{96}N_{12}O_{12}\cdot C_2H_4O_2$: C, 60.20; H, 8.09; N, 13.60. Found: C, 60.39; H, 8.05; N, 13.77.

The molecular weight by mass spectrometry was 1175 ± 1 (calcd 1176).[48]

The amino acid composition of the peptide was determined after hydrolysis (6 *N* HCl, 130–140°, 48 hr in a sealed vessel) with a method of Manning and Moore[47] which involves conversion of the amino acids to L-Leu-dipeptides followed by chromatography on an automatic amino acid analyzer.[59] Under the conditions used (Beckman-Spinco amino acid analyzer 120B, 0.9 × 55 cm column with Beckman-Spinco AA-15 resin, *T* = 56°, buffer pH 4.25, flow rate, 70 ml/hr) the elution times were L-Leu-D-Pro 87 min, L-Leu-L-Pro and L-Leu-D-Val 115 min, and L-Leu-L-Val 129 min. The peak areas found indicated amino acid ratios of D-Pro:(L-Pro and D-Val):L-Val = 3.1:5.6:3.0 (calcd 3:6:3).

Potassium Picrate Complex of *cyclo*-[-L-Val-D-Pro-D-Val-L-Pro]$_3$. *cyclo*-[-L-Val-D-Pro-D-Val-L-Pro]$_3$ (20 mg) was dissolved in 1.5 ml of CH_2Cl_2. When crystalline potassium picrate was added (10 mg) the solvent quickly turned yellow indicating an uptake of picrate. (Substituting valinomycin[56] for the cyclododecapeptide the same observation was made. However, there was no picrate solubilized in CH_2Cl_2 in the absence of either compound.) After 16-hr agitation at room temperature the solution was filtered, diluted with an equal volume of benzene, and allowed to evaporate. A residue with 5-mm long, fern-like yellow crystals remained; amino acid ratio after hydrolysis, proline:valine = 1.03:1.00; mp 335° dec.

Anal. Calcd for $C_{66}H_{98}N_{12}O_{19}K + 2C_6H_6$: C, 58.52; H, 6.93; N, 13.19. Found: C, 58.50; H, 6.95; N, 13.52.

The complex retained 2 mol of benzene per mole which was removed by redrying at 140° (0.1 mm) for 70 hr.

Anal. Calcd for $C_{66}H_{98}N_{12}O_{19}$: C, 54.87; H, 6.84; N, 14.54. Found: C, 54.55; H, 6.76; N, 14.26.

An aliquot was dissolved in 95% ethanol and the picrate content of this solution was measured spectrophotometrically at 358 nm. It contained 104% of the amount expected for a 1:1 complex.

Similar to Pressman[49] the two-phase dissociation constant of the complex was determined by equilibrating a 10^{-4} *M* solution of the peptide in CH_2Cl_2 with various concentrations of aqueous potassium

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picrate and measuring the uptake of picrate into the organic phase spectrophotometrically. According to this constant (*i.e.*, the concentration of potassium picrate in the aqueous phase that caused half-saturation of the peptide with potassium picrate in the organic phase) the peptide ($K_{D_2} = 7 \times 10^{-6}$ *M*) has a sevenfold higher affinity for potassium picrate than valinomycin ($K_{D_2} = 5 \times 10^{-5}$ *M*).

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Studies on Polypeptides. LI. Application of *S*-Ethylcarbamoylcysteine to the Synthesis of a Protected Heptatetracontapeptide Related to the Primary Sequence of Ribonuclease T_1[1-4]

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Abstract: This paper describes the synthesis of a partially protected heptatetracontapeptide hydrazide (fragment ABCD) of molecular weight 5485 corresponding to positions 1–47 of the proposed primary structure of the enzyme ribonuclease T_1. *S*-Ethylcarbamoyl-L-cysteine was used to synthesize formylalanyl-*S*-ethylcarbamoylcysteinyl-aspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycylserylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine benzyloxycarbonylhydrazide (fragment A). This peptide derivative corresponds to positions 1–11 of the ribonuclease T_1 sequence and contains three of the four half-cystine residues of the enzyme. Fragment A was exposed to hydrogen bromide in trifluoroacetic acid, and the ensuing hydrazide *via* the azide was coupled to the previously described fragment B *tert*-butoxycarbonylhydrazide to give fragment AB, which spans positions 1–23 of the enzyme sequence. Fragment AB was converted to the hydrazide by exposure to trifluoroacetic acid, and the hydrazide, in the form of the corresponding azide, was coupled to fragment CD *tert*-butoxycarbonylhydrazide to give fragment ABCD. The sequential homogeneity of fragment ABCD is based on the fact that it was obtained by linking fragments, shown to be homogeneous, by azide couplings and on the observation that the ratios of the "diagnostic" amino acid residues agreed with those expected by theory. The insolubility of both fragments AB and ABCD eliminated thin layer chromatography as a useful tool for analytical evaluation. The stability of the *S*-ethylcarbamoyl group was investigated with simple model peptides and this sulfur protecting group was found to be stable under acid conditions (trifluoroacetic acid and hydrogen bromide in trifluoroacetic acid) but was cleaved in an alkaline milieu of pH 8.5 or higher. The *S*-ethylcarbamoyl group is readily cleaved by silver and mercuric acetate with formation of mercaptides. *S*-Ethylcarbamoylcysteine and peptides containing this protected amino acid fail to react with *p*-chloromercuribenzoate at pH 4.6 but are readily cleaved by this reagent at pH 7.0 or higher. This observation enabled us to determine the *S*-ethylcarbamoyl content of the various peptide intermediates in the synthesis of fragment A. Peptides containing *S*-ethylcarbamoylcysteine do not react with Ellman reagent on thin layer plates, but exposure of developed plates to ammonia vapor results in the formation of bright yellow spots. The *S*-ethylcarbamoyl group provides suitable protection of the thiol group of cysteine in peptide synthesis but care has to be exercised not to expose peptides containing this sulfhydryl protector to strongly alkaline conditions. Oxidation with performic acid converts peptides containing *S*-ethylcarbamoylcysteine into the corresponding cysteic acid derivatives.

In previous communications,[1,3,5] we have described syntheses of two protected peptide hydrazides, *i.e.*, fragments BCD spanning positions 12–47 and fragment EF corresponding to positions 48–80 (Figure 1,

(1) See R. Camble, G. Dupuis, K. Kawasaki, H. Romovacek, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **94**, 2091 (1972), for paper L in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation.

(3) A preliminary communication of some of the results presented in this paper has appeared: H. T. Storey and K. Hofmann, *Peptides, Proc. Eur. Peptide Symp., 11th, 1971*, in press.

(4) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M, aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)]; Boc, *tert*-butoxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; EC, ethylcarbamoyl; EtOH, ethanol; F, formyl; MeOH, methanol; N_3, azide; NMM, *N*-methylmorpholine; OCP, 2,4,5-trichlorophenyl ester; ONHS, *N*-hydroxysuccinimido ester; *O-t*-Bu, *tert*-butyl ester; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; tlc, thin layer chromatography; X, *tert*-butoxycarbonylhydrazide; Y, benzyloxycarbonylhydrazide; Z, benzyloxycarbonyl. In order to simplify the designation of complex peptide derivatives, the following nomenclature is used: fragment A, the *N*-formyl benzyloxycarbonylhydrazide of the peptide corresponding to positions 1–11 of T_1; fragment B, the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 12–23 of T_1; fragment AB, the *N*-formyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 1–23 of T_1; fragment CD, the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 24–47 of T_1; fragment ABCD, the *N*-formyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 1–47 of T_1; fragments A, AB, CD, and ABCD hydrazides, the free hydrazides of the N-protected peptides; fragments B and CD *tert*-butoxycarbonylhydrazides, the amino-deprotected peptides.

(5) J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971).