Further studies along these lines of displacement reactions at heteroatoms in very basic solutions are in progress in our laboratory.

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by H_-, indicating, as the authors had suggested, that the reactions are in fact first order in hydroxide ion. The second-order rate constants we have calculated using eq 5 are 3.3×10^{-3} M^{-1} min^{-1} for the alkaline hydrolysis of phenylmethylphosphonic acid at 78° and 4.0×10^{-3} M^{-1} min^{-1} for that of *p*-nitrophenylmethylphosphonic acid at 30°. By extrapolation of their data to low hydroxide ion concentration, Behrman, *et al.*, obtained a value of 3.0×10^{-3} M^{-1} min^{-1} for the second-order rate constant in the case of the unsubstituted phosphonate at 78° and the same value for the nitro-substituted compound at 30°, in reasonable correspondence with our calculation.

Carboxyl-Catalyzed Intramolecular Aminolysis. A Side Reaction in Solid-Phase Peptide Synthesis

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Abstract: The polymer-supported peptide ester, D-valyl-L-prolyl-resin, was found to undergo intramolecular aminolysis which was catalyzed by carboxylic acids. The resulting loss of the dipeptide from the resin, which amounted to 70% during a regular coupling with *N,N'*-dicyclohexylcarbodiimide, was repressed by adding the carbodiimide reagent prior to the carboxyl component. The diketopiperazine of D-valyl-L-proline, the only detectable product of this side reaction, was isolated and characterized. The rate of the intramolecular aminolysis was dependent on the composition and configuration of the dipeptide. None of the other reagents tested were as efficient catalysts as the carboxylic acids.

In the course of the synthesis of the peptide sequence D-Pro-D-Val-L-Pro[1] by the solid-phase method,[2] we observed a considerable loss of peptide from the resin.[3] Although the yield of the protected dipeptide was nearly quantitative, only about 30% of the expected amount of tripeptide was found. A step-by-step monitoring of the synthesis indicated that the loss did not occur during deprotection or neutralization of the dipeptide-resin[4] but during the coupling with Boc-D-proline and DCC. This unexpected finding called for a closer investigation, some aspects of which are presented here.

The methods and procedures employed were essentially the established techniques of solid-phase peptide synthesis.[5] Polystyrene-*co*-1% divinylbenzene resin was chloromethylated with chloromethyl methyl ether and stannic chloride[2,6] which was converted, first, to acetoxymethyl resin[7,8] and then aminolyzed with diethylamine[3] to yield hydroxymethyl resin. Boc-L-proline was esterified to the resin by the *N,N'*-carbonyldiimidazole method[7,9] and the remaining hydroxy groups were blocked by esterification with acetic anhydride. This procedure was chosen in order to avoid the introduction of any quaternary ammonium groups into the polymer[10] which can interfere with the quantitative determination of amino groups as described below. The dipeptide-resins were prepared using two DCC couplings[11] with a twofold excess of Boc-amino acid and DCC reagent each time.

In order to monitor the loss of dipeptide from the resin, a procedure for the determination of amino groups on an insoluble polymer with picric acid[12] was adopted. The amine-containing resins were treated with a solution of picric acid to form the polymer supported amine picrate. After thorough washings to remove nonionically bound picric acid, the resins were treated with an excess of diisopropylethylamine which quantitatively released the picrate from the polymer into solution. The concentration of picrate in this solution, which was determined spectrophotometrically, reflected the amine content and therefore the amount of dipeptide on the resin. These values were used to compute apparent first-order rate constants for the decrease in amine content of dipeptide resins (Tables I and II[13]) as described in the Experimental Section.

Since diketopiperazines can be quantitatively determined by gas–liquid chromatography,[14] this method was used to measure the release of D-Val-L-Pro diketopiperazine[15] from the solid support. These experi-

(1) The abbreviations recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.*, **241**, 2491 (1966); **242**, 555 (1967) have been used throughout. In addition, TFA = trifluoroacetic acid, DMF = dimethylformamide, DCC = *N,N'*-dicyclohexylcarbodiimide.
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Table I. Apparent First-Order Rate Constants of the Disappearance of Amino Groups from H-D-Val-L-Pro-Resin with Various Reagents

Reagent	Concn,[a] M	k_{app},[b] min^{-1}	k_{rel}
None		2.5×10^{-4}	1.0[c]
Diisopropylethylamine	0.3[d]	6.0×10^{-4}	2.4
N-tert-Butyloxy-carbonyl-D-proline[e]	0.05	1.3×10^{-1}	520
Acetic acid	0.05	8.5×10^{-2}	340
Trimethylacetic acid	0.06	5.7×10^{-2}	230
Benzoic acid	0.06	7.8×10^{-2}	310
Trifluoroacetic acid	6.8[f]	1.5×10^{-3}	6.1
	2.7[g]	6.3×10^{-4}	2.5
	0.06	1.5×10^{-4}	0.6
Picric acid	0.1	6.9×10^{-4}	2.8
3,5-Dimethylpicric acid[h]	0.06	2.7×10^{-3}	11
2,4-Dinitrophenol	0.06	2.3×10^{-3}	9.2
p-Nitrophenol	0.06	1.2×10^{-2}	48
Imidazole	0.06	4.5×10^{-3}	18
2-Hydroxypyridine	0.06	4.7×10^{-3}	19
N-Hydroxysuccinimide	0.03[i]	8.9×10^{-4}	3.6
Pyridine picrate	0.03[i]	4.6×10^{-4}	1.8
Triethylamine hydrochloride	0.06	$<1.3 \times 10^{-4}$	<0.5

[a] In methylene chloride. [b] See Experimental Section. [c] Reference value. [d] 5% by volume. [e] Reference 3. [f] 50% by volume. [g] 20% by volume. [h] Reference 13. [i] Saturated solution.

Table II. Apparent First-Order Rate Constants of the Acetic Acid Catalyzed Disappearance of Amino Groups from Dipeptide-Resins[a]

Compound	k_{app}, min^{-1}	k_{rel}	Half-time, min
H-D-Val-L-Pro-resin	8.5×10^{-2}	100[b]	8.1
H-L-Val-L-Pro-resin	7.3×10^{-3}	8.6	95
H-D-Pro-L-Pro-resin	6.5×10^{-3}	7.6	107
H-L-Pro-L-Pro-resin	9.2×10^{-2}	108	7.5
H-L-Val-Gly-resin	4.7×10^{-3}	5.5	150
H-Gly-L-Val-resin	1.0×10^{-3}	1.2	690

[a] 0.1 M HOAc in methylene chloride, 25°. [b] Reference value.

ments (Figure 1) were performed in a thermostated vessel. After the cyclization reaction the released diketopiperazine was injected into the gas chromatograph without derivatization to yield the experimental data in Figure 1. Each point represents the total diketopiperazine found after the corresponding period of time.

Results

It was found (Table I) that H-D-Val-L-Pro-resin was stable as the trifluoroacetate and was nearly so in its free amine form ($k_{app} = 2.5 \times 10^{-4}$ min^{-1}, $k_{rel} = 1.0$) when suspended in methylene chloride. There was only a slight increase in the rate of loss of amino groups from the resin with 5% diisopropylethylamine in methylene chloride ($k_{rel} = 2.4$). With the carboxylic acid Boc-D-Pro-OH (0.05 M, in methylene chloride), however, amine was lost from the resin at a rate 520 times greater than with methylene chloride alone ($k_{app} = 1.3 \times 10^{-1}$ min^{-1}). We take this rate, corresponding to a half-time of 5.3 min, to account for the low yield in the preparation of D-Pro-D-Val-L-Pro-resin mentioned earlier. For, in that synthesis, the standard

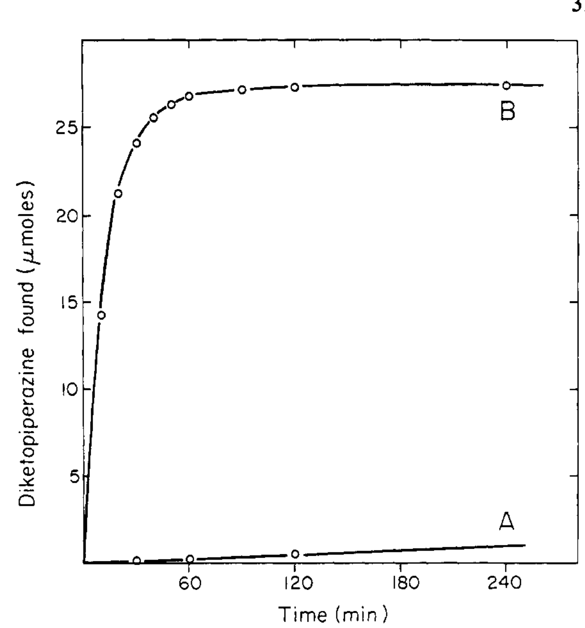

Figure 1. The cleavage of D-Val-L-Pro diketopiperazine from H-D-Val-L-Pro-resin with methylene chloride (A) and with 0.05 M acetic acid in methylene chloride (B). Experimental values (○) by glc.

procedure for DCC coupling[2] was employed, which involves the equilibration of the amino-resin with the carboxyl component for 10 min prior to the addition of the coupling agent. When the order in which the reagents were added to the amine component was reversed, namely, DCC followed by Boc-Pro-OH in several small portions, the loss was reduced and the yield of tripeptide was over 90%.

The loss of dipeptide was not only accelerated by Boc-D-Pro-OH but also by other carboxylic acids such as acetic acid ($k_{rel} = 340$, Figure 1), benzoic acid ($k_{rel} = 310$), and trimethylacetic acid ($k_{rel} = 230$). In all three instances tlc of the supernatant showed, in addition to the spot corresponding to the reagent, a single new ninhydrin negative spot which stained blue in the iodine–tolidine reaction. This suggested to us a cleavage of the anchoring bond to the resin to form the diketopiperazine of D-valyl-L-proline. In order to test this hypothesis a sample of H-D-Val-L-Pro-resin was treated with 0.1 M acetic acid in methylene chloride for 1 hr at room temperature. The resin was filtered off and the filtrate was evaporated to give a crystalline residue which, with one crystallization, had the same melting point as that reported for L-Val-D-Pro diketopiperazine.[16] The product which was obtained in 98% yield gave a satisfactory C, H, and N analysis and was also found to give an ir spectrum identical with the one of the diketopiperazine synthesized by cyclization of D-valyl-L-proline *p*-nitrophenyl ester.

Table II shows the effect of 0.1 M acetic acid in methylene chloride on the disappearance of amino groups from six different dipeptide-resins. H-L-Pro-L-Pro-resin had a sensitivity comparable to H-D-Val-L-Pro-resin whereas H-D-Pro-L-Pro-resin and H-L-Val-L-Pro-resin both were about ten times more resistant to this reagent. H-L-Val-Gly-resin was 18 times and H-Gly-L-Val-resin was 80 times more stable than H-D-

(15) Synonyms: *trans*-1,6-trimethylene-3-isopropyl-2,5-piperazinedione; *cyclo*-[D-valyl-L-prolyl].

(16) A. Stoll, A. Hofmann, and B. Becker, *Helv. Chim. Acta*, **26**, 1602 (1943).

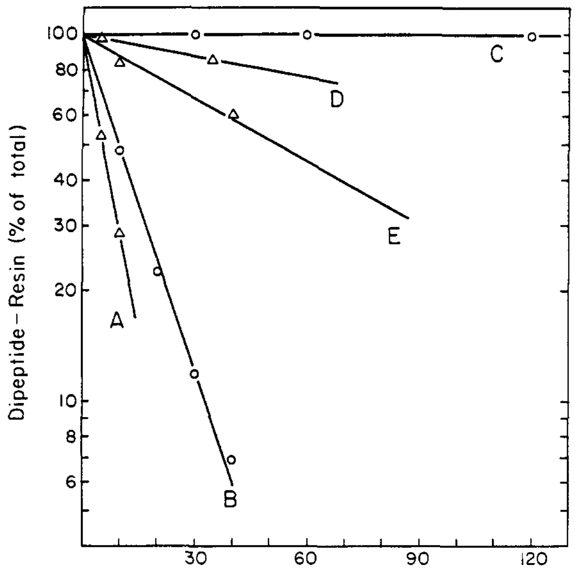

Figure 2. Semilogarithmic plot of the disappearance of amino groups from H-D-Val-L-Pro-resin upon treatment with different reagents in methylene chloride: (A) Boc-D-Pro-OH (0.05 *M*); (B) acetic acid (0.05 *M*); (C) methylene chloride; (D) imidazole (0.06 *M*); (E) *p*-nitrophenol (0.06 *M*). Experimental values by glc (○) and by picrate determination (△).

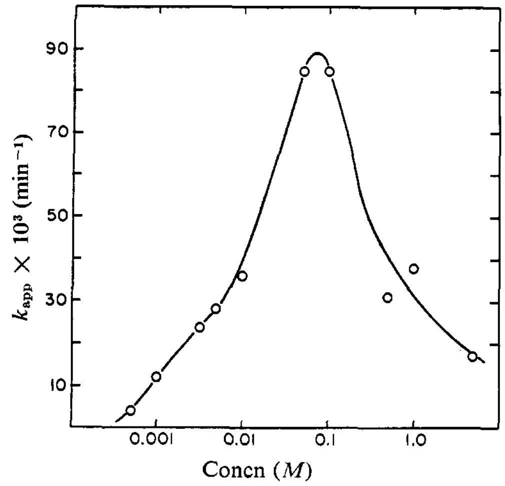

Figure 3. Semilogarithmic plot of the apparent first-order rate constants of the cleavage of D-Val-L-Pro diketopiperazine from H-D-Val-L-Pro-resins under the influence of acetic acid at various concentrations in methylene chloride determined by monitoring the disappearance of amino groups with picrate.

Val-L-Pro-resin. Based on these data we can expect losses in the order of 1–5% for a normal coupling procedure to a non-imino acid dipeptide-resin, in which the carboxyl and amine components are premixed for 10 min before the addition of DCC.

A semilogarithmic plot of the amount of diketopiperazine that was found by glc in the supernatant of an acid treated dipeptide-resin *vs.* time is consistent with pseudo-first-order kinetics (Figure 2, curve B).

The rate of cleavage is dependent on the concentration of the catalyst. Figure 3 shows how differing concentrations of acetic acid in methylene chloride affect the rate of cleavage of dipeptide from the resin at room temperature. The plot indicates a maximal efficiency of acetic acid in a concentration of approximately 0.08 *M*. By coincidence, similar concentrations are normally used in the presoaking step of the amine-resin with the Boc-amino acid prior to the addition of DCC for the coupling reaction.

Discussion

The side reaction which caused a low yield in the synthesis of Boc-D-Pro-D-Val-L-Pro-resin was a carboxylic acid catalyzed intramolecular aminolysis of the ester bond to the resin at the dipeptide stage. This conclusion was arrived at by interpretation of the following experimental results.

(a) It was shown that the cleavage occurred during the coupling step. The cleavage rates of H-D-Val-L-Pro-resin at other steps of the synthesis, *i.e.*, with 50% TFA in methylene chloride, 5% diisopropylamine in methylene chloride, or with the solvent alone, could not account for the magnitude of the loss of peptide that was observed.

(b) The yield of tripeptide was drastically improved by adding DCC to the dipeptide-resin prior to the carboxyl component.

(c) The dipeptide is also lost from the resin under the influence of acetic acid or other weak carboxylic acids at rates comparable to that with Boc-D-Pro-OH.

(d) The only detectable product of the cleavage, D-Val-L-Pro diketopiperazine, was isolated in high yield.

Experiment b was chosen with reference to the mechanism for DCC couplings which is generally thought to involve the very rapid formation of an activated derivative of the carboxyl component, which then aminolyzes more slowly to form the peptide bond. If, therefore, the DCC were added first, the subsequently added carboxyl component would be consumed almost immediately to form the active intermediate and would not catalyze the formation of diketopiperazine. With this "reversed DCC coupling" the exposure of the dipeptide-resin to carboxyl groups was expected to be minimal as compared to the standard procedure or to simultaneous addition of the two components to the resin. The increase in yield of Boc-D-Pro-D-Val-L-Pro-resin from 32% with the standard procedure to over 90% with reversed DCC coupling was compatible with that reasoning.

The cyclization can either be catalyzed or inhibited by acetic acid (Figure 3). At higher concentrations the amine becomes increasingly protonated and cannot participate as a nucleophile. This hypothesis is supported by the values for the cleavage rates with strong acids at low concentrations (picric acid, TFA) which are not significantly different from the reference rate with solvent alone (Table I). Higher concentrations of TFA (20% or 50%) lead to the linear dipeptide (verified by tlc) by the known acidolytic cleavage of the benzyl ester linkage that anchors the peptide on the resin[17] rather than to the diketopiperazine by intramolecular aminolysis.

A definite acceleration of the reaction was observed with other weak acids (3,5-dimethylpicric acid, 2,4-dinitrophenol, *p*-nitrophenol) and with "bifunctional catalysts"[18] (2-hydroxypyridine, imidazole). However,

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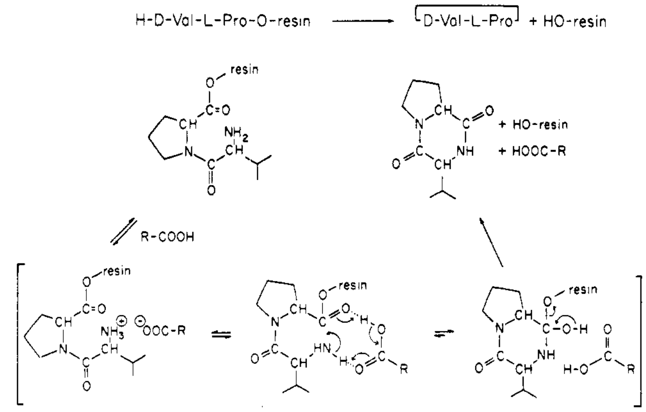
(18) C. G. Swain and J. F. Brown, Jr., *J. Amer. Chem. Soc.*, **74**, 2538 (1952); M. L. Bender, *Chem. Rev.*, **60**, 53 (1960).

the rates with these acids were much smaller than with the carboxylic acids at similar concentrations. Whether this is due to different mechanisms or to the result of a different concentration dependence or whether it reflects an inherent property of these reagents awaits a more detailed investigation.

Owing to the ease with which acylimino acids can form cis peptide bonds,[19] the tendency of diketopiperazine formation is high in the case of peptides that contain proline[20-23] or sarcosine.[22] One is also reminded of the acceleration of the aminolysis of esters[24] and "active esters"[25,26] by "catalytic amounts" of a carboxylic acid. Similarly, the cyclization of glutamic esters is catalyzed by carboxylic acids.[27] However, this type of reaction is not restricted to the aminolysis of esters.[21b,22,23,28-31] Recently, the formation of pyroglutamyl peptides from Boc-glutaminyl peptides during the deprotection step has been linked to the presence of carboxylic acids[28a] and the spontaneous decomposition of the pure tripeptide, H-D-valyl-L-prolylsarcosine, to yield D-valyl-L-proline diketopiperazine and sarcosine has been reported.[23] An explanation offered for the latter reaction was based on the limited conformational freedom of the peptide due to the bulkiness of its substituents.[29] It might now be supplemented with the conceivable participation of the carboxyl group of sarcosine that was present under the prevailing conditions. Another carboxyl-catalyzed intramolecular aminolysis reaction is the rearrangement of *N*-acyl-*N'*-α-aminoacylhydrazines into acyl-α-aminoacylhydrazides.[30,31] There,[31] as well as in the cases of the cyclization of glutamic esters,[27] and of the aminolysis of active esters,[26] a catalysis-inhibition dependence on acid concentration was demonstrated, which was similar to that found here for the disappearance of dipeptide from the resin (Figure 3). The postulated mechanism[26,27,31] might therefore also obtain in our case (Scheme I). It involves a concerted reaction in which the un-ionized carboxyl group of the catalyst acts through a hydrogen-bonded cyclic intermediate.

Although many peptides have been prepared by the solid-phase method[32] (several of them with C-terminal proline),[21a,33] this carboxyl-catalyzed side reaction has not been reported before. We presume that it reflects an unusually susceptible structure of certain dipeptide-resins under the specified conditions rather than a general phenomenon. In those instances where the cyclization is quantitatively important this side reaction can now be effectively suppressed.

Scheme I



Experimental Section

Amino acid analyses (Beckman Spinco amino acid analyzers 120B and 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 ir spectrophotometer through the courtesy of Dr. L. C. Craig of Rockefeller University. Melting points (not corrected) were determined in capillaries and optical rotations on a Schmidt & Haensch polarimeter. Solid-phase reactions were carried out in vessels that were made from screw-capped Pyrex culture tubes (screw cap fitted with Teflon interface, Scientific Glass Apparatus, Inc., Bloomfield, N. J., Catalogue No. T-2040-a). In order to obtain reactors of capacities ranging from 5 to 30 ml that could be used both for analytical and preparative purposes, the tubes were cut and fitted with a glass fritted disk (medium porosity) and a stopcock with a 1.5-mm bore Teflon plug. The volumes were adjusted so that the walls of the vessel were completely wetted during the mixing period of the standard mechanical shaker.[2] Hydrolyses of resins were with either 12 *N* HCl–dioxane (1:1, v/v) in sealed vessels at 110° for 18–20 hr followed by filtration and rehydrolysis in 6 *N* HCl (110°, 90 hr) or with propionic acid–12 *N* HCl (1:1, v/v) at 130–140° for 3–6 hr.[34]

***tert*-Butyloxycarbonyl Dipeptide-Resins.** Starting with 1.0 mmol of Boc-L-Pro-resin[3] (substitution, 370 μmol/g), Boc-dipeptide-resins were prepared in the following way: (a) deprotection with TFA–CH_2Cl_2 (1:1, v/v) 2 × 15 min, CH_2Cl_2 3 × 2 min; (b) neutralization with diisopropylethylamine–CH_2Cl_2 (1:19, v/v) 2 × 3 min, CH_2Cl_2 3 × 2 min; (c) coupling with 2.0 mmol of DCC in CH_2Cl_2 for 2 min and 2.0 mmol of Boc-amino acid (Boc-D-Pro-OH,[3] Boc-D-Val-OH,[35] Boc-L-Pro-OH,[36a] or Boc-L-Val-OH)[36b] for 2 hr, washing with alternating CH_2Cl_2 and DMF 3 × 2 min each; (d) coupling step c repeated for 5 hr. Amino acid analysis of the dried resins gave the following substitutions (in μeqiv/g): Boc-D-Pro-L-Pro-resin, Pro 650; Boc-D-Val-L-Pro-resin, Pro 350, Val 340; Boc-L-Pro-L-Pro-resin, Pro 710; Boc-L-Val-L-Pro-resin, Pro 332, Val 343.

D-Prolyl-D-valyl-L-prolyl-Resin. (a) By Regular DCC Coupling. Boc-D-Val-L-Pro-resin, 100 mg, was deprotected (2 × 15 min with TFA–CH_2Cl_2, 1:1) and neutralized (2 × 3 min with diisopropylethylamine–CH_2Cl_2, 5%). The amine content at this stage was

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340 μmol/g (by picrate determination) and amino acid analysis indicated 350 μmol of proline and 340 μmol/g of valine per gram. The resin was soaked with a 0.1 *M* solution of Boc-D-Pro-OH in CH_2Cl_2 (2 ml, 200 μmol) for 10 min prior to the addition of DCC (42.5 mg, 200 μmol). After 3-hr agitation at room temperature and thorough washing (DMF, CH_2Cl_2), the resin had a picrate value of 5 μmol/g, indicating over 98% coupling. After deprotection (2 × 15 min, TFA) only 110 μmol of free amine, 229 μmol of proline, and 127 μmol of valine per gram were found. (All of the analytical values have been corrected for the weight change of the peptide-resin and are thus expressed as μmoles per gram of Boc-D-Val-L-Pro-resin.) Therefore, the yield was 32% based on the picrate values of H-D-Val-L-Pro-resin and H-D-Pro-D-Val-L-Pro-resin, and 35% based on the averaged amino acid substitutions.

(b) By "Reversed" DCC Coupling. This experiment was identical with (a) except that the sequence of the addition of the coupling reagents was reversed. The resin was soaked in a 0.1 *M* solution of DCC in CH_2Cl_2 (2 ml, 200 μmol) for 2 min prior to the addition of Boc-D-Pro-OH (43 mg, 200 μmol). Coupling by this scheme gave the following analytical results: before coupling, free amine 340 μmol, Pro 350 μmol, and Val 340 μmol per gram; picrate value after coupling, 2 μmol per gram; tripeptide-resin after deprotection, free amine 310 μmol, Pro 685 μmol, and Val 309 μmol per gram (cor). This amounts to a yield of 91% by picrate determination and 93% by amino acid analysis.

***tert*-Butyloxycarbonyl-D-valyl-L-proline Benzyl Ester.** In 30 ml of CH_2Cl_2, L-proline benzyl ester hydrochloride[36b] (2.41 g, 10 mmol), triethylamine (1.4 ml, 10 mmol), Boc-D-Val-OH[35] (2.17 g, 10 mmol), and DCC (2.06 g, 10 mmol) were combined and agitated overnight. After addition of 1 ml of acetic acid the mixture was diluted with 100 ml of ether and filtered. The filtrate was washed (potassium bicarbonate, citric acid, water), dried (Na_2SO_4), evaporated, and crystallized from hexane: yield, 2.8 g (69%); mp 105–105.5°; $[\alpha]^{26}D$ + 16.4° (*c* 1, benzene).

Anal. Calcd for $C_{22}H_{32}N_2O_5$: C, 65.32; H, 7.97; N, 6.93. Found: C, 65.37; H, 7.92; N, 6.79.

***tert*-Butyloxycarbonyl-D-valyl-L-proline.** In 20 ml of methanol, *tert*-butyloxycarbonyl-D-valyl-L-proline benzyl ester (2.0 g, 4.95 mmol) was hydrogenated with 10% Pd on $BaSO_4$ for 18 hr at 50 psi of H_2. After filtration and evaporation of the solvent, the crude acid was dissolved in aqueous bicarbonate, extracted with ether, acidified with citric acid, and extracted into ether. The ether layer was separated, dried (Na_2SO_4), and evaporated to give 0.55 g (35%) of a colorless solid: mp 69–72°; $[\alpha]^{27}D$ − 11.6° (*c* 1, ethanol); cyclohexylammonium salt, mp 182–184° dec.

Anal. Calcd for $C_{21}H_{39}N_3O_5$: C, 60.99; H, 9.51; N, 10.16. Found: C, 60.77; H, 9.88; N, 10.07.

D-Valyl-L-proline Diketopiperazine. (a) Solution Method. Boc-D-valyl-L-proline (500 mg, 1.6 mmol), DCC (410 mg, 2.0 mmol), and *p*-nitrophenol (450 mg, 3.2 mmol) were added to 2.5 ml of CH_2Cl_2 at 5°. The mixture was agitated for 18 hr at 5° and, after addition of 1 ml of acetic acid, for 15 min at room temperature. The dicyclohexylurea was filtered off and the filtrate was diluted with ether, washed with aqueous citric acid, H_2O, aqueous bicarbonate, and H_2O, then dried (Na_2SO_4), and evaporated to dryness. By repeatedly dissolving the residue in acetone or ether, filtering, and evaporating the solvent, more dicyclohexylurea was removed. The yellow oil (Boc-D-Val-L-Pro *p*-nitrophenyl ester) was dissolved in 10 ml of TFA and after 10 min was evaporated to dryness. The residue (TFA–H-D-Val-L-Pro *p*-nitrophenyl ester) was dissolved in 200 ml of benzene and 100 ml of pyridine was added. After 18 hr at room temperature, the mixture was evaporated to dryness, dissolved in ethanol–water (1:1, v/v), and put on a 30-ml mixed-bed ion-exchange resin column (AG 501-X8 (D)).[36d] The neutral diketopiperazine was eluted from the column with 120 ml of H_2O. Evaporation of the solvent gave 190 mg (60%). After crystallization from ethyl acetate–hexane the diketopiperazine had mp 148–148.5°, $[\alpha]^{30}D$ −86° (*c* 0.9, H_2O) [lit.[16] for *cyclo*-L-Val-D-Pro: mp 147–149°, $[\alpha]^{20}D$ +88° (*c* 1, H_2O)].

Anal. Calcd for $C_{10}H_{16}N_2O_2$: C, 61.20; H, 8.22; N, 14.28. Found: C, 60.83; H, 8.26; N, 14.21.

(b) Solid-Phase Method. Boc-D-Val-L-Pro-resin (325 mg, 110 μequiv) was deprotected and neutralized as described above. It was treated with 0.1 *M* acetic acid in CH_2Cl_2 (5 ml) for 60 min, filtered, and washed with CH_2Cl_2. Filtrate and washes were combined and evaporated to give 21 mg (98%) of crystalline diketopiperazine. In aqueous solution it was passed through a short (3 ml) column of mixed-bed ion-exchange resin as above and, after evaporation of the solvent, crystallized from ethyl acetate–hexane: mp 147–148°, $[\alpha]^{30}D$ −95° (*c* 0.7, H_2O). The ir spectrum (KBr pellet) of this preparation was found to be identical with the one of the product of (a). Amino acid content after hydrolysis (6 *N* HCl, 135°, 3 hr in a sealed vessel) yielded Pro, 5.35 μmol/mg, Val, 4.85 μmol/mg (calcd 5.10 for each).

Anal. Calcd for $C_{10}H_{16}N_2O_2$: C, 61.20; H, 8.22; N, 14.28. Found: C, 61.20; H, 8.09; N, 14.26.

Determination of Amine Content of Resins. The following procedure was used.[12] The resin was allowed to swell in CH_2Cl_2 (1 × 5 min), neutralized with 5% (v/v) diisopropylethylamine (DIA) in CH_2Cl_2 (2 × 3 min), washed with CH_2Cl_2 (3 × 2 min), treated with 0.1 *M* picric acid in CH_2Cl_2 (2 × 3 min), and washed with CH_2Cl_2 (5 × 2-min). The picrate was eluted with 5% DIA in CH_2Cl_2 or 0.1 *M* pyridine hydrochloride in CH_2Cl_2 (2 × 3 min) and CH_2Cl_2 (3 × 2 min) and, after dilution with 95% ethanol, measured spectrophotometrically. The molar extinction coefficient of DIA picrate (E_{358} 14,500) was constant in the concentration range of (1–20) × 10^{-5} *M* if the ethanolic measuring solution contained less than 20% CH_2Cl_2. A convenient ratio of resin to solvent was 1:20 (w/v).

Determination of Cleavage Rates with Different Reagents. Method a (Tables I and II, Figure 2 and 3). Samples of Boc-dipeptide-resins (50–100 mg, 10–60 μequiv) were deprotected and neutralized as described. The picrate value representing the amine content of the resin was determined before and after treatment for periods of 5–60 min at room temperature with each of the reagents listed. (The chemicals were of reagent grade and were obtained from commercial sources unless specified.) Treatment with each reagent and picrate determination were repeated to give at least three points on the cleavage curve. After minor corrections for loss of peptide during the determination itself, these values were plotted semilogarithmically, and the apparent first-order rate constants[37] were deduced from the graphically averaged slope of the curve.

Method b (Figures 1 and 2). In a jacketed thermostated vessel (24 ± 0.2°) a sample of Boc-D-Val-L-Pro-resin was deprotected and neutralized. It was treated first with CH_2Cl_2 for 120 min and second with 0.05 *M* acetic acid in CH_2Cl_2 for 240 min. During both periods of time the resin was filtered periodically, washed with CH_2Cl_2, and resuspended in a fresh batch of reagent. In each case, filtrate and washes were combined, evaporated to dryness, and subjected to quantitative determination of the D-Val-L-Pro diketopiperazine by glc according to Mauger.[14] The analyses were performed on an F & M Model 402 gas chromatograph equipped with a flame detector and a U-shaped column (6 ft × 3.5 mm) containing 3% EGSP-Z on Gas Chrom Q, 100–200 mesh.[36] The flow rates of the gases were kept at 40 (H_2), 100 (He), and 350 cm^3/min (air), the temperature at 210°. The samples were dissolved in CH_2Cl_2 (*c* (2–5) × 10^{-3} *M*) which was 2.0 × 10^{-3} *M* in *p*-phenylphenol as internal standard and injected (without derivatization) in volumes of 2–5 μl. Under these conditions the diketopiperazine had an average retention time of 7.6 min with only slight tailing, allowing determination of the peak area by the height × half-width method. The amino acid content of aliquots of three samples after hydrolysis (6 *N* HCl, 130–140°, 4 hr in a sealed vessel) was determined and was found to correlate satisfactorily with the quantity of diketopiperazine found by glc (Table III).

Table III. Determination of the Concentration of Diketopiperazine by Gas–Liquid Chromatography (glc) and by Amino Acid Analysis after Hydrolysis (Val, Pro) in Three Different Samples (I, II, III)[a]

	I	II	III
Val	582	262	140
Pro	556	270	149
Glc	570	280	141

[a] nmole/ml.

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