Detection and Prevention of Urethane Acylation during Solid-Phase Peptide Synthesis by Anhydride Methods[1,2]

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Solid-phase synthesis of Leu-Ala-Gly-Val furnished Leu-Ala-Gly-Gly-Val as a by-product when urethane-protected amino acids were coupled by the mixed anhydride method. Synthesis of Gly-Val similarly produced Gly-Gly-Val. Activation of N^{α}-2-(4-biphenylyl)-2-propyloxycarbonylglycine (Bpoc-Gly) with ethyl chlorocarbonate and triethylamine in methylene chloride formed an intermediate that reacted with Val-resin to yield *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-Val-resin. Thus, two glycine residues were added during a single solid-phase cycle. Since Bpoc-Gly-Val-resin was not acylated at the urethane nitrogen by symmetrical or mixed anhydrides of Bpoc-Gly, by Bpoc-Gly activated with dicyclohexylcarbodiimide, or by leucine-*N*-carboxyanhydride, urethane acylation occurred before the activated intermediate was coupled to the Val-resin. A mechanism for this side reaction is proposed that involves disproportionation of a mixed anhydride of Bpoc-Gly to the symmetrical anhydride, and intramolecular rearrangement of the latter to form *N*-Bpoc-*N*-(Bpoc-Gly)-Gly, which is subsequently activated by anhydride interchange. Urethane acylation also occurred with Bpoc-alanine as the protected amino acid, with isobutyl chlorocarbonate as the activating agent, or with *N*-methylmorpholine as the base. Although symmetrical anhydrides rearranged slowly, the rate increased markedly on addition of triethylamine hydrochloride. Rearrangement was dependent on temperature and time of mixed anhydride formation and was undetectable after activation at −15° for 10 min and coupling at −15° for 2 hr. No urethane acylation (<0.1 mol %) was observed during coupling of Bpoc-Gly activated with dicyclohexylcarbodiimide under standard solid-phase conditions.

A solid phase synthesis of the model peptide, L-leucyl-L-alanylglycyl-L-valine, by the mixed anhydride method[3-5] produced an undesired by-product. The new substance, representing over 4% of the total product, did not correspond with any of the di- or tripeptides that might have arisen by incomplete coupling or deprotection reactions. Instead, it resulted from urethane acylation. The by-product was isolated and identified as Leu-Ala-Gly-Gly-Val. Some evidence for the mechanism of the acylation reaction has been obtained and conditions by which it can be avoided have been defined.

Mixed anhydride coupling has been applied in a few instances in solid-phase synthesis[6-8] and symmetrical anhydrides are finding increased use.[9-12] Several side reactions, including urethane acylation, are known to occur during conventional syntheses in solution with mixed carboxylic–carbonic anhydride coupling and have been discussed in a review by Albertson.[13] As far as we know, this is the first time urethane acylation has been recognized to have occurred during a solid-phase synthesis with anhydride activation.

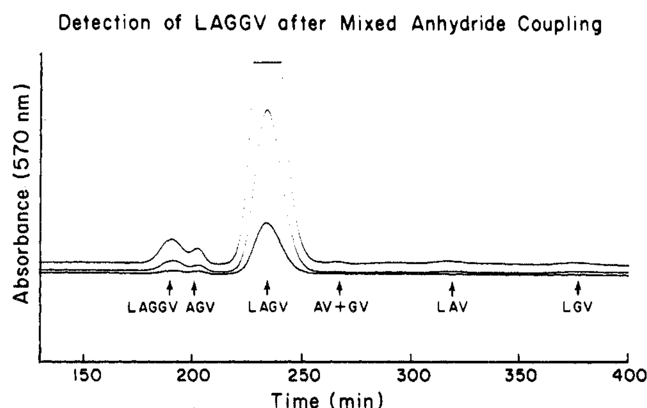


Figure 1. Detection of Leu-Ala-Gly-Gly-Val (LAGGV) after mixed anhydride coupling. Separation was on the long column (0.9 × 60 cm) of a Beckman 120B amino acid analyzer packed with AA-15 sulfonated copoly(styrene–8% divinylbenzene) beads. Elution was at 56°, 66 ml/hr, with pH 3.49 sodium citrate buffer (0.2 *N*). Peptides were detected by the ninhydrin reaction.

Results and Discussion

The initial observation was made following a synthesis of Leu-Ala-Gly-Val by solid-phase methods[14] in which mixed carboxylic–carbonic anhydrides were used in place of dicyclohexylcarbodiimide for the coupling reactions. The completed tetrapeptide resin was cleaved with HF and the crude product mixture was fractionated directly on a cation-exchange column of an amino acid analyzer as described elsewhere.[15] High loading with the peptide products gave a very large peak for the desired tetrapeptide and allowed a good separation and a sensitive measure of the various peptide by-products present. A typical chromatogram is shown in Figure 1. The peaks were identified and quantitated by comparison with synthetic standards.

Attempts were made to eliminate or reduce the amount of the peptide eluted at 189 min by varying the reaction conditions (Table I). Changes in the amino-protecting group, the washing procedure, and the alkyl group of the chlorocarbonate did not markedly change the quantity of this unknown peptide, although replacing the hydroxymethyl resin by a chloromethyl resin (runs 6 and 7), which introduced some quaternary ammonium sites, did cause a moderate increase in the by-product. Examination of the product distribution resulting from these mixed anhydride couplings also shows that, in addition to the unknown peptide at 189 min, appreciably more deletion peptides were formed under these conditions than were formed by DCC couplings. The best conditions for DCC coupling gave less than 0.1% of any of the deletion peptides or of the unknown peptide.

In order to identify the 189-min peptide a preparative run on the same ion-exchange column was made. A portion (80%) of the column effluent was diverted to a fraction collector while the remainder was passed through the ninhydrin analytical system of the analyzer. Hydrolysis and amino acid analysis of the peptide eluted at 189 min gave Gly, 1.97; Ala, 1.00; Val, 1.04; Leu, 0.85. This result suggested that 2 mol of glycine had been added during the first synthetic cycle to subsequently give Leu-Ala-Gly-Gly-Val. This pentapeptide was synthesized by the usual solid-phase method using DCC coupling and shown to elute also at 189 min. Since the isolated by-product from the mixed anhydride synthesis and the pentapeptide stan-

Table I
Peptides Detected after Solid-Phase Synthesis of Leu-Ala-Gly-Val under Various Conditions

Run	Resin derivative[a]	% TFA for deprotections[b]	2-Propanol washes[c]	Activation reagent[d]	Coupling time, min	Peptide products, mol %[e] LAGV (234)	LAV (324)	LGV (385)	AGV (201)	GV + AV (266)	LV (450)	LAGGV (189)
1	$HOCH_2R$	1, 1, 1	−	EtOCOCl	120	89.7	0.0	1.05	3.00	1.87	0.0	4.45
2	$HOCH_2R$	20, 1, 1	−	EtOCOCl	120	91.9	0.37	0.64	1.57	0.27	0.27	4.98
3	$HOCH_2R$	20, 1, 1	+	EtOCOCl	120	91.4	0.43	0.54	2.19	0.43	0.0	4.02
4	$HOCH_2R$	20, 1, 1	−	EtOCOCl	5	89.2	0.0	2.09	2.74	0.23	0.0	5.82
5	$HOCH_2R$	20, 1, 1	−	Bu^{i}OCOCl	120	92.4	0.0	0.48	2.20	0.0	0.0	4.89
6	$ClCH_2R$	20, 1, 1	+	EtOCOCl	120	87.9	0.0	0.22	2.10	1.72	0.0	8.09
7	$ClCH_2R$	20, 1, 1	+	EtOCOCl	120	90.5	0.0	0.50	2.23	0.23	0.0	6.54
8	$ClCH_2R$	20, 1, 1	+	DCC	120	98.7	0.25	0.0	0.82	0.18	0.0	0.0
9	$ClCH_2R$	10, 10, 10	+	DCC	120 × 2	99.8	0.00	0.09	0.08	0.05	0.00	0.00

[a] R represents a copoly(styrene–1% divinylbenzene) bead. The hydroxymethyl resin was esterified by the *N,N'*-carbonyldiimidazole method with Bpoc-Val for run 1 and with Boc-Val for runs 2–5. The chloromethyl resin was esterified by the triethylamine method with Boc-Val for runs 6–8 and with Bpoc-Val for run 9. [b] Percentage of TFA in CH_2Cl_2 used in the three deprotection steps of the syntheses; Boc was removed by 20% TFA and Bpoc by 1% TFA except for run 9, in which 10% TFA was used for removal of the Bpoc group. [c] Three washes with 2-propanol were inserted between the standard CH_2Cl_2 washes following the deprotection, neutralization, and coupling steps. [d] Mixed anhydrides were prepared by treating 1.0 equiv of Bpoc-amino acid with 0.9 equiv of chlorocarbonate and 1.0 equiv of triethylamine in CH_2Cl_2 at 0° for 30 min. [e] Separated on the long column (0.9 × 60 cm) of AA-15 sulfonated polystyrene on a Beckman 120 B amino acid analyzer. A sample containing about 1.4 μmol of total peptides was applied in 1.0 ml of buffer and eluted at 56° with pH 3.49 citrate buffer at 66 ml/hr. The elution time in minutes for each peptide is shown in parentheses, expressed as mole per cent of the ninhydrin-positive free peptides after correction for color constants. Blocked peptides, such as those resulting from wrong-way addition, would not be detected.

Table II
Separation of Peptide Standards on the Amino Acid Analyzer[a]

Compd	Elution time, min
Gly	74
Val	98
Gly-Gly-Gly-Gly-Gly-Gly	128
Gly-Gly-Gly-Gly-Gly	144
Gly-Gly-Gly-Gly	161
Gly-Gly	176
Gly-Gly-Gly	194
Gly-Gly-Val	218
Gly-Val	268

[a] 0.9 × 60 cm Beckman AA-15 column, sodium citrate buffer, pH 3.49, 0.2 *N*, 66 ml/hr, 56°.

dard were found to be indistinguishable when cochromatographed, it was concluded that the by-product of the synthesis was Leu-Ala-Gly-Gly-Val.

For further study of the side reaction leading to the incorporation of two glycine residues during the single glycine coupling step, the system was simplified by examining only the glycine coupling reaction. The appropriate standard peptides, Gly-Val, Gly-Gly-Val, Gly-Gly, Gly-Gly-Gly, and Gly-Gly-Gly-Gly, were shown to be separable from each other and from glycine and valine on the analyzer column (Table II). Synthesis of Gly-Val by the mixed anhydride method with ethyl chlorocarbonate and triethylamine produced appreciable amounts of Gly-Gly-Val.

For convenience and comparison with DCC experiments, the initial couplings were carried out at 25° in the presence of 5 equiv of Val-resin. Under these conditions the total yield of peptides and the proportion of Gly-Gly-Val were functions of the temperature and time of activation before addition of the Val-resin (Table III). Thus, activation at 0° for 10 min gave only 0.26% Gly-Gly-Val, but the by-product increased to 2.5% in 2 hr and to 90% in 24 hr. The overall yield of coupling decreased from 85% after 10 min to 30% after 24 hr at 0°. When the anhydride solution was held for 24 hr at 25° prior to coupling the yield dropped to only 1.5%. Formation of the anhydride at 0° for 2 hr and coupling at 0° instead of 25° decreased the yield of by-product only slightly. When the synthesis was conducted under more nearly standard conditions for the

Table III
Effect of Temperature and Time on the Formation of Gly-Gly-Val from Val-Resin and the Mixed Anhydride of Bpoc-Gly and EtOCOCl

Run	Activation Temp, °C	Activation Time, min	Coupling[a] temp, °C	% of Gly coupled	Mol % of free peptides Gly-Val	Gly-Gly-Val
1	0	10	25	85	99.7	0.26
2	0	120	25	71	97.5	2.5
3[b]	0	120	25	54	98.2	1.8
4	0	120	0	64	98.5	1.5
5	0	1440	25	30	10.0	90.0
6	0	2880	25	26	7.0	88.8[c]
7	25	1440	25	1.5	48	52
8	−15	10	−15	42	100.0	0.0

[a] Coupling time was 2 hr. [b] Et_3N was replaced by *N*-methylmorpholine. [c] In addition to the di- and tripeptides, 2.5% of Gly-Gly-Gly-Val and 1.8% of Gly-Gly-Gly-Gly-Val were found.

mixed anhydride method (activation at −15° for 10 min and coupling at −15° for 2 hr) there was no detectable Gly-Gly-Val (<0.1%). The overall yield, however, was only 42%, suggesting incomplete activation[16] or wrong-way addition.[13] Substitution of *N*-methylmorpholine for triethylamine only reduced the yield of Gly-Gly-Val from 2.5% to 1.8% when the activation was for 2 hr at 0°.

When 1.0 mmol of Bpoc-Gly was activated for 48 hr at 0° and then coupled to Val-resin (run 6, Table III), 0.009 mmol of Gly-Val and 0.116 mmol of Gly-Gly-Val were found. In addition, 0.0033 mmol of Gly-Gly-Gly-Val and 0.0024 mmol of Gly-Gly-Gly-Gly-Val were also observed. These peptides accounted for 26% of the starting Bpoc-Gly. In a separate experiment, the filtrate from the coupling reaction was treated with TFA (to deprotect Bpoc-containing components) and found by ion-exchange chromatography to contain 0.007 mmol of Gly, 0.030 mmol of Gly-Gly, 0.004 mmol of Gly-Gly-Gly, and 0.002 mmol of Gly-Gly-Gly-Gly. Thus, 8.7% of the initial Bpoc-Gly was found as free, uncoupled peptides. In addition, 18% of the original Bpoc-Gly was found in the acid-treated filtrate in the form of a ninhydrin-negative product, which was assumed to have been the diketopiperazine derived from 1,4-di-Bpoc-piperazine-2,5-dione by analogy with the results of Zaoral and Rudinger.[17] When the filtrate was subjected to gas chromatography,[18,19] the presence of a

Table IV
Formation of Gly-Gly-Val during Symmetrical Anhydride or Standard DCC Couplings

Run	Activation Temp, °C	Activation Time, min	$Et_3N \cdot HCl$	Coupling method	Yield, %[a]	Peptide products,[b] mol % Gly-Val	Peptide products,[b] mol % Gly-Gly-Val
1	25	10	−	SA[c]	100	100.0	0.0
2	25	120	−	SA[c]	70	100.0	0.0
3	25	1440	−	SA[c]	24	96.5	3.3
4	25	120	+	SA[c]	65	91.4	8.6
5	25	1440	+	SA[c]	45	84.5	15.2
6	0°	120	+	SA[c]	81	93	7
7	25°	120	−	SA[d]	62	99.7	0.34
8			−	DCC[e]	100	100.0	0.0
9			+	DCC[e]	100	99.8	0.24

[a] The yield of the symmetrical anhydride (SA) runs is based on the theoretical amount of anhydride that could be formed; if based on Bpoc-Gly, it would be $^1/_2$ of the values shown. The yield of the DCC runs is based on the Val-resin, which is limiting; if based on the excess Bpoc-Gly the values would be $^1/_4$ of those shown. [b] After coupling, the peptides were cleaved from the resin with HF containing 10% anisole at 0° for 1 hr. The crude mixture was extracted from the resin and fractionated on an ion-exchange column (0.9 × 60 cm, pH 3.49 citrate). [c] Bpoc-Gly (1 equiv) was activated with 0.5 equiv of DCC in CH_2Cl_2 either without or with 1 equiv of $Et_3N \cdot HCl$. Val-resin (2 equiv) was then added and coupling was continued for 2 hr at 25°. [d] Boc-Gly (1 equiv) was activated with DCC (1 equiv) in CH_2Cl_2 for 2 hr and then coupled with Val-resin (3 equiv) for 2 hr at 25°. [e] Bpoc-Gly (4 equiv) was added to Val-Resin (1 equiv) in CH_2Cl_2 and stirred for 10 min either without or with 4 equiv of $Et_3N \cdot HCl$. DCC (4 equiv) was then added and coupling was continued for 2 hr at 25°.

component which cochromatographed with an authentic sample of glycine diketopiperazine was demonstrated.

Urethane acylation is not limited to glycine, although it probably proceeds faster with the unhindered amino acid. For example, treatment of Bpoc-Ala with EtOCOCl and Et_3N for 25 hr at 0°, followed by coupling with Val-resin, gave a low yield of a peptide mixture composed of 14% Ala-Ala-Val and 86% Ala-Val.

Several other coupling methods were examined to determine whether or not the side reaction is limited to the mixed anhydride procedure. The symmetrical anhydride, $(Bpoc\text{-}Gly)_2O$, was prepared by treating Bpoc-Gly with 0.5 equiv of DCC in CH_2Cl_2. After various times at 0° or 25° the reagent was mixed with excess Val-resin and allowed to couple for 2 hr at 25°. In separate runs, 1 equiv of $Et_3N \cdot HCl$ was added to simulate the amount of this salt formed during a mixed anhydride coupling. Finally, a conventional DCC coupling was examined for comparison. In this experiment 4 equiv of Bpoc-Gly and 1 equiv of Val-resin were mixed and stirred for 10 min at 25° either with or without 4 equiv of $Et_3N \cdot HCl$. Then 4 equiv of DCC was added and coupling was continued for 2 hr at 25°. The results of these experiments are summarized in Table IV. In the absence of $Et_3N \cdot HCl$ the symmetrical anhydride produced no Gly-Gly-Val in 10 min or 2 hr and only 3.3% after 24 hr at 25°. The presence of $Et_3N \cdot HCl$ caused a marked increase, however, and even at 0° for 2 hr 7% Gly-Gly-Val was formed (compare run 6, Table IV with run 2, Table III). The standard DCC coupling gave no detectable Gly-Gly-Val (0.1%), but 0.24% was found when 4 equiv of $Et_3N \cdot HCl$ was present.

When 0.53 mmol of Boc-Gly was treated with 0.53 mmol of DCC for 2 hr at 25°, and the CH_2Cl_2-soluble fraction was deprotected with TFA and dissolved in water, the product consisted of a mixture of 0.24 mmol of Gly and 0.12 mmol of Gly-Gly. Since the starting material was free of Gly-Gly derivatives, it was concluded that the intermediate isourea, or symmetrical anhydride–DCC complex, underwent an acylation reaction at the urethane nitrogen in a manner analogous to that reported by De Tar, *et al.*,[20] for the benzyloxycarbonyl derivative. When 0.53 mmol of Boc-Gly was treated with 0.53 mmol of DCC for 2 hr at 25° as before, and then was treated with excess Val-resin, the resin-derived product consisted of a mixture of 0.16 mmol of Gly-Val and 0.0005 mmol of Gly-Gly-Val. These data are consistent with the view that essentially all of the Gly was still present as the reactive symmetrical anhydride after 2 hr. On the other hand, only a trace (0.4%) of the rearranged Gly-Gly product produced during the activation period was in activated form that could couple with the added Val-resin. This can be rationalized best in terms of the intramolecular mechanism to be discussed later and argues against a mechanism in which the Boc-glycyl isourea would acylate itself by an intermolecular pathway. When DCC was added to Boc-Gly that was already in the presence of Val-resin and the mixture was allowed to couple for 2 hr at 25°, no Gly-Gly-Val was produced.

Evidence for the structure of the resin-bound product leading to Gly-Gly-Val was obtained by comparing the number of Bpoc groups with the amino acid composition. The mixed anhydride was prepared by incubating 19.3 μmol of Bpoc-Gly with equivalent amounts of EtOCOCl and Et_3N in CH_2Cl_2 at 0° for 24 hr. An excess (112 μmol) of Val-resin was added and coupling was continued for 2 hr at 25°. The thoroughly washed resin was deprotected by treatment with 1% TFA in CH_2Cl_2. The increased absorbance of the TFA solution at 261 nm due to 2-(4-biphenylyl)propene (ϵ 1.74 × 10^4) corresponded to 7.60 μmol of Bpoc groups. Subsequent HF cleavage of the resin and chromatographic analysis of the resulting peptides revealed the presence of 0.34 μmol of Gly-Val, 3.39 μmol of Gly-Gly-Val, and 87.3 μmol of free Val. Assuming that 0.34 μmol of Bpoc was bound to the Gly-Val, then 7.26 μmol of Bpoc was bound to 3.39 μmol of Gly-Gly-Val (ratio 2.11:1). This indicates clearly that the protected resin-bound tripeptide contained two Bpoc groups for every Val residue, or an average of one Bpoc per Gly residue. The data are consistent with structure I. *N*-biphenylisopropyloxycarbonyl-*N*-(biphenylisopropyloxycarbonylglycyl)glycylvalyloxymethyl resin.

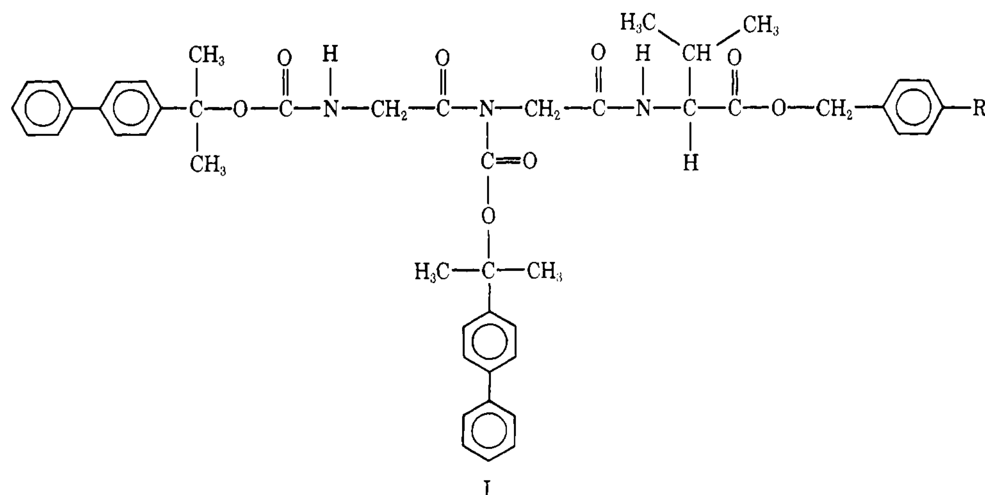
On the Mechanism of the Reaction. Four mechanisms have been considered to explain the appearance of additional amino acid residues in peptides from solid-phase syntheses in which anhydride methods were used.

A. Acylation of the Amide Nitrogen of Bpoc-Gly-Val-Resin and Insertion. Brenner[21] has proposed that net insertion of an amino acid can be effected through N-acylation of an amide bond of a peptide backbone by an activated N-protected amino acid followed by intramolecular cyclization and ring opening. Mitchell and Roeske[22] detected no triglycine, however, on treating Boc-Gly-Gly-resin with 10 equiv each of Boc-Gly and DCC followed by deprotection in acid and treatment with triethylamine. The same experiment was repeated in this study, using mixed anhydride activation of Boc-Gly, and no formation

Table V
Stability of Amide and Urethane Nitrogen Atoms toward Several Acylating Agents

No.	Amide or urethane component	Acylating agents (activating conditions)	Reaction time, hr	Products, mol %[a] Starting material	Products, mol %[a] Acylated product	Ref
1	Boc-Gly-Val—R[b] 1 equiv	Boc-Gly + EtOCOCl + Et_3N (30 min, 0°) 4 equiv 4 equiv 4 equiv	2	100	0	*c*
2	Boc-Gly-Val—R 1 equiv	Boc-Gly + DCC (10 min, 25°) 4 equiv 2 equiv	2	100	0	*c*
3	Boc-Gly-Val—R 1 equiv	Boc-Gly + DCC 4 equiv 4 equiv	2	100	0	*c*
4	Boc-Gly-Val—R 1 equiv	Leucine *N*-carboxyanhydride 10 equiv	24	100	0	*c*
5	Z-NH$(CH_2)_5$C(=O)—R 1 equiv	Boc-Gly + Bu^{i}OCOCl + Et_3N (15 min, −10°) 86 equiv 81 equiv 86 equiv	12	100	0	*c*
6	Z-NH$(CH_2)_5$C(=O)—R 1 equiv	Boc-Gly + DCC (10 min, 25°) 81 equiv 81 equiv	12	100	0	*c*
7	Boc-Gly-Gly—R 1 equiv	Boc-Gly + Bu^{i}OCOCl + Et_3N (15 min, −10°) 68 equiv 64 equiv 68 equiv	2	100	0	*c*
8	Boc-Gly-Gly—R 1 equiv	Boc-Gly + DCC 10 equiv 10 equiv	24	100	0	14
9	Z-NHCHRC(=O)-NH_2	Z-NHCHRCOOH + EtOCOCl + Et_3N			0	23
10	Z-Ala-NH_2	Z-Gly-Cl			0	23

[a] Products from runs 1–6 were separated and quantitatively measured on the amino acid analyzer following deprotection and cleavage from the resin by HF or HBr. After the attempted acylation reaction in runs 7 and 8, the peptide resin was deprotected with TFA, treated with 10% Et_3N-CH_2Cl_2, and then cleaved with HBr. A portion of the peptide resin from run 1 was similarly treated and no acylated product was detected. Limit of detection was 0.1 mol %. [b] R represents oxymethylcopoly(styrene–1% divinylbenzene). [c] This study.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of triglycine was observed. Similarly, treatment of Bpoc-Gly-Val-resin with Bpoc-Gly-OCO_2Et did not lead to the formation of Gly-Gly-Val (Table V). These results support the original contention[22] that the Brenner mechanism is not a significant side reaction during solid-phase peptide synthesis.

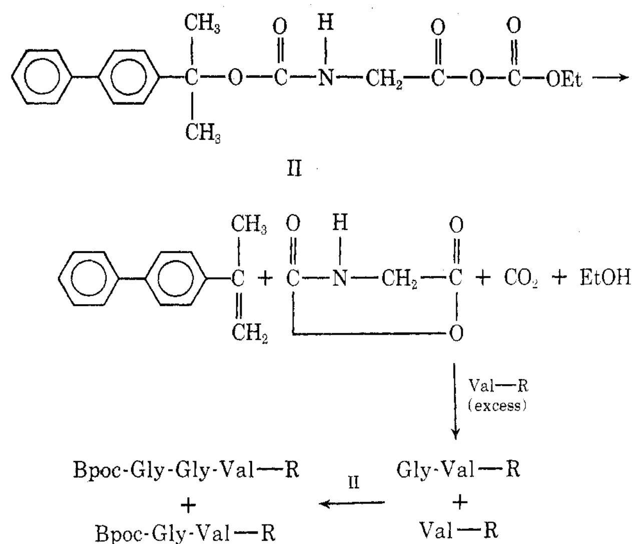
In the context of amide reactivity, Antonov and Shemyakin[24] studied the reaction of protected peptide esters with acylating agents of higher reactivity than those used in the present study. Phthalylglycyl chloride was found to acylate not the amide nitrogen, but the urethane nitrogen of *N*-benzyloxycarbonyl dipeptide esters. Acylation of the peptide bond in a phthalyl dipeptide ester was effected through the use of a more potent acylating agent, azidoacetyl chloride, at reflux in toluene for several hours. In light of the nonreactivity of amide nitrogens toward the less active acylating agents (Table V) it is interesting that Fankhauser, *et al.*,[25] have recently described a model system in which amide acylation (and subsequent insertion) was thought to have occurred during solid-phase peptide synthesis. It appears, however, that other interpretations of their data are possible.

B. Acylation of the Urethane Nitrogen of Bpoc-Gly-Val-Resin. This mechanism involves coupling of activated Bpoc-Gly to Val-resin and N-acylation of the urethane bond of the resulting Bpoc-Gly-Val-resin by a second equivalent of activated Bpoc-Gly. However, the effects of temperature and activation time of the mixed anhydride indicated that the side reaction was occurring in solution during the activation step rather than after the coupling reaction between Boc-Gly-OCO_2Et and Val-resin. This conclusion was confirmed by treatment of Boc-Gly-Val-resin with excesses of Boc-Gly-OCO_2Et, $(Boc\text{-}Gly)_2O$, Boc-Gly + DCC, or leucine *N*-carboxyanhydride. No Gly-Gly-Val or Leu-Gly-Val was obtained (Table V), indicating that the urethane nitrogen was not acylated under these conditions. Attempts to acylate the urethane bond of 6-(benzyloxycarbonylamino)hexanoyl resin with large ex-

cesses of Boc-Gly-OCO_2Bu^i or Boc-Gly + DCC were also unsuccessful.

C. Acylation of the Amino Nitrogen *via* an *N*-Carboxyanhydride. Bodanszky, *et al.*,[26] recently reported that activation of Boc-amino acids with DCC in CH_2Cl_2 gave rise to a precipitate that contained ninhydrin-positive material. They pointed out that intermediate Boc-amino acyl isourea contains a good anionic leaving group and a potential cation of high stability and that the presence of two oppositely polarized centers in such a molecule favors the formation of an *N*-carboxyanhydride (NCA) which, following hydrolysis, could account for the ninhydrin-positive product. By this reasoning Bpoc-Gly anhydride (or Boc-Gly anhydride) should also form the NCA and subsequently give rise to Gly-Gly-Val as shown in Scheme I. Thus, if a small amount of NCA were to form it would react rapidly with the large excess of Val-resin to produce a mixture of Gly-Val-resin and Val-resin (very little polyglycyl-valine-resin would be expected from an NCA polymerization process because of the excess amine initiator). The remaining Bpoc-Gly mixed anhydride would then react to give Bpoc-Gly-Val-resin and Bpoc-Gly-Gly-Val-resin. By this mechanism the resin-bound tripeptide would contain Bpoc and Val in a ratio of 1:1. However, it was found (run 6, Table III) that the ratio was 2.11:1.

Scheme I
The *N*-Carboxyanhydride as a Possible Intermediate Leading to Gly-Gly-Val



During the activation and incubation steps, before addition of the Val-resin, an appreciable amount of NCA should have accumulated if this were the mechanism of the side reaction. In an experiment (run 5, Table III) where 27% of the Boc-Gly eventually appeared as Gly-Gly-Val, no carboxyanhydride could be found by infrared spectroscopy. As little as 5% of Gly NCA would have been detected by its characteristic absorption at 1857 cm^{-1}. Thus Gly NCA did not accumulate during the activation step and does not appear to be on an important route to the observed Gly-Gly-Val.

D. Acylation of the Urethane Nitrogen of Bpoc-Gly Anhydride. The probable mechanism for the formation of *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-Val-resin involves the acylation of the urethane nitrogen of Bpoc-Gly anhydride in solution, followed by coupling of the resulting glycylglycine derivative with Val-resin. The acylation reaction might occur intermolecularly or intramolecularly.

As far as we are aware, this is the first time the urethane acylation reaction has been recognized to have occurred during a solid-phase synthesis, although it is well known to occur during conventional syntheses in solution with mixed carboxylic–carbonic anhydrides. Kopple and Renick[27] found that activation of Z-Gly with ethyl chlorocarbonate and triethylamine for 10 min at −5°, followed by coupling with glycine ethyl ester, gave rise to a 30% yield of a by-product containing two Z-Gly residues per glycine ester. Treatment with HBr–HOAc yielded the linear tripeptide, HBr·Gly-Gly-Gly-OEt. The initial product was deduced to be *N*-Z-*N*-(Z-Gly)-Gly-Gly-OEt. It was assumed that this diacylamide was formed by reaction of the mixed anhydride with glycine ethyl ester and acylation of the resulting Z-Gly-Gly-OEt by another equivalent of the mixed anhydride. Similarly, Schellenberg and Ullrich[28] found 17% of crystalline *N*-Z-*N*-(*Z*-Gly)-Gly-Glu-$(OEt)_2$ following the coupling of diethyl glutamate with the mixed anhydride of Z-Gly and isobutyl chlorocarbonate. The expected Z-Gly-Glu-$(OEt)_2$ was obtained in 66–71% yield from the filtrates. No mechanism was proposed. Determann[29] treated the reaction product of Z-Phe and ethyl chlorocarbonate with NaOH and isolated 1-benzyloxycarbonyl-3-(2-benzylacetic acid)-5-benzylhydantoin. He concluded that one molecule of the mixed anhydride had undergone an attack by the nucleophilic nitrogen of another molecule in an *inter*molecular acylation to give *N*-Z-*N*-(Z-Phe)-Phe-OCOOEt, which then underwent ring closure in alkali, with loss of benzyl alcohol, to give the hydantoin. Kotake and Saito[30] obtained an 85% yield of *N*-Z-*N*-(Z-Gly)-Gly after treatment of $(Z\text{-Gly})_2O$ with Et_3N (0.1–1 equiv). The high yield suggests, but does not prove, that the acylation reaction was *intra*molecular.

Urethane acylation is not limited to symmetrical carboxylic anhydrides and mixed carboxylic–carbonic anhydrides. This side reaction was first observed by Wieland and Heinke[31] when Z-Gly was activated by the phosphorous oxychloride method. The product, *N*-Z-*N*-(Z-Gly)-Gly, was obtained in 30% yield.

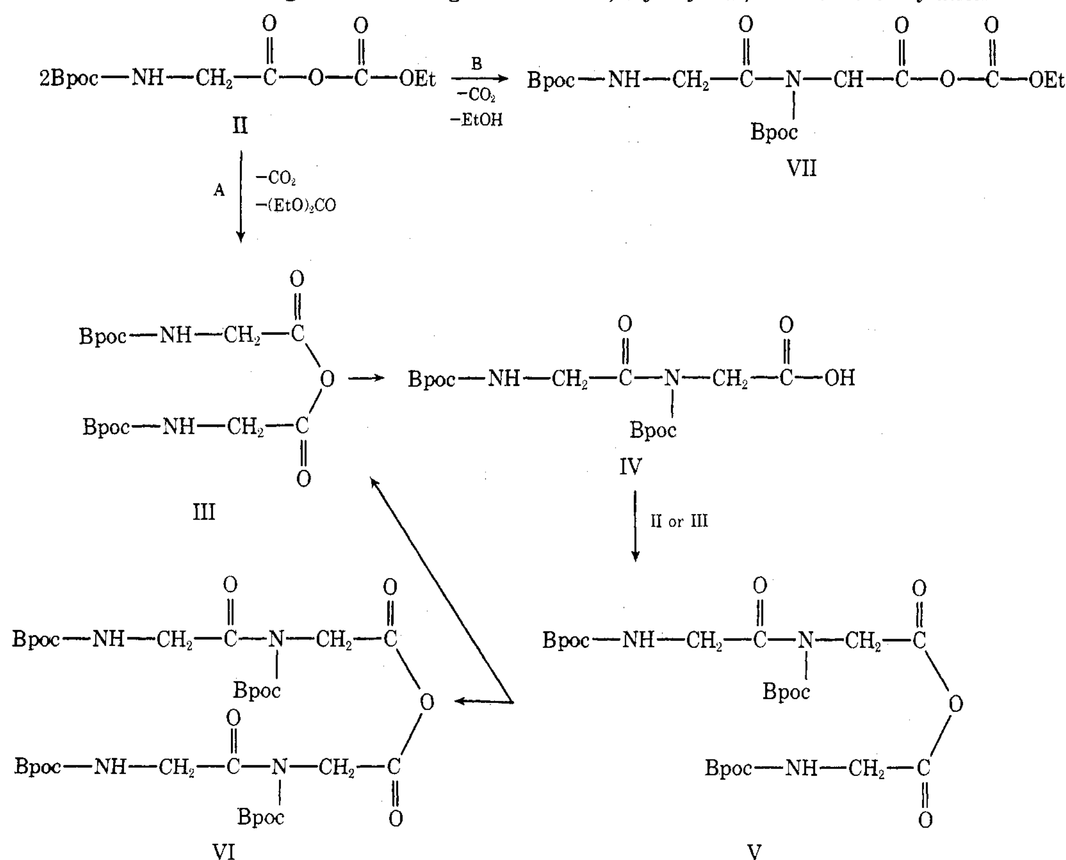
Neither is the acylation reaction limited to N^α-urethane-protected amino acids; Zaoral and Rudinger[17] observed it when the mixed anhydride formed from tosylglycine and *sec*-butyl chlorocarbonate in pyridine was treated with aniline. They actually isolated ten crystalline products from the single reaction mixture, including 15% of *N*-Tos-*N*-(Tos-Gly)-Gly anilide. Several mechanisms for the origin of the observed products were discussed in some detail. One way in which imides such as Tos-$NHCH_2C(=O)$-*N*-Tos-CH_2COOH could arise under the conditions of a mixed anhydride synthesis was *via* an O to N shift that would allow for a mobile equilibrium between Tos-$NHCH_2C(=O)OC(=O)CH_2NH$-Tos and Tos-$NHCH_2C(=O)$-*N*-Tos-CH_2COOH. This process requires first that the symmetrical anhydride be formed by disproportionation of the mixed carboxylic–carbonic anhydride. Indeed, several reactions, all of which were expected to give the symmetrical anhydride $(Tos\text{-Gly})_2O$, were found to lead invariably to *N*-Tos-*N*-(Tos-Gly)-Gly. Evidence for the existence of both species was obtained when the reaction between Tos-Gly-Cl and Tos-Gly was followed by infrared spectroscopy. They concluded that it was unlikely that disproportionation under the conditions used for the mixed anhydride would be sufficiently extensive to account for all of the imide actually isolated (~56%). In addition, disproportionation requires CO_2 evolution, but they observed none. For these reasons it appeared likely that direct acylation of tosyl-glycine, or more probably of the mixed anhydride, by a second molecule of the mixed anhydride also occurred.

Thus, the widely observed side reaction leading to the incorporation of two residues of glycine instead of only one has been attributed to acylation of the urethane nitrogen

of a protected dipeptide ester,[27] of an N-protected amino acid,[17] or of an N-protected amino acid mixed anhydride[29,31] by intermolecular reactions, or, in part, to acylation of an N-protected amino acid symmetrical anhydride by an intramolecular reaction.[17,30]

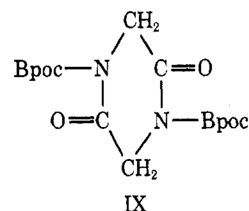
Evidence gathered in this study of the side reaction during solid-phase synthesis favors the view that it occurs by an intramolecular rearrangement as outlined in pathway A, Scheme II. Thus, Bpoc-Gly-OCO_2Et (II), first formed in solution from Bpoc-Gly and EtOCOCl, disproportionates to the symmetrical anhydride $(Bpoc\text{-}Gly)_2O$ (III), which undergoes an intramolecular acylation to give *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-OH (IV). This diacyl dipeptide, which is not activated for coupling, would then become activated by an anhydride interchange with other anhydride species present in solution, such as mixed anhydride (II) or symmetrical anhydride (III), to give *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-*O*-Gly-Bpoc (V) or $[N\text{-Bpoc-}N\text{-(Bpoc-Gly)-Gly}]_2O$ (VI), or possibly *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-OCO_2Et (VII). After the activation period, during which these reactions could occur, each of the anhydride species would then couple with the excess Val-resin to give a mixture of Bpoc-Gly-Val-resin (VIII) and *N*-Bpoc-*N*-(Bpoc-Gly)-Gly-Val-resin (I) in proportion to their concentrations. Deprotection and cleavage from the resin by HF would then give the di- and tripeptides finally observed. During the anhydride interchange reactions (*e.g.*, V → VI) additional symmetrical anhydride would be expected to form and would recycle to lead to more of the diacyl dipeptide anhydrides (V, VI, VII).

Scheme II
Mechanisms Leading to the Rearrangement Product, Gly-Gly-Val, *via* Urethane Acylation

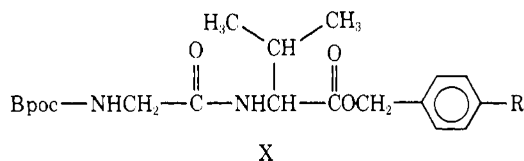


By the symmetrical anhydride route the maximum incorporation of glycine would be 50% if the product were all Gly-Val and, depending on the relative rates of the individual reactions, the maximum yields would be between 33 and 67% if the product were all Gly-Gly-Val. The alternative route (pathway B, Scheme II) would involve the intermolecular generation of VII directly from II. By this route up to 100% of glycine could be incorporated, either as Gly-Val or Gly-Gly-Val. When Bpoc-Gly, EtOCOCl, and Et_3N were incubated at 0° for 24 hr, and then treated with Val-resin, Gly-Val and Gly-Gly-Val were found in a molar ratio of 1:10. The coupling yield based on Bpoc-Gly was 37%. However, the pathway cannot be deduced from yield alone because a complete accounting of materials was not made and side reactions could markedly reduce the overall yield. It is important to note that the amino acid and peptide products observed in this study were detected by the ninhydrin reaction after chromatography. The presence of blocked products resulting from wrong way addition would not be measured by this analytical system.

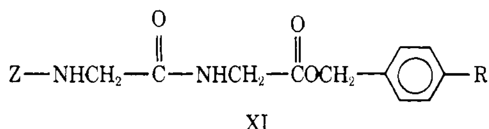
The diketopiperazine IX is thought to be another inactivation product.[17] It was observed that prolonged standing of an activation solution at 25° led to a very low coupling yield. The filtrate contained a ninhydrin-negative component which gave rise on hydrolysis to a quantity of glycine approximately equal to that which had coupled to Val-resin. The presence of the diketopiperazine was established by gas chromatography.[18,19]



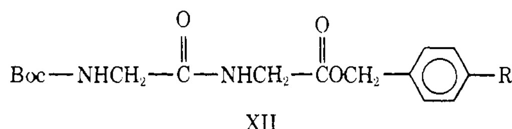
The main reason for rejecting the intermolecular acylation (pathway B) as the major route is the complete lack of reactivity of the urethane nitrogen of resin esters X, XI, and XII with various amino acid anhydrides, including



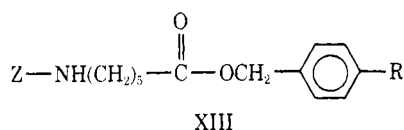
X



XI



XII



XIII

Bpoc-Gly-OCO_2Et, Boc-Gly-OCO_2Bu^i, $(Bpoc\text{-}Gly)_2O$, Bpoc-Gly-*O*-C(=NC_6H_{11})NHC_6H_{11}, and Val-NCA. In addition, large excesses of Boc-Gly-OCO_2-Bu^i or Boc-Gly + DCC were unable to effect acylation of XIII. Moreover, the reaction did not occur in solution between Z-amino acid amides or anilides and mixed anhydrides.[23] It seems unlikely that the urethane nitrogen of Bpoc-Gly-OCO_2Et would be appreciably more reactive toward acylation under these conditons. A greatly enhanced rate, however, could be expected from the *intra*molecular acylation of II to give III (Scheme II).

If pathway A is the actual route for the rearrangement of the mixed anhydride, the symmetrical anhydride should undergo a similar rearrangement at a comparable rate under the same conditions. Since the usual conditions for formation and coupling of symmetrical anhydrides are not the same as for mixed anhydrides, the results cannot be compared directly. Thus, activation of Bpoc-Gly by 0.5 equiv of DCC in CH_2Cl_2 for 10 min or 2 hr at 25°, followed by coupling with Val-resin, gave no Gly-Gly-Val, and activation for 24 hr gave only 3.3% (Table IV), whereas the mixed anhydride produced 2.5% in 2 hr at 0° and 90% after 24 hr (Table III). When 1 equiv of triethylamine hydrochloride was added to simulate the amount of this salt formed during the mixed anhydride reaction,[32] the results were quite different. Then $(Bpoc\text{-}Gly)_2O$ gave rise to 7% Gly-Gly-Val after standing for 2 hr at 0°. Therefore, the data up to 2 hr are compatible with pathway A and support the view that it is the major route to the rearrangement products. The data at longer times are complicated by low yields and undetermined by-products and are more difficult to rationalize.

The normal DCC coupling of Bpoc-Gly with Val-resin gave no detectable tripeptide, and the presence of an additional mole of DCC did not catalyze the side reaction. When $Et_3N\cdot HCl$ was present, however, even the DCC method produced 0.26% of Gly-Gly-Val.

Rearrangement of II should produce CO_2, and it was observed. The activation solution containing Bpoc-Gly + EtOCOCl + Et_3N in CH_2Cl_2 was placed at 25° in a closed cell, then underwent infrared spectroscopy. A strong, sharp peak at 2300 cm^{-1} for CO_2 was observed after 25 min and the peak was approximately twice as large after 4 hr. When the activation was carried out in a flask connected to the air by a drying tube, no CO_2 peak could be found. The infrared data also showed a steady drop in the mixed anhydride carbonyl peak at 1840 cm^{-1} with a half-time of about 45 min. The second anhydride peak at 1760 cm^{-1} decreased but was replaced by an overlapping peak at 1740 cm^{-1} which is attributed to the imide carbonyl resulting from the acylation. The urethane carbonyl peak at 1725 cm^{-1} remained unchanged.

Conclusions

The present experiments show that substantial quantities of by-product can be formed during solid-phase syntheses with the mixed anhydride method at 0°. The undesired product arises by an intramolecular acylation of the urethane nitrogen of Bpoc-glycine during the activation step in solution, rather than by acylation of a resin-bound peptide. The reaction can also occur with other amino acids, although at a slower rate. When the mixed anhydride was formed and coupled at lower temperature (−15°) the urethane acylation could not be detected. Therefore, with respect to *this side reaction,* the mixed anhydride method appears to be satisfactory, even with glycine, for solid-phase peptide synthesis. However, because the conditions are critical, the possibility of adding two residues during a single coupling step must be kept in mind.

The symmetrical anhydride of Bpoc-Gly, generated by DCC, showed far less tendency to undergo the rearrangement than the mixed anhydride. The difference is correlated with the presence of $Et_3N\cdot HCl$ during the formation of the mixed anhydride. In addition, the standard DCC coupling procedure that has been used extensively for solid-phase synthesis produced none of the rearrangement product in these model systems. Thus, urethane acylation represents a side reaction in solid-phase peptide synthesis that can be avoided under appropriate conditions.

Experimental Section

Infrared spectra were taken with a Perkin-Elmer Model 237B grating infrared spectrophotometer. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Amino acid and peptide analyses were carried out on Beckman amino acid analyzers (Model 120B and 121). Elemental analyses were performed by Mr. S. T. Bella. An F & M Model 402 gas chromatograph was used to determine glycine diketopiperazine as described elsewhere.[18,19] The solvents used for thin layer chromatography (precoated 0.25-mm silica gel G plates, Analtech) were 1-butanol-acetic acid-water (BAW) (4:1:1) and chloroform-methanol-acetic acid (CMA) (17:2:1). Boc-amino acids were obtained from Beckman Instruments, Inc. They were checked for homogeneity by thin layer chromatography and for optical purity by the Manning and Moore method.[33] Bpoc-amino acids were synthesized in this laboratory as cyclohexylamine or dicyclohexylamine salts and characterized as described recently.[34] Gly-Val, Gly-Gly, Gly-Gly-Gly, and Gly-Gly-Gly-Gly were obtained from Schwarz Bioresearch. Gly-Gly-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly-Gly, and Gly-Gly-Val were purchased from Fox Chemical Co. Chloromethyl methyl ether (Aldrich Chemical Co.) was distilled in an efficient hood before use. *N,N'*-Dicyclohexylcarbodiimide (Schwarz), *N,N'*-carbonyldiimidazole (Aldrich), and ethyl and isobutyl chlorocarbonates (Eastman) were used without further purification. The resin support was a copolymer of styrene and 1% divinylbenzene (Bio-Rad), 200–400 mesh. It was chloromethylated to the extent of 1.2 mmol/g and esterified with Boc-Val by a procedure using triethylamine[35] or by the new cesium salt method.[36] No quaternary ammonium sites were formed with the latter procedure. Hydroxymethyl resin was prepared from chloromethyl resin *via* the acetoxymethyl derivative and was esterified by the carbonyldiimidazole procedure.[37] The general procedures for solid-phase synthesis were similar to those described earlier[14,37-39] but modified as indicated.

Synthesis of Leucyl-Alanyl-Glycyl-Valine by the Mixed Anhydride Method. Boc-valine-resin (1.2 g, 0.378 mmol Val) was placed in a water-jacketed reaction vessel and deprotected with 40 ml of 20% trifluoroacetic acid in methylene chloride for 30 min

at 25°. The resin was filtered, washed with CH_2Cl_2, neutralized with 10% Et_3N in CH_2Cl_2, and washed with CH_2Cl_2. Bpoc-glycine (313 mg) was dissolved in 10 ml of CH_2Cl_2 at 0° and mixed with 10 ml of 0.1 *M* Et_3N and 9 ml of 0.1 *M* EtOCOCl. The activation mixture was stirred for 30 min at 0°, filtered, diluted to 50 ml with CH_2Cl_2, and added to the valine-resin. The coupling mixture was shaken for 2 hr at 25°, filtered, and washed with CH_2Cl_2. The Bpoc group was removed by treatment with 1% TFA in CH_2Cl_2 for 20 min and the trifluoroacetate was neutralized with 10% Et_3N in CH_2Cl_2. Bpoc-alanine (327 mg) and Bpoc-leucine (468 mg) were coupled in a similar way to give the protected tetrapeptide Bpoc-Leu-Ala-Gly-Val-resin. In this synthesis the only solvent was CH_2Cl_2, but in other syntheses (see Table I) washes with 2-propanol were inserted between the CH_2Cl_2 washes following deprotection, neutralization, and coupling.

Part of the peptide resin (500 mg) was cleaved in 5 ml of redistilled HF and 0.5 ml of anisole at 0° for 1 hr.[40] After evaporation of the HF, the peptide was extracted from the resin with 10% aqueous acetic acid and then with glacial acetic acid. The combined filtrates were lyophilized; yield, 21 mg (40% from Boc-Val-resin). Amino acid analysis of the crude peptide resin showed Gly, 1.00; Ala, 0.95; Val, 1.05; Leu, 0.95. A second synthesis, in which ethyl chlorocarbonate was replaced by isobutyl chlorocarbonate, gave 39 mg (74%) of crude product.

Isolation of Leu-Ala-Gly-Val and Leu-Ala-Gly-Gly-Val. Part (5 mg) of the crude lyophilized product from the mixed anhydride synthesis of Leu-Ala-Gly-Val was dissolved in 1 ml of water and applied to the 0.9 × 60 cm column of the Beckman 120B amino acid analyzer which had been modified to enable the simultaneous analysis and isolation of peptide.[41] The column was packed with Beckman AA-15 cation exchange resin and was equilibrated with pH 3.49 citrate buffer (0.2 *N*) at 56°. The flow rate was 66 ml/hr. A divider pump was adjusted to remove 12 ml/hr and to allow the remaining 54 ml/hr to be collected in a fraction collector (1.5 min/tube). The diverted stream was mixed with buffer pumped at the rate of 54 ml/hr and then with ninhydrin at 33 ml/hr. The solution was passed through the reaction coil and colorimeter of the analyzer as usual and the results were plotted on the recorder (Figure 1). The delay between collection of a peak in the fraction collector and its detection on the recorder was 15 min. The main peak of Leu-Ala-Gly-Val (2.9 μmol) was in tubes 139–159 (peak at 234 min). A small amount of Ala-Gly-Val (0.076 μmol) was found in tubes 127–129 (peak at 201 min) and Leu-Ala-Gly-Gly-Val (0.084 μmol) was found in tubes 118–121 (peak at 189 min). Aliquots of the three fractions were hydrolyzed and the amino acid ratios were determined.

Fraction	Amino acid ratio			
	Gly	Ala	Val	Leu
118–121	1.97	1.00	1.04	0.85
127–129	0.90	1.00	1.01	0.05
139–159	1.03	1.00	1.02	0.99

Synthesis of Leucyl-Alanyl-Glycyl-Glycyl-Valine. Boc-Val-resin (2.35 g, 0.67 mmol) was deprotected with 20% TFA in CH_2Cl_2, neutralized with 10% Et_3N in CH_2Cl_2, and coupled for 2 hr with 4 equiv of Boc-Gly and 4 equiv of DCC in 50 ml of CH_2Cl_2. A sample of the Boc-Gly-Val-resin was removed for analysis and the remainder was extended by coupling in a similar way with Boc-Gly, Boc-Ala, and Boc-Leu. The resulting Boc-Leu-Ala-Gly-Gly-Val-resin was deprotected in TFA, hydrolyzed,[38] and analyzed: Gly, 1.98; Ala, 1.00; Val, 1.06; Leu, 0.92. A 500-mg sample of the peptide resin was cleaved in 5 ml of HF at 0° for 1 hr to yield 52 mg of peptide (94% from Boc-Val-resin). The pentapeptide (0.5 mg) was chromatographed on the 0.9 × 60 cm column of the amino acid analyzer in pH 3.49 buffer. The main peak emerged at 189 min and accounted for 96% of the product. This standard preparation of Leu-Ala-Gly-Gly-Val was cochromatographed on the same column with the corresponding pentapeptide isolated from the mixed anhydride synthesis. A single component emerged as a peak at 189 min.

To follow the progress of the pentapeptide synthesis and establish its structure, samples of peptide resin were removed at each step. Amino acid ratios were determined on acid hydrolysates, and the peptides were cleaved from the resin with HF and fractionated by ion exchange chromatography on the 0.9 × 60 cm column with pH 3.49 buffer.

Peptide	Elution time, min	Amino acid ratios		
		Val	Gly	Ala
Gly-Val	268	1.00	1.00	
Gly-Gly-Val	218	1.10	2.00	
Ala-Gly-Gly-Val	167	1.02	2.00	0.97

Detection of Glycine Diketopiperazine after Mixed Anhydride Coupling with Val-Resin. Bpoc-Gly (0.045 mmol) was activated with triethylamine (0.045 mmol) and ethyl chlorocarbonate (0.040 mmol) for 24 hr at 0° in 1.3 ml of CH_2Cl_2 and shaken with 0.69 g (0.225 mmol) of valine resin in methylene chloride for 2 hr at room temperature. The filtrate was made 10% in trifluoroacetic acid and allowed to stand for 30 min, after which time the solvents were removed *in vacuo.* The resulting residue was kept overnight *in vacuo* in a desiccator containing calcium sulfate and phosphorous pentoxide. The residue was dissolved in hot DMF (0.5 ml) and allowed to cool to room temperature, whereupon some insoluble material was removed by centrifugation and samples from the supernatant were subjected to gas–liquid chromatography[18,19] in the absence and presence of glycine diketopiperazine. The column (6 ft × 3.5 mm of 3% EGSP-Z on Gas Chrom Q, 100–200 mesh) was maintained at 201° and the flow rates of the gases were kept at 44 (H_2), 60 (He), and 360 cm^3/min (air). A component was observed at 20 min, which cochromatographed with the authentic sample of glycine diketopiperazine dissolved in dimethylformamide. The filtrate was calculated to contain 0.0036 mmol of glycine diketopiperazine, which accounted for 18% of the activated Bpoc-glycine.

***N-tert*-Butyloxycarbonylglycyl-6-aminohexanoic Acid.** A modification of the Rothe and Kunitz[42] method for synthesis of 6-aminohexanoyl peptides was used in this preparation. A solution of Boc-Gly (1.40 g, 8.00 mmol) and triethylamine (1.16 ml, 8.40 mmol) in 20 ml of tetrahydrofuran was cooled to −15° in an ice–salt bath. Isobutyl chlorocarbonate (1.11 ml, 8.40 mmol) was added and the mixture was stirred for 8 min. A solution of 6-aminohexanoic acid, prepared by refluxing ε-caprolactam (0.900 g, 8.00 mmol) in 10 ml of 0.93 *N* aqueous sodium hydroxide, was added. The reaction mixture was stirred in the ice bath for 1 hr and at room temperature overnight. The clear, light brown solution was evaporated *in vacuo* to a residue, which was dissolved in water (20 ml), chilled in an ice bath, and acidified to pH 3.0 by addition of 3 *N* hydrochloric acid. The solution was saturated with sodium chloride and extracted with three 50-ml portions of ethyl acetate. The combined extracts were washed with four 50-ml portions of saturated aqueous sodium chloride, dried over magnesium sulfate, and freed of solvent to yield an oil (2.43 g). The oil contained the title compound, R_f 0.70 (CMA), contaminated with Boc-Gly, R_f 0.66 (CMA). The product was purified on a column (4.2 × 53 cm) of Sephadex LH-20 eluted with dimethylformamide. When the resulting oil, which was free of Boc-Gly by tlc, was allowed to stand under petroleum ether (bp 30–60°) in the cold for a week, a crystalline product (0.270 g) was isolated in low yield (12%), mp 87–89°.

Anal. Calcd for $C_{13}H_{24}N_2O_5$: C, 54.15; H, 8.39; N, 9.71. Found: C, 52.42; H, 8.38; N, 9.62.

A weighed portion of the above compound was treated with trifluoroacetic acid for 30 min at room temperature. The trifluoroacetic acid was removed *in vacuo* and the resulting glycyl-6-aminohexanoic acid trifluoroacetate was dissolved in an aqueous solution containing known quantities of ammonium chloride and 6-aminohexanoic acid. Glycyl-6-aminohexanoic acid cannot be resolved from 6-aminohexanoic acid on the short column (0.9 × 6 cm Beckman PA-35 cation exchange resin) of the amino acid analyzer under the usual conditions (sodium citrate, 0.35 *N*, pH 5.26, 65 ml/hr, 56°). A longer column (0.9 × 13 cm) of the same resin allowed good resolution of ammonium chloride (127 min), glycyl-6-aminohexanoic acid (149 min), and 6-aminohexanoic acid (175 min) when a modified buffer (sodium citrate, 0.20 *N*, pH 4.15, 1.5% benzyl alcohol, 2.0% 1-propanol) was used. The relative ninhydrin color values are 1.81:2.26:1.00 for ammonium chloride–glycyl-6-aminohexanoic acid–6-aminohexanoic acid.

Attempted Acylation of *N*-Benzyloxycarbonyl-6-aminohexanoyl Resin. The substituted resin was prepared by treating 6-(benzyloxycarbonylamino)hexanoic acid[42] with chloromethylated copoly(styrene–1% divinylbenzene) resin in the presence of triethylamine and dimethylformamide according to Marglin.[43] Amino acid analysis of the resin gave 0.108 mmol/g of 6-aminohexanoic acid.

A. Carbodiimide Method. A portion of the substituted resin (0.200 g, 0.0216 mmol) was placed in a 5-ml reaction vessel[19] and shaken (10 min) with 2 ml of methylene chloride containing Boc-Gly (0.306 g, 1.75 mmol). Dicyclohexylcarbodiimide (0.360 g, 1.75 mmol) in 2 ml of methylene chloride was added and shaking was continued for 12 hr. The resin was washed with methylene chloride (6 × 2 ml) and then shaken with a mixture of 2 ml of 32% HBr in acetic acid and 2 ml of TFA[44] for 100 min. The cleavage solution was filtered and the resin was washed with three 2-ml

portions of TFA, TFA–methylene chloride (1:1), and methylene chloride. The pooled filtrates were evaporated *in vacuo*. The resulting oil was suspended in methylene chloride and again evaporated *in vacuo*. This procedure was repeated several times to remove excess acid from the cleavage product. The residue was dissolved in 1 ml of glacial acetic acid. Addition of water (19 ml) produced a massive white precipitate of dicyclohexylurea. The suspension was shaken for several minutes and then filtered through a millipore disk prior to analysis with the amino acid analyzer. The presence of glycyl-6-aminohexanoic acid could not be detected (<0.1%); the presence of 20 μmol of 6-aminohexanoic acid indicated a cleavage efficiency of 93%.

B. Mixed Anhydride Method. A solution of 0.437 *M* Boc-glycine mixed anhydride was prepared by treating Boc-glycine (0.486 g, 2.78 mmol) and triethylamine (0.388 ml, 2.78 mmol) with isobutyl chlorocarbonate (0.346 ml, 2.62 mmol) in methylene chloride (6 ml) at $-10°$ for 15 min. A portion (4 ml, 1.75 mmol) of this solution was shaken with 0.200 g of resin for 12 hr at 25° and worked up as described for the carbodiimide method. The presence of glycyl-6-aminohexanoic acid could not be detected (<0.1%); the presence of 156 μmol of 6-aminohexanoic acid indicated a cleavage efficiency of 78%.

Attempted Acylation of Boc-Gly-Gly-Resin. The substituted resin was prepared by treating Boc-Gly-Gly with chloromethylated resin (1% cross linked, 1.17 mmol Cl/g) according to Marglin.[43] Amino acid analysis of the resin indicated the presence of 0.136 mmol of glycylglycine/g. A portion of Boc-Gly-Gly-resin (0.200 g, 0.00272 mmol) was placed in a 5-ml reaction vessel and shaken (2 hr, 25°) with a solution (4 ml, 1.75 mmol) of 0.437 *M* Boc-glycine mixed anhydride, which was prepared as described above. The resin was washed with methylene chloride (6 × 2 ml) and then shaken for 15 min in 50% trifluoroacetic acid–methylene chloride (4 ml). The acid treatment was repeated and the resin was washed with methylene chloride (6 × 2 ml). It was treated with 10% triethylamine in methylene chloride (10 min), and washed with methylene chloride (6 × 2 ml). Cleavage of the resin with HBr was performed as described above. The presence of 20.8 μmol of glycylglycine indicated a cleavage efficiency of 77%. Triglycine, tetraglycine, pentaglycine, and hexaglycine could not be detected (<0.1% of Gly-Gly). When the sample was concentrated tenfold and applied to the analyzer column, the presence of a trace component running at the position of triglycine was detected (0.007 μmol, 0.03%). The presence of tetraglycine, pentaglycine, and hexaglycine could not be detected in the concentrated sample (<0.03%).

Beyerman, *et al.*,[45] have reported the formation of Gly-Gly-Gly-Gly-resin from Gly-Gly-resin in 1% yield when the latter was treated with 10% triethylamine in methylene chloride for 10 min. The dimerization reaction was thought to result from an interchain aminolysis. Our results contrast with this finding, since no tetraglycine (<0.03%) was detected after the treatment of deblocked Gly-Gly-resin with 10% triethylamine in methylene chloride for 10 min followed by cleavage with HBr.

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Registry No.—Leucyl-alanyl-glycyl-valine, 17195-26-5; Bpoc-glycine, 23650-19-3; ethyl chlorocarbonate, 541-41-3; leucyl-alanyl-glycyl-glycyl-valine, 49849-82-3; Boc-glycine, 4530-20-5; *N*-*tert*-butyloxycarbonylglycyl-6-aminohexanoic acid, 40203-83-6; isobutyl chlorocarbonate, 543-27-1; 6-aminohexanoic acid, 60-32-2; glycyl-glycyl-valine, 20274-89-9.

References and Notes

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(2) The nomenclature and symbols follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966), **242**, 555 (1967), and **247**, 977 (1972). In addition, TFA = trifluoroacetic acid; Bpoc = 2-(4-biphenylyl)-2-propyloxycarbonyl = biphenylisopropyloxycarbonyl.
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