±0.05°. An inert atmosphere in the reaction vessel was maintained with helium, which not only eliminates CO_2 interference in the titrations but also removes any complications produced by the direct reaction between CO_2 and chymotrypsin.[39] The total volume of the solution used in each experiment was 10 ml. A minimum of 25 combinations of substrate and alcohol concentrations (5 × 5) was employed in the measurement of initial velocities at each temperature. Ionic strength was maintained at a constant value of 0.2 with 2.0 *M* KCl. Titrations were performed with 0.01 *M* NaOH as the titrating agent. Tris-HCl or phosphate buffers at concentrations of 1.0×10^{-4} *M* were used in all experiments to stabilize the response of the measuring system. Fresh enzyme solutions were prepared each second day in 10^{-4} *M* HCl and stored at 4°. Enzyme concentrations were in the range 10^{-8}–10^{-6} *M* and were adjusted to give roughly identical initial velocities regardless of substrate concentration. Rates were measured from product-formation data taken at less than 10% of the total reactions. No spontaneous hydrolysis of the ester was detected under conditions of the experiments. Alcohol concentrations were varied between 0.2 and 2.0 *M*. Alcohol solutions were made up by volume at 25°. No corrections were made for small electrode errors in pH measurements in alcohol solutions. Initial slopes were obtained using a computer program fitting 30–40 experimental data points read from the product–time record from each titration experiment to a series expansion of the integrated Michaelis-Menten equation written with *P* as the independent variable. The first term of this series is the true velocity at $t = 0$, if the assumption of Michaelis-Menten kinetics is correct. All kinetic and thermodynamic parameters were evaluated using a computer program designed for an iterative, appropriately weighted least-squares analysis of the data according to eq 1, and the van't Hoff and Arrhenius equations.

(39) S. Rajender, unpublished observations from this laboratory, 1967.

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Azide Solid Phase Peptide Synthesis[1]

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Abstract: The stepwise synthesis of peptides by the initial attachment of an amino acid *t*-butyloxycarbonylhydrazide through its α-amino group to a polystyrene resin was investigated. The resin–amino acid azide was generated quantitatively and coupled with another amino acid *t*-butyloxycarbonylhydrazide. The peptide chain was elongated by further azide couplings. Finally, the C-terminal amino acid was added as a *t*-butyl ester. The peptide was deprotected and removed from the resin in one step with HBr. The feasibility of the approach was demonstrated by the synthesis of L-leucylglycine and L-leucyl-L-alanylglycyl-L-valine by this stepwise procedure and also by a fragment condensation. Studies on the stability and reactivity of the intermediate azides were carried out.

Modifications in the solid phase peptides synthesis procedure[3] have been directed toward the resin,[4] the coupling reaction,[4a,c,5] the N-protecting group,[4e,5e,6] and the cleavage step.[4d,5a,6a,7] In general the method has involved the attachment of the carboxyl group of the first amino acid to a resin, followed by peptide chain elongation at the amino end.[3] There has been one instance[8,9] in which an amino acid was attached to the resin through its amino group while the carboxyl was protected as an ethyl ester. However, the procedure described in those early experiments was somewhat limited because of the danger of racemization during the subsequent deprotection and coupling steps. In order to develop this general approach further it is necessary to find suitable carboxyl protection and efficient, racemization-free coupling methods. The acid azide route of classical peptide synthesis[10] is known to yield optically pure products[11] not only when amino acids are coupled, but even when the carboxyl groups of peptides are activated. The modified approach of Rudinger and Honzl[12] to the azide reaction utilizing a nonaqueous system, and the use of 1-aminoacyl-2-*t*-butyloxy-

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(3) (a) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); (b) R. B. Merrifield, *Advan. Enzym.*, **32**, 221 (1969).

(4) (a) M. M. Shemyakin, Y. A. Ovchinnikov, A. A. Kiryushkin, and I. V. Kozhevnikova, *Tetrahedron Lett.*, **27**, 2323 (1965); (b) G. I. Tesser and B. W. J. Ellenbroek, *Proc. Eur. Peptide Symp., 8th, Noordwijk* 124 (1967); (c) M. A. Tilak and C. S. Hollinden, *Tetrahedron Lett.*, 1297 (1968); (d) F. Weygand, *Proc. Eur. Peptide Symp., 9th, Orsay*, 183 (1968); (e) N. Inukai, K. Nokano, and M. Murakami, *Bull. Chem. Soc., Jap.*, **211**, 182 (1968); (f) F. Camble, R. Garner, and G. T. Young, *Nature*, **217**, 248 (1968); (g) B. Green and L. R. Garson, *J. Chem. Soc., C*, 401 (1969).

(5) (a) M. Bodanszky and J. T. Sheehan, *Chem. Ind.*, 1597 (1966); (b) F. M. Bumpus, M. C. Khosla, and R. R. Smeby, *Abstracts*, 153rd Meeting of the American Chemical Society, Division of Medicinal Chemistry, Miami, Fla., 1967; (c) M. Rothe and H. Schneider, *Angew. Chem. Intern. Ed. Engl.*, **5**, 417 (1966); (d) H. Klostermeyer, H. Halstrøm, P. Kusch, J. Föhles, and W. Lunkenheimer, *Proc. Eur. Peptide Symp., 8th, Noordwijk*, 113 (1967); (e) F. Weygand and U. Ragnarsson, *Z. Naturforsch.*, **21b**, 1141 (1966).

(6) (a) Kessler and B. Iselin, *Helv. Chim. Acta*, **49**, 1330 (1966); (b) V. A. Najjar and R. B. Merrifield, *Biochemistry*, **5**, 3765 (1966); (c) A. Deér, *Angew. Chem. Intern. Ed. Engl.*, **5**, 1041 (1966); (d) G. Losse and K. Noubert, *Z. Physiol.*, **8**, 278 (1968); (e) P. Sieber and B. Iselin, *Helv. Chim. Acta*, **51**, 614 (1968).

(7) (a) J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967); (b) A. Loffet, *Experientia*, **23**, 406 (1967); (c) L. Stryer and R. P. Haugland, *Proc. Natl. Acad. Sci. U. S.*, **58**, 719 (1967); (d) M. Ohno and C. B. Anfinsen, *J. Amer. Chem. Soc.*, **89**, 5994 (1967); (e) H. C. Beyerman, H. Hindriks, and E. W. B. DeLeer, *Chem. Commun.*, 1668 (1968); (f) B. Halpern, L. Chew, V. Close, and W. Patton, *Tetrahedron Lett.*, 5163 (1968).

(8) R. L. Letsinger and M. J. Kornet, *J. Amer. Chem. Soc.*, **85**, 3045 (1963).

(9) R. L. Letsinger, M. J. Kornet, V. Mahadevan, and D. M. Jerina, *ibid.*, **86**, 5163 (1964).

(10) T. Curtius, *Ber.*, **35**, 3226 (1902).

(11) N. H. Smart, G. T. Young, and M. W. Williams, *J. Chem. Soc.*, 3902 (1960).

(12) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

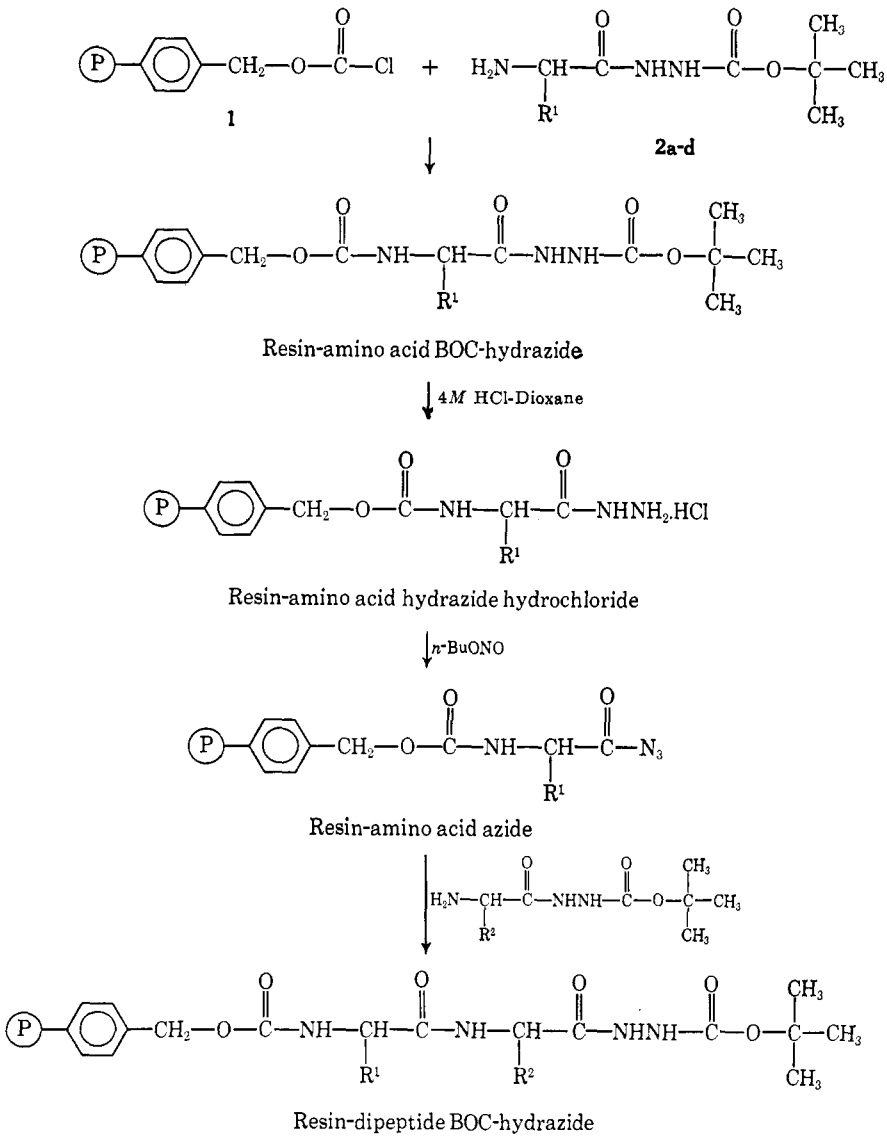

Figure 1. The general scheme for azide solid-phase peptide synthesis. P = copolymer of styrene–2% divinylbenzene.

carbonylhydrazines (amino acid Boc-hydrazides),[13] have given the acid azide method still further versatility. There are reports[7d,14] in which a peptide coupling reaction was carried out between the azide in solution and the amino component on a resin. We now report the stepwise synthesis of a tetrapeptide in which the azide is on a resin support and the amino component is in solution. An advantage of building the peptide from the carboxyl end by such an approach is the potential of coupling fragments without racemization. Moreover, this approach has application in special cases where a series of analogs with variations near the carboxyl end of the peptide is desired.

Synthesis of Peptides

The general procedure for the azide solid phase peptide synthesis is outlined in Figures 1 and 2. In the first step, 0.5 equiv of L-leucine Boc-hydrazide,[15] was allowed to react with a methylchloroformylated styrene–divinylbenzene resin, **1**.[8,9] This reaction was carried out in chloroform in the presence of triethylamine. Addition of anhydrous diethylamine or dimethylamine terminated the unreacted methylchloroformylated resin. The first cycle (Figure 1) consisted of (a) removal of the protecting group by treatment with 4 *M* HCl–dioxane at 23° for 30 min to give the resin–amino acid hydrazide hydrochloride; (b) conversion to the resin–amino acid azide by treatment with *n*-butylnitrite in tetrahydrofuran at −30° for 1 hr; and (c) coupling with an amino acid Boc-hydrazide in tetrahydrofuran using temperature steps of −30, 0, and 25°. Excess reagents and by-products were removed by washing with tetrahydrofuran and methanol. This coupling cycle was repeated with other amino acid Boc-hydrazides. The last cycle of the synthesis (Figure 2) consisted of (a) removal of the protecting group, (b) conversion to the resin–peptide azide, and (c) coupling with the amino acid *t*-butyl ester corresponding to the C-terminal residue of the peptide. The *t*-butyl ester protecting group gave better yields than the benzyl ester in our system and was removed equally well by the HBr–TFA used for the cleavage of the peptide

(13) (a) R. Schwyzer, *Angew. Chem.*, 71, 742 (1959); (b) R. Schwyzer, E. Surbeck-Wegmann, and H. Dietrich, *Chimia* (Aarau), 14, 366 (1960); (c) R. A. Boissonnas, S. Guttmann, and P. A. Jacquenoud, *Helv.*, 43, 1349 (1960).
(14) D. W. Woolley, unpublished results. See also ref 3b.
(15) E. Wünsch, F. Drees, and J. Jentsch, *Ber.*, 98, 803 (1965).

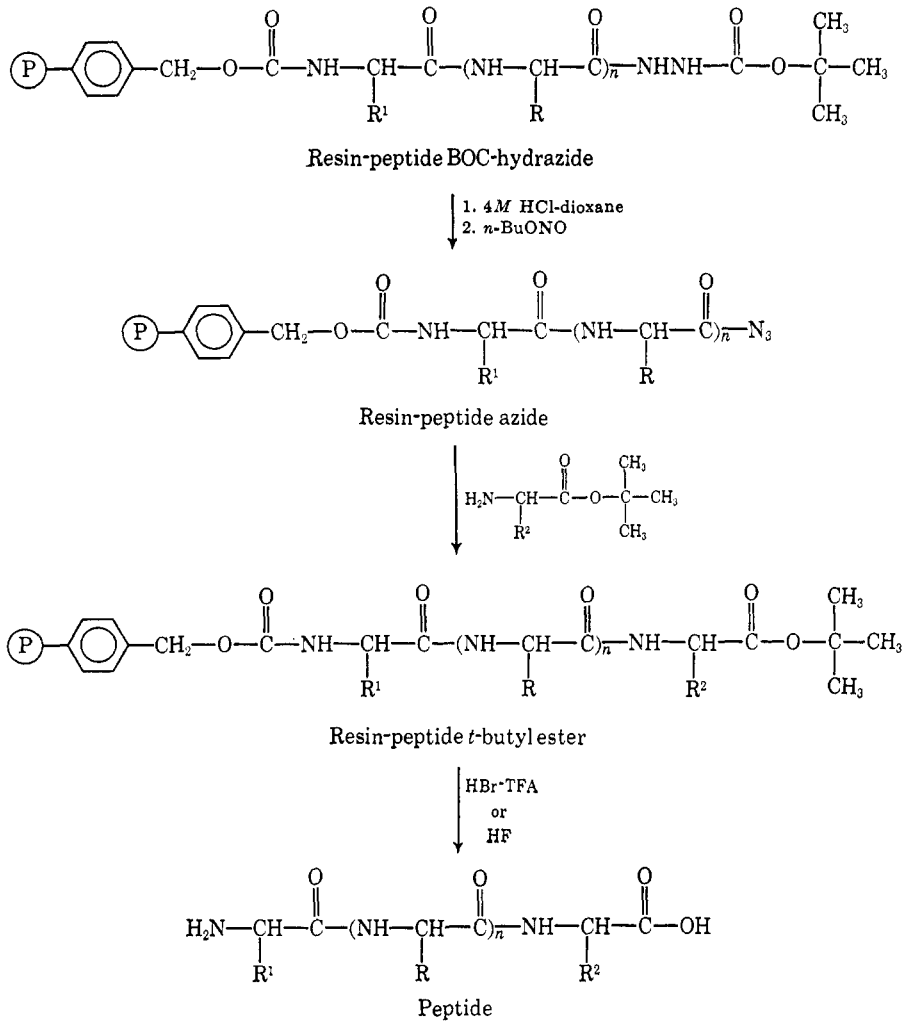

Figure 2. The termination and cleavage steps for azide solid-phase peptide synthesis.

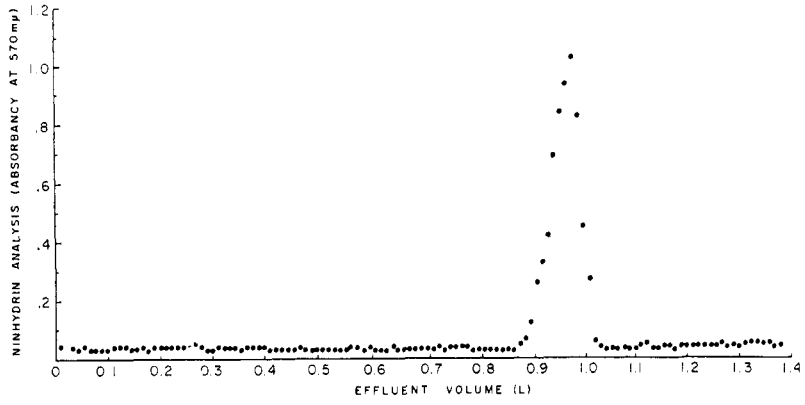

Figure 3. Chromatographic separation of crude lyophilized L-leucyl-L-alanylglycyl-L-valine on a 2 × 120 cm column of Dowex 50-X4 resin. Elution was with pyridine acetate buffer, pH 4.0, 0.5 *M* in acetate, with a gradient of 104 ml/hr. Aliquots (0.2 ml) from 5.5-ml fractions were analyzed by the ninhydrin test.

from the resin.[16] In order to demonstrate the feasibility of this approach the tetrapeptide, L-leucyl-L-alanylglycyl-L-valine, was synthesized. The crude product, after cleavage from the resin contained a minor contaminant which could be separated by column chromatography on Dowex 50-X4. An overall yield of 30% of purified tetrapeptide was obtained by this stepwise procedure.

The versatility of this method was illustrated by the synthesis of the same tetrapeptide, in better yield, by fragment condensation. In this instance, resin-L-leucyl-L-alanine azide prepared as described above, was coupled with glycyl-L-valine *t*-butyl ester. Cleavage of peptide from resin afforded the tetrapeptide. Chromatography of this product on a Dowex 50-X4 resin by elution with 0.5 *M* pyridine acetate buffer (pH 4) as shown in Figure 3 gave a single, homogeneous peak. The product gave one spot on thin-layer chromatography in each of three solvent systems. It was also

(16) R. B. Merrifield, *J. Amer. Chem. Soc.*, **86**, 304 (1964).

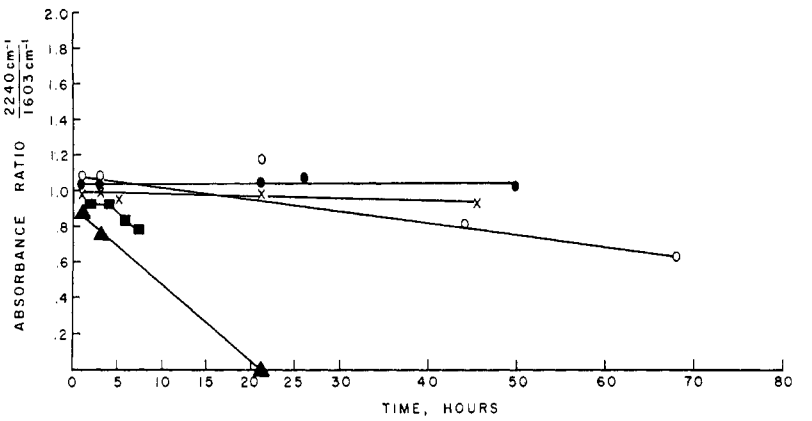

Figure 4. Stability in tetrahydrofuran: ●, resin-L-leucine azide at −30°; ×, resin-L-leucine azide at −10°; ○, resin-L-leucine azide at 4°; ▲, resin-L-leucine azide at 23°; ■, resin-L-cysteine azide at 23°.

judged to be pure by amino acid analysis and by comparison with an authentic sample.[3a] The overall yield of purified tetrapeptide was 60% (calculated from the amount of resin-L-leucine Boc-hydrazide). Fragment condensation of resin-L-leucine azide was also carried out successfully with L-alanylglycine Boc-hydrazide. This intermediate was essentially identical with that prepared by stepwise condensation.

L-Leucylglycine was prepared in a similar manner, *i.e.*, resin-L-leucine azide was coupled with glycine *t*-butyl ester. Cleavage of peptide from resin and chromatography on Dowex 50-X4 afforded a product that was identical with a commercial sample of L-leucylglycine.[17] The overall yield of purified dipeptide was 57% (calculated from the resin-L-leucine Boc-hydrazide). It was judged pure by thin-layer chromatography, amino acid analysis, and elemental analysis.

Kinetic Studies

The major problems that have been encountered in the past with the acid azide method have been the formation of isocyanates by the Curtius rearrangement and the formation of amides. The former can lead to urea-containing peptides and the latter results in chain termination. The stability of peptide azides in dimethylformamide has been studied by Schwyzer[18] and Katsoyannis[19] who showed that the azides gave isocyanates at ambient temperature but were stable at 0°. Rudinger and Honzl[12] demonstrated that although the side reactions of carbobenzoxy-S-benzyl-L-cysteine azide occurred they could be suppressed in nonaqueous homogeneous systems. It was then necessary to determine if these side reactions occurred in the solid phase system and if they could also be suppressed.

Resin-L-leucine azide was prepared as outlined above at −30° in tetrahydrofuran. It was filtered, washed with cold tetrahydrofuran, isolated in the "dry state," and allowed to come to room temperature. An infrared spectrum of this pale yellow resin in a KBr pellet revealed a strong band at 2240 cm^{-1}. The position and intensity of this band (relative to a reference band at 1603 cm^{-1} from the same spectrum) was unchanged even when the resin was left exposed in the laboratory for many days. Grinding and reforming the pellet also had no effect on the spectrum. When this resin–azide was reacted with L-alanine Boc-hydrazide, using the temperature gradient described above, resin-L-leucyl-L-alanine Boc-hydrazide was obtained in high yield. Since there was no evidence of urea formation[20] the band at 2240 cm^{-1} was assigned to the azide function, rather than to isocyanate. It was concluded that the resin-L-leucine azide was stable for more than 10 days at room temperature in the dry state.

A new sample of the resin-L-leucine azide was prepared and allowed to stand at −30° in tetrahydrofuran. Aliquots were removed at various times and infrared spectra were measured as outlined above. It was determined that resin-L-leucine azide was stable in tetrahydrofuran at −30° for at least 50 hr. Since derivatives of cysteine are known to undergo side reactions readily,[21] resin S-*p*-methoxybenzyl-L-cysteine azide was also prepared and its stability examined in the same manner. These measurements indicated that the stability of resin-S-*p*-methoxybenzyl-L-cysteine azide was parallel with that of resin-L-leucine azide (*i.e.*, no change was observed in the 2240 cm^{-1}:1603 cm^{-1} ratio for at least 24 hr). Studies were also carried out on the stability of resin-L-leucine azide and resin-S-*p*-methoxybenzyl-L-cysteine azide at higher temperature in tetrahydrofuran using the same parameters as outlined above (Figure 4). It was determined that the resin–azide was stable at −30 and −10° for longer than 40 hr but that there was some decomposition at 4° over a prolonged period of time. This decomposition was pronounced at 23° and the azide band completely disappeared within 24 hr. Since we failed to observe the formation of a new band in the 2240-cm^{-1} region, characteristic for the isocyanate, it was concluded that a Curtius rearrangement had not occurred even at the higher temperature but, rather, that the resin–azide had decomposed to give the amide.[12] It should be noted that preliminary experiments with resin-L-asparagine azide have revealed the presence of a second band at 2140 cm^{-1}, which is under further study.

The rate of reaction of resin-L-leucine azide and resin-S-*p*-methoxybenzyl-L-cysteine azide with L-alanine Boc-

(17) Mann Research Laboratories, New York, N. Y.
(18) R. Schwyzer and H. Kappeler, *Helv. Chem. Acta*, **44**, 1991 (1961).
(19) P. G. Katsoyannis, A. M. Tometsko, C. Zalut, and K. Fukuda, *J. Amer. Chem. Soc.*, **88**, 5625 (1966).
(20) Amino acid analysis of the resin hydrolysate showed equimolar amounts of leucine and alanine. If urea formation had occurred *via* an isocyanate intermediate only alanine would have been found.
(21) (a) R. Roeske, F. M. C. Stewart, R. J. Stedman, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **78**, 5883 (1956); (b) R. B. Merrifield and D. W. Woolley, *ibid.*, **80**, 6635 (1958).

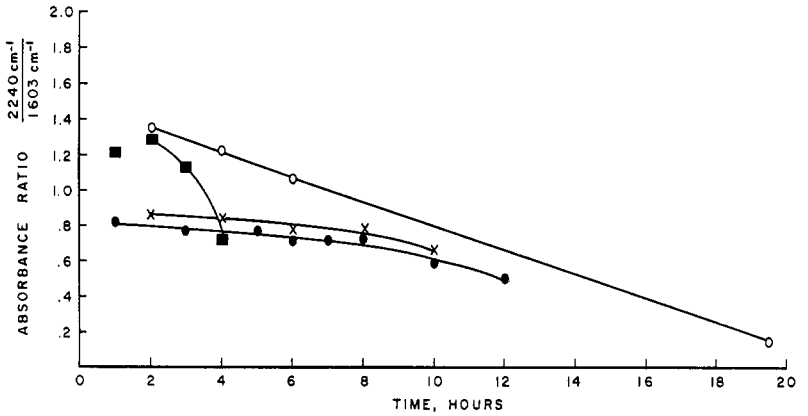

Figure 5. Reactivity in tetrahydrofuran with L-alanine Boc-hydrazide: ●, resin-L-leucine azide at −30°; ×, resin-L-leucine azide at −10° ○, resin-L-cysteine azide at 4°; ■, resin-L-cysteine azide at 23°.

hydrazide (Figure 5) was also examined by following the disappearance of the azide band at 2240 cm^{-1}. These studies revealed that the coupling reaction proceeded very slowly at −30 and −10° but that at 4° it was almost complete within 20 hr. Although coupling was very rapid at 23°, the rate of decomposition at this temperature (Figure 4) was appreciable. It was concluded from this kinetic study that the resin–azide should ideally be generated at −30° and coupled at temperatures between −10 and 4°. Under these conditions we have no evidence for rearrangement of the resin–azide.

The generation of resin-L-leucine azide and the subsequent coupling reactions were also studied in other solvent systems (dimethylformamide, dioxane, methylene chloride, and chloroform). It was found that preparation and coupling of resin azides were best carried out in tetrahydrofuran. Variable results were obtained with dimethylformamide. Dioxane, methylene chloride, and chloroform were poor solvents in this system. The overall yields are presently marginal and we do not recommend this new approach as a general replacement for solid phase peptide synthesis. However, it may be a valuable supplement to peptide synthesis and demonstrates the versatility of the solid phase method.

Experimental Section

All analytical samples were dried for 24 hr at 56° under high vacuum. All melting points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 621 grating spectrophotometer. Nuclear magnetic resonance (nmr) spectra were recorded for all compounds on a Varian Associates A-60 spectrometer with 2% tetramethylsilane as internal standard. Optical rotations were taken in a jacketed 1-dm cell on a Perkin-Elmer Model 140 polarimeter. Thin-layer chromatography was carried out on plates prepared with silica gel G and developed with buffered ninhydrin reagent.[22] Mass spectra were determined with a (CEC) 21-110 spectrometer. Tetrahydrofuran (reagent grade) was freed of peroxides by passage through a column of alumina. All other reagents were obtained commercially and used without further purification. The reaction vessel for peptide synthesis, as described previously,[8a] was jacketed and cooled by a Lauda TK-30 circulator to maintain the required temperature. Amino acid analyses were performed on the spinco Amino Acid Analyzer, Model 120B. Resin hydrolysates were carried out on 10–20-mg samples of resin with concentrated HCl, glacial acetic acid, and phenol (2:1:1) at 110° for 18–24 hr.

Methylchloroformylated Resin (1).[8,9] Chloromethylated copolystyrene–2% divinylbenzene (1.04 mmol/g; 25 g, 26 mmol), suspended in 200 ml of methyl Cellosolve, was treated with potassium acetate (7.0 g, 71.3 mmol) and heated at 130° for 64 hr. The reaction mixture was filtered, and washed with water and methanol. The product was converted to the hydroxymethylated resin by saponification with 150 ml of 0.5 *M* NaOH (75 mmol) at 23° for 47 hr. It was filtered, washed with water and methanol, and dried *in vacuo*. Treatment of this resin with 200 ml of 1.27 *M* phosgene in benzene (254 mmol) at 23° for 4 hr followed by filtration and washing with benzene and ether, afforded methylchloroformylated resin, **1** (0.718 mmol of chloride/g of resin). The infrared spectra of the hydroxymethylated and chloroformylated resins were identical with those reported by Letsinger.[9]

1-L-Leucyl-2-*t*-butyloxycarbonylhydrazine (L-Leucine Boc-hydrazide) (2a). N-Carbobenzoxy-L-leucine (15.0 g, 0.056 mol) and *t*-butylcarbazate (9.6 g, 0.072 mol) in 250 ml of ethyl acetate at 0° were treated with dicyclohexylcarbodiimide (12.4 g, 0.06 mol). The reaction mixture was stirred for 2 hr at 0° and 4 hr at 23°. It was filtered, extracted in turn with 1 *M* citric acid, saturated $NaHCO_3$, saturated KCl, and evaporated to dryness. The residue was taken up in 160 ml of methanol, treated with 4 g of 5% Pd–$BaSO_4$, and hydrogenated for 19 hr at 40 psi. Evaporation of solvent and recrystallization from ethyl acetate–petroleum ether afforded 6.6 g (47%) of product: mp 112.0–114.0°; $[\alpha]^{20}D$ 21.77° (*c*, 2.35; methanol); lit.[15] mp 114–116°; $[\alpha]^{20}D$ 21.54° (*c*, 2.2; methanol).

Anal. Calcd for $C_{11}H_{23}N_3O_3$: C, 53.86; H, 9.45; N, 17.13. Found: C, 53.95; H, 9.46; N, 17.10.

L-Alanine Boc-hydrazide (2b). N-Carbobenzoxy-L-alanine (6.7 g, 0.03 mol) and *t*-butylcarbazate (4.8 g, 0.036 mol) in 100 ml of ethyl acetate at 0° were treated with dicyclohexylcarbodiimide (6.2 g, 0.03 mol) and worked up as described above for L-leucine. Hydrogenation for 3 hr in methanol over 2 g of 5% Pd–$BaSO_4$, followed by crystallization from ethyl acetate–petroleum ether, afforded 4.0 g (66%) of product: mp 86.0–90.5° $[\alpha]^{20}D$ 6.29° (*c*, 2.07; methanol).

Anal. Calcd for $C_8H_{17}N_3O_3$: C, 47.28; H, 8.43; N, 20.67. Found: C, 47.50; H, 8.38; N, 20.48.

Glycine Boc-hydrazide (2c). N-Carbobenzoxyglycine (6.3 g, 0.03 mol) and *t*-butylcarbazate (4.8 g, 0.036 mole) in 120 ml of ethyl acetate at 0° were treated with dicyclohexylcarbodiimide (6.2 g, 0.03 mol) and worked up in the usual manner. Hydrogenation for 12 hr in methanol over 2 g of 5% Pd–$BaSO_4$, followed by crystallization from ethyl acetate–petroleum gave 3.2 g (56%) of crystalline product, mp 130–132.5°.

Anal. Calcd for $C_7H_{15}N_3O_3$: C, 44.43; H, 7.99; N, 22.21. Found: C, 44.69; H, 8.03; N, 21.98.

L-Alanylglycine Boc-hydrazide. N-Carbobenzoxy-L-alanine (1.34 g, 6.0 mmol) and glycine Boc-hydrazide (**2c**) (1.13 g, 6.0 mmole) in 50 ml of ethyl acetate at 0° were allowed to react with dicyclohexylcarbodiimide (1.24 g, 6.0 mmol). The reaction mixture was stirred for 1.5 hr at 0° and 17 hr at 23°. It was filtered, evaporated to dryness, taken up in 50 ml of methanol, treated with 2.5 g of 5% Pd–$BaSO_4$, and hydrogenated for 5 hr at 40 psi. Evaporation of solvent and crystallization from methanol–ethyl acetate–petroleum ether gave 920 mg (59%) of crystalline product: mp 129.0–132.0° dec; $[\alpha]^{20}D$ 7.52° (*c* 1.93; methanol).

Anal. Calcd for $C_{10}H_{20}N_4O_4 \cdot 1/2\ H_2O$: C, 44.65; H, 7.86; N, 20.88. Found: C, 44.88; H, 7.89; N, 20.46.

N-Trifluoroacetyl-S-*p*-methoxybenzyl-L-cysteine. This compound was prepared by the procedure of Schallenberg and Calvin.[23]

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

A mixture of S-*p*-methoxybenzyl-L-cysteine[24] (4.82 g, 20 mmol), 1.0 *N* NaOH (20 ml, 20 mmol), saturated sodium borate (80 ml), and ethyl thioltrifluoroacetate (5.54 g, 31.2 mmol) was shaken at 23° for 7 hr. The reaction mixture was extracted three times with ether, acidified with 1 *M* HCl, and reextracted three times with ether. This latter ethereal extract was dried over $MgSO_4$, evaporated to dryness, and crystallized from benzene–petroleum ether. There was obtained 2.75 g (41%) of product: mp 94.0–95.0° dec; $[\alpha]^{20}D$ −76.01° (*c* 1.82; methanol).

Anal. Calcd for $C_{13}H_{14}NO_4F_3S$: C, 46.29; H, 4.18; N, 4.15. Found: C, 46.00; H, 4.08; N, 4.12.

N-Trifluoroacetyl-S-*p*-methoxybenzyl-L-cysteine Boc-hydrazide. N-Trifluoroacetyl-S-*p*-methoxybenzyl-L-cysteine (5.63 g, 16.7 mmol) and *t*-butylcarbazate (2.21 g, 16.7 mmol) in 75 ml of ethyl acetate at 0° were treated with dicyclohexylcarbodiimide (3.45 g, 16.7 mmol). The reaction mixture was stirred for 2 hr at 0° and 20 hr at 23°. Filtration, evaporation to dryness, and crystallization from ethyl acetate–petroleum ether afforded 5.8 g (77%) of white crystals: mp 110.0–111.5°; $[\alpha]^{20}D$ −41.65° (*c* 1.82; methanol).

Anal. Calcd for $C_{18}H_{24}N_3O_5F_3S$: C, 47.89; H, 5.36; N, 9.31. Found: C, 47.59; H, 5.39; N, 9.27.

S-*p*-Methoxybenzyl-L-cysteine Boc-hydrazide (2d). Deprotection of the precursor was carried out by a modification of the procedure of Weygand and Reiher.[25,26] N-Trifluoroacetyl-S-*p*-methoxybenzyl-L-cysteine Boc-hydrazide (5.75 g, 12.7 mmol) in 60 ml of absolute ethanol was treated with 135 ml of 6.3 *M* NH_4OH and stirred for 2 hr at 23°. The reaction mixture was evaporated to dryness, treated with 25 ml of water, and reevaporated. This residue was evaporated from another 25 ml of water, taken up in ethyl acetate, and extracted three times with cold saturated aqueous Na_2CO_3. The ethyl acetate layer was dried over $MgSO_4$, filtered, evaporated to dryness, and pumped out *in vacuo*. There was obtained 3.79 g (84%) of an amorphous solid $[\alpha]^{20}D$ −1.49° (*c* 2.01; methanol); tlc showed 1 ninhydrin positive spot with R_f 0.54 (methanol:chloroform; 1:9). The infrared spectrum of **2d** in KBr was consistent with expectation. Characteristic bands were observed at 1620, 1590, 1520, 1310, and 1265 cm^{-1}. The nmr spectrum (NaOD–D_2O) was also consistent with expectation giving peaks at 3.26 (m, 2 H), 3.88 (m, 1 H), 4.14 (s, 2 H), 4.18 (s, 3 H), and 7.61 ppm (aromatic A_2B_2 type, 4 H).

Glycyl-L-valine *t*-Butyl Ester. N-Carbobenzoxyglycine (2.09 g, 10 mmol) and L-valine *t*-butyl ester[27] (1.73 g, 10 mmol) in 50 ml of tetrahydrofuran at 0° were treated with dicyclohexylcarbodiimide (2.06 g, 10 mmol). The reaction mixture was stirred for 2 hr at 0° and 48 hr at 23°. It was filtered, evaporated to dryness, taken up in ethyl acetate, and extracted in turn with saturated $NaHCO_3$, saturated KCl, and 1 *M* citric acid. The ethyl acetate layer was dried over $MgSO_4$, filtered, evaporated to dryness and pumped out *in vacuo*. The resultant oil was taken up in 100 ml of methanol, treated with 2 g of 5% Pd–C, and hydrogenated at 45 psi for 12 hr. Filtration, and evaporation, afforded 1.83 g (84%) of a viscous pale yellow oil which resisted all attempts at crystallization: tlc ($CHCl_3$) gave one major spot at R_f 0.13; $[\alpha]^{20}D$ −21.55° (*c* 2.00; methanol). The infrared spectrum in $CHCl_3$ was consistent with expectation. Characteristic bands were observed at 3415, 3340, 1735, 1675, and 1525 cm^{-1}. The nmr spectrum ($CDCl_3$) was also consistent with expectation giving peaks at 0.94 (d, 6 H), 1.48 (s, 9 H), 2.25 (m, 1 H), 3.66 (m, 1 H), 4.17 (m, 2 H), 4.67 (m, 2 H, exchangeable), and 7.92 ppm (d, 1 H, exchangeable).

Resin-L-leucine Boc-hydrazide. The methylchloroformylated resin (**1**) (5.0 g, 3.6 mmol of Cl) was suspended in 75 ml of chloroform and treated with L-leucine Boc-hydrazide (**2a**) (442 mg, 1.8 mmol), followed by triethylamine (0.25 ml, 1.8 mmol). The reaction mixture was stirred for 2 hr at 23° and was treated with dimethylamine (1.6 g, 36 mmol) in 15 ml of chloroform. After stirring for 3 more hours at 23°, the resin was filtered, washed with chloroform, and dried *in vacuo*. Amino acid analysis on a resin hydrolysate revealed a substitution of 0.297 mmol of L-leucine per gram of resin.

Addition of larger amounts of L-leucine Boc-hydrazide gave more highly substituted resin. Therefore, methylchloroformylated resin (**1**) (5.0 g, 3.6 mmol) was suspended in 50 ml of chloroform and treated with L-leucine Boc-hydrazide (**2a**) (884 mg, 3.6 mmol), followed by triethylamine (0.50 ml, 3.6 mmol). The reaction mixture was stirred for 2 hr at 23°, treated with diethylamine (2.6 g, 36 mmol) in 15 ml of chloroform, and stirred overnight at 23°. The resin was filtered, washed with chloroform, and dried *in vacuo*. Amino acid analysis of a resin hydrolysate revealed a substitution of 0.503 mmol of L-leucine per gram of resin.

Resin-S-*p*-methoxybenzyl-L-cysteine Boc-hydrazide. The methylchloroformylated resin (**1**) (3.0 g, 2.15 mmol) was suspended in 50 ml of chloroform and treated with S-*p*-methoxybenzyl-L-cysteine Boc-hydrazide (**2d**) (2.14 g, 6.02 mmol) followed by triethylamine (0.84 ml, 6.0 mmol). The reaction mixture was stirred for 42 hr at 23°. Dimethylamine (2.2 g, 49 mmol) in 10 ml of chloroform was added and stirring continued for 2 hr. The product was filtered, washed with chloroform, and dried *in vacuo*. Amino acid analysis on a resin hydrolysate revealed total cysteine substitution of 0.273 mmol/g of resin.

Resin-L-leucine Azide for Kinetic Studies. Resin-L-leucine Boc-hydrazide (100 mg, 0.03 mmol) was placed in the reaction vessel and shaken with 50 ml of 4 *M* HCl–dioxane for 30 min at 23°. The resin was filtered, and washed three times with 50-ml portions of tetrahydrofuran. Tetrahydrofuran (50 ml) was added and the reaction vessel was cooled to −30°. It was opened momentarily and *n*-butyl nitrite (0.02 ml, 0.16 mmol) followed by 4 *M* HCl–dioxane (0.04 ml, 0.16 mmol) was added. The reaction mixture was rocked at −30° for 1 hr, filtered and washed three times with 50-ml portions of precooled tetrahydrofuran at −30°. The resultant resin-L-leucine azide was used directly in the kinetic stability studies. For the kinetic reactivity studies, resin-L-leucine azide was washed three times with 50-ml portions of precooled 10% Et_3N–THF at −30°. The resin was washed with 50-ml portions of precooled tetrahydrofuran at −30°. A solution of L-alanine Boc-hydrazide (**2b**) (31 mg, 0.15 mmol) in 50 ml of precooled tetrahydrofuran at −30° was added. The required temperature for the kinetic reactivity study was attained, aliquots were removed, rapidly filtered, and washed with precooled tetrahydrofuran.

Resin-S-*p*-methoxybenzyl-L-cysteine Azide for Kinetic Studies. Resin-S-*p*-methoxybenzyl-L-cysteine Boc-hydrazide (400 mg, 0.11 mmol) was placed in the reaction vessel and shaken with 50 ml of 4 *M* HCl–dioxane for 30 min at 23°. The resin was filtered and washed three times with 50-ml portions of tetrahydrofuran. Tetrahydrofuran (50 ml) was added and the reaction vessel was cooled to −30°. It was opened momentarily and *n*-butyl nitrite (0.08 ml, 0.60 mmol) followed by 4 *M* HCl–dioxane (0.15 ml, 0.60 mmol) was added. The reaction mixture was rocked at −30° for 1 hr, filtered and washed three times with 50-ml portions of precooled tetrahydrofuran at −30°. The resultant resin-S-*p*-methoxybenzyl-L-cysteine azide was used directly in the kinetic stability studies. For the kinetic reactivity studies, resin-S-*p*-methoxybenzyl-L-cysteine azide (150 mg, 0.041 mmol) was washed three times with 50-ml portions of precooled 10% Et_3N–THF at −30°. The resin was then washed with 50-ml portions of precooled tetrahydrofuran at −30°. A solution of L-alanine Boc-hydrazide (**2b**) (41 mg, 0.20 mmol) in 50 ml of precooled tetrahydrofuran at −30° was added. The required temperature was attained, aliquots were removed, rapidly filtered, and washed with precooled tetrahydrofuran.

L-Leucylglycine. Resin-L-leucine Boc-hydrazide (1.5 g, 0.446 mmol) was introduced into the jacketed reaction vessel. The following cycle of reactions was employed: (1) *t*-Boc group cleaved by 4 *M* HCl–dioxane (50 ml) for 30 min; (2) washed with three 50-ml portions of tetrahydrofuran; (3) added 50 ml of tetrahydrofuran and cooled the vessel to −30°; (4) generated the azide by addition of 4 *M* HCl–dioxane (0.56 ml, 2.23 mmol; 5 meq), followed by *n*-butylnitrite (0.28 ml, 2.23 mmol, 5 meq) for 1 hr; (5) washed with three 50-ml portions of precooled 10% Et_3N–THF at −30°; (6) washed with three 50-ml portions of precooled tetrahydrofuran at −30°; (7) added glycine *t*-butyl ester[28] (292 mg, 2.23 mmol, 5 meq) in 50 ml of tetrahydrofuran precooled to −30°; (8) rocked at −30° for 2 hr, 0° for 16 hr and 23° for 3 hr; (9) washed with three 50-ml portions of tetrahydrofuran; (10) washed with three 50-ml portions of methanol. The resin was cleaved with HBr in trifluoroacetic acid as described previously.[16] The crude peptide was lyophilized twice from water and purified on a 2 × 120 cm Dowex 50-X4 column in a pH 4.0 pyridine acetate cycle. The sample was applied to the column in 1 ml of water. Elution

(23) E. E. Schallenberg and M. Calvin, *J. Amer. Chem. Soc.*, **77**, 2779 (1955).

(24) S. Akabori, S. Sakakibara, Y. Shimonishi, and Y. Nobuhara, *Bull. Chem. Soc. Jap.*, **37**, 433 (1964).

(25) F. Weygand and M. Reiher, *Chem. Ber.*, **88**, 26 (1955).

(26) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961, p 1251.

(27) R. W. Roeske, *Chem. Ind.*, 1121 (1959).

(28) G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, **82**, 3359 (1960).

with pH 4.0 pyridine acetate (0.5 *M* in acetic acid) proceeded at a rate of 118 ml/hr for a total of 16 hr. Aliquots (0.2 ml) from 5.5-ml fractions were analyzed by the ninhydrin test. The fractions between 0.88 and 1.16 l. were combined and lyophilized; yield 52 mg (57% calculated from the amount of resin-L-leucine Boc-hydrazide); $[M]^{25}D$ 170.7° (*c* 1.40; water); lit.[29] $[M]^{25}D$ 161.3° (*c* 2.4; water); amino acid ratios: Leu, 1.00; Gly, 0.98.

Anal. Calcd for $C_8H_{16}N_2O_3 \cdot H_2O$: C, 46.59; H, 8.80; N, 13.58. Found: C, 46.45; H, 8.52; N, 13.77.

Infrared and nmr spectra of the product were identical with that of a commercial sample.[17]

L-Leucyl-L-alanylglycyl-L-valine (Stepwise Condensation). Resin-L-leucine Boc-hydrazide (2.0 g, 0.544 mmol) was placed in the reaction vessel. The following cycle of reactions was employed: (1) *t*-Boc group cleaved by 4 *M* HCl–dioxane (50 ml) for 30 min; (2) washed with three 50-ml portions of tetrahydrofuran; (3) added 50 ml of tetrahydrofuran and cooled the vessel to −30°; (4) generated the azide by addition of 4 *M* HCl–dioxane (0.68 ml, 2.72 mmol, 5 meq) followed by *n*-butylnitrite (0.34 ml, 2.72 mmol, 5 meq) for 1 hr; (5) washed with three 50-ml portions of precooled 10% Et_3N–THF at −30°; (6) washed with three 50-ml portions of precooled tetrahydrofuran at −30°; (7) added L-alanine Boc-hydrazide (**2b**) (552 mg, 2.72 mmol, 5 meq) in 50 ml of tetrahydrofuran precooled to −30°; (8) rocked at −30° for 2 hr, 0° for 4 hr and 23° for 16 hr; (9) washed with three 50-ml portions of tetrahydrofuran; (10) washed with three 50-ml portions of methanol. The second and third cycles were carried out exactly as described above with the exception that in step 7 glycine Boc-hydrazide (**2c**) (481 mg, 2.72 mmol, 5 meq) or L-valine *t*-butyl ester (471 mg, 2.72 mmol, 5 meq) was used. A portion of the peptide–resin (1.13 g) was cleaved with HBr in trifluoroacetic acid.[16] The crude peptide was lyophilized twice from water and purified on a 2 × 120 cm Dowex 50-X4 column in a pH 4.0 pyridine acetate cycle. The sample was applied to the column in 1 ml of water. Elution with pH 4 pyridine acetate proceeded at a rate of 81 ml/hr for a total of 25 hr. Aliquots (0.2 ml) from 5.5-ml fractions were analyzed by the ninhydrin test. The fractions between 0.85 and 0.96 l. were combined and lyophilized: yield, 33 mg (30% calculated from amount of resin-L-leucine Boc-hydrazide); $[\alpha]^{25}D$ 23.55° (*c* 0.15%, ethanol); lit.[30] $[\alpha]^{20}D$ 24.55° (*c* 0.68%; ethanol); lit.[4f] $[\alpha]^{20}D$ 22.5–23.7° (*c* 1–2%; ethanol); amino acid ratios: Leu, 0.97; Ala, 0.96; Gly, 1.07; Val, 1.00.

Anal. Calcd for $C_{16}H_{30}N_4O_5 \cdot 1.5H_2O$: C, 49.89; H, 8.63; N, 14.54. Found: C, 49.81; H, 8.37; N, 14.40.

Tlc of the tetrapeptide in three systems was identical with that of a sample previously synthesized by the standard solid phase procedure.[3a] It moved with R_f 0.64 (butanol–acetic acid–water; 8:2:2); R_f 0.71 (butanol–acetic acid–water–pyridine, 15:3:12:10); R_4 0.71 (butanol–acetic acid–ethyl acetate–water; 1:1:1).

The peptide was homogeneous when run as an analytical sample on the amino acid analyzer. The pH 3.28 buffer was changed to pH 4.25 buffer at 120 min. The peptide peak emerged 17 min after the buffer artifact (187 min total time).

Leucyl-L-alanylglycyl-L-valine (Fragment Condensation). Resin-L-leucine Boc-hydrazide (1.5 g, 0.446 mmol) was placed in the reaction vessel. The following cycle of reactions was employed: (1) Boc group cleaved by 4 *M* HCl–dioxane (50 ml) for 30 min; (2) washed with three 50-ml portions of tetrahydrofuran; (3) added 50 ml of tetrahydrofuran and cooled the vessel to −30°; (4) generated the azide by addition of 4 *M* HCl–dioxane (0.56 ml, 2.23 mmol) followed by *n*-butylnitrite (0.28 ml, 2.23 mmol) for 1 hr; (5) washed with three 50-ml portions of precooled 10% Et_3N–THF at −30°; (6) washed with three 50-ml portions of precooled tetrahydrofuran at −30°; (7) added L-alanine Boc-hydrazide (**2b**) (453 mg, 2.23 mmol) in 50 ml of tetrahydrofuran precooled to −30°; (8) rocked at −30° for 4 hr, 0° for 12 hr and 23° for 2 hr; (9) washed with three 50-ml portions of tetrahydrofuran; (10) washed with three 50-ml portions of methanol. The second cycle was carried out exactly as described above with the exception of step 7 in which glycyl-L-valine *t*-butyl ester (514 mg, 2.23 mmol) was added. The resin was cleaved with HBr in trifluoroacetic acid.[16] The crude peptide was lyophilized from water and purified on a 2 × 120 cm Dowex 50-X4 column in a pH 4.0 pyridine acetate cycle. The sample was applied to the column in 1 ml of water. Elution with pH 4 pyridine acetate proceeded at a rate of 103 ml/hr for a total of 16 hr. Aliquots (0.2 ml) from 5.5-ml fractions were analyzed by the ninhydrin test (Figure 3). The fractions between 0.87 and 1.06 l. were combined and lyophilized: yield, 102 mg (60% calculated from the amount of resin-L-leucine Boc-hydrazide); $[\alpha]^{20}D$ 26.77° (*c* 0.96%, ethanol); lit.[30] $[\alpha]^{20}D$ 24.55° (*c*, 0.68%; ethanol), lit.[4f] $[\alpha]^{20}D$ 22.5–23.7° (*c* 1–2%; ethanol); amino acid ratios: Leu, 0.97; Ala, 1.00; Gly, 0.95; Val, 1.01.

Anal. Found: C, 50.51; H, 8.01; N, 14.40.

This tetrapeptide moved with the same R_f in three tlc solvent systems as the peptide made by the stepwise route. This peptide also emerged on the amino acid analyzer at 17 min after the buffer artifact. A small peak containing 1.0% of the ninhydrin color of the main peak was found at 9 min.

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(29) M. Bergmann, L. Zervas, and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).

(30) This value was obtained on the sample prepared by Merrifield (ref 3a) when remeasured on the Perkin-Elmer Model 140 polarimeter.