deuteration and does not depend on n, the number of bonding sites.

In order to extend eq II.6 into the consideration of those systems where there exists an isotope effect, eq I.9 derived in Appendix I must be used. If eq II.6 is rewritten as

$$(\langle H^2\rangle_{nd} - \langle H^2\rangle_{nh})/(\langle H^2(\mathrm{pD})\rangle_n - \langle H^2\rangle_{nh}) = (100/\mathrm{pD})_{\mathrm{calcd}} \quad \text{(II.7)}$$

substitution of eq I.9 yields

$$\alpha = K(100/\mathrm{pD})_{\mathrm{expt}} + (1 - K) \quad \text{(II.8)}$$

where

$$\alpha = (\langle H^2\rangle_{nd} - \langle H^2\rangle_{nh})/(\langle H^2(\mathrm{pD})\rangle_n - \langle H^2\rangle_{nh})$$

Therefore, plotting α vs. $(100/\mathrm{pD})_{\mathrm{expt}}$ should yield a straight line of slope K and intercept $(1 - K)$. This means that the variation of both the second moment and the spectral line width (see Appendix I) can be used to calculate the proton–deuteron hydrogen-bonding equilibrium constant. The attainment of K by these two nonrelated techniques is highly significant. The use of line widths to determine K requires knowledge of the number of bonding sites. On the other hand, the use of the spectrum's second moment yields K directly, but states nothing about the number of bonding sites. Therefore, both of these techniques are necessary for the unequivocal determination of both the equilibrium constant and the number of hydrogen-bonding sites.

References and Notes

(1) (a) Supported in part by a Cottrell Research Grant from Research Corporation and Grant No. GM23272 by the Public Health Services. (b) First paper in this series: B. J. Hales, *J. Am. Chem. Soc.*, **97**, 5993 (1975).
(2) J. R. Harbour and G. Tollin, *Proc. Natl. Acad. Sci., U.S.A.*, **69**, 2066 (1972).
(3) M. S. Blois, H. W. Brown, R. M. Lemmon, R. O. Lindblom, and M. Weissbluth, Ed., "Free Radicals in Biological Systems", Academic Press, New York, N.Y., 1961.
(4) E. Charney and E. Becker, *J. Chem. Phys.*, **42**, 3314 (1972).
(5) B. J. Hales and J. R. Bolton, *J. Am. Chem. Soc.*, **94**, 3314 (1972).
(6) J. R. Harbour and G. Tollin, *Photochem. Photobiol.*, **19**, 147 (1974).
(7) E. W. Stone and A. H. Maki, *J. Chem. Phys.*, **36**, 1944 (1962).
(8) J. Gendell, J. H. Freed, and G. K. Fraenkel, *J. Chem. Phys.*, **37**, 2832 (1962).
(9) E. W. Stone and A. H. Maki, *J. Am. Chem. Soc.*, **87**, 454 (1965).
(10) M. R. Das and G. K. Fraenkel, *J. Chem. Phys.*, **42**, 1350 (1965).
(11) W. M. Gulick and D. H. Geske, *J. Am. Chem. Soc.*, **88**, 4119 (1966).
(12) M. Broze, Z. Luz, and B. L. Silver, *J. Chem. Phys.*, **46**, 4891 (1967).
(13) W. E. Geiger and W. M. Gulick, *J. Am. Chem. Soc.*, **91**, 4657 (1969).
(14) T. Yonezawa, T. Kawamura, M. Ushio, and Y. Nakao, *Bull. Chem. Soc. Jpn.*, **43**, 1022 (1970).
(15) C. P. Poole, "Electron Spin Resonance. A Comprehensive Treatise on Experimental Techniques", Interscience, New York, N.Y., 1967.
(16) M. W. Hanna, A. D. McLachlan, H. H. Dearman, and H. M. McConnell, *J. Chem. Phys.*, **37**, 361 (1962).
(17) G. Vincow and P. M. Johnson, *J. Chem. Phys.*, **39**, 1143 (1963).
(18) I. Morishima, K. Endo, and T. Yomezawa, *J. Am. Chem. Soc.*, **93**, 2048 (1971).
(19) I. Morishima, K. Endo, and T. Yomezawa, *Chem. Phys. Lett.*, **9**, 143, 203 (1971).
(20) I. Morishima, K. Endo, and T. Yomezawa, *J. Chem. Phys.*, **58**, 3146 (1973).
(21) A. S. Kabankin, G. M. Zhidomirov, and A. L. Bachachenko, *J. Magn. Reson.*, **9**, 199 (1973).
(22) G. M. Zhidomirov and A. S. Kabankin, *J. Magn. Reson.*, **19**, 47 (1975).
(23) J. Koller and A. Azman, *Croat. Chem. Acta*, **42**, 583 (1970).
(24) M. A. Slifkin and R. H. Walmsley, *Spectrochim. Acta, Part A*, **26**, 1237 (1970).
(25) H. Fritzsche, *Z. Phys. Chem.*, **43**, 154 (1964).
(26) T. Sakurai and H. Tagawa, *Acta Crystallogr., Part B*, **27**, 1453 (1971).

tert-Butoxycarbonylaminoacyl-4-(oxymethyl)-phenylacetamidomethyl-Resin, a More Acid-Resistant Support for Solid-Phase Peptide Synthesis[1]

A. R. Mitchell,* B. W. Erickson,* M. N. Ryabtsev,[2] R. S. Hodges, and R. B. Merrifield

Contribution from The Rockefeller University, New York, New York 10021.

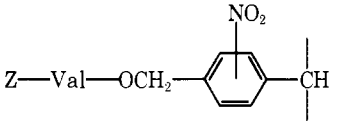
Received February 24, 1976

Abstract: Some of the peptide chains esterified to the hydroxymethyl-poly(styrene-*co*-divinylbenzene) resin are lost by acidolysis during solid-phase peptide synthesis. This loss has been minimized by using 4-(hydroxymethyl)phenylacetamidomethyl-poly(styrene-*co*-divinylbenzene) as the solid support. The phenylacetamidomethyl (Pam) bridge between the peptide and the resin is sufficiently electron withdrawing that the peptidyl-OCH_2-Pam-resin is 100 times more stable than the conventional peptidyl-OCH_2-resin to cleavage of the ester bond by 50% trifluoroacetic acid in dichloromethane. Boc-Val-OCH_2-Pam-resin, which was prepared by two routes, compared favorably with Boc-Val-OCH_2-resin for synthesis of the model peptides leucyl-alanylglycylvaline and decalysylvaline. The greater acid stability of the Pam-resin is expected to result in much higher yields of large peptides prepared by solid-phase peptide synthesis.

The solid support commonly used for solid-phase peptide synthesis,[3] *tert*-butoxycarbonylaminoacyloxymethyl-poly(styrene-*co*-divinylbenzene), is not completely stable under the acidic conditions required to remove the *tert*-butoxycarbonyl (Boc) group.[4-7] Acidolysis of the benzyl ester link between a peptide acid and the hydroxymethyl-resin is undesirable during stepwise assembly of the peptide because the yield of crude peptide obtained at the end of the synthesis is thereby decreased. In addition, the production of new hydroxymethyl sites due to premature release of peptide chains from the resin can result in the late initiation of peptides and formation of deletion peptides lacking one or more residues at the carboxyl terminus. Early acidolysis of peptide chains during solid-phase peptide synthesis can be minimized or prevented by use of a more acid-resistant bond between the peptide and the solid support as described in this paper.

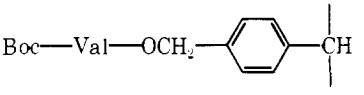
The benzyl ester link has been rendered more resistant to acid by attachment of electron-withdrawing substituents to the benzene ring.[8] This approach was originally described by Merrifield,[3] who nitrated (or brominated) every aromatic ring

of chloromethyl-poly(styrene-*co*-divinylbenzene). Subsequent esterification with N^{α}-benzyloxycarbonyl-L-valine furnished the support **1**, which served as the starting material for synthesis of the test peptide Leu-Ala-Gly-Val. N^{α}-Deprotection was accomplished with 30% HBr in acetic acid. Since the tetrapeptide nitrobenzyl ester link was not readily cleaved by acidolysis with HBr in acetic acid, the peptide was removed from the nitrated support by saponification with NaOH in aqueous ethanol. Subsequent modifications and improvements for attachment of amino acids and removal of peptides from solid supports have been reviewed.[8-11]

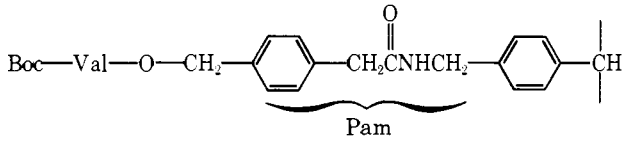


1

Gutte and Merrifield[7] began assembly of the 124-residue chain of ribonuclease A with the support **2**, a Boc-valyloxymethyl derivative of poly(styrene-*co*-1% divinylbenzene). At the end of the synthesis, about 80% of the peptide chains initially attached to the resin had been cleaved by repetitive treatment with CF_3CO_2H in CH_2Cl_2, which was used to remove the N^{α}-Boc group during each of the 123 synthetic cycles. A substituted benzyl ester support having lower stability in acid than the nitrated support **1** but greater stability than support **2** would have the desired level of stability. Thus, the benzyl ester support **3** bearing a phenylacetamidomethyl (Pam)



2



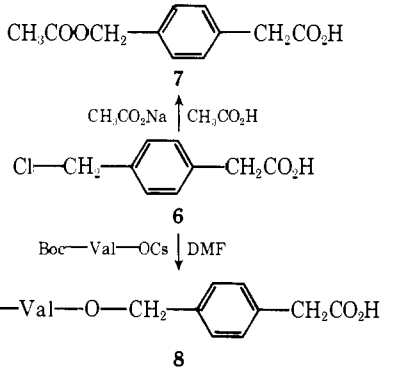
3

bridge between the polystyrene matrix and the peptide sites was designed, prepared, and evaluated.

Results and Discussion

Preparation of Boc-Val-OCH_2-Pam-Resins. Preparation of the support **3a** from chloromethyl-poly(styrene-*co*-1% divinylbenzene) is shown in Scheme I. The chloromethyl-resin (0.45 mmol of Cl/g) was converted into the phthalimidomethyl-resin **4** (0.36 mmol of N/g) and then into the aminomethyl-resin **5** (0.38 mmol of N/g). Coupling of the latter with Boc-valyl-4-(oxymethyl)phenylacetic acid (**8**) using dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 provided the desired support **3a** (0.38 mmol of Val/g). Any remaining free amino groups were blocked by acetylation with acetic anhydride–pyridine.

The key to this scheme was the successful preparation of the acid **8** by alkylation of Boc-Val cesium salt[12] with 4-(chloromethyl)phenylacetic acid[13] (**6**). Separation of the desired Boc-valyl-4-(oxymethyl)phenylacetic acid (**8**) from the starting acid **6**, Boc-Val, and other reaction products was more difficult than expected. Attempts to purify **8** by crystallization, by column chromatography on Sephadex LH-20 or silica gel, and by countercurrent distribution were not entirely satisfactory. The cyclohexylammonium salt of **8**, however, was conveniently isolated in 34% yield (based on **6**) and in analytically pure condition after preparative layer chromatography in 9:1 hexane–acetic acid.

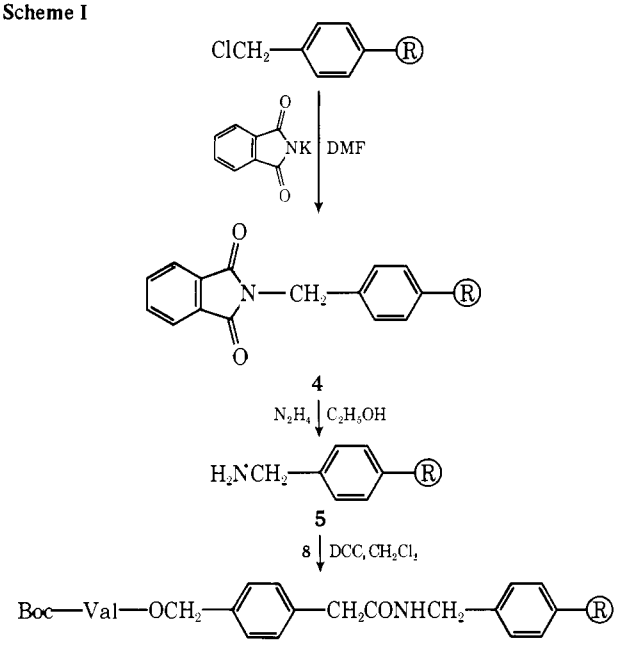


7

6

8

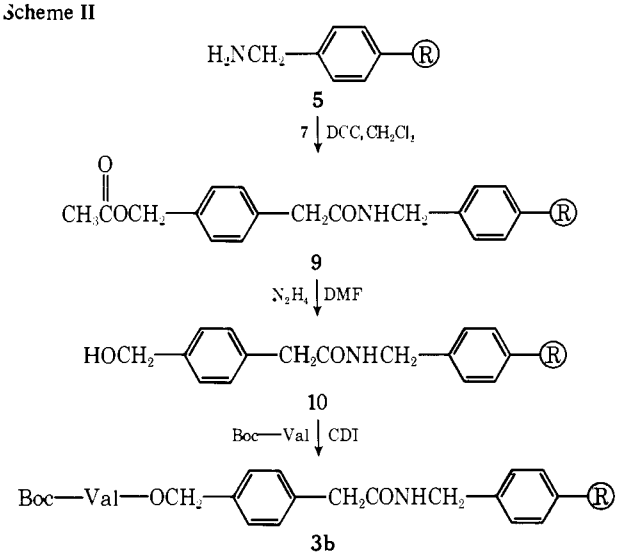
Scheme I



3a

A more general preparation of Boc-aminoacyloxymethyl-Pam-resin is illustrated in Scheme II. Aminomethyl-polystyrene-*co*-2% divinylbenzene) (**5**) was acylated with 4-(acetoxymethyl)phenylacetic acid (**7**) and DCC; the resulting acetoxymethyl-Pam-resin (**9**) was deacetylated with hydrazine in dimethylformamide. Subsequent acylation of $HOCH_2$-Pam-resin (**10**) with Boc-Val activated by *N,N'*-carbonyldiimidazole afforded the support **3b**. Any remaining aminomethyl or hydroxymethyl-Pam groups were permanently blocked by acetylation. This scheme should permit the preparation of other Boc-aminoacyloxymethyl-Pam-resins from the common intermediate **10**.

Scheme II



3b

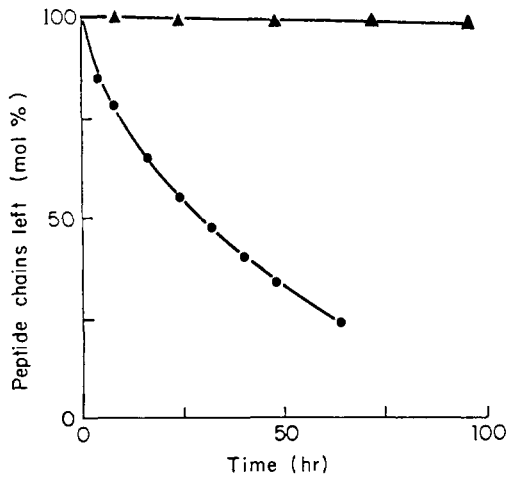


Figure 1. Loss of peptide chains from two resins in 50% CF_3CO_2H–CH_2Cl_2 at room temperature: (●) Leu-Ala-Gly-Val-OCH_2-resin from **2**; (▲) Leu-Ala-Gly-Val-OCH_2-Pam-resin from **3b**.

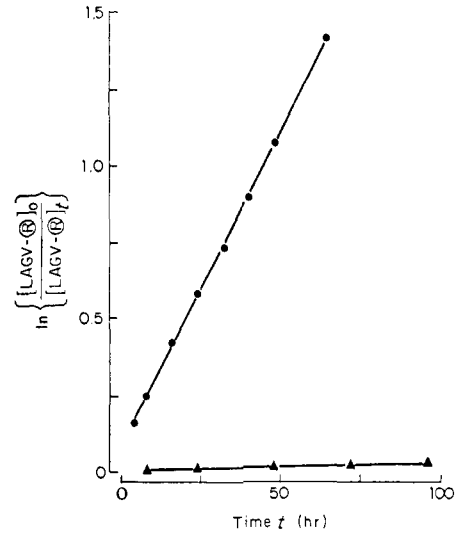
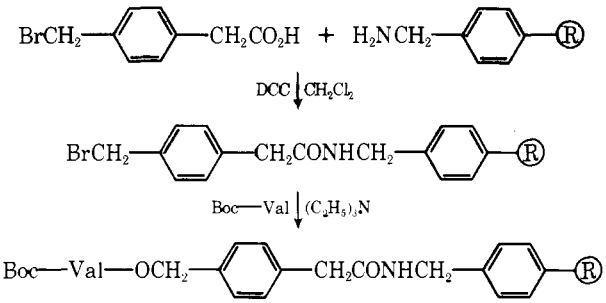


Figure 2. Calculated least-squares lines of best fit for the apparent first-order loss of Leu-Ala-Gly-Val in 50% CF_3CO_2H–CH_2Cl_2: (●) Leu-Ala-Gly-Val-OCH_2-resin from **2**; (▲) Leu-Ala-Gly-Val-OCH_2-Pam-resin from **3b**.

The acid-resistant supports originally employed in solid-phase peptide synthesis[3] contained a bulky nitro or bromo substituent on every aromatic ring of the polystyrene beads. Thus, less space was available in the nitrated or brominated resins than in the unsubstituted resin to accommodate the synthetic peptide chains. But only 1–5% of the aromatic rings of the cross-linked polystyrene support normally serve as sites for attachment of peptide chains. The schemes outlined above for preparation of the Pam-resin specifically involve the substitution of only those aromatic rings needed as sites for peptide assembly. The lack of extraneous substituents on the remaining 95–99% of the aromatic rings effectively minimizes steric hindrance by the support and maximizes the space available within the beads for the assembly of peptide chains. In contrast to the unknown position of ring nitration or bromination, the acetamidomethyl group of the Pam-resin is specifically located para to the peptidyloxymethyl group. In addition, the acetamidomethyl group serves not only as the covalent link between the benzyl ester and the solid support but also as the electron-withdrawing substituent that increases the acid stability of the benzyl ester bond.

The third route for preparation of Boc-aminoacyloxymethyl-Pam-resins shown in Scheme III employs 4-(bromomethyl)phenylacetic acid. In preliminary experiments this route furnished a model tetrapeptide in less satisfactory purity than did the previous routes and was not pursued further. Although the predominant reaction was N-acylation of the aminomethyl-resin by the DCC-activated 4-(bromomethyl)-phenylacetic acid, N-benzylation of some aminomethyl sites by the bromomethylphenylacetic acid may also have occurred. In addition, bromomethyl-Pam sites that did not react with the first Boc-amino acid may have participated in undesirable benzylation reactions later in the synthesis. A preliminary report by Sparrow[14] described the preparation of a Boc-aminoacyl-4-(oxymethyl)phenylacetamido-11-undecanoylaminomethyl-resin by a variation of Scheme III. The use of the aminoundecanoyl spacer was thought necessary to overcome possible steric hindrance by the polystyrene backbone to peptide synthesis,[15] but the acid stability of this resin was not investigated.

Scheme III



Synthesis of Model Peptides Using Pam-Resins. Boc-Valyl-4-(oxymethyl)phenylacetamidomethyl-resins **3a** and **3b** were used to prepare the model tetrapeptide Leu-Ala-Gly-Val[3,16] by solid-phase procedures. The Leu-Ala-Gly-Val-OCH_2-Pam-resins were cleaved with anhydrous HBr or, preferably, with liquid HF. The unpurified peptide obtained from each resin contained 98.0 mol % of Leu-Ala-Gly-Val as measured by ion-exchange peptide analysis.[16] The model tetrapeptide was obtained in the same purity (97.7 mol %) when the conventional support, Boc-Val-oxymethyl-poly(styrene-*co*-1% divinylbenzene) (**2**) was used. Thus, the tetrapeptide can be prepared on the Pam-resin in high purity using either 1 or 2% cross-linking and using relatively high loadings of the first amino acid (0.33, 0.65 mmol/g). Much lower loadings (0.05–0.2 mmol/g), however, are advisable for the solid-phase synthesis of large polypeptides.

The test peptide decalysylvaline was also prepared using a Boc-Val-oxymethyl-Pam-resin (0.07 mmol of Val/g). Ten lysine residues were added sequentially as N^{α}-Boc-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine as previously described.[17] The peptide mixture was cleaved from the resin in 9:1 HF–anisole and analyzed by ion-exchange chromatography on carboxymethylcellulose. The chromatogram was nearly identical with the published chromatogram[17] obtained using Boc-Val-OCH_2-resin and those obtained from several related syntheses of decalysylvaline.[18,19] The predominant product was $Lys_{10}Val$; larger peptides due to premature deprotection and subsequent growth of peptide branches from the side-chain amino groups were not present. The advantages of the Pam-resin for the synthesis of substantially larger peptides are currently being examined.

Acid Stability of Leu-Ala-Gly-Val-OCH_2-Pam-Resin. The tetrapeptide-OCH_2-resin **2** and the tetrapeptide-OCH_2-Pam-resin **3b** were treated separately with 50% trifluoroacetic acid in dichloromethane. The stability of each resin to this moderately acidic medium was determined by measuring the loss of Leu-Ala-Gly-Val with time, as shown in Figure 1. The loss of peptide chains followed apparent first-order kinetics for both resins (Figure 2). The Leu-Ala-Gly-Val-OCH_2-Pam-

Table I. Cleavage of Peptide Chains from Two Leu-Ala-Gly-Val-Resins by 50% Trifluoroacetic Acid

Solid support	k_t,[a] 10^{-8} s^{-1}	k_{rel}	% loss per cycle[b]
-OCH_2-resin **2**	578.2 ± 6.2 (6)	100	0.70
-OCH_2-Pam-resin **3b**	5.80 ± 0.92 (3)	1.0	0.007

[a] Apparent first-order rate constant and 90% confidence limits for cleavage of Leu-Ala-Gly-Val from the solid support in 1:1 (v/v) CF_3CO_2H–CH_2Cl_2 at room temperature; degrees of freedom are shown in parentheses. [b] Based on N^{α}-deprotection for 20 min/cycle.

Table II. Relative Stability of Benzylic Protecting Groups in 50% Trifluoroacetic Acid

Benzylic derivative[c]	k_t,[a] 10^{-8} s^{-1}	k_{rel}	% loss in 20 min	Ref
· · · Val-OCH_2-resin	578	[100]	0.70	*b*
Lys(Z)	396	69	0.47	17
Ser(Bzl)	11	1.9	0.013	20
Asp(OBzl)	9	1.6	0.011	20
Glu(OBzl)	7	1.2	0.009	20
Thr(Bzl)	6.7	1.2	0.008	20
· · · Val-OCH_2-Pam-resin	5.8	1.0	0.007	*b*
Lys(2,4-Cl_2Z)	4.9	0.8	0.006	17

[a] Apparent first-order rate constant. [b] This work. [c] Z, benzyloxycarbonyl; Bzl, benzyl.

resin was found to be about 100 times more stable than the conventional Leu-Ala-Gly-Val-OCH_2-resin in this medium (Table I). The loss of peptide chains per 20-min N^{α}-deprotection step is calculated to be 0.7% for the usual resin but only 0.007% for the Pam-resin. Once assembly of the desired peptide is complete, however, the benzyl ester bond of the Pam-resin is readily cleaved in high yield (87%) by treatment with anhydrous HF. Thus the advantages of increased acid stability of the anchoring bond can be achieved without sacrificing the high yield of peptide obtained by HF cleavage.

As shown in Table II, the 4-alkylbenzyl ester link of the tetrapeptide-OCH_2-resin is somewhat more labile than the N^{ϵ}-benzyloxycarbonyl group of lysine. The addition of two electron-withdrawing chloro groups to the lysine protecting group increased its acid stability about 80-fold.[17] Similarly, the presence of the electron-withdrawing Pam bridge increased the stability of the solid support 100-fold. In both cases, the modified protecting group was found to be more stable than the side-chain benzyl esters of aspartic acid and glutamic acid and the benzyl ethers of serine and threonine.[20]

This greater acid stability of the Pam-resin will result in substantially higher yields of large polypeptides. For example, the use of the Pam-resin during assembly of proteins containing about 120 residues, such as ribonuclease[7] or an immunoglobulin domain,[21] should reduce the loss of peptide chains from the support from 80% to about 4%. In addition, the late initiation and growth of shorter peptide by-products on the resulting hydroxymethyl sites will also be decreased.[8]

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (ir) spectra were taken with a Perkin-Elmer Model 237B grating infrared spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Model A-60 spectrometer. Amino acid and peptide analyses were conducted with Beckman Model 120B or 121 amino acid analyzers. Elemental analyses were performed by Mr. S. T. Bella of the Microanalytical Laboratory, The Rockefeller University. The solvents used for thin-layer chromatography (TLC) (precoated 0.25-mm silica gel GF plates, Analtech) were 99:1 chloroform–acetic acid (CA), 9:1 hexane–acetic acid (HA), and 9:1 petroleum ether (bp 30–60 °C)–acetic acid (PA). Spots were visualized with ultraviolet light (254 nm) followed by spraying with 0.2% ninhydrin in 1-butanol and heating. Preparative layer chromatography was performed using 30 × 30 × 0.5 cm plates[22] prepared with silica gel PF-254 containing $CaSO_4$ (Brinkman Instruments). All solvents and bulk chemicals were reagent grade. Boc-amino acids were obtained from Beckman Instruments. Poly(styrene-*co*-1% divinylbenzene) beads (200–400 mesh) were purchased from Bio-Rad Laboratories. Chloromethyl-poly(styrene-*co*-divinylbenzene) resin was prepared according to Feinberg and Merrifield[23] or was obtained from Schwarz BioResearch or Lab Systems. The materials and methods for solid-phase synthesis were similar to those described earlier.[3,10,16,17]

Boc-Valyloxymethyl-Resin (2). Chloromethyl-poly(styrene-*co*-1% divinylbenzene) (0.24 mmol of Cl/g, 200–400 mesh, Lab Systems; 10.0 g) was stirred for 16 h at 50 °C with a solution of the cesium salt of Boc-Val[12] (3.15 mmol) in DMF (60 ml). Resin **2** was collected, washed with DMF, DMF–H_2O (9:1), DMF, and ethanol, and dried under vacuum. Hydrolysis in 1:1 (v/v) HCl–propionic acid[24] showed the presence of 0.158 mmol of Val/g of substituted resin (0.164 mmol of Val/g of polystyrene resin).

Phthalimidomethyl-Resin (4). The reaction conditions used by Sheehan and Bolhofer[25] for the Gabriel synthesis of primary amines were adapted for the preparation of phthalimidomethyl-resin. Similar results were obtained by Weinshenker and Shen.[26] Chloromethyl-poly(styrene-*co*-2% divinylbenzene) (3.7 mmol of Cl/g, >250 mesh, Schwarz/Mann; 20.0 g), potassium phthalimide (22.2 g, 120 mmol), and DMF (400 ml) were stirred at 120 °C for 5.5 h. The resin was collected, washed successively with hot DMF, DMF–H_2O (1:1), H_2O, H_2O–dioxane (1:1), dioxane, ethanol, and methanol, and vacuum dried to give resin **4** (23.9 g): ir (KBr) 5.63 and 5.81 μm. Anal.: N, 2.56 (1.83 mmol of N/g).

Aminomethyl-Resin (5). Resin **4** (20.0 g, 36.6 mmol) and a solution of hydrazine (>95%; 16 ml, 480 mmol) in ethanol (400 ml) were heated at reflux for 3 h. The resin was collected, washed with hot ethanol, DMF, DMF–H_2O (1:1), H_2O, H_2O–dioxane (1:1), dioxane, ethanol, and methanol, and vacuum dried to provide the resin **5**, which contained 3.47% N (2.48 mmol of N/g) by elemental analysis, 2.13 mmol of NH_2/g by picric acid titration,[27] and no carbonyl groups by infrared spectroscopy. Similar procedures were used to convert chloromethyl-poly(styrene-*co*-1% divinylbenzene) beads (0.45 mmol of Cl/g, 200–400 mesh) into phthalimidomethyl-resin (0.36 mmol of N/g) and then into aminomethyl-resin (0.38 mmol of N/g).

4-(Chloromethyl)phenylacetic acid (6) was prepared by the method of Bogdanov.[13] A mixture of phenylacetic acid (64 g, 0.47 mol, 1.0 equiv), aqueous 37% formaldehyde (220 ml, 3 mol, 6 equiv), and zinc chloride (25 g, 0.17 mol, 0.4 equiv) was warmed to 75 °C. The resulting solution was stirred at 75 to 80 °C for 8 h as a slow stream of HCl gas was bubbled through the mixture, which was turbid after 30 min. The cooled mixture was partitioned between water (400 ml) and chloroform (400 ml), and the organic phase was washed with water (two 250-ml portions), dried over anhydrous sodium sulfate, and freed of solvent. The residue was crystallized from chloroform to afford the acid **6** (9.5 g, 11% yield) as white needles: mp 153.0–154.0 °C (lit.[13] mp 152–153 °C); ir (CH_2Cl_2) 3.3 (m, OH), 5.83 (s, C=O), and 7.13 μm (CH_2 bend). It was completely the para isomer by NMR ($CDCl_3$): 3.65 (s, 2 H, CH_2CO), 4.57 (s, 2 H, CH_2Cl), and 7.32 ppm (s, 4 H, *p*-C_6H_4).

4-(Acetoxymethyl)phenylacetic Acid (7). 4-(Chloromethyl)phenylacetic acid (**6**, 0.39 g, 2.1 mmol) was dissolved in a warm solution of anhydrous sodium acetate (3.4 g, 42 mmol) in acetic acid (15 ml, 260 mmol). The solution was heated near 107 °C for 9 h in a 75-ml test tube sealed with a Teflon-lined screw cap. The cooled solution was diluted with water (25 ml) and evaporated to dryness. The residue was dissolved in 0.5 M HCl (50 ml) and extracted with ethyl acetate (two 25-ml portions). The organic phase was washed with brine (10 ml), dried over anhydrous magnesium sulfate, and freed of solvent. The residual solid (0.50 g) was heated at reflux with 1:9 (v/v) benzene–hexane (40 ml) until the solid dissolved. After decantation from the insoluble oil, the clear supernatant deposited the acid **7** (0.27 g, 63%) as fine white needles: mp 84.0–86.0 °C; ir (CCl_4) 3.4 (m, OH), 5.73 (s, ester C=O), 5.83 (s, acid C=O), 7.13 (m, CH_2 bend), 7.29 and 7.38 (m, CH_3 bend), and 8.22 μm (s, COC); NMR (CF_3CO_2H) 2.10 (s, 3 H, CH_3CO), 3.70 (s, 2 H, CH_2CO), 5.08 (s, 2 H, CH_2O), and

7.19 ppm (s, 4 H, C_6H_4). Anal. Calcd for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.40; H, 5.95.

Boc-Valyl-4-(oxymethyl)phenylacetic Acid (8). The cesium salt of Boc-Val, prepared from Boc-Val (1.09 g, 5.00 mmol) and cesium bicarbonate as described by Gisin,[12] was dissolved in dimethylformamide (DMF; 15 ml) and added to a solution of 4-(chloromethyl)-phenylacetic acid (0.462 g, 2.5 mmol) in DMF (10 ml). After the solution had been stirred at 45 °C for 48 h, a fine white precipitate was removed by filtration and the filtrate was freed of solvent. The resulting oil was shown by analytical TLC (PA, two developments) to contain the product **8** (R_f 0.20) and both starting acids (R_f 0.36). It was dissolved in chloroform–acetic acid (95:5), applied to a preparative layer chromatographic plate, and developed three times with PA. Elution of the product band with ethyl acetate and evaporation of the solvent gave a clear yellow oil (0.466 g). The product was isolated as the cyclohexylammonium salt, which was recrystallized from dichloromethane–hexane to furnish the CHA salt of acid **8** (0.395 g, 34% from **6**): mp 153–154 °C; $[\alpha]^{24}D$ −22.5° (*c* 2, CH_3OH). Anal. Calcd for $C_{25}H_{40}N_2O_6$: C, 64.63; H, 8.68; N, 6.03. Found: C, 64.42; H, 8.64; N, 5.94.

Boc-Valyl-4-(oxymethyl)phenylacetamidomethyl-Resin (3a). Aminomethyl-resin (**5**; 0.200 g, 0.076 mmol) and a solution of Boc-valyl-4-(oxymethyl)phenylacetic acid (**8**; 110 mg, 0.302 mmol) in dichloromethane (2.5 ml) was shaken in a 6-ml reaction vessel[28] for 10 min. A solution of dicyclohexylcarbodiimide (62 mg, 0.302 mmol) in dichloromethane (2.5 ml) was added and the suspension was shaken for 2 h at room temperature. The resin was filtered, washed with dichloromethane (six 4-ml portions), suspended in 1:1 (v/v) pyridine–acetic anhydride (4 ml), and shaken for 2 h to acetylate the remaining free amino groups. The resin was filtered and washed with dichloromethane, dichloromethane–acetic acid (1:1), acetic acid, 2-propanol and dichloromethane, and vacuum dried to furnish the resin **3a** (0.237 g), which contained 0.332 mmol of Val/g of substituted resin (0.359 mmol of Val/g of Pam-polystyrene resin).

4-(Acetoxymethyl)phenylacetamidomethyl-Resin (9). Aminomethyl-resin (1.00 g, 2.48 mmol) was shaken for 10 min with a solution of 4-(acetoxymethyl)phenylacetic acid (**7**; 0.520 g, 2.48 mmol) in dichloromethane (10 ml). *N,N'*-Dicyclohexylcarbodiimide (0.510 g, 2.48 mmol) in dichloromethane (2 ml) was added and the suspension was shaken for 20 h at room temperature. The resin was filtered, washed with dichloromethane, and shaken in 20 ml of 1:1 pyridine–acetic anhydride for 2 h. The resin was filtered, washed, and dried as described for resin **3a.** Resin **9** showed carbonyl bands at 5.75 (ester) and 6.06 μm (amide) and contained 1.90 mmol of acetoxy/g by the method of Goddu et al.[29]

4-(Hydroxymethyl)phenylacetamidomethyl-Resin (10). The acetoxymethyl-Pam-resin **9** was shaken with 10% hydrazine in DMF for 23 h at room temperature. Resin **10** was filtered, washed with DMF, 2-propanol, and dichloromethane, and vacuum dried. It showed no infrared absorption due to ester carbonyl and contained <0.02 mmol of acetoxy/g.

Boc-Valyl-4-(oxymethyl)phenylacetamidomethyl-Resin (3b). A solution of Boc-Val (0.413 g, 1.90 mmol) and carbonyldiimidazole (0.276 g, 1.70 mmol) in dichloromethane (10 ml) was kept at −5 °C for 30 min and added to a reaction vessel containing resin **10** (1.00 g). The residual solution was washed in with CH_2Cl_2 (2 ml) and the suspension was shaken for 8 h at room temperature. The resin was filtered, washed with dichloromethane, and acetylated by being shaken with 1:1 pyridine–acetic anhydride (20 ml) for 40 min. Subsequent filtration, washing with DMF, dichloromethane, 2-propanol, and dichloromethane, and drying under vacuum afforded the resin **3b,** which contained 0.647 mmol of Val/g of substituted resin (0.760 mmol of Val/g of Pam-polystyrene resin).

Synthesis of Leucylalanylglycylvaline. A. Using Resin 2. Boc-Valyloxymethyl-resin (**2**; 2.00 g, 0.316 mmol) was placed in a reaction vessel on a shaker and treated as follows for the incorporation of each residue: (1) washed with dichloromethane (3 × 20 ml), (2) shaken with trifluoroacetic acid–dichloromethane (1:1) (20 ml) for 30 min, (3) washed with dichloromethane (3 × 20 ml), (4) shaken twice with 5% ethyldiisopropylamine in dichloromethane (20 ml) for 10 min; (5) washed with dichloromethane (6 × 20 ml), (6) 3.2 equiv of Boc-Gly in dichloromethane (15 ml) was added and mixed for 10 min, (7) 3.2 equiv of dicyclohexylcarbodiimide in dichloromethane (3 ml) was added and shaken for 2 h, and (8) washed with dichloromethane (6 × 20 ml). The cycle was repeated with 3.2 equiv of Boc-Ala in step 6, and with 3.2 equiv of Boc-Leu in the last cycle. The Boc-Leu-Ala-Gly-Val-resin was washed with 1:1 dichloromethane–acetic acid, acetic acid, 2-propanol, and dichloromethane and vacuum dried. Amino acid analysis indicated that this material contained 0.134 mmol of peptide/g of substituted resin (0.143 mmol of peptide/g of Pam-polystyrene resin) and had an amino acid composition of $Leu_{1.12}Ala_{1.00}Gly_{0.91}Val_{1.07}$.

Table III. Picric Acid Monitoring[a] during Synthesis of Boc-Leu-Ala-Gly-Val-Resin from the Boc-Val-Resin **3b**

Synthetic Cycle	Boc-Amino acid	Coupling step[b]	% coupling
1	Boc-Gly	First	99.5
1	Boc-Gly	Second	100
2	Boc-Ala	First	98.5
2	Boc-Ala	Second	99.6
3	Boc-Leu	First	99.4
3	Boc-Leu	Second	99.8

[a] See ref 27 and 31 for experimental procedure. [b] Coupling for 30 min with 2.9 equiv each of Boc-amino acid and dicyclohexylcarbodiimide.

A portion (212 mg, 28.4 μmol) of the peptide-resin was shaken with a mixture[30] of 32% hbr in acetic acid (2 ml) and trifluoroacetic acid (2 ml) for 1 h at room temperature. The liquid phase was filtered and the resin was washed with trifluoroacetic acid, trifluoroacetic acid–dichloromethane (1:1), and dichloromethane (three 2-ml portions each). After the pooled filtrates were freed of solvent, the residue was dissolved in water (5 ml) and part (1 ml) was applied on the long column (0.9 × 60 cm, AA-15 sulfonated polystyrene) of a Beckman 120B amino acid analyzer. The column was eluted at 56 °C with pH 3.49 citrate buffer (0.2 N) at 66 ml/h.[16] The presence of 21.7 μmol of Leu-Ala-Gly-Val indicated a cleavage efficiency of 77%. The desired product Leu-Ala-Gly-Val comprised 97.7 mol% of the unpurified peptide product. When the Leu-Ala-Gly-Val-resin (100 mg, 13.4 μmol) was treated with anhydrous HF (10 ml) containing anisole (1 ml) for 1 h at 0 °C, 11.6 μmol (86%) of Leu-Ala-Gly-Val was released.

B. Using Pam-Resin 3a. Boc-Valyl-4-(oxymethyl)phenylacetamidomethyl-resin **3a** (0.224 g, 0.0744 mmol) was placed in a reaction vessel and treated as described for resin **2**, except that 4 equiv of Boc-amino acid and dicyclohexylcarbodiimide were used for a 30-min coupling reaction. Amino acid analysis of the tetrapeptide-resin gave 0.288 mmol of peptide/g of substituted resin (0.333 mmol of peptide/g of Pam-polystyrene resin) and an amino acid composition of $Leu_{1.00}Ala_{1.00}Gly_{1.02}Val_{1.02}$. Cleavage of the resin in 16% HBr in 1:1 (v/v) acetic acid–trifluoroacetic acid for only 30 min released Leu-Ala-Gly-Val in 28% yield. The model tetrapeptide constituted 98.0 mol % of the total peptide mixture.

C. Using Pam-Resin 3b. Boc-valyl-4-(oxymethyl)phenylacetamido-resin **3b** (0.600 g, 0.388 mmol) was placed in the reaction vessel of a Beckman Model 990 peptide synthesizer. The synthetic procedure was similar to that described for resins **2** and **3a** except that 1 h (two 30-min reactions) of deprotection with 1:1 trifluoroacetic acid-dichloromethane was used. In addition, 2.9 equiv of Boc-amino acid and dicyclohexylcarbodiimide were coupled for 30 min followed by picric acid titration[27,31] to determine the extent of coupling; then a second coupling reaction followed by a picric acid titration was performed. The coupling yields as determined by picric acid titration are given in Table III.

Amino acid analysis of the tetrapeptide-resin gave 0.538 mmol of peptide/g of substituted resin (0.720 mmol of peptide/g of Pam-polystyrene resin) and an amino acid composition of $Leu_{0.88}Ala_{1.00}Gly_{0.95}Val_{0.97}$. Cleavage of the resin with 16% HBr in 1:1 (v/v) acetic acid–trifluoroacetic acid for 75 min released Leu-Ala-Gly-Val in 35% yield. Peptide analysis showed that the tetrapeptide represented 98.0 mol % of the mixture. Another preparation of Leu-Ala-Gly-Val-OCH_2-Pam-resin was treated with 9:1 HF–anisole at 0 °C for 1 h to afford the tetrapeptide in 87% yield.

Stability of Leu-Ala-Gly-Val-oxymethyl-Resins in 50% Trifluoroacetic Acid–Dichloromethane. Boc-Leu-Ala-Gly-Val-oxymethyl-resin (100 mg, 13.4 μmol) prepared from resin **2** was shaken with 1:1 (v/v) trifluoroacetic acid–dichloromethane (4 ml). At a given time, the resin was filtered and washed with 1:1 (v/v) trifluoroacetic

acid–dichloromethane (3 × 4 ml). The combined filtrates were evaporated in vacuo and the residue was dissolved in water for the chromatography of Leu-Ala-Gly-Val on the long column of the Beckman 120B amino acid analyzer. The color value for Leu-Ala-Gly-Val was found to be 0.71 that of valine. The resin was resuspended in 50% trifluoroacetic acid–dichloromethane (4 ml) and shaking was continued. The combined loss of Leu-Ala-Gly-Val from the resin was determined at 4 h (14.7%), 8 h (21.7%), 16 h (34.4%), 24 h (44.3%), 32 h (52.1%), 40 h (59.3%), 48 h (65.9%), and 64 h (75.8%). Figure 1 shows a plot of the peptide remaining vs. time.

Boc-Leu-Ala-Gly-Val-4-(oxymethyl)phenylacetamidomethyl-resin (100 mg, 53.8 μmol) prepared from resin **3b** was shaken with 50% trifluoroacetic acid–dichloromethane (4 ml) as described above. The combined loss of Leu-Ala-Gly-Val from the resin was monitored at 8 h (0.43%), 24 h (0.96%), 48 h (1.53%), 72 h (1.96%), and 96 h (2.26%); see Figure 1.

Acknowledgments. This work was supported in part by Grant No. AM 01260 from the U.S. Public Health Service, by funds from the Hoffmann-La Roche Foundation, by Grant-in-Aid No. 74-844 from the American Heart Association, and by funds contributed by the New York Heart Association. We thank Mr. I. C. Chu for technical assistance.

References and Notes

(1) Presented in part at the 170th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1975, Abstract BIOL-091.
(2) U.S.–U.S.S.R. Health Exchange Visitor, 1974.
(3) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(4) S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, **24**, 1010 (1970).
(5) U. Ragnarsson, S. Karlsson, and G. Lindeberg, *Acta Chem. Scand.*, **24**, 2821 (1970).
(6) P. Fankhauser, B. Schilling, P. Fries, and M. Brenner, *Pept. Proc. Eur. Pept. Symp., 11th, 1971*, 153–161 (1973).
(7) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).
(8) B. W. Erickson and R. B. Merrifield, in "The Proteins", 3rd Ed., Vol. 2, H. Neurath and R. L. Hill, Ed., Academic Press, New York, N.Y., 1976, pp 255–527.
(9) R. B. Merrifield, *Adv. Enzymol. Relat. Areas Mol. Biol.*, **32**, 221 (1969).
(10) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969.
(11) J. Meienhofer in "Hormonal Proteins and Peptides", Vol. 2, C. H. Li, Ed., Academic Press, New York, N.Y., 1973, pp 45–267.
(12) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(13) M. N. Bogdanov, *J. Gen. Chem. USSR (Engl. Transl.)*, **28**, 1670 (1958).
(14) J. T. Sparrow, *Pept.: Chem. Struct. Biol., Proc. Am. Pept. Symp., 4th*, 419–424 (1976).
(15) A. R. Mitchell, *Pept.: Chem. Struct. Biol., Proc. Am. Pept. Symp., 3rd*, 98 (1972).
(16) R. B. Merrifield, A. R. Mitchell, and J. E. Clarke, *J. Org. Chem.*, **39**, 660 (1974).
(17) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3757 (1973).
(18) B. W. Erickson and R. B. Merrifield, *Isr. J. Chem.*, **12**, 79 (1974).
(19) B. W. Erickson and C. Y. Wang, 170th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1975, Abstract ORGN-081.
(20) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).
(21) B. W. Erickson and D. E. Krieger in "Antibodies in Human Diagnosis and Therapy", E. Haber and R. M. Krause, Ed., Raven Press, New York, N.Y., 1976, pp 159–190.
(22) G. W. Clark, *J. Chromatogr.*, **34**, 262 (1968).
(23) R. S. Feinberg and R. B. Merrifield, *Tetrahedron*, **30**, 3209 (1974).
(24) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 315 (1970).
(25) J. C. Sheehan and W. A. Bolhofer, *J. Am. Chem. Soc.*, **72**, 2786 (1950).
(26) N. M. Weinshenker and C. M. Shen, *Tetrahedron Lett.*, 3281 (1972).
(27) B. F. Gisin, *Anal. Chim. Acta*, **58**, 278 (1972).
(28) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).
(29) R. F. Goddu, N. F. LeBlanc, and C. M. Wright, *Anal. Chem.*, **27**, 1251 (1955).
(30) B. F. Gisin, unpublished procedure. *Caution!* Pressure develops.
(31) R. S. Hodges and R. B. Merrifield, *Anal. Biochem.*, **65**, 241 (1975).

Reaction of Hydroxylamine with Ethyl Acetoacetate. Details of the Addition and Cyclization Steps Studied by Flow Nuclear Magnetic Resonance[1]

Michael Cocivera,* Adan Effio, Holger E. Chen, and Shiv Vaish

Contribution from the Guelph-Waterloo Center for Graduate Work in Chemistry, University of Guelph, Guelph, Ontario, Canada. *Received April 9, 1976*

Abstract: In the pH range 6.5 to 8.5, hydroxylamine adds to the keto carbonyl of ethyl acetoacetate (EAA) to form a carbinolamine intermediate, which subsequently dehydrates to form the syn and anti oxime. The syn isomer is unstable and cyclizes to form 3-methylisoxazol-5-one (MI). The conversion of the anti isomer is much slower and presumably must isomerize before cyclizing. Because the proton NMR spectra of all of these compounds can be resolved, it is possible to measure rate constants for each step. The rapid addition step causes NMR line broadening, which can be measured while flowing the H_2O solution, after mixing, to create steady-state conditions for the transients. The dehydration and cyclization rates are measured after the flow is stopped. The pH and buffer concentration appear to have no effect on the addition step, but do affect the dehydration and cyclization rates. Since the cyclization is an intramolecular reaction that involves the expulsion of ethanol, its rate is compared with hydrolysis of the *O*-methyloxime of EAA in the presence of the oxime of acetone.

Introduction

Although the synthesis and properties of pyrazoles and isoxazoles have been the subjects of many studies,[2] a survey of the literature reveals little concerning the details of the kinetics and mechanism of their formation. Recently,[3] we have reported evidence to indicate that hydroxylamine adds to acetylacetone (ACAC) to give 3,5-dimethyl-5-hydroxylisoxazoline without the intermediacy of the oxime, i.e., cyclization of the carbinolamine intermediate occurs more rapidly than dehydration. In fact, the cyclization is sufficiently rapid to affect the proton nuclear magnetic resonance (NMR) line shapes. In the present paper, we report a study of the reaction of hydroxylamine (**HA**) with ethyl acetoacetate (EAA) using the NMR of flowing liquids. The acyl carbonyl of EAA is not as active as the keto carbonyl of **ACAC**, and cyclization occurs via the syn oxime to form 3-methylisoxazol-5-one (MI), i.e., dehydration of the carbinolamine (CA) is faster than cyclization. Because of the detailed information available in the NMR spectra during and after flowing, it has been possible