Use of Chlorinated Benzyloxycarbonyl Protecting Groups to Eliminate N$^{\epsilon}$-Branching at Lysine during Solid-Phase Peptide Synthesis[1]

Bruce W. Erickson and R. B. Merrifield*

Contribution from The Rockefeller University, New York, New York 10021.
Received December 13, 1972

Abstract: Six ring-chlorinated derivatives of N^{ϵ}-benzyloxycarbonyl-L-lysine were synthesized and their apparent first-order rates of deprotection in 50:50 (v/v) trifluoroacetic acid–dichloromethane at 20° were quantitatively determined by ion-exchange chromatography. The lysine derivatives and their rate constants (k, 10^{-8} sec^{-1}) follow in order of increasing stability: N^{ϵ}-benzyloxycarbonyl-L-lysine (396) < N^{ϵ}-(4-chlorobenzyloxycarbonyl)-L-lysine (138) ≪ N^{ϵ}-(2-chlorobenzyloxycarbonyl)-L-lysine (6.3) < N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine (4.9) < N^{ϵ}-(3,4-dichlorobenzyloxycarbonyl)-L-lysine (2) < N^{ϵ}-(3-chlorobenzyloxycarbonyl)-L-lysine (0.49) < N^{ϵ}-(2,6-dichlorobenzyloxycarbonyl)-L-lysine (0.4). Each of these derivatives except the last was completely deprotected by treatment with liquid HF for 60 min at 0°. Although stronger conditions (60 min at 20°) were needed to remove the 2,6-dichlorobenzyloxycarbonyl group, it may be useful in the synthesis of large, acid-insensitive peptides. Both the benzyloxycarbonyl group and its 4-chloro analog are too unstable in 50% trifluoroacetic acid–dichloromethane to provide adequate N^{ϵ}-protection of lysine during the synthesis of large peptides. The 2-chloro, 3-chloro, 2,4-dichloro, and 3,4-dichloro analogs are recommended for N$^{\epsilon}$-protection of lysine during solid-phase peptide synthesis. The N$^{\epsilon}$-protecting group best suited for synthesis of a given peptide will vary with the acid sensitivity of the peptide, the number and location of its lysine residues, and the acid stability of the N$^{\alpha}$-protecting group used. The extent of branching at lysine residues during solid-phase synthesis was examined by synthesis of deca-(L-lysyl)-L-valine, once using N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine and once using N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine. N$^{\epsilon}$-Deprotection and subsequent N$^{\epsilon}$-branching were enhanced by removal of the *tert*-butyloxycarbonyl group during each cycle by treatment for 1 hr with 50% trifluoroacetic acid–dichloromethane. When the benzyloxycarbonyl group was used for N$^{\epsilon}$-protection, 30 mol % of the peptides synthesized were branched at lysine. In contrast, when the 2,4-dichlorobenzyloxycarbonyl group was used, no branched peptides (<0.2 mol %) were detected by carboxymethylcellulose chromatography.

The solid-phase synthesis of a peptide containing a lysine residue requires the use of two types of amino-protecting groups. The relatively stable N$^{\epsilon}$-protecting group must survive repeated removal of the relatively labile N$^{\alpha}$-protecting group during the synthesis and yet be removed at the end of the synthesis. The purity of the desired peptide is strongly influenced by the relative stability of these protecting groups. If the N$^{\epsilon}$-protecting group were lost during removal of the N$^{\alpha}$-protecting group, the next amino acid would also couple with the free ϵ-amino group. The resulting N$^{\epsilon}$-branched peptide would couple with subsequent amino acids at both the α-amino group of the main chain and the α-amino group of the peptide side chain.

The benzyloxycarbonyl (Z) group has generally been used for N$^{\epsilon}$-protection of lysine when the *tert*-butyloxycarbonyl (Boc) group was used for N$^{\alpha}$-protection. The Z group is removed by acidolysis in a strongly acidic medium such as liquid HF or trifluoroacetic acid saturated with HBr. The more labile Boc group is cleaved in a moderately acidic medium such as dilute HCl in acetic acid or trifluoroacetic acid in dichloromethane.

This choice of amino-protecting groups has not proven entirely satisfactory. Yaron and Schlossman[2] investigated the solid-phase synthesis of a nonalysine derivative from Boc-Lys(Z) using 1.0 *M* HCl in acetic acid for N$^{\alpha}$-deprotection; about 40% of the peptides formed were branched at lysine due to premature loss of Z groups during acidolysis of the Boc groups. Yet only about 5% of the peptides were N$^{\epsilon}$-branched when 0.5 *M* HCl in acetic acid was used for N$^{\alpha}$-deprotection. Grahl-Nielsen and Tritsch,[3] however, observed no formation of N$^{\epsilon}$-branched peptides during solid-phase synthesis of a decalysine using 1.0 *M* HCl in acetic acid for N$^{\alpha}$-deprotection. Ontjes and Anfinsen[4] found that 24-hr treatment of Boc-Lys(Z) with 4 *M* HCl in dioxane afforded 20% free lysine, with 1 *M* HCl in acetic acid furnished 39% lysine, and with trifluoroacetic acid produced over 60% lysine.

Kinetics of N$^{\epsilon}$-Deprotection in Solution. A quantitative kinetic study[5] of the acidolysis of several benzylic protecting groups commonly used in solid-phase peptide synthesis has shown that the rate of deprotection of N^{ϵ}-benzyloxycarbonyllysine is exceeded only by that of *O*-benzyltyrosine. In 50:50 (v/v) trifluoroacetic acid–dichloromethane (50% CF_3COOH), a medium[6] often used in solid-phase synthesis for removal of the N^{α}-Boc group in the presence of side-chain protecting groups, N^{ϵ}-benzyloxycarbonyllysine was 37–59 times less stable than *O*-benzylserine, aspartic

(1) Preliminary communication: B. W. Erickson and R. B. Merrifield in "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium, Boston, Mass., June 1972," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 191. This work was supported in part by Grant No. AM 01260 from the U. S. Public Health Service and by funds from the Hoffmann-La-Roche Foundation.

(2) A. Yaron and S. F. Schlossman, *Biochemistry*, 7, 2673 (1968).

(3) O. Grahl-Nielsen and G. L. Tritsch, *Biochemistry*, **8**, 187 (1969).

(4) D. A. Ontjes and C. B. Anfinsen in "Peptides, Chemistry and Biochemistry, Proceedings of the First American Peptide Symposium, Yale University, August 1968," B. Weinstein and S. Lande, Ed., Marcel Dekker, New York, N. Y., 1970, p 79.

(5) B. W. Erickson and R. B. Merrifield, *J. Amer. Chem. Soc.*, **95**, 3750 (1973).

(6) B. Gutte and R. B. Merrifield, *J. Biol. Chem.*, **246**, 1922 (1971).

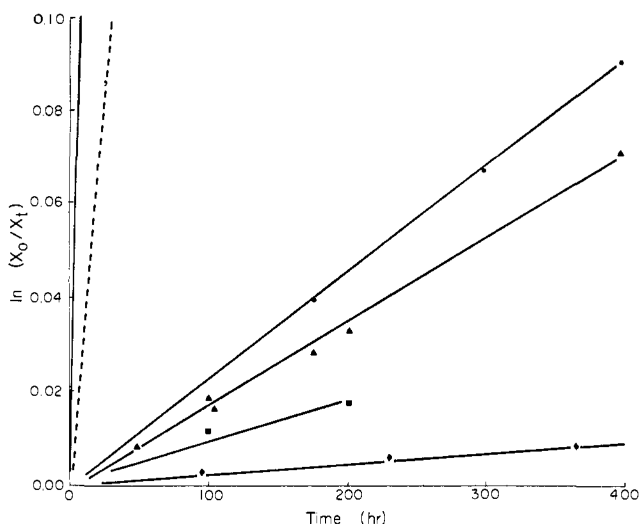

Figure 1. Calculated least-squares lines of best fit for the apparent first-order deprotection of lysine derivative X by 50% CH_3COOH at 20°: (—) Lys(Z), (- -) Lys(4-ClZ), (●) Lys(2-ClZ), (▲) Lys (2,4-Cl_2Z), (■) Lys(3,4-Cl_2Z), (◆) Lys(3-ClZ).

acid β-benzyl ester, glutamic acid γ-benzyl ester, and *O*-benzylthreonine.

These results prompted a systematic search for N^ϵ-protecting groups more stable in 50% CF_3COOH and yet labile in HF. Several kinetic studies[7-12] have demonstrated that benzyloxycarbonyl groups bearing electron-withdrawing substituents on the aromatic ring undergo acidolysis more slowly than Z itself. Certain substituents render the Z group so stable that the lysine residues are resistant to acidolysis at the end of the synthesis. Lys(4-NO_2Z) and Lys(4-CNZ), for example, are stable in liquid HF at 0° for at least 12 hr.[10] The chloro substituent, however, is small, chemically stable, and weakly, moderately, or strongly electron-withdrawing depending on the position of substitution. Thus the acid stability of several ring-chlorinated derivatives of Lys(Z) was examined.

a. 50% CF_3COOH. The 2-chloro, 3-chloro,[10,12] 4-chloro,[10-12] 2,4-dichloro, 2,6-dichloro, and 3,4-dichloro derivatives of Lys(Z) were prepared and subjected to 50% CF_3COOH at 20° for periods up to 985 hr. The relative amounts of the Lys(Z) derivative, X, and free lysine were quantitatively measured by ion-exchange chromatography. A column of sulfonated polystyrene was eluted with pH 7 citrate buffer at elevated temperatures (76–95°) to overcome partially the strong π bonding of the Lys(ClZ) and Lys(Cl_2Z) derivatives to the resin. Using the rate law

$$kt = \ln (X_0/X_t)$$

and the conservation equation

$$X_0 = X_t + (\mathrm{Lys})_t$$

the apparent first-order rate constant, *k*, for deprotection of X was determined from the slope of the least-squares line of best fit through data points plotted on a grid of ln (X_0/X_t) *vs.* *t* (Figure 1). The rate constant and the rate relative to the rate of deprotection of Lys(Z) are given in Table I. Also shown is the calculated percentage of the protecting group lost during exposure to 50% CF_3COOH for 20 min, which is sufficient time to remove the N^α-Boc group during solid-phase synthesis.[6,13]

Table I. Deprotection of Chlorinated Derivatives of N^ϵ-Benzyloxycarbonyllysine in 50% CF_3COOH

Lysine derivative	k,[a] 10^{-8} sec^{-1}	k_{rel}	Loss per cycle,[b] %
Lys(Z)	395.6 ± 4.0 (10)	[100]	0.473
Lys(4-ClZ)	137.9 ± 1.7 (3)	35	0.166
Lys(2-ClZ)	6.32 ± 0.56 (2)	1.6	0.0076
Lys(2,4-Cl_2Z)	4.91 ± 0.24 (5)	1.2	0.0059
Lys(3,4-Cl_2Z)	2.4 ± 1.3 (1)	0.6	0.003
Lys(3-ClZ)	0.485 ± 0.023 (3)	0.12	0.00062
Lys(2,6-Cl_2Z)	0.39 ± 0.18 (1)	0.1	0.0005

[a] Apparent first-order rate constant and 90% confidence limits for deprotection with 50% (v/v) CF_3COOH–CH_2Cl_2 at 20°; degrees of freedom are shown in parentheses. [b] Based on treatment with 50% CF_3COOH for 20 min/cycle.

b. HF. The addition of chloro substituents to the benzyloxycarbonyl group increased its stability not only in 50% CF_3COOH but also in the strongly acidic medium used for side-chain deprotection at the end of the synthesis. The first six lysine derivatives listed in Table I were completely deprotected by treatment with liquid HF for 1 hr at 0°. About 1.3% of Lys(3-ClZ) remained protected after treatment with HF for 40 min at 0°, but deprotection was complete at 60 min. After treatment of Lys(2,6-Cl_2Z) with HF for 60 min at 0°, however, about 15% was still protected; complete deprotection was achieved in 60 min at 20°.

Kinetics of N^ϵ-Deprotection on a Solid Support. The validity of using kinetic data measured in solution to predict the stability of N^ϵ-protecting groups under solid-phase conditions was tested by measuring the loss of N^ϵ-benzyloxycarbonyl groups during solid-phase synthesis of decalysylvaline. Valine was selected as the C-terminal residue of the test peptide to allow determination of the number of lysine residues in an isolated peptide by measurement of the Lys:Val ratio after hydrolysis.

N^α-Boc-valine was esterified[14] to hydroxymethyl-resin, which had been prepared from chloromethyl-copoly(styrene–1% divinylbenzene) beads by a new procedure. The Boc-Val-OCH_2-resin was deprotected for 1 hr with 50% CF_3COOH, neutralized with ethyldiisopropylamine, coupled for 1 hr with 3 equiv each of Boc-Lys(Z) and dicyclohexylcarbodiimide, and again neutralized and coupled in the same way. These excessive deprotection conditions were purposely used to enhance the formation of branched peptides and

(7) K. Blaha and J. Rudinger, *Collect. Czech. Chem. Commun.*, 30, 585 (1965).
(8) R. B. Homer, R. B. Moodie, and H. N. Rydon, *J. Chem. Soc.*, 4403 (1965).
(9) J. Meienhofer in "Peptides, Proceedings of the Sixth European Peptide Symposium, Athens, Greece, September 1963," L. Zervas, Ed., Pergamon Press, London, 1966, p 55.
(10) K. Noda, S. Terada, and N. Izumiya, *Bull. Chem. Soc. Jap.*, 43, 1883 (1970).
(11) S. Sakakibara, T. Fukuda, Y. Kishida, and I. Honda, *Bull. Chem. Soc. Jap.*, 43, 3322 (1970).
(12) E. Schnabel, H. Klostermeyer, and H. Berndt, *Justus Liebigs Ann. Chem.*, 749, 90 (1971).
(13) S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, 24, 1010 (1970).
(14) M. Bodanszky and J. T. Sheehan, *Chem. Ind. (London)*, 1597 (1966).

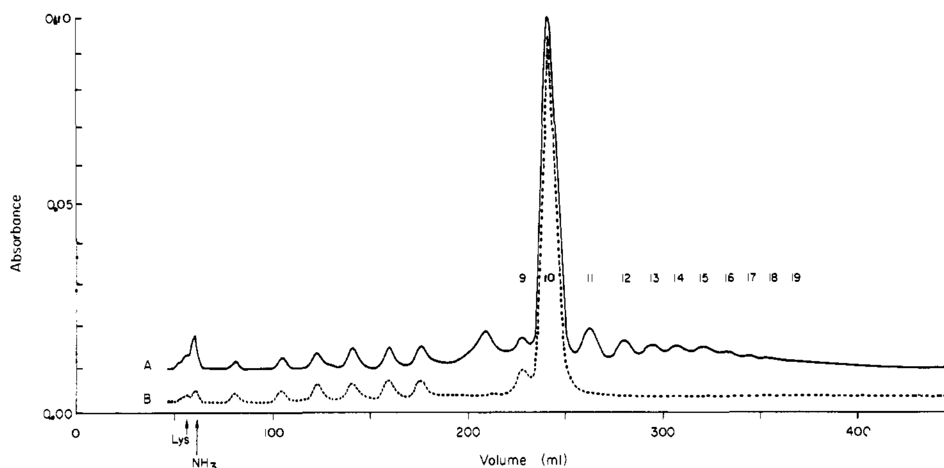

Figure 2. Carboxymethylcellulose chromatography of peptide mixtures formed during synthesis of decalysylvaline. The column (0.9 × 50 cm) was eluted at 65 ml/hr with a 250-ml constant-volume gradient of 0.2 M NaCl to 1.0 M NaCl buffered at pH 5.5. The eluate was continuously reacted with ninhydrin and monitored spectrophotometrically at 570 nm. Curve A shows peptides formed using Boc-Lys(Z); curve B shows peptides produced using BocLys(2,4-Cl_2Z). The Lys:Val molar ratios are shown above later peaks.

thus give more accurate rate data. Repetition of this cycle nine more times and treatment of the resin with HF and anisole for 1 hr at 25° furnished a mixture of peptides.

This mixture of synthetic peptides was chromatographed on carboxymethylcellulose[15] and quantitatively detected by continuous ninhydrin assay (curve A of Figure 2), a system previously employed[2] for separation and analysis of N^{α}-(2,4-dinitrophenyl)-oligolysine peptides. The elution volume of an oligolysine peptide in this system is primarily dependent on its total number of free amino groups and is independent of the number or length of its N^{ϵ} branches. The relative molar amounts of decalysylvaline and the branched peptides were obtained by dividing the area of the Lys_xVal peak by the relative molar ninhydrin color value[16] of Lys_x. The mole fraction, $\chi_n = \chi_{20-x}$, of the Lys_xVal peptides was calculated by dividing the relative molar amount of Lys_xVal by the total relative molar amount of all Lys_xVal, x = 10–19 (Table II).

If, after n residues of Lys(Z) have been coupled to the valyl-resin, one of the Lys(Z) residues loses its N^{ϵ}-protecting group during acidolysis of the N^{α}-Boc group, both the α-amino group of the N-terminal lysine residue and the free ϵ-amino group of the prematurely deprotected lysine residue will be available for coupling with Boc-Lys(Z). The N^{ϵ}-branched peptides thus formed will possess two α-amino groups and will couple with 2 equiv of Boc-Lys(Z) during subsequent cycles of the synthesis to generate a family of branched peptides containing $20 - n$ lysine residues. If in the course of the synthesis each peptide chain loses no more than one Z group,[18] then during treatment of the Boc-[Lys(Z)]$_n$Val-OCH_2-resin with 50% CF_3COOH

(15) E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, **78**, 751 (1956).

(16) For $x > 7$, the molar ninhydrin color value, C_x, of the linear peptide Lys_x relative to $C_{Lys} = 1.00$ is given by the expression[17] $C_x = 0.387x + 0.441$. The value of C_x increases linearly from $C_{10} = 4.31$ to $C_{19} = 7.79$.

(17) Derived from an equivalent expression based on peptide weight; see A. Yaron, M. C. Otey, H. A. Sober, E. Katchalski, S. Ehrlich-Rogozinski, and A. Berger, *Biopolymers*, **11**, 607 (1972).

(18) This assumption is justified because, based on the average rate of deprotection of Lys(Z) residues calculated from χ_n, only 3.4 mol % of the chains lost more than one Z group.

Table II. Mole Fractions of Branched Peptides and Rates of Deprotection of Lys(Z) Residues by 50% CF_3COOH during a Solid-Phase Synthesis of Decalysylvaline using Boc-Lys(Z)

Cycle n[a]	Lys:Val ratio[b]	$10^2\chi_n$[c] Obsd	$10^2\chi_n$[c] Calcd[d]	k_n,[e] 10^{-6} sec^{-1}
1	19	0.8	0.7	2.2
2	18	1.3	1.4	1.9
3	17	1.8	2.1	1.8
4	16	2.4	2.7	1.8
5	15	3.4	3.4	1.9
6	14	3.9	4.0	1.7
7	13	4.4	4.8	1.7
8	12	5.6	5.5	1.8
9	11	7.2	6.2	2.2
	10	69.0	69.2	

[a] Number of Lys(Z) residues that were coupled to Val-resin when the Z group was lost. [b] For the family of peptides derived from branching during the nth cycle. [c] Mole fraction of all peptides containing 10–19 lysine residues is taken as one. [d] Based on an average rate of deprotection per Lys(Z) residue of 1.9 × 10^{-6} sec^{-1}, the average of the observed k_n. [e] Average apparent first-order rate of loss of Z from the Lys(Z) residues present during the nth cycle; see text.

the average apparent first-order rate constant for the loss of Z from a Lys(Z) residue is given by the equation

$$k_n = \chi_n/nt$$

Based on the observed χ_n and the deprotection time t of 1.00 hr, the values of k_n fall in the range of 1.7–2.2 × 10^{-6} sec^{-1} (Table II).

The average value of k_n (1.9 × 10^{-6} sec^{-1}) for removal of Z from a Lys(Z) residue by 50% CF_3COOH during the solid-phase synthesis of decalysylvaline is about half of the value of k (3.96 × 10^{-6} sec^{-1}) for removal of Z from free Lys(Z) dissolved in 50% CF_3COOH. The agreement of these rate constants is reasonable, since the α-amino and α-carboxyl groups of lysine were involved in peptide bonds during the synthesis but were unprotected during the kinetic experiments in solution. These concordant results show that the stability of a side-chain-protected residue within a resin-bound peptide chain is predicted satisfactorily by the stability of the corresponding side-chain-protected amino acid in solution.

Evaluation of N$^{\epsilon}$-Protecting Groups. The usefulness of these ring-chlorinated benzyloxycarbonyl groups for N$^{\epsilon}$-protection of lysine during synthesis of a large peptide can be predicted (a) by estimating their deprotection rates relative to that of N^{α}-Boc or (b) by estimating the fraction of the peptide that would not undergo branching at lysine.

a. **Relative Rates.** As a practical criterion, an N$^{\epsilon}$-protecting group can be considered sufficiently stable for use in solid-phase synthesis if less than 0.05% of this group is lost during removal of more than 99.95% of the N$^{\alpha}$-protecting group. Since the N$^{\alpha}$-deprotecting reagent is usually present in great excess, the ratio of the apparent first-order rate constants for loss of the amino-protecting groups should be at least

$$k_{\alpha}/k_{\epsilon} = \ln(0.0005)/\ln(0.9995) = 1.5 \times 10^4$$

This rate ratio should permit adequate discrimination between the amino-protecting groups.

The deprotection of N^{α}-Z-glycine benzylamide by 0.1 *M* HBr in acetic acid at 20° is reported[19] to be about 1700 times slower than deprotection of N^{α}-Boc-glycine benzylamide. If the deprotection of N^{ϵ}-Z-lysine in 50% CF_3COOH at 20° is thus assumed to be about 1700 times slower than deprotection of an N^{α}-Boc peptide, then from Table I the 4-chloro, 2-chloro, and 3-chloro derivatives of N^{ϵ}-Z-lysine are deprotected about 5×10^3, 10^5, and 10^6 times slower than an N^{α}-Boc peptide, respectively. Thus the Z and 4-ClZ groups are too acid labile for adequate N$^{\epsilon}$-protection when Boc is used for N$^{\alpha}$-protection. The 2-ClZ, 2,4-Cl_2Z, and 3,4-Cl_2Z groups should each provide sufficient N$^{\epsilon}$-protection during the solid-phase synthesis of relatively large peptides. Finally, the 3-ClZ and 2,6-Cl_2Z groups should afford suitable N$^{\epsilon}$-protection for the synthesis of very large peptides, since these groups are calculated to suffer less than 0.01% acidolysis during removal of greater than 99.99% of the N^{α}-Boc group with 50% CF_3COOH.

b. **N$^{\epsilon}$-Branching in Model Peptides.** The second method for predicting the usefulness of an N$^{\epsilon}$-protecting group is estimation of the fraction of peptide chains that would *not* undergo branching at lysine during synthesis of a large model peptide. In this context, three large peptides derived from the human immunoglobulin Eu[20] are useful models:[5,21] the 108-residue variable region of the light chain, the 214-residue light chain, and the 440-residue heavy chain, which is one of the largest peptides sequenced to date.

When using the Boc group for N$^{\alpha}$-protection and a 20-min treatment with 50% CF_3COOH for N$^{\alpha}$-deprotection,[6] the solid-phase synthesis of these large model peptides will succeed for only certain choices of the N$^{\epsilon}$-protection group (Table III). Based on the deprotection rates measured in solution (Table I), the Z and 4-ClZ groups are sufficiently acid labile that most peptide chains would undergo branching at lysine during synthesis of the light-chain variable region if either group were used for N$^{\epsilon}$-protection. An attempt to synthesize the heavy chain using these groups would result in essentially every chain being branched at one or more lysine residues. In contrast, use of the 3-ClZ or 2,6-Cl_2Z group during synthesis of the heavy chain would result in less than 5% of the chains being N$^{\epsilon}$-branched. Use of the 2-ClZ, 2,4-Cl_2Z, or 3,4-Cl_2Z group would be acceptable for synthesis of the 108-residue peptide, marginally acceptable for synthesis of the 214-residue light chain, and unacceptable for synthesis of the 440-residue heavy chain.

These predictions should not require substantial modification (a) if the rates of deprotection of residues incorporated in resin-bound peptide chains were 2–3 times slower than the rates in solution, (b) if the N^{α}-Boc groups were deprotected with a medium of somewhat lower acidity, such as 20–25% CF_3COOH,[6,22,23] or (c) if a slightly more acid-labile group, such as 4-methoxybenzyloxycarbonyl or furfuryloxycarbonyl, were used for N$^{\alpha}$-protection. The estimated values in Table III are percentages of peptides unbranched at lysine before purification. Many, but not all, of the N$^{\epsilon}$-branched peptides, however, would have molecular weights, shapes, or net charges sufficiently different from the desired peptide that they could be removed by present separation techniques at the end of the synthesis.

A substantially different synthetic strategy is the use of a highly acid-labile N$^{\alpha}$-protecting group, such as the 2-nitrophenylsulfenyl (Nps) group or the 2-(4-biphenylyl)-2-propyloxycarbonyl (Bpoc) group. For example, Paul and Kask[24] observed no branching at lysine during solid-phase synthesis of nonalysine and lower oligomers from Nps-Lys(Z) using 1 *M* HCl in acetic acid for N$^{\alpha}$-deprotection. The N^{α}-Bpoc

Table III. Yields of Peptides Unbranched at Lysine Calculated for Solid-Phase Syntheses of Three Peptides from Human Immunoglobulin Eu[a]

Peptide	Variable region of light chain		Complete light chain		Complete heavy chain	
Mol wt	11,788 d		23,399 d		48,492 d	
Sequence	1–108		1–214		1–440	
Lysine residues	7		15		31	
Acidic cycles[b]	475		1824		7595	
	Mol % of unbranched peptides					
Lysine derivative	50% TFA	1% TFA	50% TFA	1% TFA	50% TFA	1% TFA
Lys(Z)	10.6	95.6	0.0	84.1	0.0	48.6
Lys(4-ClZ)	45.5	98.4	4.8	94.1	0.0	77.7
Lys(2-ClZ)	96.5	99.9	87.1	99.7	56.2	98.9
Lys(2,4-Cl_2Z)	97.2	99.9	89.8	99.8	63.9	99.1
Lys(3,4-Cl_2Z)	98.7	99.9	94.9	99.9	80.4	99.6
Lys(3-ClZ)	99.7	99.9	98.9	99.9	95.7	99.9
Lys(2,6-Cl_2Z)	99.8	99.9	99.2	99.9	96.5	99.9

[a] Based on deprotection by 50% CF_3COOH (TFA) (for rate *k*, see Table I) or by 1% TFA (assumed rate of *k*/50) for 20 min/cycle. [b] Sum of the acid exposures of the individual lysine residues, where the acid exposure of the lysine residue at position *P* from the N-terminus is *P* − 1 cycles.

(19) G. Losse, D. Zeidler, and T. Grieshaber, *Justus Liebigs Ann. Chem.*, 715, 196 (1968).
(20) G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, and M. J. Waxdal, *Proc. Nat. Acad. Sci. U. S.*, 63, 78 (1969).
(21) The percentages of lysine residues found in these peptides (6.5, 7.0, and 7.0%) are very close to the average percentage of lysine residues (7.0%) found in representative sequences of 108 different types of peptides and proteins; see M. O. Dayhoff and L. T. Hunt in "Atlas of Protein Sequence and Structure 1972," Vol. 5, M. O. Dayhoff, Ed., National Biomedical Research Foundation, Washington, D. C., 1972, p D-355.
(22) C. L. Krumdieck and C. M. Baugh, *Biochemistry*, 8, 1568 (1969).
(23) A. Marglin, *Tetrahedron Lett.*, 3145 (1971).
(24) W. E. Paul and A. M. Kask, *Immunology*, 21, 575 (1971).

group, which is about 3000 times less stable than N^{α}-Boc in 80% aqueous acetic acid,[25] can be removed within 10 min under mildly acidic anhydrous conditions such as 0.5% CF_3COOH–CH_2Cl_2.[26] The percentages given in Table III indicate that the strategy of deprotecting the N^{α}-Bpoc group with 1% CF_3COOH for 20 min while using Z or 4-ClZ for N^{ϵ}-protection would still furnish an unacceptably low yield of peptides unbranched at lysine during synthesis of the light or heavy chains. The other chlorinated Z groups would provide adequate N^{ϵ}-protection under these conditions.

Thus even when using a highly acid-labile N^{α}-protecting group, the Z or 4-ClZ group would generally not provide suitable N^{ϵ}-protection during the solid-phase synthesis of large peptides. Since the removal of the 2,6-Cl_2Z group by HF is relatively slow, this group is not useful for N^{ϵ}-protection of acid-sensitive peptides, although it may be beneficial in the synthesis of very large acid-insensitive peptides. The 2-ClZ, 3-ClZ, 2,4-Cl_2Z, and 3,4-Cl_2Z groups are generally recommended for N^{ϵ}-protection of lysine residues and prevention of N^{ϵ}-branching during the solid-phase synthesis of peptides. The group best suited for a given peptide will depend on the acid sensitivity of the peptide, the number and location of its lysine residues, and the acid lability of the N^{α}-protecting group used.

Elimination of N^{ϵ}-Branching in Decalysylvaline. When a 1-hr treatment with 50% CF_3COOH was used for N^{α}-deprotection, about 30% of the peptides formed during the solid-phase synthesis of decalysylvaline from Boc-Lys(Z) were branched at lysine (Table II). Since the lysine residue at position P from the N-terminus was treated with 50% CF_3COOH for $P - 1$ hr, the total time of acid exposure of the individual lysine residues was 45 hr. Based on the deprotection rate of Lys(2,4-Cl_2Z) in solution (Table I), we calculated that N^{ϵ}-branching would be below the present limit of detection (0.2 mol %) if 2,4-Cl_2Z were used for N^{ϵ}-protection. Specifically, the Lys_{11}Val peak was expected to be less than 0.16 mol %.

This prediction was tested by repeating the solid-phase synthesis of decalysylvaline using the same resin, reagents, and procedures as before, except that Boc-Lys(2,4-Cl_2Z) was used instead of Boc-Lys(Z). Indeed, no peptides containing eleven or more lysine residues were detected by chromatography of the resulting peptide mixture (curve B of Figure 2). Use of the 2-ClZ, 3-ClZ, 2,6-Cl_2Z, or 3,4-Cl_2Z group for N^{ϵ}-protection would also reduce N^{ϵ}-branching in this synthesis to the point that the branched peptides would be undetectable in this sensitive analytical system. Thus N^{ϵ}-branching in solid-phase synthesis can be eliminated by use of a sufficiently acid-stable N^{ϵ}-protecting group.

The source of the six small peaks following the ammonia peak has not yet been determined. Among the possible sources are late chain initiation at newly formed hydroxymethyl sites or premature chain termination. The relative distribution of these peaks rules out incomplete N^{α}-deprotection or incomplete coupling as their source. However, incomplete N^{α}-deprotection and/or coupling was the source of the deletion peptides Lys_9Val and Lys_8Val. Based on the relative amounts of Lys_{10}Val (94.2 mol %), Lys_9Val (5.0 mol %), and Lys_8Val (0.8 mol %) and the absence of Lys_7Val (< 0.2 mol %), the combined efficiency of the N^{α}-deprotection and coupling steps is calculated to have averaged 99.4% per cycle.

(25) P. Sieber and B. Iselin, *Helv. Chim. Acta*, **51**, 614, 622 (1968).
(26) S. S. Wang and R. B. Merrifield, *Int. J. Protein Res.*, **1**, 235 (1969).

Experimental Section

Instrumentation. Infrared spectra were taken with a Perkin-Elmer Model 257 grating infrared spectrophotometer using KBr pellets. Melting points were determined in glass capillary tubes with a Thomas-Hoover melting-point apparatus. Automated ion-exchange separation and ninhydrin assay of amino acids, protected amino acids, and peptides were conduced with a Beckman Model 120B amino acid analyzer. Thin layer chromatography was conducted with Analtech plates precoated with a 0.25-mm layer of silica gel G. Spots were visualized by spraying with 0.2% ninhydrin in 1-butanol and heating. Elemental analyses were performed by Mr. S. T. Bella of the Microanalytical Laboratory, The Rockefeller University.

Materials. All solvents and bulk chemicals were reagent grade. Dichloromethane was distilled from sodium carbonate and stored in amber bottles. Dioxane and anisole were dried by storage over activated molecular sieves (Linde Type 4A). Tetrahydrofuran was freshly distilled from lithium aluminum hydride. Ethyldiisopropylamine (Aldrich Chemical Co.), bp 126–129°, was distilled from sodium hydride through a 20-cm Widmer column. 2-Propanol, bp 82.8–83.3°, was distilled from calcium hydride through a 20-cm Widmer column.

Trifluoroacetic acid (Halocarbon Chemical Co.) was distilled and stored in 100-ml amber bottles with polyethylene-lined screw caps. Traces of low-boiling impurities were shown by combined gas chromatography–mass spectrometry to be fluorocarbons and not trifluoroacetic anhydride. The pH 7 citrate buffer (0.35 M) was prepared by dissolving sodium citrate dihydrate (34.3 g), poly(oxyethylene)lauryl alcohol (BRIJ-35, 1.3 g), and phenol (1.0 g) in distilled water (1.0 l.) and adjusting the pH to 7.00 with a few drops of 12 M aqueous HCl. Buffered 0.2 M sodium chloride was prepared by dissolving sodium chloride (222 g, 3.80 mol), sodium acetate (16.4 g, 0.20 mol), poly(oxyethylene)lauryl alcohol (BRIJ-35 detergent; 26.7 g), and phenol (bacteriocide; 20 ml) in sufficient distilled water to bring the final volume to 20.0 l.; the pH was adjusted to 5.50 with 12 M hydrochloric acid. Buffered 1.0 M sodium chloride was prepared similarly using 1.155 kg (19.8 mol) of sodium chloride.

Synthetic Procedures. Derivatives of N^{ϵ}-benzyloxycarbonyl-L-lysine were prepared from the copper salt of L-lysine and the appropriate benzyl chloroformate, which was prepared from phosgene and the corresponding benzyl alcohol. The cupric ion was removed as the water-soluble complex with ethylenediaminetetraacetic acid.[27] The N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine procedure is typical; physical constants for new compounds are shown in Table IV. Each derivative was homogeneous by ion-exchange chromatography (see Table V). Most of the benzyl alcohols were obtained from Aldrich Chemical Co. Reduction of 2-chlorobenzaldehyde with lithium aluminum hydride in tetrahydrofuran furnished 2-chlorobenzyl alcohol in 97% yield as white

Table IV. Physical Constants for New Derivatives of Lys(Z)

Compd	Crystn solvent	Mp,[a] °C dec	Anal., %: C	H	N
Lys(2-ClZ)[b]	90% aq EtOH	219–222	53.65	5.90	8.87
Lys(2,4-Cl_2Z)[c]	70% aq EtOH	225–228	47.99	5.42	8.01
Lys(2,6-Cl_2Z)[c]	30% aq EtOH	221–223	47.85	5.41	7.66
Lys(3,4-Cl_2Z)[c]	80% aq EtOH	222–224	48.15	5.32	8.22

[a] Heating rate was 1.5°/min; in each case the white crystals decomposed to a clear red liquid with gas evolution. [b] Calcd for $C_{14}H_{19}ClN_2O_4$: C, 53.42; H, 6.08; N, 8.90. [c] Calcd for $C_{14}H_{18}Cl_2N_2O_4$: C, 48.15; H, 5.19; N, 8.02.

(27) S. Kuwata and H. Watanabe, *Bull. Chem. Soc. Jap.*, **38**, 676 (1965); R. Ledger and F. H. C. Stewart, *Aust. J. Chem.*, **18**, 933 (1965).

Table V. Conditions[a] for Ion-Exchange Chromatographic Analysis of N^{ϵ}-Benzyloxycarbonyl-L-lysine and Derivatives

Protected lysine	Column temp, °C	Retention time, min: Protected lysine	Lysine
Lys(Z)	55	32	55
Lys(2-ClZ)	85	38	52
Lys(3-ClZ)	85	41	52
Lys(4-ClZ)	85	32	52
Lys(2,4-Cl_2Z)	76	73	53
Lys(2,6-Cl_2Z)	95	36	51
Lys(3,4-Cl_2Z)	85	63	52

[a] Sulfonated polystyrene (Beckman PA-35; 0.9 × 13 cm) was eluted with pH 7 citrate buffer at 65 ml/hr. The eluate was continuously reacted with ninhydrin and monitored spectrophotometrically at 570 nm.

needles from hexane, mp 68.9–70.1° (lit.[28] mp 70–71°). Similarly. reduction of 3,4-dichlorobenzoic acid afforded 3,4-dichlorobenzyl alcohol in 87% yield as a colorless liquid, bp 97–100° (0.07 Torr) [lit.[29] bp 97° (0.4 Torr)], which crystallized on standing as white needles, mp 35.8–36.6° (lit.[30] mp 38°).

2,4-Dichlorobenzyl Alcohol. 2,4-Dichlorobenzoic acid was recrystallized from 1:1 (v/v) tetrachloromethane–benzene to afford a white powder, mp 160.2–161.0°. A solution of 2,4-dichlorobenzoic acid (52.0 g, 0.272 mol) in dry tetrahydrofuran (250 ml) was added slowly over 20 min to lithium aluminum hydride (9.5 g, 0.25 mol, 1.2 equiv) in dry tetrahydrofuran (750 ml) with stirring under nitrogen. After the mixture was heated at reflux for 21 hr, the starting acid was not detected by tlc. The cooled mixture was cautiously acidified with 6 *M* aqueous HCl and extracted with ethyl acetate. The extract was washed with water and 0.1 *M* aqueous sodium bicarbonate, dried, and freed of solvent. The residue was crystallized from hexane in three crops to afford 2,4-dichlorobenzyl alcohol (32.7 g, 68% yield) as white needles, mp 57.2–58.4° (lit.[31] mp 56–57°).

Anal. Calcd for $C_7H_6Cl_2O$: C, 47.49; H, 3.42; Cl, 40.0. Found: C, 47.62; H, 3.53; Cl, 39.6.

N^{ϵ}-(2,4-Dichlorobenzyloxycarbonyl)-L-lysine. A solution of 2,4-dichlorobenzyl alcohol (35.4 g, 0.200 mol) in 1.25 *m* phosgene in benzene (Matheson Coleman and Bell; 176 g, 0.22 mol) was stored near 20° in a glass-stoppered flask for 37 hr. Solvent was removed on a rotary evaporator protected from water with an acetone–Dry Ice trap. Traces of phosgene were removed by twice adding dry dioxane (50 ml) and reevaporating the solvent The clear colorless residual liquid (52 g; calcd for 2,4-dichlorobenzyl chloroformate: 48 g) was diluted to 120 ml with dry dioxane.

Basic cupric carbonate powder (30.8 g, 0.129 mol) was slowly added to a solution of L-lysine hydrochloride (Ajinomoto; 44.1 g, 0.242 mol) in hot water (500 ml). The dark mixture was briefly heated at reflux and filtered through a glass frit into a 2-l. suction flask; the last traces of the blue copper salt of lysine were eluted from the filter cake with hot water (20 ml). The blue filtrate was diluted with 2.0 *M* aqueous potassium bicarbonate (120 ml) and dioxane (300 ml) and cooled to 0° by partly immersing the rubber-stoppered flask in a 20-l. bucket of ice–water and shaking it with a large wrist-action shaker.

A 10-ml portion of the solution of 2,4-dichlorobenzyl chloroformate in dioxane and a 4.2-ml portion of 4.0 *M* aqueous potassium hydroxide were added to the blue solution and the mixture was shaken at 0° for 10 min; this process was repeated 11 more times over 2 hr. After the mixture had shaken another 10 hr at 0°, the blue precipitate was collected on a medium-porosity glass frit, washed well twice each with water, 95% ethanol, and ether, and vacuum dried to afford the copper salt of N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine in 85% yield as a blue powder (65.1 g).

This powder was added in small portions over 30 min to the vortex cone of a rapidly stirring suspension of ethylenediaminetetraacetic acid (30.0 g; 0.10 mol) in 0.33 *M* aqueous hydrochloric acid (1.2 l.). The mixture was stirred rapidly for 1.5 hr and filtered. The filter cake was suspended in 7:3 (v/v) ethanol–water (1 l.), heated to reflux, and filtered. On slow cooling, the filtrate deposited white needles of N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine (25.7 g); see Table IV for melting point and elemental analysis. This material contained less than 0.08% of the D isomer by Manning–Moore assay[32] and no lysine by tlc assay: R_f 0.02 in 17:2:1 (v/v/v) chloroform–methanol–acetic acid and R_f 0.65 in 15:12:10:3 (by volume) 1-butanol–water–pyridine–acetic acid. Two further crops of needles (22.9 g, 4.7 g) raised the combined yield of Lys(2,4-Cl_2Z) to 76% based on the starting alcohol.

N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine. A slurry of N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine (18.80 g, 53.8 mmol) in 1:1 (v/v) dioxane–water (110 ml) was adjusted to pH 10.2 with 4.0 *M* aqueous sodium hydroxide. *tert*-Butyl azidoformate (Pierce Chemical Co.; 9.0 ml, 63.5 mmol) was added and the mixture was stirred near 20° for 16 hr; the pH was kept at 10.2 by intermittent addition of 4.0 *M* aqueous sodium hydroxide with a Radiometer Autotitrator.[33] The clear pale-yellow solution was washed with four 50-ml portions of ether to remove excess *tert*-butyl azidoformate and was acidified to pH 3.9 by addition of powdered citric acid. The mixture was extracted with two 100-ml portions of ether and 100 ml of ethyl acetate. The combined extracts were washed with five 20-ml portions of water and 20 ml of saturated aqueous sodium chloride, dried over anhydrous magnesium sulfate, and freed of solvent. The clear colorless liquid was vacuum dried for 15 hr to provide N^{α}-Boc-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine in 97% yield as a colorless viscous oil (23.4 g). It was homogeneous by tlc in 17:2:1 (v/v/v) chloroform–methanol–acetic acid (R_f 0.60) and in 15:12:10:3 (by volume) 1-butanol–water–pyridine–acetic acid (R_f 0.79).

Anal. Calcd for $C_{19}H_{26}Cl_2N_2O_6$: C, 50.78; H, 5.83; Cl, 15.8; N, 6.23. Found: C, 50.84; H, 5.75; Cl, 15.3; N, 5.93.

Kinetic Procedures. The protected lysine (50 μmol) was dissolved in 50:50 (v/v) trifluoroacetic acid–dichloromethane (5.0 ml); 0.5-ml aliquots of this solution were kept at 20 ± 1° in dry glass tubes sealed with Teflon-lined screw caps. After a period of reaction between 5 min and 985 hr, the solution within a single tube was freed of CH_2Cl_2 and most TFA by evaporation under a gentle stream of dry air. The residue was dissolved in pH 7 citrate buffer; this quenching procedure required less than 1 min. An aliquot of this solution was analyzed for both the protected lysine and free lysine.

At the usual column temperature (55°) the Lys(ClZ) peaks overlapped the lysine peak while the Lys(Cl_2Z) peaks were strongly retarded, extremely broad, and nongaussian (Table VI). At elevated column temperatures (76–95°) the Lys(ClZ) peaks appeared well before the temperature-insensitive lysine peak and the Lys (Cl_2Z) peaks appeared sooner as narrower peaks that were more amenable to integration. The analytical conditions finally selected for the kinetic work and the observed retention times are given in Table V.

Table VI. Retention Time *vs.* Column Temperature[a]

Compd	Retention time, min: 55°	69°	80°	90°
Lys	55	53	52	51
Lys(Z)	32	26	23	20
Lys(4-ClZ)	64	46	37	30
Lys(3-ClZ)	66	48	38	30
Lys(2,6-Cl_2Z)	95	61	48	37
Lys(3,4-Cl_2Z)	156	96	71	53

[a] Sulfonated polystyrene (Beckman PA-35; 0.9 × 13 cm) eluted with pH 7 citrate buffer at 65 ml/hr.

Hydroxymethyl-resin. Copoly(styrene–1% divinylbenzene) beads (Bio-Beads SX-1, 200–400 mesh) were thoroughly washed[34] and chloromethylated[35] as described previously, except that the resin was allowed to swell in dichloromethane (6 ml/g of resin) before addition of the chloromethyl methyl ether (6 ml/g of resin). A slurry of the chloromethyl-resin (25.0 g, 34.0 mmol Cl) and 1.0 *M* potassium acetate in 2-methoxyethanol (150 ml) was heated gently at reflux for 7

(28) A. G. G. Leonard, *J. Chem. Soc.*, 109, 570 (1916).
(29) R. A. Herrett and R. V. Berthold, French Patent 1,397,525 (1965); *Chem. Abstr.*, 63, 4207 (1965).
(30) G. M. Kraay, *Recl. Trav. Chim. Pays-Bas*, 49, 1082 (1930).
(31) M. Metayer and N. Dat-Xuong, *Bull. Soc. Chim. Fr.*, 615 (1954).
(32) J. M. Manning and S. Moore, *J. Biol. Chem.*, 243, 5591 (1968).
(33) E. Schnabel, *Justus Liebigs Ann. Chem.*, 702, 188 (1967).
(34) R. B. Merrifield, *J. Amer. Chem. Soc.*, 85, 1249 (1963).
(35) R. B. Merrifield, *Biochemistry*, 3, 1385 (1964).

hr (130° bath). The resin was collected on a coarse glass frit, washed with methanol (10 ml), resuspended in 1.0 *M* potassium acetate in 2-methoxyethanol (150 ml), and heated gently at reflux for 10 hr; this cycle was repeated again. After the third reflux period, the resin was collected, washed with 2-methoxyethanol, water, and methanol (two 100-ml portions each), and vacuum dried to furnish the hydroxymethyl-resin (22.2 g). The infrared spectrum showed a strong hydroxyl band at 2.8 μ, new bands at 7.3, 8.5, and 12.3 μ, and the absence of carbonyl absorption.

Boc-L-valyloxymethyl-resin. The hydroxymethyl-resin (5.00 g, 6.8 mmol OH) was allowed to swell in dichloromethane, filtered to remove excess solvent, and shaken for 99 hr at room temperature with a solution of N^{α}-*tert*-butyloxycarbonyl-L-valine (1.48 g, 6.8 mmol) and *N,N'*-carbonyldiimidazole (1.10 g, 6.8 mmol) in a mixture of dichloromethane (50 ml), chloroform (10 ml), and dry dimethylformamide (3 ml). The resin was collected, washed with four 50-ml portions of dichloromethane, and shaken for 21 hr with a solution of acetic anhydride (20 ml, 0.21 mol) and pyridine (40 ml, 0.5 mol) in benzene (40 ml). The resin was collected, washed with four 100-ml portions of dichloromethane and three 10-ml portions of methanol, and vacuum dried. The resulting Boc-L-valyloxymethyl-resin (4.89 g) exhibited infrared bands at 2.93 (w, secondary urethane NH), 5.73 (s, ester carbonyl), and 5.80 μ (s, urethane carbonyl). The absence of hydroxyl absorption at 2.7–2.9 μ indicated that the excess hydroxymethyl sites had been completely acetylated. Four 10-mg resin samples were suspended in 12 *M* hydrochloric acid (2.0 ml), acetic acid (1.0 ml), and phenol (1.0 ml) and hydrolyzed in evacuated, sealed tubes for 24 hr at 110°. By quadruplicate amino acid analysis, 384 $\pm$ 4 μmol of valine was present per gram of Boc-L-valyloxymethyl-resin.

Deca(L-lysyl)-L-valine. A. Using Boc-Lys(2,4-Cl_2Z). The Boc-L-valyloxymethyl-resin (0.851 g, 328 μmol Val) was placed in a 20-ml reaction vessel and converted into N^{α}-Boc-deca[N^{ϵ}-(2,4-Cl_2Z)-L-lysyl]-L-valyloxymethyl-resin by the stepwise addition of ten protected lysine residues. Each residue was added manually by the following five-step cycle: (a) deprotection for 60 min with 50% TFA–CH_2Cl_2, (b) neutralization with 0.50 *M* ethyldiisopropylamine in CH_2Cl_2, (c) coupling for 60 min with a CH_2Cl_2 solution 0.12 *M* in N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-(2,4-dichlorobenzyloxycarbonyl)-L-lysine (1.00 mmol) and 0.12 *M* in *N,N'*-dicyclohexylcarbodiimide (1.00 mmol), (d) neutralization as in step b, and (e) coupling as in step c. Two residues were added per day; the synthesis was stopped at night only after step c or e.

One cycle of the synthesis required the resin to be washed 41 times with 10-ml washes over 5.5 hr as follows: (a) washing three times for 1 min with CH_2Cl_2, 1 min with 50% CF_3COOH–CH_2Cl_2, 60 min with 50% CF_3COOH–CH_2Cl_2, three times for 1 min with CH_2Cl_2, 1 min with 1.3 *M* 2-propanol in CH_2Cl_2 (to react with any strong electrophiles present before addition of the amine), and six times for 1 min with CH_2Cl_2; (b) washing three times for 1 min with 0.5 *M* ethyldiisopropylamine in CH_2Cl_2 and six times for 1 min with CH_2Cl_2; (c) shaking for 5 min with 0.25 *M* Boc-Lys(2,4-Cl_2Z) in CH_2Cl_2 (4.0 ml) and CH_2Cl_2 (0.5 ml), adding to this 0.26 *M* DCC in CH_2Cl_2 (3.8 ml) and CH_2Cl_2 (0.5 ml) and shaking for 60 min, draining, and washing three times for 1 min with CH_2Cl_2; (d) repetition of step b; and (e) repetition of step c.

After ten cycles, part of the dried resin (50 mg) was treated with anhydrous HF (2.7 ml) and dry anisole (0.3 ml) for 60 min at 0°. The HF was evaporated under water aspiration and most of the anisole was evaporated under high vacuum. The resin was collected on a glass frit, washed with three 3-ml portions of ether to remove anisole and its by-products, and washed with five 2-ml portions of CF_3COOH to remove the crude peptide. The CF_3COOH solution was evaporated to dryness under water aspiration; the residue was slurried with water (5.0 ml) and microfiltered through a Millipore filter. Part of this solution (0.30 ml) was diluted with buffered 0.2 *M* sodium chloride (4.20 ml) and adjusted to pH 5.5 with 12 *M* hydrochloric acid.

Part of this solution (1.00 ml) was chromatographed on an 0.9 $\times$ 50 cm column of carboxymethylcellulose preequilibrated with buffered 0.2 *M* sodium chloride. The column was eluted with an aqueous sodium chloride gradient delivered by a Beckman Accu-Flo pump at 65 ml/hr. The constant-volume gradient was formed by placing buffered 0.2 *M* sodium chloride (250 ml) in a stoppered vessel and continuously replacing this well-stirred solution by buffered 1.0 *M* sodium chloride from a second vessel. The column eluate was continuously reacted with ninhydrin and monitored spectrophotometrically at 570 nm to provide curve B of Figure 2.

In a semipreparative chromatographic run, 20% of the column eluate was utilized to monitor the separation and 80% was delivered to a fraction collector. The fraction corresponding to the midpoint of the $Lys_{10}Val$ peak was evaporated to dryness, hydrolyzed under vacuum with 6 *M* aqueous HCl (110°, 24 hr), and analyzed; the observed Lys:Val ratio was 10.1:1.00.

B. Using Boc-Lys(Z). The synthesis of decalysylvaline was repeated using the same resin, solutions, equipment, and procedures described above, except that N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysine was the lysine reagent. Chromatographic analysis of a sample of the crude peptide mixture furnished curve A of Figure 2. In a second chromatographic run the branched-peptide peaks appearing after the peak due to $Lys_{10}Val$ were enhanced for more accurate integration by analyzing a fivefold larger sample of the peptide mixture.

Crystal and Molecular Structure of the Salt (1-Methylnicotinamide)(+) Aden-9-ylacetate(−) Dihydrate. A Model for the Intramolecular Interactions of Oxidized Nicotinamide Adenine Dinucleotide

Donald Voet

Contribution from the Department of Chemistry and the Laboratory for Research on the Structure of Matter, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received September 25, 1972*

Abstract: The X-ray crystal structure of the salt 1-methylnicotinamide(+) aden-9-ylacetate(−) dihydrate has been determined. The aromatic rings form layered walls in which ribbons of coplanar adenine rings alternate with ribbons of coplanar nicotinamide rings. Short contacts between the stacked parallel rings suggest that the intermolecular forces between the stacked rings are partially charge transfer in character. There are no hydrogen bonds between the nicotinamide and the adenine residues. The significance of these observations in terms of the structure and biological function of NAD^+ is discussed.

The pyridine nucleotides, NAD^+ and $NADP^+$, are required by various biochemical processes in all known forms of life. These coenzymes function as electron carriers in metabolic processes through the reversible reduction of their nicotinamide moiety. Thus a molecule of NAD^+ that had been oxidized in one