Critical Examination of a Method for the Analysis of α and ω Linkages in Peptides Containing Aspartic Acid and Glutamic Acid

John T. Capecchi, Marvin J. Miller, and G. Marc Loudon*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

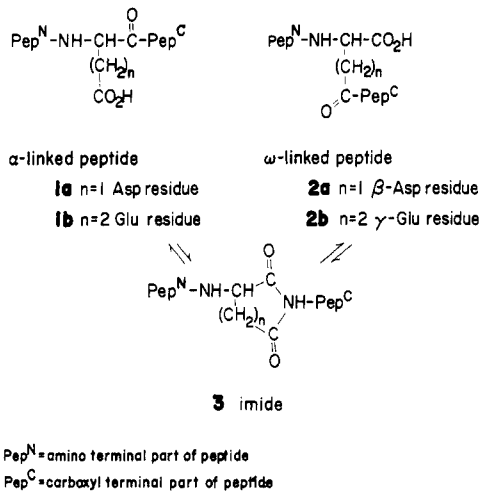
Received August 30, 1982

Coupling of *O*-pivaloylhydroxylamine (4) and subsequent Lossen rearrangement under mild conditions led to the disappearance of β-aspartyl and γ-glutamyl residues from subsequent amino acid analysis in a variety of peptides. Residues from the usual α linkage rearrange much more sluggishly to products that are detectable by amino acid analysis. An interesting complication in the procedure is that α-linked glutamyl residues are converted in part to a 2-oxohexahydropyrimidine-4-carboxylic acid derivative which is stable to extended acid hydrolysis. After base hydrolysis this derivative yields 2,4-diaminobutanoic acid. This reaction explains aberrant results in the linkage analysis of collagen that have been reported in the literature.

In the delineation of a peptide or protein sequence a problem arises when the dicarboxylic amino acids aspartic acid (Asp) and glutamic acid (Glu) or their respective amides asparagine (Asn) and glutamine (Gln) are present in the peptide. In principle, either of the carboxylic acid groups can be involved in the peptide linkage (Scheme I). Therefore, in the structural elucidation of aspartyl or glutamyl peptides, the nature of the linkage at these residues must be determined. This point is of particular importance in the case of synthetic Asp-containing peptides, since the two isomers **1** and **2** can interconvert via imide **3** either during synthesis or during subsequent purification. The large amount of attention[1-4] that this problem has received attests to its importance. In the case of Glu-containing peptides, transpeptidation is less of a problem; however, there do exist a large number of naturally occurring γ-glutamyl peptides[5,6] as well as some protracted arguments in the literature on the nature of the peptide linkage in these species.[7]

There have been numerous attempts to develop methods to analyze the nature of the linkage at the dicarboxylic amino acid residue of peptides. Various spectroscopic techniques including IR spectroscopy,[8] NMR spectrometry,[9] and mass spectrometry[10] have been applied to the problem. A number of chemical methods have also been investigated. These include the reduction of esterified groups to alcohols,[11] hydrazinolysis,[12] tritium exchange

Scheme I



following azlactone formation,[13] and oxidative rearrangements (Curtius,[14] Hofmann,[15] and Lossen[16] rearrangements). In addition, peptidases have been used to perform linkage analyses,[7,17] since these are known to catalyze the hydrolysis of peptides at the α but not the ω linkage. Finally, the pK_a difference between the α- and ω-carboxylic acid groups has been utilized to determine the linkage type either by titration[8a,18] or by behavior on electrophoresis and ion-exchange chromatography.[8b,19]

Although all of these methods have been used successfully in particular cases, few are applicable to a wide range

(1) Battersby, A. R.; Robinson, J. C. *J. Chem. Soc.* **1955**, 259-69.
(2) Ondetti, M. A.; Deer, A.; Sheehan, J. T.; Plusčec, J.; Kocy, O. *Biochemistry* **1978**, *7*, 4069-75.
(3) (a) Bodanszky, M.; Natarajan, S. *J. Org. Chem.* **1975**, *40*, 2495-9. (b) Tam, J. P.; Wong, T.-W.; Reiman, M. W.; Tjoeng, F.-S.; Merrifield, R. B. *Tetrahedron Lett.* **1979**, 4033-6.
(4) (a) Korngruth, M. L.; Neidle, A.; Waelsch, H. *Biochemistry* **1963**, *2*, 740-65. (b) Harris, C. M.; Harris, T. H. *J. Am. Chem. Soc.* **1982**, *104*, 4293-5.
(5) Nitecki, D. E.; Goodman, J. W. "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins"; Weinstein, B., Ed.; Marcel Dekker: New York, 1971; Vol. 1, Chapter 4.
(6) Jeschkeit, H.; Losse, G. *Z. Chem.* **1965**, *3*, 81-96.
(7) See: Bensusan, H. B. *Biochemistry* **1969**, *8*, 4723-7 and references therein; this controversy will be considered later in this paper.
(8) (a) Clayton, D. W.; Kenner, G. W. *Chem. Ind. (London)* **1953**, 1205. (b) Kasai, T.; Larsen, P. O.; Sorenson, H. *Phytochemistry* **1978**, *17*, 1911-51. (c) Kawai, T.; Sakamura, S. *Nippon Nogei Kagaku Kaishi* **1974**, *48*, 521-3. (d) Liwschitz, Y.; Zilkha, A.; Amiel, Y. *J. Am. Chem. Soc.* **1956**, *78*, 3067-8. (e) Battersby, A. R.; Reynolds, J. J. *J. Chem. Soc.* **1961**, 534-30.
(9) Kasai, T.; Sakamura, S. *Agric. Biol. Chem.* **1973**, *37*, 2155-7. (b) Kristensen, I.; Larsen, P. O. *Acta Chem. Scand.* **1973**, *27*, 3123-5. (c) Kasai, T.; Sakamura, S. *Agric. Biol. Chem.* **1974**, *38*, 1257-8; **1975**, *39*, 239-41. (d) Baba, T.; Sugiyama, H.; Seto, S. *Chem. Pharm. Bull.* **1975**, *21*, 207-9.
(10) Okada, K.; Kawase, M. *Chem. Pharm. Bull.* **1977**, *25*, 1497-1508.
(11) (a) Jolles, P.; Fromageot, C. *Biochem. Biophys. Acta* **1952** *9*, 287-92. (b) Bruckner, V.; Medzihradsky, K.; Kandel, I.; Kandel, M. *Acta Chim. Acad. Sci. Hung.* **1959**, *21*, 105-20.
(12) Ohno, K. *J. Biochem.* **1954**, *41*, 345-50.
(13) Ramponi, G.; Cappugi, G.; Nassi, P. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 642-6.
(14) Kovacs, J.; Bruckner, V. *J. Chem. Soc.* **1952**, 4255-9.
(15) (a) Bruckner, V.; Kovacs, J.; Nagy, H. *J. Chem. Soc.* **1953**, 148-50. (b) King, F. E.; Kidd, D. A. A. *J. Chem. Soc.* **1949**, 3315-9.
(16) (a) Gallop, P. M.; Seifter, S.; Lukin, M.; Meilman, E. *J. Biol. Chem.* **1960**, *235*, 2619-27. (b) Franzblau, C.; Gallop, P. M.; Seifter, S. *Biopolymers* **1963**, *1*, 79-97. (c) Urivetsky, M.; Lendavai, S.; Wiener, S.; Meilman, F. *Biochem. Biophys. Acta* **1969**, *181*, 208-18.
(17) Bensusan, H. B.; Dixit, S. M.; McKnight, S. P. *Biochem. Biophys. Acta* **1971**, *25*, 100-8.
(18) Greenstein, J. P.; Klemperer, F. W. *J. Biol. Chem.* **1939**, *128*, 245-50.
(19) (a) Riniker, B.; Schwyzer, R. *Helv. Chim. Acta* **1964**, *47*, 2357-74. (b) Lieflander, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1960**, *320*, 35-57. (c) Law, H. D.; Millar, I. T.; Springal, H. D. *J. Chem. Soc.* **1961**, 279-84. (d) Bornstein, P. *Biochemistry* **1967**, *6*, 3082-93. (e) Waley, S. G. *Biochem. J.* **1957**, *67*, 172-6; **1958**, *68*, 189-92.

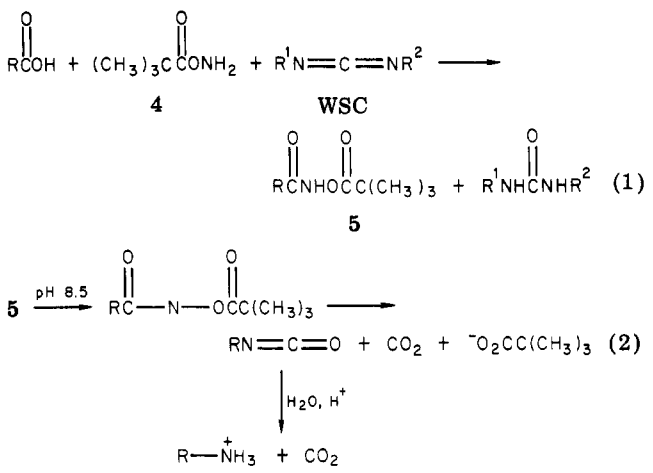
0022-3263/83/1948-2014$01.50/0 © 1983 American Chemical Society

of peptides. Those methods that rely on physical differences between isomers are generally applicable only to small peptides in which these differences are readily detectable. In many cases, both peptide isomers are needed to make a definitive assignment. Several of the methods, especially the degradative methods, subject the peptide to dehydrating conditions that can lead to the formation of imide **3** (Scheme I) and resulting transpeptidation. Some methods also require isolation and quantitation of the resulting derivative which makes the procedure difficult to perform on a small scale.

Recently in our laboratory, a procedure for the carboxyl-terminal analysis of peptides has been developed.[20] This method is based on a Lossen rearrangement of a modified peptide carboxylate group under extremely mild conditions. In this paper, we examine the extension of this procedure to the analysis of the type of aspartyl and glutamyl linkages in peptides.[21] The results reported here have resolved a long-standing conflict in the literature on the nature of the glutamyl peptide linkages in collagen.[7,16]

Results and Discussion

Chemical Basis of the Method. The procedure for linkage analysis takes advantage of the fact that every ω-linked Asp or Glu residue represents a carboxyl-terminal amino acid. Our method for carboxyl-terminal residue analysis[20] can therefore be applied to the peptide by using the chemistry shown in eq 1 and 2. Carboxylic acid groups



are converted to O-substituted *N*-hydroxy amides by using a water-solube carbodiimide (WSC) such as 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and the hydroxylamine derivative *O*-pivaloylhydroxylamine (**4**). The resulting *O*-pivaloylhydroxamate **5** is then subjected to mildly alkaline conditions (pH 8.5) to deprotonate the hydroxamate, and the resulting anion is allowed to undergo the Lossen rearrangement at 50 °C.

The fates of Asp and Glu residues involved in the α vs. the ω linkage depends on a detailed consideration of both the mechanism and outcome of the Lossen rearrangement in each case (Scheme II). Asp or Glu residues which are ω linked will be converted by this process (Scheme IIA) to aldehydes, which will not be detected by amino acid analysis. (In principle, analysis of these aldehydes can provide a positive indication of the presence of ω linkages.) Asp or Glu residues which are α linked will be converted on rearrangement (Scheme IIB) to diamino acid residues, which can be quantitated by amino acid analysis.

A consideration of the mechanism of the Lossen rearrangement, however, leads us to anticipate some selectivity in the rearrangement of ω-linked residues. It is known that the rate of the Lossen rearrangement is accelerated by electron-donating groups on the migrating center.[22] This fact, and the use of a relatively poor pivalate leaving group, suggests that the ω-linked residue will rearrange much more rapidly than α-linked residues. To the extent that the rearrangement of α-linked residues is suppressed by a poor migrating group, these residues will also be detected as unchanged Asp or Glu on subsequent amino acid analysis.

In principle, then, Asp or Glu residues lost on rearrangement and not accounted for as the diamino acids shown in Scheme IIB would be those which are ω linked in the starting peptide. This analysis depends, therefore, on the selective absence from the amino acid analysis of Asp or Glu originally occurring in ω linkages. It further depends on the utilization of conditions which themselves do not induce transpeptidation ($\alpha \rightleftharpoons \omega$ interconversion). To test these ideas, we synthesized model peptides containing Asp or Glu residues in a defined type of linkage and subjected them to the conditions outlinked above. The method was also applied to copolymers of unknown linkage.

Results with Aspartic Acid Peptides. The results of the degradation of α- and β-aspartyl peptides are shown in Tables I and II, respectively. The loss of Asp from the α-linked aspartyl peptides is, as expected, quite low and is in most cases within the error of the amino acid analyzer. In each case a trace of 2,3-diaminopropionic acid (**9a**,

Table I. Degradation of α-Aspartyl Peptides

peptide[a]	degradation yield of Asp,[b] %
Ac-Phe-Asp-Val-amide	4.5
pyroGlu-Asp-Phe-amide	15.0
Ac-Phe-Asp-Pro-amide	5.3
His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys	0[c]
Gln-Tyr-Trp-Pro-Phe-Ser-Ala-Ser-Asp-Leu-Trp	9[d]
Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	0[e]
Pro-Leu-Arg-Ala-Ile-Gly-Pro-Pro-Ala-Glu-Pro-Asn-Gly-Leu-Val-Pro-Leu-Gln-Tyr-Trp-Pro-Phe-Ser-Ser-Ala-Asp-Leu-Tyr	4[d,f]

[a] Ac = acetyl. [b] Degradation yield = 100 (Asp residues in starting peptide − Asp residues in degraded peptide)/(residues of Asp in degraded peptide). [c] Tryptic fragment TS-1 of glucagon; C-terminal lysine also 85% degraded, as expected.[20] [d] Synthetic peptides provided by Dr. S. Sallay (see Acknowledgment). [e] Delta-sleep-inducing peptide; C-terminal Glu also 58% degraded, as expected.[20] [f] Tyr is 88% degraded.[20b]

Table II. Degradation of β-Aspartyl Peptides

peptide[a]	degradation yield of Asp, %
Ac-Gly-Asp$_\beta$-diethylamide	74
Ac-Phe-Asp$_\beta$-Val-amide	64
Ac-Phe-Asp$_\beta$-Pro-amide	84
Ac-Phe-Asp$_\beta$-Gly-amide	48

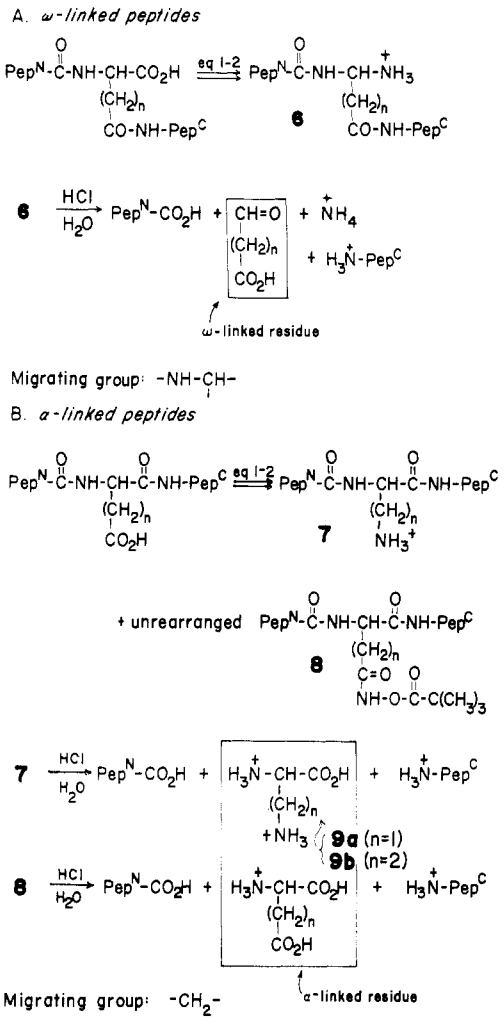
[a] See footnotes *a* and *b* of Table I for definitions.

(20) (a) Miller, M. J.; Loudon, G. M. *J. Am. Chem. Soc.* **1975**, *97*, 5295–7. (b) Miller, M. J.; DeBons, F. E.; Loudon, G. M. *J. Org. Chem.* **1977**, *42*, 1750–61.

(21) A preliminary account of this work has appeared: Capecchi, J. T.; Loudon, G. M. In "Peptides: Structure and Biological Function"; Gross, E., Meienhofer, J., Eds.; Pierce: Rockford, IL, 1979; pp 157–60.

(22) (a) Renfrow, W. B., Jr.; Hauser, C. R. *J. Am. Chem. Soc.* **1937**, *59*, 2308–14. (b) Bright, R. D.; Hauser, C. R. *Ibid.* **1939**, *61*, 618–29. (c) Berndt, D. C.; Schechter, H. *J. Org. Chem.* **1964**, *29*, 916–7.

Scheme II

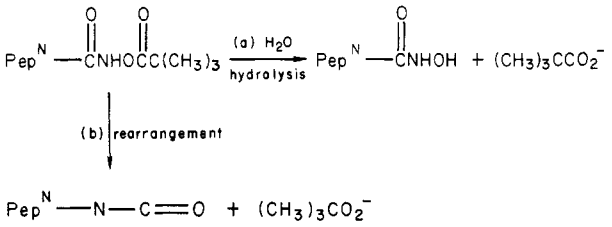


DAPA), the expected degradation product derived from an α-Asp residue (Scheme IIB) was observed. The small amount of degradation and the low ninhydrin color value of this amino acid,[23] however, ruled out quantitation. A further point worthy of note is that larger peptides, which are probably better models for "real" peptides of interest, appear to undergo the least amount of α-Asp degradation. When the carboxyl-terminal residue is not blocked at the carboxylic acid group (e.g., as an amide), this residue is degraded, as expected.[20]

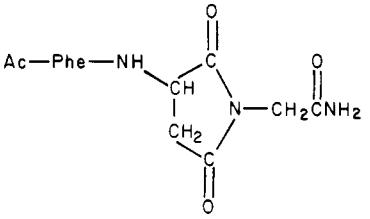
The degradation yield of β-aspartyl peptides is, as anticipated, much higher than that for α-aspartyl peptides. However, the degradation of β-Asp residues is not quantitative and is somewhat sequence dependent. Therefore, the method does not provide an exact quantitative determination of the percent of β linkage in a peptide, but it does appear to provide a reasonable qualitative and semiquantitative assay for this linkage.

The percent degradation of β-Asp residues does not increase measurably with time beyond the 20 h used in the standard procedure. A small amount of hydrolysis of the *O*-pivaloyl ester bond (eq 3) probably competes with the rearrangement; the slower the rearrangement, the more hydrolysis occurs.

The degradation yield of the β-Asp-Gly peptide is significantly lower than that for other β-Asp residues. A similar observation was made for the structurally similar β-*N*-methylamide peptide (not shown in Table II), which



degraded to the extent of 55%. The fact that the Asp-Gly sequence is very prone toward imide formation and transpeptidation has been well documented in the literature.[2,24] It was considered possible that this reaction, occurring during the carbodiimide-mediated coupling of 4 with the peptide, might account for the lower degradation yield of β-Asp-Gly peptides. To test this idea, we subjected the peptides Ac-Phe-β-Asp-Gly-amide and Ac-Phe-β-Asp-Gly-amide to the coupling conditions, and the samples were analyzed by thin-layer chromatography (TLC). The hypothetical imide **10** was synthesized independently, and



10

could be visualized by a very sensitive and specific fluorescence technique (see Experimental Section). A trace of imide was observed in both experiments, but it accounted for less than 2% of the peptide present in the coupling reaction. A control experiment in which the imide was subjected to the coupling procedure showed that it was stable under these conditions. Were any imide formed in the coupling procedure, it would have been detected. Although the exact reasons for the lower degradation yield in the case of the Asp-Gly peptides is not known, it is possible that the competition shown in eq 3 is somewhat more favorable to hydrolysis (path a) for the less sterically hindered Asp-Gly sequence.

The degradation sequence was applied to synthetic copolymers containing Asp residues. A copolymer of Glu (γ-benzyl ester) and Asp (β-*tert*-butyl ester) was synthesized[25] by copolymerizing glutamic acid glutamic acid γ-benzyl ester N_α-carboxyanhydride and aspartic acid β-*tert*-butyl ester N_α-carboxyanhydride. The resulting polymer, by amino acid analysis, contained 91 mol % of Glu residues (presumably as γ-benzyl esters) and 9 mol % of Asp residues (presumably as β-*tert*-butyl esters). Samples of this copolymer were treated respectively in two different ways (Scheme III). One sample was first treated with 4-amino-1-butanol, ostensibly to convert the γ-benzyl esters to *N*-4-hydroxybutanamides. Acidic cleavage of the β-*tert*-butyl esters and purification gave a copolymer (copolymer I, Scheme III) which was intended to contain 9 mol % of Asp residues and 91 mol % of N_α-(4-hydroxybutyl)glutamine residues. Titration, however, revealed that copolymer I contained 5.3 mol % of carboxyl groups instead of the 9 mol % expected. Furthermore, saponification of copolymer I yielded a peptide which, by titration, contained 9.4 mol % of carboxyls, as expected.

(23) Yang, C. C.; Merrifield, R. B. *J. Org. Chem.* **1976**, *41*, 1032–41.

(24) (a) Bodanszky, M.; Kwei, J. Z. *Int. J. Pept. Protein Res.* **1978**, *12*, 69–74. (b) Harding, J. J. *Adv. Protein Chem.* **1965**, *20*, 109–90.

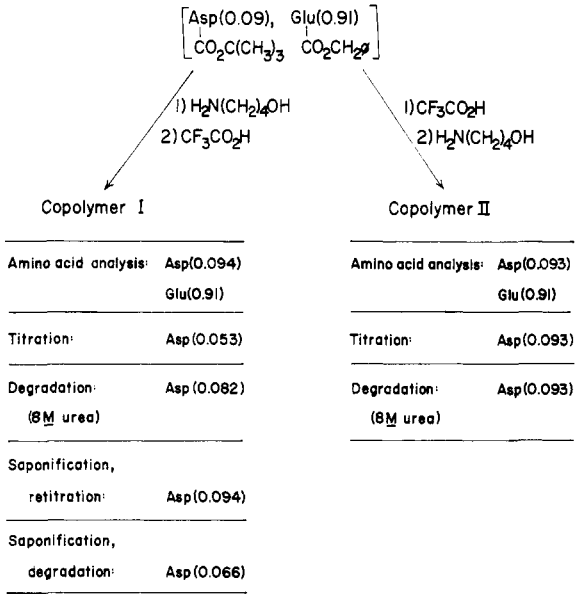
(25) Kobayashi, Y.; Cardinaux, F.; Zweifel, B. O.; Scheraga, H. A. *Macromolecules* **1977**, *10*, 1271–83. (b) Scheule, R. K.; Cardinaux, F.; Taylor, G. T.; Scheraga, H. A. *Ibid.* **1976**, *9*, 23.

Table III. Degradation of α-Glutamyl Peptides[a]

peptide[b]	after 6 N HCl hydrolysis: degradation yield, %	after 6 N HCl hydrolysis: yield of DABA,[c] %	after 6 N $Ba(OH)_2$ hydrolysis: degradation yield, %	after 6 N $Ba(OH)_2$ hydrolysis: yield of DABA,[c] %
Ac-Gly-Glu-Phe	31	13		
Ac-Gly-Glu-Val-Phe	22	7		
Ac-Gly-Glu-Gly-Phe	27	9		
Ac-Phe-Gly-Glu-Gly-Ile[d]	24, 23	4, 4	18	18
Ac-Phe-Glu-Val[d]	30, 29	3, 2	26	12
Ac-Phe-Glu-Ala-Leu[d]	19, 19	3, 3	14	10
Ac-Phe-Glu-Leu-amide[d]	21, 23	3, 3	22	23

[a] For definitions, see notes *a* and *b* of Table I. [b] In all peptides with free C-terminal carboxyl groups, the C-terminal residue is, as expected,[20] degraded in 72–99% yield. [c] DABA = 2,4-diaminobutanoic acid (**9b**, Scheme II). [d] Two values indicate duplicate experiments.

Scheme III



Scheme IV

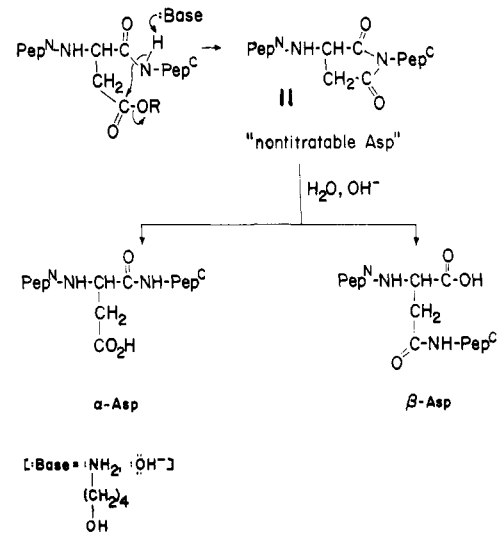


Table IV. Degradation of γ-Glutamyl Peptides[a]

peptide[b]	degradation yield,[c] %
Ac-Gly-Glu_γ-Met	92
Ac-Gly-Glu_γ-diethylamide	87[d]
Ac-Phe-Glu_γ-Val	71
Ac-Phe-Glu_γ-Ala-Leu	78
Ac-Phe-Glu_γ-Leu-amide	84

[a] For definitions, see notes *a* and *b* of Table I. [b] In peptides with free carboxyl groups at the C terminus, the C-terminal residue is lost, as expected,[20] in 90–98% yield. [c] No DABA (2,4-diaminobutanoic acid) was observed in the degradation (see note *d*). [d] Trace of DABA observed.

When, however, the starting protected polymer was treated first with trifluoroacetic acid to remove the *tert*-butyl ester groups and subsequently subjected to aminolysis with 4-amino-1-butanol followed by purification, copolymer II (Scheme III) was formed, for which titration and amino acid analysis gave concordant results: 9.3 mol % of carboxylic acid groups and 9.3 mol % of Asp residues.

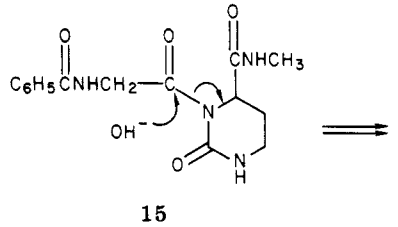
The titration results for copolymer I show that there are some "nontitratable" carboxylic acid groups, but the linkage analysis both before and after saponification shows clearly what has happened (Scheme IV). When the Asp residues are esterified, treatment of the peptides with a base such as 4-amino-1-butanol induces transpeptidation via the imide 11. Prior to the complete saponification, some of the residues exist as imides and are therefore nontitratable, whereas others have opened to Asp residues at either an α or a β linkage by attack of water at either of the imide carbonyl groups. Following saponification, all imides are opened; however, the linkage analysis reveals a significant and, as expected, increased fraction of β linkage in the peptide. This result shows that the use of base in the presence of Asp ester residues, even when the ester is a *tert*-butyl ester, leads to significant transpeptidation and linkage mixing. Copolymer II is devoid of Asp esters at the time of base treatment and is therefore not prone toward transpeptidation. The linkage analysis of this copolymer is consistent with a homogeneous α-linked peptide. Obviously, small amounts of β linkage would go undetected by our method, as we have shown in the model compounds above. The linkage analysis has, however, clearly defined the difference between copolymer I and copolymer II, showing that copolymer I contains a significant amount of β-Asp residues.

Results with Glutamic Acid Peptides. The results of degradation of α-glutamyl and γ-glutamyl peptides are shown in Tables III and IV, respectively. In contrast to the α-aspartyl peptides discussed above, in which little or no rearrangement is observed, the α-glutamyl peptides are consistently degraded in 20–30% yield. This difference is presumably due to the weaker electron-withdrawing inductive effect of the peptide backbone, which is one carbon further away from the migrating terminus in the Glu peptides. The migrating group in the rearrangement of the Glu side chain is therefore somewhat superior to that in the Asp side chain, so more rearrangement is observed. This rearrangement is expected to produce 2,4-diaminobutanoic acid (DABA, **9b**, Scheme II). This product is indeed observed, as shown in Table III (6 N HCl results); the yield of this product will be further considered in the

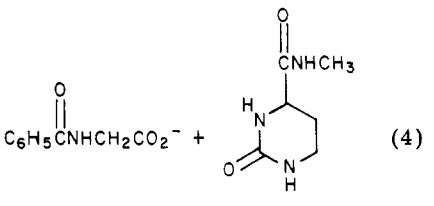
discussion below. The degradation of γ-glutamyl peptides (Table IV) occurs in consistently high yield, and essentially no DABA is observed. This is the result expected from consideration of Scheme IIA.

A disturbing feature of the rearrangement of α-glutamyl peptides is the fact that DABA is not recovered in the same amount that Glu residues are lost. Scheme IIB leads one to expect that the loss of Glu, on the contrary, should be completely accounted for by the appearance of DABA. A proposal which resolves this discrepancy is shown in Scheme V. If the isocyanate intermediate 12 in the Lossen rearrangement is intercepted by the N-peptide nitrogen, a cyclic derivative, 13, would form that, upon hydrolysis, would yield as a product 2-oxohexahydropyrimidine-4-carboxylic acid (14). If this compound were stable to the 6 N HCl hydrolysis conditions, it would not be observed on amino acid analysis. The result would be an apparent loss of Glu not accounted for by appearance of DABA, as observed.

Several experiments indicated that Scheme V can in fact account for the discrepancy in the analysis of α-glutamyl residues. An authentic sample of 14 was prepared by allowing 2,4-diaminobutanoic acid to react with 1,1′-carbonyldiimidazole. This could be used as an authentic standard in the search for this material in peptide hydrolyzates or degradation reaction mixtures. The peptide *N*-benzoyl-glycyl-α-glutamyl-*N*-methylamide was then coupled to *O*-pivaloylhydroxylamine (4), and the coupled material was isolated. This was then subjected to the usual Lossen rearrangement conditions, and the reaction mixture was hydrolyzed in 6 N HCl. The presence of 14 in the hydrolyzate was demonstrated by electrophoresis. In a subsequent experiment, the *O*-pivaloyl derivative was subjected to the Lossen rearrangement procedure, and some of the products were identified without hydrolysis of the reaction mixture. In addition to the starting peptide, *N*-benzoylglycine and the *N*-methylamide of 14 were also isolated. The latter two products were evidently formed by hydrolysis of the labile imide linkage of 15 (eq 4) under the alkaline rearrangement conditions.



15



16 (4)

As noted by Spande et al.,[26] this reaction suggests the possibility of a specific cleavage at the amino carbonyl of Glu residues. Such a cleavage would be useful only if the resulting hexahydropyrimidinone (in essence, a modified glutamic acid) could either be opened or removed to expose a free amino terminus for subsequent sequencing. Noting the close structural similarity of this residue to a pyroglutamyl residue, we subjected peptides bearing the amino-terminal hexahydropyrimidinone residue to hydrolysis in the presence of pyroglutamyl aminopeptidase, an exopeptidase known to hydrolyze amino-terminal pyroglutamyl peptide bonds.[27] Unfortunately, these residues were virtually inert to the enzyme, nor did appropriate hexahydropyrimidinone peptides inhibit the action of the enzyme with its normal substrates.

If Scheme V is to account for the observed discrepancy in the analysis of α-glutamyl residues, it is further required that compound 14 be stable toward the 6 N HCl conditions used in preparation of the peptide for amino acid analysis. This stability was confirmed by a direct demonstration

Scheme V

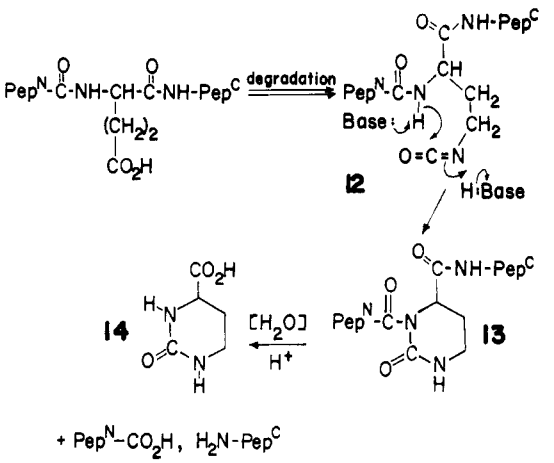
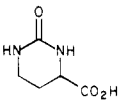
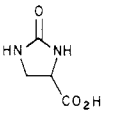
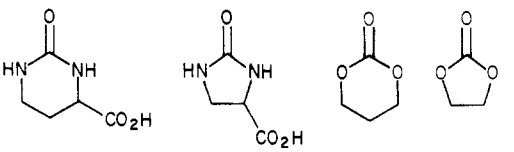


Table V. Hydrolysis of Ureas in Acid and Base[a]

compd	half-life (h) under hydrolysis conditions: 6 N HCl, 110 °C	6 N $Ba(OH)_2$, 110 °C
 14	340	1.3
 17	<0.1	0.3
$CH_3NHC(O)NH_2$	22	
$H_2NC(O)NH(CH_2)_3CH(NH_2)CO_2H$	33	

[a] Followed by the appearance of the amino acid or amine product in amino acid analysis.

Chart I



14 17 18 19

k_{14}/k_{17} (this work, 6 N HCl) < 3×10^{-4}
k_{18}/k_{19} (ref 28, H^+ catalysis) ≃ 91

(26) Spande, T. F.; Witkop, B.; Degani, Y.; Patchornik, A. *Adv. Protein Chem.* **1970**, *24*, 97–260; see p 153, specifically conversions no. 182 and 183.

(27) Podell, D. W.; Abraham, G. N. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 176–85.

(28) Levin, I.; Pohoryles, L. A.; Sarel, S.; Usiell, V. *J. Chem. Soc.* **1963**, 3949–54.

(Table V), along with a similar study on other ureas. The relative inertness of six-membered ureas toward acid hydrolysis is very similar to that of open-chain ureas but stands in marked contrast to the extreme hydrolytic lability of the five-membered cyclic urea. A search for analogies to this behavior revealed that there appears to be no parallel between the relative hydrolysis rates of five- vs. six-membered cyclic ureas and the analogous carbonates (Chart I). Moreover, relative to the open-chain compounds, the *six-membered cyclic carbonate* appears to hydrolyze abnormally rapidly, whereas the *five-membered cyclic urea* appears to hydrolyze abnormally rapidly. Whether this effect is an isolated one from the comparison of rates at a single acidity or whether it is general is not presently known, but it would seem to warrant further inquiry. The lability of the five-membered urea shows that if α-aspartyl residues were to undergo a rearrangement analogous to that shown in Scheme V, the resulting urea would be unstable and would yield 2,3-diaminopropanoic acid (DAPA) on amino acid analysis.

As we shall discuss further below, there has not been a general appreciation of the fact that the rate of hydrolysis of ureas (a) is less than that of amides at high acidity and (b) *decreases* with increasing acid concentration even at acidities corresponding to a few percent H_2SO_4. These points have been clearly demonstrated by the work of Armstrong, Farlow, and Moodie.[29] Indeed, the hydrolysis of the various ureas encountered in this study was faster in dilute acid solution than in 6 N HCl but not sufficiently so to be useful in our work. Therefore, urea derivatives were quantitated by hydrolysis in base. The ureas are opened in concentrated $Ba(OH)_2$ (Table V) to yield the corresponding amino acids, which can then be quantitated on amino acid analysis.

Some of the peptides in Table III were degraded again; separate aliquots of the degradation mixture were subjected to acid and base hydrolysis. The results in Table III show that base hydrolysis liberates more DABA in every case and that the liberation of DABA is nearly quantitative, especially for the longer peptides.

Since α-linked Glu residues can therefore be recovered as either undegraded Glu or as 2,4-diaminobutanoic acid (DABA) following base hydrolysis, whereas γ-linked Glu residues are not recovered in either procedure, it appears that these results can be used for the identification of γ-glutamyl residues in peptides.

Explanation of Anomalous Literature Results in the Degradation of Glutamic Acid Containing Peptides. There are a number of instances described in the literature in which the Lossen or Curtius rearrangements has been used either in synthesis or to investigate the linkage in glutamyl peptides. In some of these cases, loss of Glu following the Lossen rearrangement has been taken to be indicative of the presence of γ-linked Glu residues; the possibility of the cyclization shown in Scheme V and the stability of the resulting ureas has not been taken into account. Karrer et al.[30] used the Hofmann rearrangement to convert N_α-acetylglutamine to 2-(acetylamino)-4-aminobutanoic acid which was subsequently hydrolyzed to DABA in acid. The isolated yield of DABA was only 18%. This low yield can be explained by the urea formation discussed above.

One of the most protracted controversies in the literature involved the nature of the Glu linkages in collagen. In an extensive series of papers, Gallop et al.[16a,b] demonstrated that the Lossen rearrangement of hydroxamate derivatives of collagen showed a loss of Glu after acid hydrolysis which was not accounted for by the recovery of DABA. These authors further claimed to have identified succinic semialdehyde (the degradation product expected from a γ-Glu residue; Scheme IIA) as the 2,4-dinitrophenylhydrazone. Subsequently, it was shown by Bensusan[7,17] that proteolytic digestion of collagen takes place at all of the Glu residues and furthermore that the compound claimed to be succinic semialdehyde was not identical with authentic material. The discrepancy between the two studies was not investigated further. Our work provides a clear explanation for this discrepancy. Cyclization of the isocyanate intermediates from the Lossen rearrangement to cyclic ureas under the alkaline degradation conditions, as we have observed, readily accounts for the observed results.

Conclusions

The results reported in this paper provide the basis for an analysis of the linkage in both α- and ω-linked Asp- and Glu-containing peptides and particularly define the limitations within which these linkage types can be quantitated by the Lossen rearrangement. An additional base hydrolysis is necessary in the investigation of glutamyl peptides. The Lossen rearrangement of side-chain carboxylic acid groups in proteins under even mildly alkaline conditions invariably results not only in the formation of DABA but also in significant isocyanate trapping by the N-peptide nitrogen to yield a cyclic urea which is virtually refractory to acid hydrolysis. *Those using the Lossen rearrangement strategy in peptide modifications should be particularly cognizant of this latter reaction.*

The method used in this work is, of course, a difference method and is not so sensitive as a direct method. In principle, detection of the aldehyde produced in the degradation of ω linkages would provide a much more sensitive direct assay. As noted above, efforts along this line led to an erroneous interpretation of the linkage type in collagen. We are currently considering methods of detecting the aldehyde degradation product. Even without these, however, the results with synthetic peptides show that an indirect procedure is of value.

Experimental Section

The synthesis of peptides used in this study was carried out by standard solution methods (see supplementary material). Other peptides used were as follows: TS-1 of glucagon (Table I), prepared by F. E. DeBons from glucagon which was a gift of Dr. W. W. Bromer, Eli Lilly and Co.; peptides in note *d* of Table I, gifts of Dr. S. I. Sallay, Indiana University–Purdue University, Fort Wayne; delta-sleep-inducing peptide (Table I), obtained from U.S. Biochemical Corp.; pyroGlu-Asp-Phe-amide, synthesized by F. E. DeBons. All starting amino acids are of the L configuration unless otherwise noted. Amino acid analyses were carried out on a Beckman 119Cl analyzer (ninhydrin detection). Microanalyses were carried out by the Microanalytical Laboratory of the Purdue University Chemistry Department. Melting points are uncorrected.

Coupling and Lossen Degradation of Peptides. A solution of *O*-pivaloylhydroxylamine[20] (4, 1 mmol) in 2 mL of water adjusted to pH 3.5, and a sample of the peptide to be degraded (3–5 mol) was added. The pH was kept at 3.5 (0.1 N HCl titrant) during the addition of 250-μL aliquots of a 1.25 M aqueous solution of 1-ethyl-3-[2-(diethylamino)ethyl]carbodiimide (EDC). Four aliquots of this solution were added at 15-min intervals. Fifteen minutes after the last aliquot of EDC was added, the coupling reaction was quenched by the addition of 500 μL of a 5 M pH 3.5 sodium formate buffer. This was allowed to react for 30 min. After this time, the pH was adjusted to 8.5, and the temperature was raised to 50 °C. These conditions were maintained on the

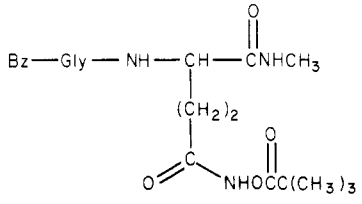
(29) (a) Moodie, R. B.; Farlow, D. W. *J. Chem. Soc.* B **1971**, 407–410. (b) Armstrong, V. C.; Farlow, D. W.; Moodie, R. B. *Ibid.* **1968**, 1099–103.
(30) Karrer, P.; Escher, K.; Widner, R. *Helv. Chim. Acta* **1926**, *9*, 301–23.

pH stat (1 N NaOH titrant). After the required time of degradation, samples containing 0.1–0.5 μmol of the peptide were hydrolyzed. The amino acid analysis was then carried out in the normal way.

Base Hydrolysis of Peptides. It was found that an open-tube, rather than a sealed-tube, base hydrolysis procedure reduced mechanical losses. The sample to be hydrolyzed was placed in a 15-mL Nalgene tube, and the appropriate amount of $Ba(OH)_2 \cdot 6H_2O$ (to make a solution of the desired normality and volume) was added. Enough boiled water was added to the Nalgene tube to achieve 80–90% of the desired volume. This was then either fitted with a glass tube (ca. 12 in. long, to act as an air condenser) and protected with a LiOH trap or sealed with a septum cap. This was placed in a 110 °C oil bath, and, after the sample reached this temperature, any further dilution with H_2O was made. The normality was checked by titration of the hydrolyzate. At the appropriate time, a 0.1-mL sample was removed (using a hot pipet to prevent precipitation), diluted with 1 equiv of acid, and then further diluted with sodium citrate buffer (pH 2.2). This sample was then applied directly to the analyzer.

2-Oxohexahydropyrimidine-4-carboxylic Acid (14). 2,4-Diaminobutanoic acid dihydrochloride (1.91 g, 10 mmol) was dissolved in 400 mL of H_2O and adjusted to pH 8.0. This pH was maintained (pH stat, 25% NaOH titrant) during the reaction. The solution was cooled in an ice bath, and 1,1′-carbonyldiimidazole (CDI; 1.62 g, 1 equiv) in 25 mL of CH_3CN was added slowly over a 20-min period (1.28 mmol of base consumed). The reaction was then stirred at room temperature for 7 h (an additional 4.38 mmol of base consumed). The addition of 1 equiv of CDI was repeated after 24 h. After a 48-h total reaction time, the reaction mixture was applied to a 5.0 × 23 cm Bio-Rad AG 50W-X2 column (cation exchange) and eluted with water. The product **14** was located by TLC and isolated by lyophilization of the appropriate fractions. The product was then purified by chromatography on a 2.5 × 18 cm DEAE-Sephadex column (gradient elution made from 0.75 L of H_2O and 0.75 L of 0.5 M AcOH/pyridine, pH 3.9 buffer): yield 0.38 g (26%); mp 203.5–205 °C (lit.[31] mp 200–201 °C); NMR (90 MHz, Me_2SO-d_6); δ 1.85–2.0 (m, 2 H), 2.95–3.15 (m, 2 H), 3.8–4.0 (m, 1 H), 6.1, 6.2 (2 br s, NH); IR (KBr) 1650, 1734 cm^{-1}; mass spectrum, *m/e* 144 (M^+), 99, 56. Anal. Calcd: C, 41.68; H, 5.59; N, 19.44. Found: C, 41.69; H, 5.67; N, 19.34.

Benzoylglycyl-N^γ-(pivaloyloxy)glutamine *N*-Methylamide (20). Bz-Gly-Glu-*N*-methylamide (0.304 g, 0.95 mmol)



20, Bz = benzoyl

was dissolved in 25 mL of water, and 4·HCl (0.30 g, 1.95 mmol) was added. The pH was adjusted to 3.5 and maintained with the pH stat. The carbodiimide EDC (see above) was added in aliquots at various times as follows: *t* (min) = 0, 0.093 g (0.49 mmol); *t* = 30, 0.148 g (0.776 mmol); *t* = 60, 0.062 g (0.326 mmol); *t* = 95, 0.102 g (0.531 mmol) (total 2.12 mmol, 2.25 equiv). After being stirred for an additional 1.5 h, the solution was acidified, saturated with NaCl, and extracted with EtOAc, and the organic extracts were dried ($MgSO_4$) and concentrated in vacuo. The residue was dissolved in 2 mL of MeOH, and 2 g of silica gel was added. This was allowed to air-dry and was packed at the end of a 1.5 × 125 cm silica gel column (medium-pressure liquid chromatography, MPLC). This was eluted at a flow rate of 4 mL/min with EtOAc/MeOH/hexanes (1:1:3). The product was located by monitoring the effluent of the column at 250 nm (elution volume 310–450 mL). The solvent was removed in vacuo, and the residue was recrystallized from EtOAc/hexane: 0.230 g (82%); mp 142–145 °C; amino acid analysis Glu (0.96), Gly (1.0), $MeNH_2$ (0.94); NMR (90 MHz, Me_2SO-d_6) δ 1.2 (s, 9 H), 1.7–2.25 (m, 4 or 5 H), 2.57–2.62 (d, 3 H), 3.78–3.87 (d, 2 H), 4.1–4.4 (m, 1 H), 7.6–8.0 (m, 6 H), 8.07–8.2 (d, 1 H), 8.68–8.85 (t, 1 H). Anal. Calcd for $C_{20}H_{28}H_4O_6 \cdot H_2O$: C, 54.78; H, 6.90; N, 12.78. Found: C, 54.93; H, 6.92; N, 12.69.

Lossen Degradation of 20. A sample of compound **20** (1.044 g, 2.48 mmol) was dissolved in 100 mL of H_2O and adjusted to pH 8.5 at 50 °C. These conditions were maintained on the pH stat (1.0 N NaOH titrant). After 36 h, 1.06 mL of titrant had been consumed. The solution was then acidified, and samples were taken for both acid (6 N HCl/110 °C/22 h) and base (5.95 N $Ba(OH)_2$/110 °C/10 h) hydrolysis. The amino acid analysis of these hydrolyzates gave the following results: acid hydrolyzate, Glu (0.507), Gly (1.0), DABA (0.152), $MeNH_2$ (1.07); base hydrolyzate[32] (duplicate determinations), Glu (0.50, 0.55), Gly (1.0, 1.0), DABA (0.319, 0.360), $MeNH_2$ (0.061, 0.045). The reaction solution was analyzed by thin-layer electrophoresis (TLE). At least seven spots were detected under various conditions. A sample of the acid hydrolyzate was also applied to an Eastman cellulose TLE plate along with a sample of **14**. After electrophoresis (pH 3.5, 430 V, 1 h) and staining (Cl_2/starch–KI)[33] a spot was seen which corresponded to **14**. A sample of the acid hydrolyzate was then applied to a TLE plate and developed as above, but only the edges were stained. The band corresponding to **14** was removed and suspended in 5 mL of H_2O (containing 0.24 μmol/mL of phenylalanine). Samples of this solution were subjected to both acid (6 N HCl/110 °C/22 h) and base (4.0 N $Ba(OH)_2$/110 °C/10 h) hydrolysis. The DABA/Phe ratio was 0.013 and 0.270 in the two hydrolyzates, respectively.

The degraded sample of **20** was lyophilized and the residue dissolved in 15 mL of 25% (v/v) $AcOH/H_2O$. This was applied to a 2.5 × 77.5 cm Sephadex G-10 column and eluted with 25% (v/v) $AcOH/H_2O$ (UV monitoring at 250 nm). Three unresolved peaks were seen in an elution volume of 225–420 mL. A fourth resolved peak eluted at 480–550 mL and was found to be *N*-benzoylglycine: mp 187–190 °C; yield 0.8 g (18.4% based on starting peptide).

The three unresolved peaks from the G-10 column were lyophilized, the residue was dissolved in 9 mL of 95% EtOH, and the resulting solution was applied to a 1.5 × 125 cm silica gel column (MPLC). This was eluted at 1.5 mL/min with 95% EtOH. Three unresolved peaks (elution volume 140–180 mL) and a fourth resolved peak (elution volume 540–650 mL) were seen. The early fractions (elution volume 140–180 mL) by TLC contained one major component and a few minor impurities. These were rechromatographed under the same conditions to yield a second pure product. There were a number of other products which were not obtained in pure form. The purified products, their physical properties, and their structural assignments are given below.

Compound **20**: elution volume 140–180 mL; yield 0.118 g (11.6%). More of this compound was present in the reaction mixture but was not isolated.

Compound **16**: elution volume 540–650 mL; yield 0.042 g (11.1% of starting peptide).

Thin-Layer Chromatographic Analysis for the Possible Formation of Imide 10 in the Coupling Reaction of the α and β Isomers of Ac-Phe-Asp-Gly-amide. Each peptide was subjected to the normal coupling conditions (1 mmol of *O*-pivaloylhydroxylamine hydrochloride, 1.1 mmol of EDC, and 10 μmol of peptide). After the coupling, however, the reaction was not quenched with formate. The concentration of the peptide in the final solution was determined by amino acid analysis (α isomer, 2.73 μmol/mL; β isomer, 2.08 μmol/mL). Samples (10 μL each) were then analyzed by TLC (silica gel). The R_f of an authentic sample of the imide (synthesis in supplementary material) in various solvents was found to be as follows: 1-butanol/acetic acid/water (4:1:1), 0.55; EtOAc/MeOH (4:1), 0.33; 95% EtOH, 0.57; 1-butanol/pyridine/acetic acid/water (4:1:1:1), 0.62. Because of the difficulties in separating the imide from other compounds on TLC, two-dimensional TLC with the above solvents was employed. After Cl_2/starch–KI strain, no imide **10** could be observed. A control plate was also run on which both the sample and imide

(31) Oohira, T. *Bull. Agric. Chem. Soc. Jpn.* **1942**, *18*, 74–6.

(32) In this case a sealed-tube procedure was used; the experiment was carried out prior to the development of the open-tube procedure.

(33) Rydon, H. N.; Smith, P. W. G. *Nature (London)* **1952**, *169*, 922–3.

were applied to the plate, and the imide was found to migrate as expected. It was observed that the imide fluoresced under long-wavelength (366 nm) excitation after chlorination with Cl_2. This observation gave a very sensitive assay for imide (<0.5 nmol of an authentic sample could be detected). In the analysis of the coupled product, a trace of imide was barley detectable on the plate. A comparison of this plate to standards showed that this amount of imide corresponds to <2% of the total peptide.

Acknowledgment. Support of this work by the National Science Foundation and the National Institute of General Medical Sciences is gratefully acknowledged. We also acknowledge with appreciation the gifts of peptides by Dr. W. W. Bromer of Eli Lilly and Co., Indianapolis, Dr. S. I. Sallay, Indiana University–Purdue University, Fort Wayne, and Dr. F. E. DeBons of our laboratory.

Registry No. **4**, 35657-34-2; **9a**, 515-94-6; **9b**, 305-62-4; **14**, 85701-35-5; **15**, 85701-36-6; **20**, 85701-37-7; **21**, 85701-38-8; **22**, 85701-39-9; **23a**, 85701-40-2; **23b**, 85701-41-3; **24**, 55604-95-0; **25**, 85701-42-4; **26**, 85701-43-5; **27a**, 75865-40-6; **27b**, 75851-15-9; **28a**, 85701-44-6; **28b**, 85709-74-6; **29**, 85701-45-7; **30**, 85701-46-8; **31**, 85701-47-9; **32a**, 75851-19-3; **32b**, 85701-48-0; **33**, 24715-24-0; **34**, 85701-49-1; **35**, 75851-26-2; **36**, 75851-25-1; **37**, 75851-24-0; **39**, 85701-50-4; **40**, 85701-51-5; **41**, 85701-52-6; **42**, 75851-27-3; **43**, 77529-79-4; **44**, 85701-53-7; **45**, 85701-54-8; **46**, 85701-55-9; **47**, 66134-74-5; **48**, 85701-56-0; **49**, 85701-57-1; **50**, 85701-58-2; **51**, 13574-13-5; **52**, 7536-59-6; **53**, 85701-59-3; **54**, 85701-60-6; **55**, 85701-61-7; **56**, 85701-62-8; **57**, 3392-07-2; **58**, 85701-63-9; **59**, 85701-64-0; **60**, 85701-65-1; **61**, 4124-76-9; **65a**, 85701-66-2; **65b**, 85701-67-3; **66a**, 85701-68-4; **66b**, 85701-69-5; **67a**, 85701-70-8; **67b**, 85701-71-9; **68a**, 5879-06-1; **68b**, 2746-34-1; **69a**, 85701-72-0; **69b**, 85701-73-1; **70a**, 85701-74-2; **70b**, 85701-75-3; **71a**, 85701-76-4; **71b**, 85701-77-5; **72a**, 85701-78-6; **72b**, 85701-79-7; **73a**, 85701-80-0; **73b**, 85701-81-1; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, 1892-57-5; Glu(γ-benzyl ester)·Asp(β-*tert*-butyl ester) copolymer, 65045-19-4; EDC, 85701-82-2; 2,4-diaminobutanoic acid dihydrochloride, 6970-28-1; CDI, 530-62-1; Bz-Gly-Glu-*N*-methylamide, 85701-58-2; pyroGlu-Asp-Phe-amide, 73322-74-4; His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys, 24870-77-7; Gln-Tyr-Trp-Pro-Phe-Ser-Ala-Ser-Asp-Leu-Trp, 85701-85-5; Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu, 62568-57-4; Pro-Leu-Arg-Ala-Ile-Gly-Pro-Pro-Ala-Glu-Pro-Asn-Gly-Leu-Val-Pro-Leu-Gln-Tyr-Trp-Pro-Phe-Ser-Ser-Ala-Asp-Leu-Tyr, 75851-17-1; Ac-Gly-Aspβ-diethylamide, 75851-14-8; Bz-Gly-Asp(β-benzyl ester), 85701-83-3; methylamine hydrochloride, 593-51-1; acetylphenylalanine, 2018-61-3; aspartic acid dibenzyl ester *p*-toluenesulfonate, 2886-33-1; Ac-Phe-Asp, 61884-19-3; valinamide, 13474-14-1; prolinamide, 2812-47-7; Asp β-benzyl ester, 2177-63-1; glycinamide hydrochloride, 1668-10-6; Ac-Gly, 543-24-8; Gluα-Phe-Ala, 85701-84-4; Glu-Val-Phe, 31461-61-7; Glu-Gly-Phe, 42155-93-1; Gluγ-Met, 17663-87-5; *N*-*tert*-butoxycarbonylglutamic acid γ-benzyl ester, 13574-13-5; diethylamine, 109-89-7; glutamic acid γ-benzyl ester, 1676-73-9; *p*-nitrophenyl trifluoroacetate, 658-78-6; Gly-Ile, 19461-38-2; *tert*-butoxycarbonylglycine, 4530-20-5; Gluα-Gly-Ile, 85701-61-7; Val, 72-18-4; Ala-Leu, 3303-34-2; leucinamide hydrochloride, 10466-61-2.

Supplementary Material Available: A description of the synthetic procedures and the corresponding experimental details (29 pages). Ordering information is given on any current masthead page.

Catalyzed Rearrangements of Ten-Membered-Ring Allenes

Richard W. Thies,* Janice L. Boop, Michael Schiedler, David C. Zimmerman, and Theodore H. LaPage

Department of Chemistry, Oregon State University, Corvallis, Oregon 97331

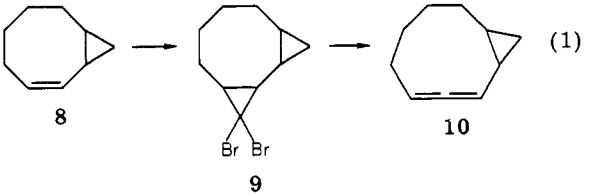
Received September 3, 1982

Cyclodeca-1,2,5,8-tetraene (**1**) is shown to rearrange in the presence of various catalysts in acetic acid to *cis,syn*-tricyclo[4.4.0.$0^{2,4}$]deca-5,8-diene (**5**) which is accompanied in some cases by rearranged acetate products, principally *cis,syn*-bicyclo[4.4.0]deca-4,8-dien-2-yl acetate (**3**). Cyclodeca-1,2,5-triene (**6**) rearranges in a similar way, except that acetate product was only observed for Ag(I) catalysts. A related allene, bicyclo[7.1.0]deca-2,3-diene underwent normal oxymercuration without rearrangement.

Earlier work[1] revealed that treatment of allene **1** with mercuric ion in acetic acid did not give the expected oxymercuration[2] product **2** (Scheme I) but instead gave only the rearranged products **3**–**5** (43:3:54 ratio). Similar treatment of allene **6** gave only the tricyclic compound **7**; no acetates corresponding to **3** or **4** were observed. The present study examined the effect of various other catalysts and also the oxymercuration of an isomeric system **10**.

Results and Discussion

Allene **10** was prepared from bicyclo[6.1.0]non-2-ene[1] (**8**) by the method[3,4] which involves addition of dibromocarbenoid to generate **9** (eq 1) which is then treated with



(1)

methyllithium to produce allene **10** as a 70:30 mixture of diastereomers. The exclusive formation of allene product is an interesting contrast with 9,9-dibromobicyclo[6.1.0]-non-2-ene which reacts with methyllithium to give only tricyclic products resulting from transannular insertion into C–H bonds across the ring.[5]

Treatment of **10** with mercuric acetate in acetic acid, followed by lithium aluminum hydride reduction, gives a

(1) Thies, R. W.; Hong, P.-K.; Buswell, R.; Boop, J. L. *J. Org. Chem.* **1975**, *40*, 585.
(2) Fukuzumi, S.; Kochi, J. K. *J. Am. Chem. Soc.* **1981**, *103*, 2783 and references therein.
(3) Skattebol, L. *Acta Chem. Scand.* **1963** *17*, 1683.
(4) Moore, W. R.; Ward, H. R. *J. Org. Chem.* **1962**, *27*, 4179.
(5) Cardenas, C. G.; Shoulders, B. A.; Gardner, P. D. *J. Org. Chem.* **1967**, *32*, 1220.

0022-3263/83/1948-2021$01.50/0 © 1983 American Chemical Society