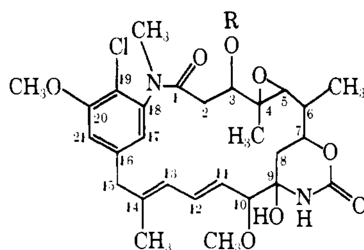


1, R = CO·CHCH$_3$·NCH$_3$·COCH$_3$
2, R = CO·CH$_3$
3, R = H
4, R = CO·CHCH$_3$·NCH$_3$·COCH$_2$CH$_3$
5, R = CO·CHCH$_3$·NCH$_3$·COCH(CH$_3$)$_2$
6, R = CO·CH$_2$CH$_3$
7, R = CO·CH$_2$Br
8, R = CO·CH=CHCH$_3$
9, R = CO·CF$_3$

ment of **3** with acetic anhydride-pyridine at 53° for 18 hr gave maytanacine (53% yield).

To evaluate the effects on biological activity of variations in structure of the ester moiety, several semisynthetic esters of maytansinol were prepared. The propionate (**6**, 38%, mp 187.2-188.6°, $[\alpha]^{22}$D −119° (*c* 0.133, CHCl$_3$), ir (KBr) 5.71 μ), bromoacetate (**7**, 43%, mp >300°, $[\alpha]^{22}$D −149° (*c* 0.107, CHCl$_3$), ir (KBr) 5.68 μ), crotonate (**8**, 13%, mp 214.1-216.0, $[\alpha]^{24}$D −70° (*c* 0.037, CHCl$_3$), ir (KBr) 5.71 μ), and trifluoroacetate (**9**, 30%, mp 162.2-163.0°, $[\alpha]^{23}$D −289° (*c* 0.080, CHCl$_3$), ir (KBr) 5.58 μ) esters of maytansinol were made by procedures involving either anhydride-pyridine (**6, 7, 8**)[15] or anhydride-acid (**9**)[16] treatment. The esters **2, 6, 7**, and **8** were found to show antileukemic activity[11] comparable to those of the naturally occurring substituted alanyl esters. The trifluoroacetate ester **9** showed no antileukemic activity, possibly because of ready solvolysis in vivo to inactive maytansides; cf. ref 6. Approaches to other synthetic modifications are in progress, to elucidate further the requirements for biological activity among the antileukemic maytansinoids.

References and Notes

(1) Tumor Inhibitors. 107. Part 106 is ref 2.
(2) S. Remillard, L. I. Rebhun, G. A. Howie, and S. M. Kupchan, *Science*, in press.
(3) Supported by grants from the National Cancer Institute (CA-11718 and CA-12059) and American Cancer Society (CI-102-J) and a contract with the Division of Cancer Treatment, National Cancer Institute (NO1-CM-12099).
(4) S. M. Kupchan, Y. Komoda, W. A. Court, G. J. Thomas, R. M. Smith, A. Karim, C. J. Gilmore, R. C. Haltiwanger, and R. F. Bryan, *J. Am. Chem. Soc.*, **94**, 1354 (1972).
(5) S. M. Kupchan, Y. Komoda, G. J. Thomas, and H. P. J. Hintz, *J. Chem. Soc., Chem. Commun.*, 1065 (1972).
(6) S. M. Kupchan, Y. Komoda, A. R. Branfman, R. G. Dailey, Jr., and V. A. Zimmerly, *J. Am. Chem. Soc.*, **96**, 3706 (1974).
(7) S. M. Kupchan, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **33**, 2288 (1974).
(8) M. K. Wolpert-Defilippes, R. H. Adamson, R. L. Cysyk, and D. G. Johns, *Biochem. Pharmacol.*, **24**, 751 (1975).
(9) T. E. O'Connor, C. Aldrich, A. Hadidi, N. Lomax, P. Okano, S. Sethi, and H. B. Wood, Proceedings of the 66th Annual Meeting of the American Association of Cancer Researchers, 29 (1975).
(10) Stems were collected in South Africa in 1972. We acknowledge with thanks receipt of the dried plant material from Dr. R. E. Perdue, Jr., U.S. Department of Agriculture, in accordance with the program developed by the National Cancer Institute.
(11) Tumor-inhibitory activity and cytotoxicity were assayed under the auspices of the National Cancer Institute, by the procedures described by R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep., Part 3*, **3**, 1 (1972).
(12) Maytanacine (**2**) showed significant antileukemic activity against the P-388 lymphocytic leukemia over a 50–100-fold dosage range at the μg/kg level (with a T/C value of 230 at its optimal dose of 100 μg/kg), and cytotoxicity (ED$_{50}$) against KB cell culture at 10^{-5} μg/ml. Maytansinol (**3**) showed no antileukemic activity at doses up to 70 times the optimal dose (12.5 μg/kg; T/C 258) of maytansine (**1**).
(13) The isolation of maytanacine (**2**) and the other maytanside esters was guided by assays of fractions for antileukemic and cytotoxic activity. However, the isolation of the minor and biologically inactive constituent maytansinol (**3**) was effected by TLC and HPLC comparisons of fractions with a reference sample of semisynthetic origin. The isolation procedure omitted the acetylation step used earlier (ref 4).
(14) We thank Professor A. I. Meyers, Colorado State University, for unpublished information concerning LiAlH$_4$ reductive cleavage of esters in synthetic model compounds.
(15) M. P. Mertes, P. E. Hanna, and A. A. Ramsey, *J. Med. Chem.*, **13**, 125 (1970).
(16) J. C. Fairlie, R. McCrindle, and R. D. H. Murray, *J. Chem. Soc. C*, 2115 (1969).
(17) N.I.H. Postdoctoral Fellow, 1972–1975.

S. Morris Kupchan,* Alan R. Branfman,[17] Albert T. Sneden
Ashok K. Verma, Richard G. Dailey, Jr.
Yasuo Komoda, Yoshimitsu Nagao
Department of Chemistry, University of Virginia
Charlottesville, Virginia 22901

Received May 16, 1975

Carboxyl-Terminal Amino Acid Residue Analysis of Peptides. A New Method

Sir:

We wish to report a general and efficient method for the determination of the carboxyl-terminal amino acid residue of peptides. Efficient methods have been developed for both sequential and single residue determinations at the amino terminus;[1-4] however, the methods currently available for carboxyl terminal analysis suffer from limitations.[5-7]

Compound **5**, *O*-pivaloylhydroxylamine (OPHA), was found, of many derivatives surveyed,[8,9] to represent a sufficient compromise of stability, water solubility, and nucleophilicity to form *O*-pivaloylhydroxamic acids quantitatively from carbodiimide-activated carboxylic acids. Ionization of the hydroxamate **6** N-H bond (pK_a 6.4-7.4)[10] initiates on peptides a Lossen rearrangement[11,12] and eventual conversion of the carboxyl-terminal amino acid to an aldehyde, as shown in Scheme I. Such a scheme has appealed to others,[13] but could not be reduced to practice on a small scale because of the large number of reactions hitherto required to generate the O-acylated hydroxamic acid.

Table I. Results of the Carboxyl–Terminal Amino Acid Analysis

Peptide[a,d]	% degradation	Peptide[a,d]	% degradation
Ac·Gly·Asn	71	Ac·Ala·Leu·Gly	77
Ala·Ser	72	Ac·Gly·Leu·Tyr[d]	92
Ac·Ala·Glu	40(60)[b]	Ac·Pro·Phe·Gly·Lys(Ac)	94
Ac·Ala·Asp	40(50)[b]	Ac·Met·Arg·Phe·Ala	92
Pro·Gly	77	Ac·Phe·Asp·Ala·Ser·Val[c]	93
Gly·Met	81	Ac·Leu·Trp·Met·Arg·Phe[d]	94
Ac·Ala·Pro	82	Ac·γ-Glu·Cys(SO$_3$H)·Gly[c]	
Gly·Phe	87	(Oxidized acetylglutathione)	95 (Glu) 75 (Gly)
Ac·Gly·Leu	90		
Bz·Gly·Arg	93	Insulin A-chain[f] (C-terminal Asn)	75
Gly·Trp[e]	86	Insulin B-chain[f] (C-terminal Ala)	99

[a] Abbreviations: Ac = acetyl, Bz = benzoyl; see text for definition of degradation yield. [b] Yields in parentheses result from carrying out coupling step at pH 0.75; see text. [c] The side-chain of Asp is coupled to OPHA in this procedure, but does not rearrange; model studies to be reported also show that Glu side-chains behave similarly. Thus, these residues analyze for Asp and Glu, respectively, after degradation. γ-Glu, however, is readily lost in the degradation, because it presents a new C-terminus. The difference in α- and ω-linked residues is readily accounted for by the substantially different migrating groups in the two cases. [d] See Footnote 15. [e] The determination of Trp by amino acid analysis presented the usual difficulties. In order to carry out this degradation, chloride ion was avoided. Thus, free OPHA and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate were used in the degradation, and amino acid analyses were carried out by the procedure of Liu and Chang[20] using methanesulfonic acid. [f] The entire analysis was carried out in 8 *M* urea.

Scheme I

$$\text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—NH—}\boxed{\text{CHR}}\text{—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{OH} \;+\; \text{R}_1\text{N{=}C{=}NR}_2 \xrightarrow[\text{H}_2\text{O}]{\text{pH 3.5}} \left[\text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—NHCHR}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{OC}\begin{smallmatrix}\nearrow\text{NR}_1\\ \searrow\text{NHR}_2\end{smallmatrix}\right] \qquad (1a)$$

1 2 3

$$3 \underset{2}{\overset{\text{H}_2\text{O}}{\rightleftharpoons}} 1 + \text{R}_1\text{NH}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHR}_2 \qquad (1b)$$

4

$$3 + \text{H}_2\text{NO}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{C(CH}_3)_3 \longrightarrow \text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHR}\overset{\text{O}}{\overset{\|}{\text{C}}}\underset{\text{H}}{\underset{|}{\text{N}}}\text{O}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{C(CH}_3)_3 + 4 \qquad (1c)$$

5 6

$$6 \xrightarrow{\text{pH 8.5}} \left[\text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHR}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—}\overset{-}{\text{N}}\text{—O—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{C(CH}_3)_3\right] \xrightarrow[\text{rearr}]{\text{Lossen}} \text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHRN{=}C{=}O} \qquad (1d)$$

7 8

$$8 \xrightarrow{\text{H}_2\text{O};\ -\text{CO}_2} \text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHRNH}_2 \qquad (1e)$$

9

$$8 \xrightarrow{\text{NH}_3 \;\leftarrow\leftarrow\; \text{H}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHOCC(CH}_3)_3\ (10)} \text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHRNH}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NH}_2\ (11); \qquad 9 \xrightarrow{8} (\text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NHCHRNH})_2\overset{\text{O}}{\overset{\|}{\text{C}}}\ (12) \qquad (1f, 1g)$$

11 12

$$11, 12 \xrightarrow{\text{pH 1-2, 50°}} \text{Pep—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{NH}_2 + \text{NH}_3 + \boxed{\text{RCH{=}O}} \qquad\qquad 11, 12 \xrightarrow{6\ N\ \text{HCl, 110°, 22 hr}} \boxed{\text{RCH{=}O}} + \text{NH}_3 + \text{amino acids} \qquad (1h, 1i)$$

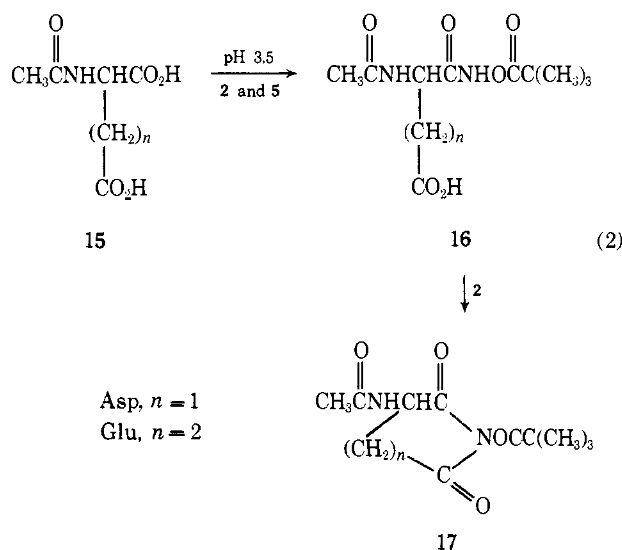
In a typical experiment 154 mg (1 mmol) of OPHA·HCl, **5**, is dissolved in 4 ml of H_2O, and the solution adjusted to pH 3.5 with 1 N NaOH. A solution of the peptide (previously amino acid analyzed;[14] ≥1 μmol) in 2 ml of H_2O is added. A 0.4-ml aliquot of a 0.5 M aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (**2**) is added immediately. The pH is maintained at 3.5 with 0.1 N HCl using a pH-stat. Three more aliquots of the carbodiimide solution are added at 15-min intervals. The addition of 0.5 ml of a 5.0 N pH 3.5 formate buffer and stirring for 20 min quenches the carbodiimide and OPHA by the formation of **10.** The Lossen rearrangement is initiated by adjustment of the pH to 8.5 and the temperature to 50°; the pH is maintained with 1.0 N NaOH. Since the O-pivaloylpeptidohydroxamate (**6**, **7**) is present in only small amounts, the base consumption of the O-pivaloylformohydroxamate, **10,** serves as an indicator for the progress of the reaction, which requires ≤20 hr. The result is the formation of a mixture of ureas **11** and **12** from the reaction of isocyanate **8** with aminoamide **9**, or with NH_3 generated from rearrangement of **10**. An aliquot of suitable size for amino acid analysis is then removed, made 6 N in HCl, hydrolyzed directly, and analyzed. The results in Table I were derived from this procedure.

Alternatively, after the rearrangement, the pH of the reaction mixture can be adjusted to pH 1-2 and the mixture stirred for 50° for 2 or more hr to hydrolyze selectively the ureas **11** and **12,** and generate the aldehyde **14,** which contains the side-chain of the original C-terminal residue. Under these conditions, a primary amide is formed at the penultimate amino acid residue.

The results of the subtractive amino acid analyses with the peptides shown in Table I illustrate the efficiency of the method. *Degradation yield* in this table is defined as the per cent loss of the C-terminal residue relative to the other residues after difference amino acid analysis.[15] These analyses could all be performed on a micromolar scale; since the analysis facilities available to us were not sensitive below this level, smaller scale determinations were not made. We observed, however, slight increases in our degradation yield as the scale of our experiments was decreased, probably because of the decreased interferences from intermolecular processes. Thus, there should be no difficulty in the analysis of considerably smaller amounts of material. In all cases a positive aldehyde test[16] was obtained following the degradation; in several cases, the aldehyde was qualitatively identified by paper chromatography of the 2,4-dinitrophenylhydrazone.

The advantages of our procedure are (1) the use of aqueous solutions (or mixed solvents; note that the insulin degradation was successful in 8 *M* urea); (2) the high yields of the procedure; (3) the fact that peptides need not (but may) be protected at the amino terminus[17] or at side-chain functionality; (4) the successful removal of proline,[7] asparagine, and presumably glutamine[18] in high yield; (5) successful analyses of peptides as large as the insulin chains; and (6) the overall simplicity of the method.

Our procedure currently has the following limitations. (1) Carboxyl-terminal Glu and Asp suffer low degradation yield (ca. 40%) under the conditions described above because they form a cyclized intermediate in the coupling step (**17**, eq 2) which regenerates Glu or Asp after hydrolysis.[19]



(The structure of **17** is based on actual isolation and identification.) However, carrying out the coupling procedure at pH 0.75 increases the yield somewhat (Table I). (2) Peptides currently must be small enough to analyze by difference amino acid analysis, although we are developing methods to identify the aldehyde on a submicro scale; however, no other size limitation appears to exist. (3) That the Lossen rearrangement step takes more time than we consider optimum, although an object of further development, is not a serious problem, since the rearrangement can be monitored by a pH-stat and left essentially unattended.

Variations of this method which will permit extension of this chemistry to a sequential C-terminal degradation are currently under study.

Acknowledgments. We gratefully acknowledge support of this work by the National Institute of General Medical Sciences. We also wish to thank Professor Vincent duVigneaud for use of amino acid analysis facilities and Mrs. Patricia Miller for assistance with some of the analyses.

References and Notes

(1) P. Edman, *Acta Chem. Scand.*, **4**, 277, 283 (1950); **7**, 700 (1953).
(2) H. Fraenkel-Conrat, J. I. Harris, and H. L. Levy, *Methods Biochem. Anal.*, **2**, 359 (1955).
(3) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
(4) W. R. Gray and B. S. Hartley, *Biochem. J.*, **89**, 59P (1963).
(5) L. Bailey, "Techniques in Protein Chemistry", 2nd ed, Elsevier, New York, N.Y., 1967, pp 223–249.
(6) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids", Wiley, New York, N.Y., 1961, pp 1577–1687.
(7) G. R. Stark, *Biochemistry*, **7**, 1796 (1968).
(8) L. A. Carpino, *J. Am. Chem. Soc.*, **82**, 3133 (1960).
(9) (a) Y. Tamura et al., *J. Org. Chem.*, **38**, 1239 (1973); (b) W. N. Marmer and G. Maerker, *J. Org. Chem.*, **37**, 3520 (1972).
(10) M. A. Stolberg, R. C. Tweit, G. M. Steinberg, and T. Wagner-Jauregg, *J. Am. Chem. Soc.*, **77**, 765 (1955).
(11) H. L. Yale, *Chem. Rev.*, **33**, 209 (1943).
(12) L. Bauer and O. Exener, *Angew. Chem., Int. Ed. Engl.*, 376 (1974).
(13) T. Wieland and H. Fritz, *Chem. Ber.*, **86**, 1186 (1953).
(14) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(15) No other non-C-terminal residues were affected by the method, except for Tyr (ca. 30% loss), the amino-terminal residue (ca. 25% loss), and Trp (ca. 50% loss). These losses were also incurred in the presence of OPHA, but *absence* of carbodiimide (conditions under which the C-terminal degradation does not take place); this observation suggests that the N-terminal amino group and the activated aromatic rings were undergoing electrophilic amination by OPHA. Consistent with this suggestion is the complete protection of the amino terminal residue from loss by prior acetylation. The amination of the amino terminus suggests the possibility of a simultaneous C- and N-terminal degradation.
(16) R. G. Dickinson and N. W. Jacobsen, *Chem. Commun.*, 1719 (1970).
(17) The large excess of OPHA over free amino terminus assures the virtual absence of peptide–peptide coupling; see also note 15.
(18) A full kinetic study was carried out for degradation of *N*-acetylglutamine. This amino acid was degraded in 99.5% yield. Similarly, the γ-Glu residue can be thought of as a "substituted Gln"; it degrades normally (Table I); this observation is the basis for an analysis for ω-linked dicarboxylic amino acids.
(19) Obviously, either the side-chain or α-carboxyl may be activated first, and **16** represents only the latter possibility; the derivative **17** is of course obtained in either case.
(20) T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

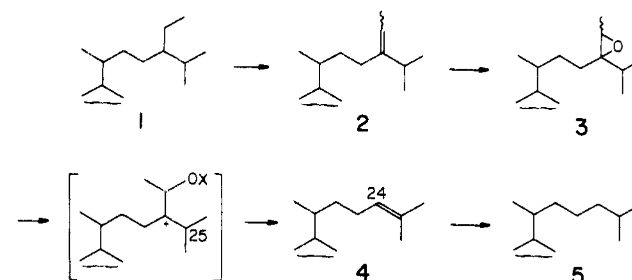
Marvin J. Miller, Gordon Marc Loudon*
Spencer Olin Laboratories of Chemistry, Cornell University
Ithaca, New York 14853
Received May 22, 1975

Stereospecificity in the Conversion of Fucosterol 24,28-Epoxide to Desmosterol in the Silkworm, *Bombyx mori*

Sir:

Insects depend on phytosterols as a steroid source since they lack the ability to synthesize cholesterol.[1] The general scheme of sitosterol (**1**) dealkylation is summarized in Scheme I.[2-4] Intermediacy of the epoxide **3** has been demonstrated by isotope incorporation techniques[3] and is also supported by the recent findings showing that ^3H in [25-^3H]-24-ethylcholesterol migrates to C-24 during conversion to desmosterol **4** in *Bombyx mori*[4] and *Tenebrio molitor.*[5]

Scheme I



The absolute stereochemistry of the intermediate epoxide involved in the transformation of sitosterol **1** to cholesterol **5** in *B. mori* has been determined to be (24*S*,28*S*)-**7a** as follows. Selective epoxidation of fucosteryl acetate **6** with *m*-chloroperbenzoic acid (in chloroform, 0°, 15 min) gave a nonseparable 1:1 mixture of the epoxides **7a**/**7b** (with 3-OAc). Presence of a mixture was clear from the ^{13}C NMR which gave paired peaks for the asterisked carbons in **7**; the two C-29 methyl signals, however, overlapped at 14.3 ppm, a point which proved to be of diagnositc value (see below).

Acid catalyzed (H_2SO_4 in aqueous THF) cleavage of epoxides **7**(3-OAc) gave the glycol mixture **8**(3-OAc), still nonseparable. However, treatment of mixture **8** with (+)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) chloride[6] yielded the MTPA ester which could now be separated