[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Polyvalyl-proteins[1]

BY A. STRACHER[2] AND R. R. BECKER

RECEIVED AUGUST 21, 1958

Poly-DL-valyl-chymotrypsin, poly-DL-valyl-lysozyme and poly-DL-valyl-insulin have been prepared by reaction of the proteins with N-carboxy-DL-valine anhydride. The number of valine residues added to the proteins and the number of sites acylated by the anhydride have been studied by the DNP method. The dinitrophenyl derivatives of the modified proteins showed unusual stability toward acid hydrolysis, with periods of as long as 96 hr. being required for complete hydrolysis. The peptides isolated from shorter periods of hydrolysis have been shown to contain amino acids from the protein chain, in addition to the valine added in the modification reaction. After total hydrolysis, essentially all of the amino groups of the native protein which have been acylated can be accounted for as DNP-valine. The poly-DL-valyl-chymotrypsin retained about 60% of the esterolytic activity of the native enzyme.

Introduction

The reaction of proteins with α-amino acid-N-carboxyanhydrides (NCAs) has been employed for the preparation of a variety of polypeptidyl-proteins using NCAs derived from several of the amino acids.[3-7] The results of studies of the biological properties of some of these modified proteins[8-10] can be explained in part on the basis of steric effects in the interaction of the polypeptidyl-proteins with other proteins or substrates. The unusual stability of valine peptides toward acid hydrolysis is well known[11,12] and has been attributed to steric shielding of the susceptible bonds by the isopropyl side chain of valine. It therefore appeared of interest to study the effects of the introduction of valine peptides into proteins upon both their biological and chemical properties. In this regard, preliminary experiments reported here have shown that the esterolytic activity of poly-DL-valyl-chymotrypsin is decreased when compared to the native protein; polyglycyl-chymotrypsin, on the other hand, has been shown to retain the full activity of the native enzyme.[3] However, before the results of experiments of this sort can be reasonably interpreted, knowledge of the structure of the modified proteins is necessary. The preceding paper[13] provides information regarding the specificity of the reaction of the NCAs of glycine and phenylalanine with several proteins, the results indicating that only amino groups are sites of acylation. This paper reports the synthesis of poly-DL-valyl-chymotrypsin, the results of end group studies by Sanger's method[14] and the results of preliminary experiments testing the enzymatic activity of the modified enzyme. N-terminal studies of poly-DL-valyl-lysozyme and poly-DL-valyl-insulin were carried out in order to obtain additional information on the specificity of the reaction of DL-valine NCA with proteins.

Results and Discussion

Poly-DL-valyl derivatives of chymotrypsin, lysozyme and insulin were prepared by reaction of the proteins in aqueous buffered solutions with DL-valine NCA. Chymotrypsin was chosen for study in order that tests for enzymatic activity could be carried out and compared with results obtained with polyglycyl-chymotrypsin.[3] Lysozyme and insulin were used since both are relatively low molecular weight, well-characterized proteins suitable for N-terminal end group experiments.

Table I summarizes data on the number of moles of valine added per mole of protein as determined by the method of Levy[15] and the number of ϵ-amino groups of lysine acylated per mole of protein. The literature values for the moles of lysine per mole of protein are included for comparison with those found by Sanger's method. For example, with chymotrypsin, 9.2 moles of the 13 present were found. This value is in good agreement with the value of 9 found by Fraenkel-Conrat and Singer[16] under similar conditions. That some of the lysine residues in chymotrypsin are unavailable for reaction is well known.[16,17] For calculation of the number of moles of ϵ-amino groups acylated per mole, a value of 4.7, the difference between 9.2 and 4.5, will be used in the discussion, since it is unlikely that groups unavailable for reaction with fluorodinitrobenzene (FDNB) would be available for reaction with valine NCA.

In the case of lysozyme, only 5 of the 6 lysine residues present in the protein should give rise to ϵ-DNP-lysine after hydrolysis of the DNP-protein, since one of them is N-terminal.[18] A value of 5.2 was found in these experiments. After reaction with DL-valine NCA, 2.7 moles of ϵ-DNP-lysine per mole of protein was found, indicating acylation of either 2.5 or 3.5 of the non-terminal ϵ-amino groups per mole. The uncertainty is due to the fact that it is not known whether the N-

(1) Supported in part by grants from the National Science Foundation (NSF-G-2132), and The Nutrition Foundation, Inc., New York, N. Y.

(2) Taken in part from the Ph.D. dissertation of A. Stracher, presented to the Faculty of Pure Science, Columbia University.

(3) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 745 (1953).

(4) M. Green and M. A. Stahmann, *ibid.*, **213**, 259 (1955).

(5) H. Tsuyuki, H. Van Kley and M. A. Stahmann, THIS JOURNAL, **78**, 764 (1956).

(6) H. Fraenkel-Conrat, *Biochim. Biophys. Acta*, **10**, 180 (1953).

(7) M. Sela, *Bull. Res. Council Israel*, **4**, 109 (1954).

(8) M. Green and M. A. Stahmann, *J. Biol. Chem.*, **231**, 259 (1955).

(9) T. Makinodan, R. R. Becker, H. R. Wolfe and M. A. Stahmann, *J. Immunol.*, **73**, 159 (1954).

(10) M. A. Stahmann, H. Tsuyuki, K. Weinke, C. Lapresle and P. Grabar, *Compt. rend.*, **241**, 1528 (1955).

(11) R. L. M. Synge, *Biochem. J.*, **39**, 351 (1945).

(12) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 609 (1957).

(13) W. H. Konigsberg and R. R. Becker, THIS JOURNAL, **81**, 1428 (1959).

(14) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(15) A. L. Levy, *Nature*, **174**, 126 (1954).

(16) H. Fraenkel-Conrat and B. Singer, *Arch. Biochem. Biophys.*, **65**, 296 (1956).

(17) V. Kneeslova, B. Keil and F. Sorm, *Chem. Listy*, **48**, 599 (1954).

(18) F. C. Green and W. A. Schroeder, THIS JOURNAL, **73**, 1385 (1951).

TABLE I

POLY-DL-VALYL-PROTEINS

Protein	Mol. wt.	Moles valine added per mole[a]	Moles lysine per mole (lit.)	Moles ε-DNP-lysine per mole[b]
Chymotrypsin	24,500[c]	..	13[d]	9.2
Poly-DL-valyl-chymotrypsin	29,400[e]	50	13	4.5
Lysozyme	14,700[f]	..	6[g]	5.2
Poly-DL-valyl-lysozyme	16,200[e]	15	6[h]	2.7
Insulin	6,000	..	1[i]	1.0
Poly-DL-valyl-insulin	7,400[e]	14	1	0.3

[a] Determined by method of Levy.[15] [b] Determined by Sanger's method.[13] [c] Calculated from molecular weight of 25,000 reported by P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. Biophys. Acta*, **24**, 72 (1957), and peptides released in activation process. See H. Neurath, *Advances in Protein Chem.*, **12**, 319 (1957). [d] Based on amino acid analysis of chymotrypsinogen by P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1957), and the fact that no lysine occurs in the activation peptides. [e] Calculated from moles of amino acid added. [f] C. Fromageot and M. P. de Garilhe, *Biochim. Biophys. Acta*, **4**, 509 (1950). [g] J. C. Lewis, N. S. Snell, D. J. Hirshman and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950). [h] D. W. Kupke and K. Linderstrøm-Lang, *Biochim. Biophys. Acta*, **13**, 153 (1954). [i] G. R. Tristram, *Advances in Protein Chem.*, **5**, 83 (1949).

terminal lysine was acylated on the α-amino group only or on both amino groups. If the α-amino group only were acylated, hydrolysis of the DNP-protein would yield ε-DNP-lysine; if both amino groups were acylated, free lysine would be expected upon hydrolysis. Acylation of the ε-amino group only is not considered likely. In the case of insulin, no such uncertainties exist, and the data indicate that 0.7 mole of ε-amino groups has been acylated per mole.

If only amino groups are sites of acylation by DL-valine NCA, then for every amino group acylated, an N-terminal DNP-valine should be found upon hydrolysis of the DNP-poly-DL-valyl-proteins. If groups other than amino groups should initiate polymerization and thus become sites of attachment of polypeptide chains, then the number of DNP-valine residues determined should exceed the number of amino groups acylated. Quantitative DNP-analyses of the modified proteins would then show, within the limits of this method, whether the amino groups are exclusively acylated or not.

Since studies of enzymatic activity were carried out with the modified chymotrypsin, it was not possible to use the diisopropylfluorophosphate inhibited enzyme[19] for these studies, and submolar amounts (about 0.1 mole per mole) of several DNP-compounds other than the N-terminal DNP-isoleucine and DNP-alanine[19] were found in the hydrolysates of the DNP-protein. However, it appears likely that these impurities were removed prior to the isolation of the poly-DL-valyl-proteins, since similar DNP-amino acids were not noted upon analysis of hydrolysates of DNP-poly-DL-valyl-chymotrypsin. The same amounts (about 0.7 mole per mole) of DNP-isoleucine were isolated from hydrolysates of DNP-poly-DL-valyl-chymotrypsin and DNP-chymotrypsin in recent experiments,[20] indicating that the N-terminal isoleucine was not acylated during the modification reaction. Of pertinence in this regard is the observation of Massey[21] who reported that the N-terminal isoleucine of α-chymotrypsin does not react with FDNB when the protein is in the native state. The absence of DNP-alanine in hydrolysates of DNP-poly-DL-valyl-chymotrypsin indicates that this amino acid residue was acylated. No attempt to determine the half-cystine N-terminal group[22] either before or after the modification reaction was made. It is reasonable to assume that this group was acylated in the modification reaction, and the data of Table II support this assumption. Thus, in the reaction of chymotrypsin with DL-valine NCA, 4.7 ε-amino groups (Table I), the N-terminal alanine residue, and presumably the N-terminal half-cystine residue, a total of 6.7 residues, have been acylated. It will be noted that a total of 6.6 moles of DNP-valine are recovered per mole of protein upon complete hydrolysis (96 hr.), a value in close agreement with the number of moles of amino groups acylated. A total of 11.1 of the 16 amino groups (13 ε-amino groups and 3 N-terminal groups) present in chymotrypsin are found as either ε-DNP-lysine or DNP-valine. Apparently, the 4 ε-amino groups which are unreactive toward FDNB are not acylated by DL-valine NCA. In addition, the N-terminal isoleucine is unreactive toward the NCA, and the over-all recovery of amino groups is well within the limits of the experimental method.

TABLE II

HYDROLYSIS OF DNP-POLY-DL-VALYL-PROTEINS

Hydrolysis time (hr.)	Moles DNP-valine per mole	Moles DNP-peptides per mole	Moles ε-DNP-lysine per mole	Total amino groups found
DNP-Poly-DL-valyl-chymotrypsin				
16	2.5	1.9	4.1	8.5
27	4.7	1.2	4.5	10.4
35	5.3	0.6	4.5	10.4
72	5.1	0.4	4.5	10.0
96	6.6	None	4.5	11.1
DNP-Poly-DL-valyl-lysozyme				
16	1.6	0.9	2.4	4.9
24	2.3	.8	2.7	5.8
27	2.4	.6	2.7	5.7
35	2.7	.4	2.7	5.8
72	3.4	None	2.7	6.1
DNP-Poly-DL-valyl-insulin				
16	1.4	0.6	0.3	2.3
96	2.7	None	0.3	3.0

The fact that peptides were isolated from the shorter period hydrolysates was not unexpected, since valine peptides are known to be quite stable to acid hydrolysis.[11,12] Surprisingly, however, the peptides isolated contained as many as nine amino acids from the protein chain, in addition to the valine added in the modification reaction. Structural studies have shown that these peptides contain lysine with a polyvalyl chain attached to the ε-amino group, thus providing direct evidence for this group as a point of attachment of poly-

(19) M. Rover, C. Fabre and P. Desnuelle, *Biochim. Biophys. Acta*, **12**, 547 (1953).

(20) J. H. Hash and R. R. Becker, unpublished.

(21) V. Massey, *Disc. Faraday Soc.*, **20**, 66 (1955).

(22) F. R. Bettelheim, *J. Biol. Chem.*, **212**, 235 (1955).

peptides in proteins modified with NCAs. A brief account of the isolation of peptides in this manner has been reported,[23] and a more detailed description will be presented in a forthcoming paper. It will be noted from the data of Table II that the per cent. of amino groups accounted for is low for the shorter hydrolysis periods. These low recoveries can be accounted for in part on the basis that DNP-peptides were lost as stationary bands at the top of the silica gel columns used for separation. Paper electrophoresis of this material indicated the presence of small amounts of at least three additional peptides. Some material, presumably large peptides, was lost as a residue insoluble in the solvents used for chromatography.

The results of similar experiments with lysozyme are shown in Table II. The only N-terminal group found in hydrolysates of DNP-poly-DL-valyl lysozyme was DNP-valine, indicating that the α-amino group of the N-terminal lysine was acylated in the modification reaction. However, the experiments do not provide information regarding the possibility that both amino groups of the N-terminal lysine were acylated by the anhydride. Isolation of peptides containing the N-terminal lysine followed by structural studies will be necessary to clarify this point. Although this point remains uncertain, the data show an over-all recovery of amino groups of 6.1 of the 7 amino groups present in lysozyme, again indicating that it is unlikely that groups other than amino groups are sites of acylation in this protein.

Table II also summarizes the results of N-terminal experiments with insulin. Insulin was chosen as one of the proteins for study, since the amino acid sequence is known[24,25] and since it contains only one lysine residue and two N-terminal groups per molecule. The results show that all of the amino groups can be accounted for as either DNP-valine or ϵ-DNP-lysine after complete hydrolysis. These data indicate that both N-terminal amino groups have been acylated and, further, that no other functional groups have been acylated by the anhydride. The peptides isolated after hydrolysis for 16 hours contained lysine, and no peptides from the N-terminal ends of the protein were isolated.[23]

The results of the N-terminal experiments of these three proteins show in all cases that the number of moles of DNP-valine found in the DNP-poly-DL-valyl-proteins was either equal to or slightly less than the number of amino groups acylated. It thus appears very unlikely that other groups in these proteins form stable acylated derivatives upon reaction with DL-valine NCA. It is probable, on the basis of this evidence and that presented in the previous paper,[13] that the specificity for amino groups of proteins in the reaction with NCAs is general.

Poly-DL-valyl-chymotrypsin was found to retain enzymatic activity when assayed for esterolytic activity[26] against control chymotrypsin which had been carried through the manipulations involved in the preparation of the modified enzyme. However, in contrast with the results obtained with polyglycyl-chymotrypsin which was shown to retain complete activity,[3] a decrease in activity to about 56% of the control was observed. It is known that neither the ϵ-amino groups nor the α-amino groups are required for the activity of this enzyme.[27,28] The basis for the decreased activity noted in polyvalyl-chymotrypsin is currently being investigated.

Experimental

Carbobenzoxy-DL-valine.—The procedure described by Hinman[29] was used for this preparation. The yield was 60%; m.p. 75–76°; lit. value, 72–73°.

N-Carboxy-DL-valine Anhydride.—This compound was synthesized using the same procedure as described for N-carboxyglycine anhydride.[30] The product was recrystallized from anhydrous ether:petroleum ether (1:2). The yield was 50%; m.p., 78.5–79.5°; lit. value, 77–79°.[31]

Poly-DL-valyl-chymotrypsin and Lysozyme.—Five hundred milligrams of crystalline chymotrypsin (purchased from Worthington Biochemical Corp.) was dissolved in 50 ml. of 0.067 *M* phosphate buffer, *p*H 7.6. Two hundred and eighty milligrams of N-carboxy-DL-valine anhydride was added to the cooled solution and the reaction was allowed to proceed for 24 hr. at 4°. During this time a precipitate of poly-DL-valine formed.

The mixture then was centrifuged at 17,000 × *g* for 1 hr. at 5°. The supernatant was transferred to a dialysis sac (Visking) and dialyzed for 5 days against cold, distilled water. The solution was then either lyophilized or treated directly with 1-fluoro-2,4-dinitrobenzene (FDNB).

Poly-DL-valyl-lysozyme was prepared in the same manner using the same molar excess of anhydride to protein. This modified protein was treated directly with FDNB.

Poly-DL-valyl-insulin.—Five hundred milligrams of crystalline Zn-insulin[32] was dissolved in 60 ml. of a solution containing 45 ml. of borate buffer, *p*H 8.5, and 15 ml. of purified dioxane. The solution was cooled in an ice-bath and 200 mg. of N-carboxy-DL-valine anhydride was added. The mixture was stirred for 24 hr. at 4°. The solution was centrifuged and then dialyzed for 4 days and treated directly with FDNB.

Enzymatic Activity of Poly-DL-valyl-chymotrypsin.—The esterolytic activity of poly-DL-valyl-chymotrypsin was determined by the manometric method of Parks and Plaut.[26] Equal enzyme concentrations based upon the absorption at 280 mμ were used in the control and experimental flasks.

End Group Studies.—The DNP-derivatives of the modified proteins were prepared according to the method of Sanger.[33] Twenty mg. of DNP-modified protein per ml. of distilled constant boiling HCl was used for the hydrolysis of the DNP-modified proteins. The sample was weighed and placed in a Pyrex test-tube of appropriate size. The HCl was added and the tube was evacuated and sealed. The tubes were then placed in an oven and incubated at 110° for the appropriate length of time. Suitable controls of DNP-valine and ϵ-DNP-lysine were hydrolyzed simultaneously to determine the amount of destruction of these DNP-amino acids at the various times of hydrolysis as recommended by Fraenkel-Conrat, *et al.*[34] After hydrolysis the tube was broken open and the hydrolysate diluted until the concentration of acid was 1 *N*. This solution was then extracted three times with anhydrous ether and three

(23) A. Stracher, W. H. Konigsberg and R. R. Becker, *Biochim. Biophys. Acta*, **20**, 595 (1956).

(24) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(25) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353 (1953).

(26) R. E. Parks and G. W. E. Plaut, *J. Biol. Chem.*, **203**, 755 (1953).

(27) I. W. Sizer, *ibid.*, **160**, 547 (1945).

(28) C. H. Chervenka and P. E. Wilcox, *ibid.*, **222**, 635 (1956).

(29) J. W. Hinman, E. L. Caron and H. N. Christensen, THIS JOURNAL, **72**, 1620 (1950).

(30) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).

(31) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3009 (1950).

(32) Sample kindly furnished by Dr. Otto K. Behrens of the Eli Lilly and Co.

(33) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(34) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, in "Methods of Biochemical Analysis," Vol. II, ed. D. Glick, Interscience Publishers, Inc., New York, N. Y., 1955.

times with ethyl acetate. The remaining aqueous portion then was filtered to remove the brown humin and diluted to a known volume with 1 *N* HCl. The optical density of the aqueous portion was determined at 390 mμ and the amount of ε-DNP-lysine calculated by comparison with a standard curve.[35] In some cases, the method of Porter[36] and that of Levy[15] were used.

The ether–ethyl acetate solution was evaporated to dryness in a current of warm air. The residue was taken up in a small amount of water-saturated chloroform. This solution then was placed on a silica gel column as described by Porter[36] and the DNP-valine separated from dinitrophenol, dinitroaniline and DNP-peptides. After separation was complete, the column was extruded, and the DNP-derivatives eluted with acetone, evaporated to dryness, dissolved in 1% $NaHCO_3$ solution and the optical density determined at 350 mμ. The moles of DNP-residue were determined using 17,000 as the molar extinction coefficient for DNP-valine. The extinction coefficient for the DNP-peptide was also assumed to be 17,000.[34]

The results of recovery experiments of DL-valine when hydrolyzed in the presence of DNP-chymotrypsin and DNP-lysozyme are recorded in Table III. Approximately 1 mg. of DNP-valine and 20 mg. of DNP-protein together with 1 ml. of constant boiling HCl were hydrolyzed for the periods of time indicated. The results represent the average of at least 4, and in some cases 6, determinations, as do the results of Tables II.

TABLE III

DNP-DL-VALINE RECOVERIES

Hydrolysis time (hr.)	% Recovery DNP-chymotrypsin	% Recovery DNP-lysozyme
16	82	80
27	78	75
35	72	69
72	65	60
96	61	..

The results reported here show slightly less destruction of DNP-valine than observed by Porter and Sanger[37] and Masri and Singer.[38] However, the losses are somewhat higher than the 13% reported recently by Rhinesmith, Schroeder and Pauling[12] for a 22-hour hydrolysis in the presence or absence of DNP-globin.

(35) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

(36) R. R. Porter, in "Methods in Medical Research," Vol. III, ed. R. W. Gerard, Yearbook Publishers, Inc., Chicago, Ill., 1950, p. 256.

(37) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(38) M. S. Masri and K. Singer, *Arch. Biochem. Biophys.*, **58**, 414 (1955).

NEW YORK, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WAYNE STATE UNIVERSITY AND THE RESEARCH LABORATORIES OF PARKE, DAVIS AND CO.]

Elaiomycin. II. Determination of the D-*threo* Configuration

BY CALVIN L. STEVENS, BERNARD T. GILLIS[1] AND THEODORE H. HASKELL

RECEIVED SEPTEMBER 26, 1958

The tuberculostatic antibiotic Elaiomycin (I) has previously been shown to contain two asymmetric carbon atoms, both situated in a C_5-moiety, which has been degraded to a derivative II of 3-amino-4-methoxy-2-butanol. Elaiomycin has been shown by independent synthesis of an optically active derivative II to have the D-*threo* configuration.

In the previous paper in this series[2] the structure of Elaiomycin was proved to be I by degradation experiments. Complete hydrogenation of I resulted in hydrogenolysis of the N-to-N bonds with the formation of octylamine and a methoxyamino alcohol. The structure of this amino alcohol was confirmed by the synthesis of a diastereoisomeric mixture of II.

In this work the amino alcohol was first shown to be in the *threo* series by synthesis of DL-II starting from DL-threonine. The ethyl ester hydrochloride of DL-threonine was treated with methyl iminobenzoate by the method of Elliott[3] to give *threo*-DL-5-methyl-2-phenyl-2-oxazoline-4-carboxylic acid ethyl ester (III), which has been shown to be formed with retention of the *threo* configuration.

The reduction of III with excess lithium aluminum hydride resulted in the cleavage of the oxazoline ring and formation of N-benzyl-DL-threoninol (IV) in 87.5% yield. By careful reduction of III with an equivalent amount of lithium aluminum hydride *threo*-DL-5-methyl-2-phenyl-2-oxazoline-4-methanol (V) was obtained in 88% yield. Lithium aluminum hydride has previously been shown to reduce esters of optically active aminoacids to the corresponding alcohols without racemization.[4]

Tosylation of V in pyridine at 0° gave the *p*-toluenesulfonate of V in 75–99% yields. Subsequent treatment of the tosylate VI for 84 hours with sodium methoxide in absolute methanol resulted in the formation of the *threo*-DL-5-methyl-2-phenyl-4-methoxymethyl-2-oxazoline(VII) in good yield. Hydrolysis of the oxazoline ring in VII, and acetylation of the resulting amino alcohol gave the desired DL-*threo*-3-acetamido-4-methoxy-2-butanol acetate (II), m.p. 78.2–79°. The infrared absorption spectra of a solution of II and of the acetylated amino alcohol from Elaiomycin were superimposable. A mixture melting point determination of the two was depressed to 70°. The identity of the spectra of the two compounds definitely placed the degradation product from Elaiomycin in the *threo* series.

The utilization of L-threonine and the procedures developed for synthesis of the DL-*threo* compounds resulted in the isolation of L-*threo*-3-acetamido-4-methoxy-2-butanol acetate, which derivative had the same melting point, 76.4–76.8°, as that from Elaiomycin but opposite rotation. These data and the fact that the infrared spectra of the two compounds were identical prove that the natural

(1) This work was supported in part by a Parke–Davis Research Fellowship.

(2) C. L. Stevens, B. T. Gillis, J. C. French and T. H. Haskell, THIS JOURNAL, **80**, 6088 (1958).

(3) D. F. Elliott, *J. Chem. Soc.*, 589 (1949).

(4) P. Karrer, P. Portmann and M. Suter, *Helv. Chim. Acta*, **31**, 1617 (1948).