The Structure of the Peptide Antibiotic Stendomycin

Miklos Bodanszky, Jan Izdebski, and Ichiro Muramatsu

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received November 22, 1968*

Abstract: Stendomycin, an antifungal peptide antibiotic, is a mixture of closely related compounds. Their amino acid sequence was elucidated mainly through the study of peptides from partial acid hydrolysates. In all members of the stendomycin family the N-terminal amino acid, proline, is acylated by a branched-chain fatty acid; the C-terminal amino acid, a cyclic derivative of arginine, forms a lactone bond with the hydroxyl group of one of the allothreonine residues. The configurations of the amino acid components and conformational aspects of the whole antibiotic are also discussed.

The antifungal antibiotic stendomycin was isolated from cultures of *Streptomyces endus* by Thompson and Hughes[1] in the Lilly Research Laboratories. These investigators described some physical and chemical properties of stendomycin, recognized its peptide nature, and identified several of the amino acid constituents. A generous sample from Eli Lilly and Co., Indianapolis, Ind., allowed us to elucidate the structure of stendomycin.[2]

Microheterogeneity. In the first report on stendomycin[1] two varieties of the antibiotic were described, stendomycin A and stendomycin B. These were separated by countercurrent distribution. In our experiments the same separation of two major peaks could be observed. However, the two products from the countercurrent distribution showed the same mobility on thin layer chromatograms and were indistinguishable from each other by amino acid analysis, uv, ir, and nmr spectra, and the ratio of their fatty acid constituents. Therefore, it was assumed that the difference between the two species, A and B, might be in the anion that is paired with the single but strong cationic center of the molecule. This assumption was supported by observations made on thin layer chromatograms of the antibiotic. In the solvent systems containing an excess of an acid, such as acetic acid, only a single spot was revealed.

These observations would suggest that stendomycin is a single entity. Yet, when acid hydrolysates of stendomycin were extracted with an organic solvent such as ether or hexane, a mixture of five different fatty acids[3] was obtained. Since the molecule of the antibiotic contains only 1 mol of fatty acid, it became obvious that we were dealing with a family of closely related compounds; each member of the group contains a different fatty acid. Soon afterwards it was found that some of the amino acid constituents[4] are present in nonstoichiometric ratios. The amino acid ratios were calculated on the assumption that in 1 mol of the antibiotic 1 mol of alanine, glycine, and proline is present. Then about 3.5 mol of valine, 1.4 mol of alloisoleucine, and 0.1 mol of leucine were found in the hydrolysate, the total of the three amino acids being 5 mol. These values were found only if the hydrolysis time was extended well beyond the usual 16 or 24 hr. The conclusion that in the dominant component of the stendomycin family 3 mol of valine and 2 mol of alloisoleucine occur and in other members one or both alloisoleucines are replaced by valine or leucine was born out later in the examination of the products of partial hydrolysis. It became clear that a very complex mixture was at hand; the components in this mixture differ mostly in the presence or absence of a methyl group at one point, and this difference is easily counterbalanced by a similar difference but in the opposite direction in another part of the structure. It is understandable, therefore, that our vigorous and tenacious attempts for the separation of individual components must have failed and also that the antibiotic appeared as a single spot on thin layer chromatograms in several solvent systems.

The experiments for the separation of single entities were seriously hampered by the tendency of stendomycin to form stable emulsions and also by the marked concentration dependence of its partition coefficients in a number of solvent systems tried for countercurrent distribution. For these reasons the attempts for the separation of individual members of the stendomycin family had to be abandoned and the structure of the antibiotic was studied in the mixture, which for the sake of simplicity is designated as stendomycin.

Fatty Acids. On hydrolysis of stendomycin with hydrochloric acid an oily material separated from the solution. Extraction with ether or with hexane gave a mixture of fatty acids, which were shown[3] to consist of isomyristic and isotridecanoic (or 11-methyllauric) acids, and their lower homologs.

Amino Acid Composition. The results of the quantitative amino acid analysis[5] of the antibiotic were reported earlier.[4] Amino acids with bulky nonpolar side chains occur (as it was shown later) as neighbors in the sequence. The peptide bond between two such residues is resistant to hydrolysis and correct amino acid ratios were obtained only if hydrolysis with con-

(1) R. Q. Thompson and M. S. Hughes, *J. Antibiot.* (Tokyo), *Ser. A*, **16**, 187 (1963).

(2) A preliminary report on the chemistry of stendomycin was presented at the Ninth European Peptide Symposium in Orsay, France, April 1968. *Cf.* "Peptides 1968," E. Bricas, Ed., North Holland Publishing Co., Amsterdam, 1968, p 306. The studies on stendomycin were conducted as part of a continued effort toward the understanding of the origin of microbial peptides, of their role in the life processes of the microorganisms, and of the causes of their antimicrobial activities. *Cf.* M. Bodanszky and D. Perlman, *Nature*, 204, 840 (1964); D. Perlman and M. Bodanszky, *Antimicrobial Agents Chemotherapy*, **1965**, 122 (1966); M. Bodanszky and D. Perlman, *ibid.*, **1967**, 464 (1968); M. Bodanszky and D. Perlman, *Science*, **163**, 352 (1969).

(3) M. Bodanszky, I. Muramatsu, and A. Bodanszky, *J. Antibiot.* (Tokyo), *Ser. A*, 20, 384 (1967).

(4) M. Bodanszky, I. Muramatsu, A. Bodanszky, M. Lukin, and M. R. Doubler, *ibid.*, **21**, 77 (1968).

(5) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

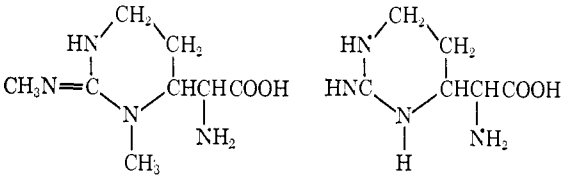
stant boiling hydrochloric acid at 110° was carried out for about 90 hr. From the common amino acids serine (1 mol), proline (1 mol), glycine (1 mol), alanine (1 mol), valine (3.5 mol), and leucine (*ca.* 0.1 mol) were found. Allothreonine (2 mol) and allosioleucine (1.4 mol) were also recognized. The elution values on the Spackman–Stein–Moore chromatograms[5] were considered as preliminary evidence for the identification of the amino acid constituents. Final evidence was provided by the isolation of preparative quantities through ion-exchange chromatography and countercurrent distribution and comparison (elemental analysis, specific rotation, ir and nmr spectra, paper chromatographic behavior) with authentic samples.

Among the hydrolysis products ammonia (somewhat more than 1 mol) can be detected. Since there are no dicarboxylic acids in the molecule and its C-terminal carboxyl was found (later) to be engaged in a lactone bond, the origin of ammonia first was not obvious. In conjunction with the uv absorption of stendomycin the assumption was made that a dehydroamino acid is present in the sequence. Dehydroamino acids (substituted α-aminoacrylic acids) are stable only in form of the N-acyl derivatives and once liberated by hydrolysis are further hydrolyzed to α-keto acids. Therefore, 2,4-dinitrophenylhydrazine was added to an acid hydrolysate of stendomycin and the 2,4-dinitrophenylhydrazone of α-ketobutyric acid was isolated.[6] To prove the presence of the thus indicated dehydrobutyrine (α-aminodehydrobutyric acid) moiety stendomycin was hydrogenated in the presence of a palladium-on-charcoal catalyst. Hydrolysis of the resulting dihydrostendomycin[7] yielded only negligible amounts of ammonia, but on the other hand butyrine (α-aminobutyric acid, 1 mol) appeared in the hydrolysate. This leaves no doubt about the presence in stendomycin of a dehydrobutyrine residue,[6] to our best knowledge, not found so far in microbial products.

An additional building component of stendomycin, N-methyl-L-threonine, almost escaped detection. Recordings of the amino acid analysis[5] of hydrolysates revealed a trace of aspartic acid, and also a second small peak in the same region. No special attention was paid to this almost negligible peak. Subsequently, in the preparative separation of serine from allothreonine a spot somewhat faster than threonine but with poor, slowly developing ninhydrin color was observed on the paper chromatograms which were used to monitor the countercurrent distribution. The material that gave this spot did not separate from allothreonine during the distribution, but separation was possible by fractional crystallization from methanol. The amino acid was identified as N-methyl-L-threonine[8] by elemental analysis, N-methyl determination, nmr spectrum, measurements of optical rotation, and comparison with an authentic sample synthesized for this purpose.

On the short-column recordings of the amino acid analysis[5] only one basic amino acid was revealed.[9] It seemed to be different from the commonly known basic amino acids. The new amino acid was isolated from the acid hydrolysates through ion-exchange chromatography and was further purified by countercurrent distribution. The dihydrochloride of a compound with the elemental composition $C_8H_{16}N_4O_2$ was obtained. This formula was confirmed through the analysis of the crystalline diflavianate. The latter compound is insoluble enough to allow its direct crystallization from the acid hydrolysate of stendomycin simply by addition of flavianic acid. The chromatographically and analytically pure material thus obtained was converted to the crystalline but hygroscopic dihydrochloride, since the latter was more readily examined by nmr spectroscopy. The nmr spectra suggested a cyclic derivative of arginine with structure I (Chart I). The related compound II (capreomycidine), a component of the antibiotic capreomycin,[10] exhibits an nmr spectrum which is very similar to that of I. A detailed comparison of the spectra of I or II in conjunction with the examination of some model compounds led to the depicted assignment of the position of the N-methyl groups. Details of the determination of the structure of compound I, for which the name "stendomycidine" is proposed, will be published separately.[11]

Chart I



stendomycidine (Ste, I) capreomycidine (II)

Configuration of the Amino Acid Constituents. The configuration of the amino acid constituents was established by determination of the specific rotation of the amino acids isolated from an acid hydrolysate of stendomycin. Alanine was not separated from glycine since the rotation of their mixture could give the necessary information. In the case of stendomycidine optical rotatory dispersion of the copper complex of the amino acid[12,13] allowed the assignment of the L configuration. The same result was obtained by application of the Clough–Lutz–Jirgensons rule.[14] The stereochemistry at the β-carbon atom remains to be established.[11]

Each member of the stendomycin family contains 14 amino acid residues. Of these, two (glycine and dehydrobutyrine) have no center of asymmetry, five

(6) I. Muramatsu and M. Bodanszky, *J. Antibiot.* (Tokyo), *Ser. A*, **21**, 68 (1968). In the nmr spectrum of the antibiotic (in CD_3COOD) a doublet (three protons) at 1.84 ppm and a quartet (one proton) at 6.64 ppm can be assigned to the hydrogens on the side chain of the dehydrobutyrine residue. This assignment was confirmed by spin-decoupling experiments.

(7) Dihydrostendomycin shows inhibitory activity against yeast, comparable with that of the parent compound. We thank Professor D. Perlman (School of Pharmacy, University of Wisconsin) for this microbiological test.

(8) M. Bodanszky, G. G. Marconi, and G. C. Colman, *J. Antibiot.* (Tokyo), **21**, 668 (1968).

(9) A basic, unidentified amino acid was mentioned already in the first report on stendomycin.[1] It was revealed in paper chromatograms run in a system of butanol–acetic acid–water (3:1:1) as a slow moving spot, near the position of histidine.

(10) E. B. Herr, Jr., *Antimicrobial Agents Chemotherapy*, **1962**, 201 (1963).

(11) M. Bodanszky, G. Marconi, and A. Bodanszky, *J. Antibiot.* (Tokyo), **22**, 40 (1969).

(12) P. Pfeiffer and W. Christeleit, *J. Physiol. Chem.*, **245**, 197 (1937).

(13) N. Izumiya, M. Winitz, S. M. Birnbaum, and J. P. Greenstein, *J. Am. Chem. Soc.*, **78**, 1602 (1956).

(14) G. W. Clough, *J. Chem. Soc.*, **113**, 526 (1918); O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931).

(proline, N-methylthreonine, serine, stendomycidine, and one of the valine residues) belong to the L family, and seven (alanine, two of the valines, the two alloisoleucines, and the two allothreonines) are D-amino acids. A remarkable feature in the distribution of configurations is that four residues with two centers of asymmetry are D acids and also are members of the allo series. A scrutiny of the literature revealed[15] that isoleucine occurs in peptide antibiotics only in the L form, while the alloisoleucine residues have D configuration. This observation suggests that the D-amino acid constituents of microbial peptides originate from L-amino acids.

The Lactone Ring. In the ir spectrum of stendomycin the sharp CO band at 5.7 μ served as an indication for the presence of an ester or lactone bond in the molecule. Saponification with a dilute solution of sodium hydroxide in aqueous ethanol followed by acidification yielded stendomycin acid; ammonolysis in methanol resulted in the amide of this acid. Both the free acid and the amide show the same amino acid composition as the parent molecule; hence no elimination of a building component took place. The opening of the lactone bond in these reactions was also shown by the absence of the 5.7-μ band in the ir spectra of the products.

Dakin–West degradation[16] of stendomycin acid followed by amino acid analysis revealed that stendomycidine might be the C-terminal residue which participates in the lactone bond. On the other hand, the destruction of the hydroxyamino acids in this procedure left some doubt about this assignment. Firmer evidence was obtained from the reduction[17] of the antibiotic with $LiAlH_4$. Although in addition to stendomycidine proline was also found to be absent from the hydrolysate of the reduced product, it became obvious later that proline is reduced because it is followed by an imino acid (N-methylthreonine) in the sequence. Moreover, proline remained intact in the Dakin–West treatment of stendomycin acid; hence only stendomycidine can be the C-terminal residue.

The second bridgehead of the lactone bond could be any of the four hydroxyamino acids. The antibiotic was oxidized with chromic acid in acetic acid and the oxidation product hydrolyzed. Amino acid analysis showed that from the hydroxyamino acids only one allothreonine residue survived the reaction. It still remained to be established which of the two allothreonine moieties is involved in the lactone bond. Partial acid hydrolysis of the oxidation product of dihydrostendomycin and separation of the resulting peptides by column chromatography and countercurrent distribution allowed the isolation of the tripeptide allothreonylvalylvaline. Therefore, the bridgehead allothreonine must be that in position 8, and the one in position 11, which is followed by serine in the sequence, has a free hydroxyl group. Of course, this exact assignment in the sequence became possible only when the sequence of the amino acids was elucidated. Edman degradation[18] of the tripeptide gave valylvaline which was compared on Stein–Moore chromatograms and thin layer chromatograms with authentic L-valyl-L-valine and L-valyl-D-valine and was shown to be the "hetero" dipeptide. Optical rotation measurements, including ORD spectra, proved that the dipeptide corresponds to D-valyl-L-valine and therefore this experiment also established the position of the single L-valine residue.

Sequence Studies. Exploratory experiments with several proteolytic enzymes gave no encouraging results. Partial hydrolysis with hydrochloric acid yielded fragments which made it possible to establish the sequence of the amino acid constituents in the antibiotic. In order to determine the position of the dehydrobutyrine residue, a similar degradation had to be carried out on dihydrostendomycin. These experiments resulted in extremely complex mixtures of peptides corresponding to partial sequences. The unusual complexity followed from the unfortunate circumstance that not a single entity was degraded but a mixture of closely related compounds. Hence in the case of alloisoleucine-containing peptides there were present in the mixture also two analogous peptides in which alloisoleucine was replaced by valine or leucine. Because of this complexity the isolation of peptides from the partial hydrolysates required subsequent purification steps. After initial separation by ion-exchange chromatography the peptides were further purified by preparative thin layer chromatography or, especially in the case of stendomycidine-containing peptides, by high-voltage electrophoresis.

The sequence of amino acids within the small peptides was assigned by dinitrophenylation[19] of their N-terminal residue and/or by determination of their C-terminal acid through the Dakin–West method.[16] In some cases the results of the quantitative amino acid analysis could furnish the needed information. Tables I and II give a summary of the thus elucidated partial sequences.[20] The only complete sequence compatible with these partial sequences is the one shown in Chart II, which represents the full structure of stendomycin, or more exactly, the structure of the dominant component in this antibiotic family.

The proposed sequence of amino acids in stendomycin was confirmed by examination of the mass spectra[21] of the permethyl derivative of stendomycin acid and of dihydrostendomycin acid. The investigations through mass spectra were carried out in Professor Lederer's laboratory at Gif-sur-Yvette. The assign-

(15) M. Bodanszky and D. Perlman, *Nature*, **218**, 291 (1968).

(16) R. A. Turner and G. Schmerzler, *J. Am. Chem. Soc.*, **76**, 949 (1954); E. Bullock and A. W. Johnson, *J. Chem. Soc.*, 3280 (1957).

(17) M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **3**, 758 (1964).

(18) J. L. Bailey, "Techniques in Protein Chemistry," Elsevier Publishing Co., New York, N. Y., 1967, p 204.

(19) G. Pataki, "Dunnschichtchromatographie in der Aminosaure und Peptid-Chemie," W. de Gruyter and Co., Berlin, 1966, p 150.

(20) In addition to these peptides, "homo" (LL or DD) and "hetero" (LD or DL) valylvalines, valylalloisoleucine, and alloisoleucylvaline were found both in the partial hydrolysate and in the hydrolysates prepared with constant boiling hydrochloric acid at 100° for 16 hr. Comparison with authentic samples, in conjunction with the knowledge that alloisoleucine residues in stendomycin have the D configuration, showed that the two alloisoleucine-containing peptides have the D,D configuration. However, stendomycin was found later to have only one sequence with alloisoleucine and valine. In order to resolve this problem, the possibility of sequence reversal of dipeptides had to be considered. Reversal of the sequence probably through diketopiperazine intermediates is more likely in sequences which are relatively resistant to hydrolysis; *cf.* also T. Sanger and E. O. P. Thompson, *Biochim. Biophys. Acta*, **9**, 225 (1952). A study of the dipeptide, D-valyl-D-alloisoleucine, synthesized for this purpose and exposed to similar hydrolytic conditions confirmed that such a reversal of the sequence indeed occurs.

(21) D. W. Thomas, E. Lederer, M. Bodanszky, J. Izdebski, and I. Muramatsu, *Nature*, **220**, 580 (1968).

Table I. Peptides from Partial Acid Hydrolysis of Stendomycin[a]

Method for separation[b]	Fract no. in ion-exchange chromatography	Peptides
E, TC		FA-Pro
IC, TC	781–840	(NMeThr,Gly)
IC, TC	106–125	(NMeThr,Gly,Val,aIle,Ala)
IC, TC	106–125	(Gly,Val,aIle)-Ala
IC, DNP, TC	711–780	Val-(aIle,Ala)
IC, TC	170–200	aThr-Val
IC, CC, EP	1271–1430	Ser-aIle-Ste[c]
IC, CC, EP	1271–1430	(aIle,Ste)[c]
	Proposed sequence:	FA-Pro-NMeThr-Gly-Val-aIle-Ala-ΔBut-aThr-Val-Val-Thr-Ser-aIle-Ste

[a] From the very numerous peptides detected in the hydrolysate, only those which could be isolated in pure form and gave satisfactory amino acid analysis are listed. [b] E = extraction with hexane, TC = thin layer chromatography, IC = ion-exchange chromatography, DNP = dinitrophenylation, CC = chromatography on silica gel column, EP = high-voltage electrophoresis. [c] A second compound with valine replacing alloisoleucine has also been found.

Table II. Peptides from Partial Acid Hydrolysis of Dihydrostendomycin[a]

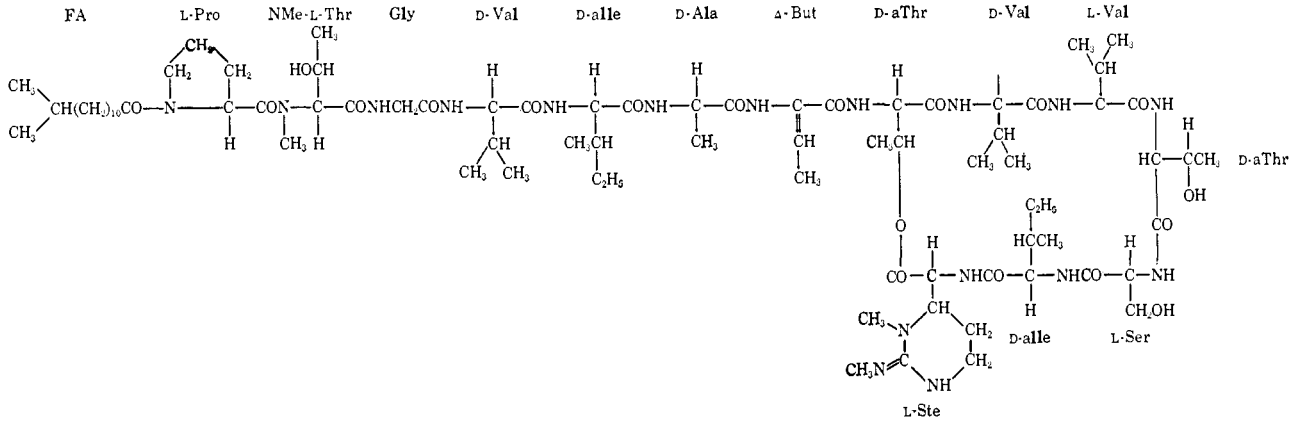
Method for separation[b]	Fract no. in ion-exchange chromatography	
E, TC		FA-Pro
IC, TC	766–835	(NMeThr,Gly)
IC, TC	706–765	(Gly,Val,aIle,Ala,But)
IC, TC	601–665	(Gly,Val,Val,Ala)-But
IC,TC or EP	1541–1750	Val-(aIle,Ala,But)
IC, TC	980–1030	(Val,Val,Ala,But)
IC, EP	1031–1151	(aThr,Val)
IC, EP	2271–2310	(aThr,Val,Val,aThr,Ser,aIle,Ste)
IC, EP	2271–2310	(aThr,Ser,aIle,Ste)[c]
IC, EP	2331–2450	(Ser,aIle,Ste)[c]
	Proposed sequence:	FA-Pro-NMeThr-Gly-Val-aIle-Ala-But-aThr-Val-Val-Thr-Ser-aIle-Ste

[a–c] For explanation, *cf.* Table I.

ment of sequence based on mass spectra was intentionally done without the information available from chemical experiments.

solubility properties of the antibiotic and from the lack of color reaction with a modified[22] Rydon reagent[23] the conclusion was drawn[24] that the "side chain"

Chart II. Structure of Stendomycin[a]



[a] This structure represents the dominant compound of the stendomycin family. In other members of the stendomycin group isomyristic acid is replaced by its lower homologs and alloisoleucine by valine or leucine.

Conformation of Stendomycin. In organic solvents the optical rotatory dispersion of the antibiotic exhibits a Cotton effect at about 300 mμ not related to the single chromophore, the double bond of the dehydrobutyrine residue. Dihydrostendomycin shows the same Cotton effect, while it is missing in stendomycin acid. Although from these observations no direct conclusion can be drawn for the conformation of the antibiotic, they create the impression that the lactone is present in a preferred conformation. From the unusual (sequence 1–7) bends over the lactone ring. The compact globular structure which results from this folding seems to hide the amide bonds while the numerous hydrophobic amino acid side chains and the fatty acid residue are on the outside of the molecule. The anion associated with the strong cationic center of the

(22) R. H. Mazur, B. W. Ellis, and P. S. Cammarata, *J. Biol. Chem.*, 237, 1619 (1962).
(23) H. N. Rydon and P. W. Smith, *Nature*, 169, 922 (1952).
(24) M. Bodanszky and A. Bodanszky, *ibid.*, 220, 73 (1968).

stendomycidine residue is located in a sheltered niche. This architecture prompted speculations[24] about the role of some microbial peptides in the life processes of the microorganisms.

Experimental Section

Stendomycin Salicylate.[1] Salicyclic acid (0.90 g) was dissolved in water with the addition of 2 *N* NaOH to give a solution of pH 6. A solution of stendomycin (1.0 g) in water (100 ml) was added. The precipitate was separated by centrifugation and washed with water (three 80-ml portions) in the centrifuge tube. The product, dried *in vacuo*, weighed 0.90 g. For an analysis a sample was dried at 125° *in vacuo* for 3 hr.

Anal. Calcd for $C_{35}H_{141}N_{17}O_{22}$ (1753.2) (assuming isotridecanoic acid, two alloisoleucines, and three valines in the molecule): C, 58.24; H, 8.11; N, 13.58. Found: C, 59.86; H, 8.31; N, 13.87.

For amino acid analysis *cf.* ref 4. From the recoveries of amino acids in the analysis the molecular weight of 1720 was calculated for stendomycin salicylate.

Dihydrostendomycin. To a solution of stendomycin (3.0 g) in 95% ethanol (120 ml) acetic acid (15 drops) and a 10% palladium-on-charcoal catalyst (1.0 g) were added. The mixture was stirred in an atmosphere of hydrogen for about 24 hr. The uv absorption of the solution at 240 mμ decreased to a minimum representing about 10% of the original absorption. The catalyst was removed by filtration aided by Celite. After evaporation of the solvent the residue was dried in a desiccator over silica gel to constant weight (2.8 g). Amino acid analysis after hydrolysis with constant boiling hydrochloric acid in an evacuated sealed ampoule for 16 hr gave the following results: aThr, 1.9; Ser, 0.9; Pro, 0.9; Gly, 1.0; Ala, 1.1; But, 1.0; Val, 2.8; aIle, 1.2; Leu, 0.1; Ste (stendomycidine), 0.8; NH_3, trace.

From the combined values of the recoveries in the amino acid analyses of glycine, proline, and alanine a molecular weight of about 1600 could be calculated for dihydrostendomycin.

A sample (0.80 g) was converted to the salicylate (0.69 g) as described above for stendomycin. The amino acid analysis of this salt gave similar results as those from the material obtained after reduction. From the recoveries a molecular weight of 1760 was calculated for dihydrostendomycin salicylate.

Stendomycin Acid. A solution of 0.19 *N* sodium hydroxide (0.85 ml) was added drop by drop to a stirred solution of stendomycin (300 mg) in water (25 ml). About 1 hr was required for the addition, after which the solution was acidified with excess 0.1 *N* hydrochloric acid (9 ml) and diluted with water to 50 ml. Chloroform (10 ml) was added; the precipitate did not dissolve but appeared at the interphase. It was isolated by centrifugation, washed with a mixture of chloroform and water, and dried *in vacuo* (158 mg). On thin layer chromatograms (silica gel, butanol–acetic acid–water, 4:1:1) stendomycin acid travels as a single spot with an R_f of 0.45.[25] Stendomycin has an R_f value of 0.69 in this system. In the ir spectrum of the product the 5.7-μ band characteristic for stendomycin is absent. Amino acid analyses gave about the same results as analysis of the starting material. A sample of stendomycin acid was used for sequence determination through mass spectra.[21]

Stendomycinamide and Dihydrostendomycinamide. Stendomycin (0.30 g) was dissolved in a *ca.* 4 *N* solution of ammonia in methanol (20 ml). On the following day the solvent was evaporated with a stream of nitrogen and residue dried. The 5.7-μ band was absent in the product. No solvent system was found for its purification; amino acid analysis: aThr, 1.9; Ser, 1.0; Pro, 1.0; Gly, 1.0; Ala, 1.1; Val, 2.7; aIle, 1.1; Leu, 0.1; NH_3, 1.6 (stendomycidine and methylamine were not determined).

Dihydrostendomycin amide was prepared similarly; amino acid analysis: aThr, 1.9; Ser, 0.9; Pro, 0.9; Gly, 1.0; Ala, 1.0; But, 1.0; Val, 2.9; aIle, 1.2; Leu, 0.1; NH_3, 1.2 (stendomycidine and methylamine were not determined).

(25) Opening of the lactone provides some information on the molecular weight of the antibiotic. This procedure can be considered as a version of the partial substitution method of A. R. Battersby and L. C. Craig (*J. Am. Chem. Soc.*, 73, 1887 (1951)). Since only a single spot was revealed on thin layer chromatograms and a part of the intact lactone was recovered from the chloroform layer, it is very likely that the antibiotic is an acyl tetradecapeptide lactone and not a dimeric lactone or a higher polymer. In these latter cases the formation of more than one acidic species would be expected.

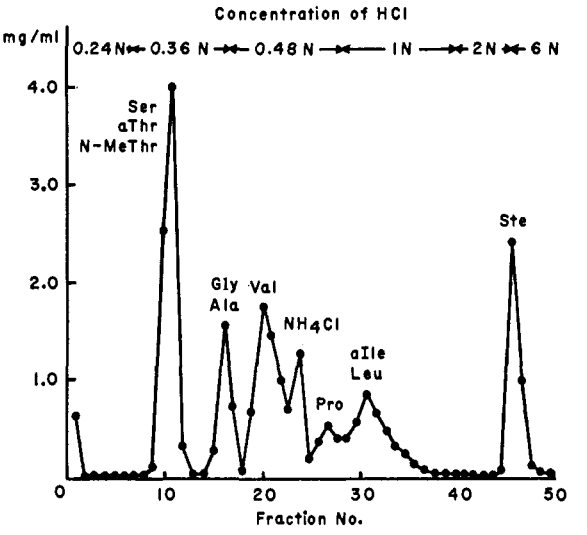


Figure 1.

Separation and Identification of the Amino Acid Constituents. Stendomycin (10 g) was dissolved in concentrated hydrochloric acid (100 ml), and the solution was diluted with water (100 ml) and heated under reflux in an atmosphere of nitrogen for 66 hr. The mixture was cooled to room temperature and extracted with hexane (150 ml, in three portions) in order to remove the fatty acids.[3] The hydrochloric acid was evaporated on a steam bath with a stream of nitrogen, the residue was dissolved in water (50 ml) and reevaporated, and this procedure was repeated twice more. The residue was dissolved in water (about 35 ml) and applied on a column (4.5 cm × 40.6 cm) of Dowex 50W-X12 ion-exchange resin, H^+ cycle. For elution hydrochloric acid was used in increasing concentration. First, 0.24 *N* acid (2.5 l.) was applied followed by 0.36 *N* (5 l.), 0.48 *N* (6.5 l.), 1 *N* (6 l.), 2 *N* (2 l.), and 6 *N* (2.5 l.). Fractions of 500 ml were collected and examined on paper chromatograms. The hydrochloric acid was removed by evaporation under nitrogen from each fraction and the weight of the residue plotted. The elution pattern is shown in Figure 1.

L-Serine. From fractions 9–12 of the ion-exchange chromatogram serine was isolated by countercurrent distribution in a system consisting of 1-butanol and 0.01 *N* hydrochloric acid. An automatic Craig apparatus[26] with 520 tubes was used with 3-ml upper and 3-ml lower layers. After 1000 transfers recycling was started. After a total of 6600 transfers serine hydrochloride was isolated by evaporation of the solvents from the contents of tubes 120–200. The hydrochloride was dissolved in ethanol and the solution made slightly alkaline with triethylamine, then weakly acid with 1 drop of acetic acid. Serine was secured as a white crystalline solid by filtration and washing with ethanol. The ir and nmr spectra were identical with those of an authentic sample of L-serine; $[\alpha]^{24}D$ +13.4° (*c* 2, 1 *N* HCl); lit.[27] $[\alpha]^{25}D$ +15.1°.

Anal. Calcd for $C_3H_7NO_3$: C, 34.24; H, 6.71; N, 13.33. Found: C, 34.34; H, 6.80; N, 13.25.

D-Allothreonine. This amino acid was obtained from fractions 220–300 of the above countercurrent distribution (6600 transfers) as described for L-serine. The ir and nmr spectra were found to be identical with those of an authentic sample of L-allothreonine; $[\alpha]^{24}D$ −31.0° (*c* 2, 5 *N* HCl); lit.[27] $[\alpha]^{25}D$ −31.7°.

Anal. Calcd for $C_4H_9NO_3$: C, 40.33; H, 7.62; N, 11.76. Found: C, 40.43; H, 7.73; N, 11.72.

N-Methyl-L-threonine. Addition of chloroform to the mother liquors from the crystallization of D-allothreonine gave crystals of the N-methylamino acid which were recrystallized from methanol. In the nmr spectrum a three-proton singlet at 2.74 ppm (in D_2O with DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as internal standard) suggested the presence of an N-methyl group.

Anal. Calcd for $C_5H_{11}NO_3$: C, 45.10; H, 8.33; N, 10.52; N-Me, 11.3; neut equiv, 133.15. Found: C, 45.05; H, 8.54; N, 10.53; N-Me, 11.1; neut equiv, 132 (as acid), 136 (as base).

(26) L. C. Craig and T. P. King, *Federation Proc.*, 17, 1126 (1958).

(27) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley & Sons, Inc., New York, N. Y., 1961.

The determination of the configuration of the N-methyl-L-threonine residue was described in a separate communication.[8]

Glycine and D-Alanine. Quantitative amino acid analysis of fractions 14–17 (*cf.* Figure 1) showed that these contain glycine and alanine in equimolar amounts. The optical rotation of the mixture of hydrochlorides was determined and the calculation of the specific rotation was based on the alanine content; $[\alpha]^{24}D$ −11° (*c* 3.76, 5 *N* HCl); lit.[27] $[\alpha]D$ −14.4° (for D-alanine).

The identity of these two amino acids was established through the elution volumes on Stein–Moore chromatograms and by comparison with authentic samples on paper and thin layer chromatograms. No attempt was made for the isolation of pure glycine and alanine.

Valine. The residue from the valine-containing fractions (19–24) was dissolved in ethanol and treated with triethylamine. The valine thus obtained showed $[\alpha]^{27}D$ −8° (*c* 3, 5 *N* HCl), and a second crop, $[\alpha]^{24}D$ −13° (*c* 0.8, 5 *N* HCl); lit.[27] $[\alpha]^{25}D$ (for D-valine) −28.3° (*c* 1–2, 5 *N* HCl). A mixture of 2 mol of D-valine and 1 mol of L-valine would give $[\alpha]D$ −9.4°; a mixture of 2.5 mol of D-valine and 1 mol of L-valine $[\alpha]D$ −12.1°. The nmr spectrum is indistinguishable from that of an authentic sample of valine.

Anal. Calcd for $C_5H_{11}NO_2$: C, 51.26; H, 9.46; N, 11.96. Found: C, 51.11; H, 9.29; N, 11.87.

L-Proline. The hydrochloride from tubes 26–29 was dissolved in a small volume of water, and the solution was neutralized with 1 *N* NaOH to about pH 6 and evaporated to dryness. The residue was extracted with hot ethanol and the solvent evaporated. The crude semicrystalline residue gave the ir spectrum of proline and was indistinguishable from proline on paper chromatograms and thin layer chromatograms; $[\alpha]^{24}D$ −82° (*c* 1.23, H_2O) (the concentration was determined by amino acid analysis of the solution); lit.[27] $[\alpha]^{25}D$ −86° (*c* 1–2, H_2O).

D-Alloisoleucine. This amino acid was isolated from fractions 31–36 as described for valine. The crude product was dissolved in a small volume of hot water and crystallized by the addition of ten volumes of ethanol. The ir and nmr spectra were those of alloisoleucine, but quantitative amino acid analysis revealed the presence of about 10% leucine; $[\alpha]^{24}D$ −36° (*c* 0.8, 5 *N* HCl); lit.[27] $[\alpha]^{25}D$ −39.6° (*c* 2, 5 *N* HCl) for D-alloisoleucine and −16° (*c* 2, 5 *N* HCl) for D-leucine. A commercial sample of D-alloisoleucine had $[\alpha]^{24}D$ −36° (*c* 0.5, 5 *N* HCl).

Anal. Calcd for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.68. Found: C, 54.95; H, 10.10; N, 10.69.

Stendomycidine. Evaporation to dryness of fractions 45–48 left a residue that was further purified by a short countercurrent distribution (30 transfers) in a system of 1-butanol–0.001 *N* hydrochloric acid. Stendomycidine dihydrochloride was recovered by evaporation from tubes 0–5.

Anal. Calcd for $C_8H_{16}N_4O_2 \cdot 2HCl$: C, 35.2; H, 6.6; N, 20.5; Cl, 26.0. Found: C, 34.9; H, 6.0; N, 20.3; Cl, 26.1.

The dihydrochloride was converted to the crystalline diflavianate by addition of an aqueous solution of excess flavianic acid to the solution of the dihydrochloride in water. The same crystalline salt was prepared by addition of a solution of excess flavianic acid in water to a hydrolysate of stendomycin (after the removal of fatty acids).

Anal. Calcd for $C_{28}H_{28}N_8O_{18}S_2$: C, 40.6; H, 3.4; N, 13.5; S, 7.7. Found: C, 40.3; H, 3.6; N, 13.7; S, 7.7.

Oxidation of Stendomycin and Its Derivatives with Chromic Acid.[28] Samples (about 10 mg) were dissolved in acetic acid (0.3 ml) containing CrO_3 (0.01 g) and pyridine (0.01 ml). After 16 hr at room temperature 95% ethanol (5 ml) was added to each mixture. The solvents were evaporated with a stream of nitrogen and the residues hydrolyzed (16 hr) for amino acid analysis as described in previous paragraphs, to give (oxidized peptide, aThr, Ser): stendomycin, 1.1, 0.3; dihydrostendomycin, 1.0, 0.1; stendomycin acid, 0.2, 0.1; stendomycinamide, 0.1, 0.1; dihydrostendomycinamide, 0.1, 0.1.

These values are calculated on the basis of alanine and glycine being 1.0. The amount of N-methyl-L-threonine could not be measured, but the appearance of significant amounts of methylamine in the hydrolysate of the oxidized products indicated that this amino acid was also destroyed during oxidation. Since before oxidation stendomycin contained two allothreonines and one serine residue, it becomes obvious from this experiment that the hydroxyl group of one of the allothreonines is involved in the lactone bond.

(28) J. C. Sheehan, H. G. Zachau, and W. B. Lawson, *J. Am. Chem. Soc.*, 80, 3349 (1958).

Chromic Acid Oxidation of Stendomycin Followed by Partial Acid Hydrolysis. Isolation and Edman Degradation of D-Allothreonyl-D-valyl-L-valine. Stendomycin (3 g) was oxidized as described above. After the addition of ethanol and evaporation the residue was dissolved in 1-butanol (120 ml) and the resulting solution extracted 20 times with 1% acetic acid (80 ml) saturated with butanol and twice with butanol-saturated water (80 ml). Most of the oxidized peptide remained in the butanol layer. After removal of the solvent *in vacuo* the residue (2.3 g) was analyzed for amino acids. The results of the analysis confirmed that one allothreonine residue remained intact, while the second allothreonine and the serine moiety were destroyed. The oxidized material (2.2 g) was dissolved in 2 *N* hydrochloric acid (100 ml) and the solution heated to boiling under reflux for 3 hr. After cooling and filtration the hydrochloric acid was removed *in vacuo* to leave a residue which in turn was dissolved in a mixture (5 ml) of 1-butanol–acetic acid–water (4:1:1), applied to a column of silica gel (300 g of silica gel, 42 cm high), and chromatographed (flow rate 30–35 ml/hr) with the same solvent mixture as eluent. Fractions of 6 ml were collected and examined by thin layer chromatography. Fractions 79–99 were combined, the solvent was removed, and the residue (0.47 g) was rechromatographed on a second silica gel column (420 g, 104 cm) with a mixture of 1-propanol–acetic acid–water (16:1:1) as eluent.

The flow rate was kept at about 30 ml/hr; 6-ml fractions were collected. Fractions 161–222 were pooled and the solvent was removed. The residue (0.40 g) was dissolved in a mixture of 1 butanol–acetic acid–water (4:1:5) and placed in the first tube of a 520-tube automatic countercurrent distribution apparatus[26] with 3-ml lower and 3-ml upper layers. The single-withdrawal method was used. The contents of tubes 641–860 of the withdrawn series were pooled and the solvent was evaporated. The residue (60 mg) gave a single spot on thin layer chromatograms (silica gel, 1-butanol–acetic acid–water, 4:1:1) with a modified[22] Rydon[23] reagent. Some of the same peptide could be detected also in tubes up to about 1020 but mixed with a second peptide, and therefore the contents of tubes 860–1020 were not included in the subsequent experiments.

On amino acid analysis (64-hr hydrolysis) the purified peptide showed the following composition: aThr, 1.0; Val, 2.1.

The tripeptide (49 mg) was treated at room temperature for 2.5 hr with a mixture of phenyl isothiocyanate (0.15 ml) in a buffer solution (5 ml) prepared from ethylmorpholine (60 ml), acetic acid (1.5 ml), 95% ethanol (500 ml), and water (440 ml). Most of the ethanol was removed by evaporation *in vacuo* and the residual solution washed with benzene three times. The aqueous layer was concentrated almost to dryness and diluted with water (2 ml). The pH of the solution was adjusted to 2.0 by the addition of 2 *N* hydrochloric acid. An oil separated and was extracted with ethyl acetate. The ethyl acetate was removed *in vacuo*, the residue was dissolved in trifluoroacetic acid (10 ml), and the solution was left to stand at room temperature for 1.5 hr. After removal of the trifluoroacetic acid *in vacuo* water (2 ml) was added to the residue and the mixture extracted twice with ethyl acetate (2 ml). The aqueous layer was concentrated to a syrup from which a white solid was secured by treatment with a mixture of ethanol, ether, and hexane. On thin layer chromatograms the presence of valylvaline (DL or LD) and of a small amount of valine was revealed. After hydrolysis only valine could be detected. The ORD curve of a solution (0.014% in water) of the dipeptide showed a negative Cotton effect at 213 mμ. A sample of L-valyl-D-valine synthesized for this purpose (see below) gave a similar but positive Cotton effect. Hence, the natural peptide is D-valyl-L-valine. (The ORD curve of L-valyl-L-valine is quite different from the ones here discussed.)

Dakin–West Degradation[16] of Stendomycin Acid. A sample (8.2 mg) of stendomycin acid was dissolved in a mixture of acetic anhydride (0.75 ml) and pyridine (0.5 ml) and heated in a sealed ampoule at 150° for 3 hr. After cooling a few milliliters of water was added and the mixture evaporated to dryness on a steam bath. The residue was hydrolyzed with hydrochloric acid for amino acid analysis which revealed the complete absence of stendomycidine. However, the amounts of serine, threonine, and proline were also greatly reduced.

Reduction of Stendomycin and Dihydrostendomycin with Lithium Aluminum Hydride.[17] Lithium aluminum hydride (500 mg) was dissolved in 55 ml of tetrahydrofuran and the insoluble material was filtered off. Stendomycin (30 mg) was dissolved in tetrahydrofuran (25 ml) and mixed with the lithium aluminum hydride solution (11 ml). The mixture was stirred at room temperature. After 1

Table III. Protected Peptides

	Yield, %	Mp, °C	$[\alpha]^{25}D$, deg (c, 95% ethanol)	Solvent of crystallization
Z-L-Val-L-Val-OBZL	63	115–116[a]		Acetone-petroleum ether[b]
Z-L-Val-D-Val-OBZL	81	145–147	+11.8 (2)	Acetone–ether–petroleum ether
Z-D-Val-D-aIle-OBZL	80	133–135	+34.5 (0.9)	Acetone–ether–petroleum ether
Z-L-Val-D-aIle-OBZL	71	133–135	+8.7 (0.9)	Acetone–ether–petroleum ether
Z-D-aIle-D-Val-OBZL	84	120–121	+32.3 (0.9)	Acetone–ether–petroleum ether
Z-D-aIle-L-Val-OBZL	72	129–131	−14.9 (2)	Ethyl acetate-petroleum ether

[a] Lit. (ref 27, p 1139) mp 116°. [b] Petroleum ether, bp 40–50°.

hr the excess reducing reagent was decomposed by the addition of 6 *N* hydrochloric acid (*ca.* 1 ml) and the pH of the mixture was adjusted to about 6. The precipitate was collected on a filter, washed with tetrahydrofuran, and dried. The same procedure was carried out with dihydrostendomycin. For amino acid analysis the reduction products were hydrolyzed with 6 *N* hydrochloric acid for 48 hr. The results of the analysis show that proline and stendomycidine were lost during reduction; stendomycin: aThr, 2.2; Ser, 1.0; Pro, 0.2; Gly, 0.9; Ala, 0.9; But, 0.1; Val, 3.9; aIle, 1.9; Leu, 0.1; Ste, 0.0; dihydrostendomycin: aThr, 2.3; Ser, 1.0; Pro, 0.2; Gly, 1.0; Ala, 1.0; But, 0.9; Val, 3.7; aIle, 1.9; Leu, 0.1; Ste, 0.0.

Partial Acid Hydrolysis of Stendomycin. A sample of the antibiotic (1.0 g) was dissolved in concentrated hydrochloric acid (20 ml). After 5 days at room temperature the solution was placed in a vacuum desiccator over NaOH and concentrated to about 5 ml. The residue was diluted with water (20 ml) and extracted with hexane (150 ml). Evaporation of the hexane extract to dryness left an oily residue (0.23 g). The aqueous layer was evaporated again and after dilution with water (20 ml) extracted with ethyl acetate (150 ml). The extract after evaporation left an oily residue (0.01 g), which was not further studied. The aqueous solution was evaporated to dryness (weight about 1 g).

The oily residue from the extraction with hexane was purified by preparative thin layer chromatography. The main product gave a yellow spot with Rydon reagent and was ninhydrin negative. After hydrolysis it gave proline; from the hydrolysate fatty acids were extracted and identified (ir spectra) as discussed earlier.[3]

Separation of Peptides from Partial Hydrolysis of Stendomycin. Dowex 50-X12, 200–400 mesh, H^+ cycle (450 g), was washed with 2 *N* pyridine and then with buffer 1 (pH 3.0; see below). The resin was poured into a 4 × 75 cm column. The column was equilibrated with buffer 1 until the pH of the effluent was 3.0.

A sample (0.6 g) of the water-soluble fraction (*cf.* preceeding paragraph) in buffer 1 (100 ml) was applied to the column. Fractions of 7 ml were collected at a flow rate of about 20 ml/hr.

For elution a series of buffers were used: (1) 0.05 *M* pyridine, formic acid to pH 3.0, for fractions 1–551; (2) 0.10 *M* pyridine, acetic acid to pH 3.5, for fractions 552–1225; (3) 0.40 *M* pyridine, acetic acid to pH 5.0, for fractions 1226–1260; (4) 0.60 *M* pyridine, acetic acid to pH 6.0, for fractions 1261–1520.

The content of every fifth tube was evaporated and the residue examined on thin layer chromatograms (silica gel, butanol-acetic acid-water, 3:1:1). Fractions containing peptides with identical R_f values were pooled and the solvents removed *in vacuo*. For final purification preparative thin layer chromatography on silica gel plates in the above-mentioned solvent system was used. The peptides were eluted from the plates according to guide strips revealed with a modified Rydon reagent.[22,23] For a few peptides chromatography on silica gel columns was applied. The fragments that contained stendomycidine were purified by electrophoresis on Whatman 3MM paper (104 × 46 cm) at 4000 V, 5 hr, in a buffer (pH 6.4) made of pyridine-acetic acid-water (25:1:375).

The purified peptides were analyzed for amino acids; in some cases their N-terminal amino acid was determined by dinitrophenylation[19] while the Dakin-West procedure[16] was applied for the determination of the C-terminal amino acid. The results of these analyses are summarized in Table I.

Partial Acid Hydrolysis of Dihydrostendomycin and Separation of the Resulting Peptides. For hydrolysis the same conditions were used as in the case of stendomycin except that the reaction was terminated, by evaporation, after 3 days. For the separation of peptides the methods described above for peptides from stendomycin were applied. However, the ion-exchange resin Dowex 50W-X2 and a somewhat different series of buffers were used: (1) 0.05 *M* pyridine, formic acid to pH 3.0, fractions 1–2000; (2) 0.30 *M* pyridine, acetic acid to pH 4.5, fractions 2001–2150; (3) 0.40 *M* pyridine, acetic acid to pH 5.0, fractions 2151–2600.

The fractionation was completed by preparative thin layer chromatography and high-voltage paper electrophoresis as discussed before. The results of the analyses of the purified peptides are summarized in Table II.

Acylproline-N-methylamide from Dihydrostendomycin. Dihydrostendomycin (0.3 g) was oxidized with chromic acid as described in the oxidation of stendomycin. The residue from the evaporation of the butanol layer (0.24 g) was suspended in water (15 ml) and the mixture extracted with hexane. Evaporation of the hexane left a residue (27 mg) which was purified by preparative thin layer chromatography on silica gel in the system butanol-acetic acid-water (4:1:1). The band which was detected on guide strips with a modified Rydon reagent[22,23] as R_f 0.7 was eluted with ethyl acetate and hydrolyzed with 6 *N* HCl in the usual manner. Amino acid analyses revealed the presence of proline and methylamine in the hydrolysate. This fragment, acylproline-N-methylamide, indicates that proline is followed by the N-methyl-L-threonine residue in the sequence of the parent molecule.

Synthesis of Dipeptides. Valylvaline (L,L and L,D), alloisoleucylvaline (D,D and D,L), and valylalloisoleucine (D,D and L,D) were synthesized for comparison with dipeptides in hydrolysates of stendomycin. Coupling of N-carbobenzyloxy amino acids and amino acid benzyl esters by dicyclohexylcarbodiimide was followed by hydrogenolysis of the protecting groups. The free peptides were crystallized from water–ethanol. Yields and properties of the protected intermediates and of the free peptides are shown in Tables III and IV.

Table IV. Free Peptides

	Yield, %	$[\alpha]^{25}D$, deg (c, water)	R_f[b]	Elution time,[c] min
L-Val-L-Val	69	+10.6[a] (2.45)	0.50	54
L-Val-D-Val	80	+64.2[a] (0.95)	0.41	34
D-Val-D-aIle	82	−21.6 (0.57)	0.59	103
L-Val-D-aIle	76	+47.4 (0.59)	0.48	74
D-aIle-D-Val	68	−22.1 (0.66)	0.59	78
D-aIle-L-Val	82	−63.3 (1.26)	0.48	65

[a] Lit. (ref 27, p 1229) $[\alpha]^{25}D$ +10.8 and +59.5°, respectively. [b] On silica gel thin layer chromatograms, with freshly prepared mixture of butanol–acetic acid–water (4:1:1). [c] Elution times measured from the leucine peak, on the Beckman Spinco amino acid analyzer, Model 120C with buffer B (*cf.* ref 5).

Treatment of D-Valyl-D-alloisoleucine with Hot 6 *N* Hydrochloric Acid. A sample of the synthetic dipeptide (10 mg) was heated with 1 ml of 6 *N* hydrochloric acid in an evacuated sealed ampoule at 110° for 10 hr. After evaporation to dryness the residue was dissolved in water and chromatographed on thin layer plates of silica gel with a butanol-acetic acid-water (4:1:1) mixture. The band containing the mixture of dipeptides was eluted with 1% acetic acid and the eluted material treated in aqueous ethanol, with an excess of 2,4-dinitrofluorobenzene in the presence of sodium bicarbonate. After extraction with ether, the mixture was acidified and DNP-peptides were extracted with ether. They were hydrolyzed with 6 *N* hydrochloric acid at 110° for 18 and 54 hr. From the hydrolysate the DNP-amino acids were extracted with ether and examined on silica gel plates (benzene–pyridine–acetic

acid, 80:20:2). Yellow spots corresponding to DNP-valine and DNP-alloisoleucine were detected. The aqueous layer after extraction of DNP-amino acids contained valine and alloisoleucine, as shown by silica gel thin layer chromatography (butanol–acetic acid–water, 4:1:1).

Treatment of L-Valyl-L-valine with Hot Hydrochloric Acid. The dipeptide (11.8 mg) was dissolved in 6 *N* hydrochloric acid (1 ml) and treated as described for valylalloisoleucine. Thin layer chromatography (silica gel, butanol–acetic acid–water (4:1:1)) showed that the hydrolysate contained valine and homovalylvaline but no heterovalylvaline; hence no detectable racemization occurred. Thus the heterovalylvaline in the hydrolysate of stendomycin does not originate from homovalylvaline.

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Diffusion-Controlled Proton Transfer in Intramolecular Thiol Ester Aminolysis and Thiazoline Hydrolysis[1]

Ronald E. Barnett and William P. Jencks

Contribution No. 636 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154.
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Abstract: The following evidence supports the conclusion that a simple proton transfer step is rate determining in the intramolecular aminolysis of S-acetylmercaptoethylamine above pH 2.3 and determines the products of thiazoline hydrolysis in this pH region. (1) The value of α for general-acid catalysis by carboxylic acids and phosphate monoanion of the formation of amide from the uncharged thiol ester is experimentally indistinguishable from zero, as expected for a diffusion-controlled proton transfer. (2) The catalytic constant for the solvated proton is 24 times higher than for carboxylic acids and phosphate monoanion, corresponding to the known rapid rate of proton transfer from this species. (3) Catalytic constants for acids with pK values larger than 7.4 approach the expected Brønsted slope of $\alpha = 1.0$ expected for weak acids in a simple proton transfer reaction in the thermodynamically unfavorable direction. (4) The break in the corresponding Brønsted plot for S-chloroacetylmercaptoethylamine occurs 1.5 pH units lower than that for the acetyl compound, as predicted from the reduced basicity of the addition intermediate in this reaction. (5) Water and bicarbonate ion, which can act as bifunctional acid–base catalysts, exhibit enhanced catalytic activity, analogous to the known behavior of water in simple intramolecular proton transfer reactions. (6) The yield of thiol ester in the hydrolysis of thiazoline decreases with increasing pH, with a half-maximum yield at pH 2.37 corresponding to the change in the rate-determining step of the S to N transfer reaction at pH 2.30. (7) The proposed mechanism provides a satisfactory solution to the "thiazoline dilemma." Rate and equilibrium constants have been estimated for all of the steps of the reaction and appear to be within reasonable limits.

General acid–base catalyzed carbonyl addition and acyl transfer reactions differ from simple proton transfer reactions, such as ketone enolization, in that bonds between carbon and oxygen, nitrogen, or sulfur must be formed and broken in the former class of reactions; in fact, it is the formation or cleavage of such bonds rather than the proton transfer which is generally thought to provide the principal energy barrier in these reactions. It has been suggested that the proton transfer is the rate-determining step in reactions of this kind, by analogy with simple proton transfer reactions of carbon acids.[2-5] Recently, however, the view has become increasingly accepted that many such reactions are "concerted" or "coupled," in the sense that formation or cleavage of bonds to carbon takes place in the rate-determining step; the exact role of the associated proton transfer is not yet clear.[6-10] The strongest experimental evidence for this conclusion is (1) the fact that such reactions frequently exhibit Brønsted slopes other than 0 or 1 over considerable ranges of catalyst acidity or basicity, whereas if proton transfer alone were involved the Brønsted slopes should approach the values of 0 or 1.0 characteristic of diffusion-controlled proton transfer between electronegative atoms,[8] (2) the occurrence of heavy atom isotope effects in reactions of this kind,[11] and (3) the require-

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