The Structure of Telomycin

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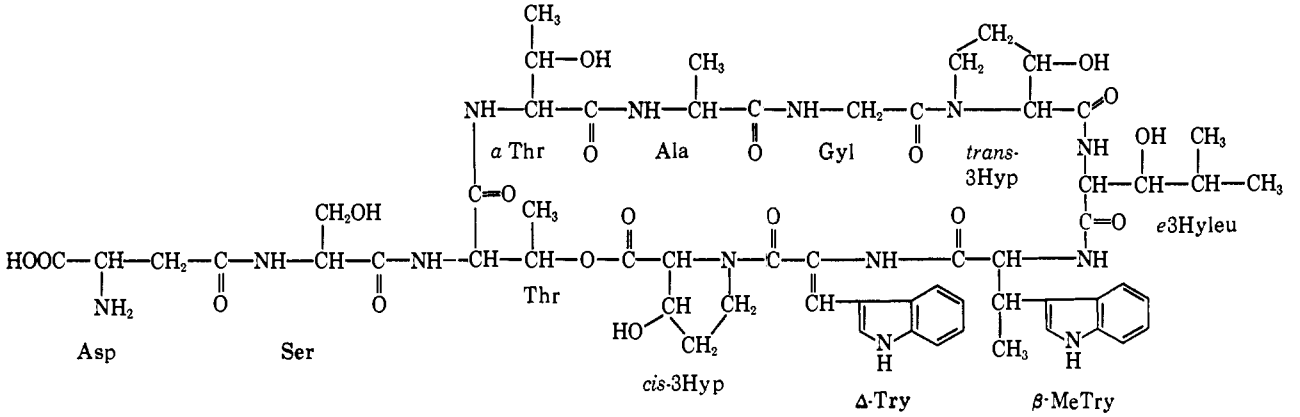
Abstract: The structure of Telomycin, a new antibiotic, is shown to be the macrocyclic peptide lactone, as shown in structure I. Telomycin contains eleven amino acids, two of which are not known as components of natural proteins. In the determination of the amino acid sequence, partial basic hydrolysis was used successfully to provide five water-soluble peptides and one water-insoluble hexapeptide. On these, a combination of the Edman, Sanger (DNFB method), and Akabori (hydrazinolysis) degradations were applied for sequence information. The presence of β-methyltryptophan was established mass spectrometrically. Potentiometric pK measurements and comparison of model compounds were used to assign the type of peptide bond of the N-terminal aspartic acid to be β. The structural unit in Telomycin responsible for the 339-mμ ultraviolet maximum is shown by degradative studies as well as by the synthesis of model compounds to be dehydrotryptophan. A mechanism is proposed for the unusual products obtained from the chromophore by basic hydrolysis of Telomycin.

Telomycin, a new peptide antibiotic, was isolated in 1958 by Misiek, *et al.*,[2] from the culture broth of an unidentified Streptomyces. This antibiotic, a colorless solid, either amorphous or crystalline, is sparingly soluble in water and relatively stable at room temperature over a wide range of pH values. It is active against Gram-positive organisms, including antibiotic-resistant strains of *Micrococcus pyogenes var. aureus*, but has little or no effect against most Gram-negative bacteria.[3] The high activity against Gram-positive bacteria and the relatively low toxicity, together with the fact that Telomycin gives upon hydrolysis four new amino acids, encouraged a more detailed study.



Telomycin (I)

Telomycin can be detected on chromatograms and electropherograms by a characteristic blue ultraviolet fluorescence, by ninhydrin, or by the *t*-butyl hypochlorite–starch–iodide reagent.[4]

The Telomycin samples studied appear to be homogeneous, both by electrophoresis (at pH 1.9 and 6.4) and by thin layer chromatography (isopropyl alcohol–3% ammonia on silica gel G). Countercurrent distribution between an upper phase of *t*-butyl alcohol and a lower phase of water containing 4% sodium chloride gives a single symmetrical peak.[2a]

Our study confirms and extends the published ultraviolet spectral data.[2a] In ethanol–water (2:1) maxima occur at 339 mμ (ε 22,058), 290 (11,890), 277 (13,746), and 222.5 (63,732). The last three maxima quoted above are typical of tryptophan which exhibits peaks at 288 mμ (ε 5012), 281 (6310), and 219 (31,630). The nature of the structural unit responsible for the 339-mμ maximum will be discussed later. This maximum shifts neither in 0.1 *N* sodium hydroxide nor in 0.1 *N* hydrochloric acid.

The infrared spectrum shows broad bands at 1640 and 1525 cm^{-1} (amide I + II) and a pronounced peak at 1745 cm^{-1}, which is characteristic of an ester or lactone structure.

The molecular weight, as measured by the Signer isothermal distillation method,[5] was reported near 1000.[2a] Determination in this laboratory by the same method gave values of 1130 and 1280. Formol titration[6] against sodium hydroxide gave a mean value of 1260.

Potentiometric titration of Telomycin indicated a molecular weight of approximately 1280, with pK values of 2.1 and 8.7.

This paper reports the isolation of β-methyltryptophan, *cis*- and *trans*-3-hydroxyprolines, indole-3-alde-

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(2) (a) M. Misiek, O. B. Fardig, A. Gourevitch, D. L. Johnson, I. R. Hooper, and L. Lein, *Antibiot. Ann.*, 852 (1957–1958); (b) J. C. Sheehan, J. Gardner, K. Maeda, D. Mania, S. Nakamura, A. K. Sen, and J. A. Stock, *J. Am. Chem. Soc.*, **85**, 2867 (1963).

(3) A. Gourevitch, G. A. Hunt, A. J. Moses, V. Zangari, T. Puglisi, and L. Lein, *Antibiot. Ann.*, 856 (1957–1958).

(4) D. Schwartz and M. Pallansch, *Anal. Chem.*, **30**, 219 (1958).

(5) R. Signer, *Ann.*, **478**, 246 (1930).

(6) M. Levy, *J. Biol. Chem.*, **99**, 767 (1933).

hyde, and a yellow, crystalline product (*vide infra*) from a basic hydrolysate of Telomycin. On the basis of degradation experiments and ultraviolet studies structure I for Telomycin is proposed.

Amino Acid Composition

Electropherograms or two-dimensional paper chromatograms of Telomycin total acid hydrolysates, developed with ninhydrin at room temperature for 3 hr, showed the presence of glycine, alanine, aspartic acid, serine, threonine, *allo*-threonine,[7] and three new amino acids subsequently identified as *erythro*-β-hydroxyleucine[8] and *cis*- and *trans*-3-hydroxyproline.[9-11]

Alkaline hydrolysis of Telomycin with barium hydroxide and separation of the products by electrophoresis at pH 1.9 give, in addition to tryptophan and β-methyltryptophan, small amounts of α-aminobutyric acid and leucine. As expected,[12] serine, threonine, *allo*-threonine, and *erythro*-β-hydroxyleucine were largely destroyed during basic hydrolysis. Leucine and α-aminobutyric acid were formed from the *erythro*-β-hydroxyleucine and threonine,[13] respectively. The identity of the α-aminobutyric acid was demonstrated by isolation and comparison of R_f value with that of an authentic sample. In addition, a DNP derivative of the isolated sample was prepared. A mixture melting point with DNP-α-aminobutyric acid remained undepressed. The β-methyltryptophan was isolated by ion-exchange chromatography from an alkaline hydrolysate of Telomycin and was identified by ultraviolet and mass spectrometry.[14] By electrophoretic and paper chromatographic comparison β-methyltryptophan corresponded to synthetic "A" racemate (kindly provided by Professor H. R. Snyder).[15] The *cis*- and *trans*-3-hydroxyprolines were isolated by ion-exchange chromatography of an acid and alkaline hydrolysate, respectively. Analytical data for these compounds correspond to $C_5H_9NO_3$ for the *trans*-3-hydroxyprolone and $C_5H_9NO_3 \cdot {}^1/_2H_2O$ for the *cis*-3-hydroxyproline. Treatment with phosphorus and hydrogen iodide converted both compounds to proline. Both 3-hydroxyprolines were also synthesized by an independent method.[11]

Tryptophan was isolated by ion-exchange chromatography from a basic hydrolysate of Telomycin. The infrared spectra of the isolated material and the dinitrophenyl derivative were identical with those of DL-tryptophan and DNP-DL-tryptophan, respectively.

Quantitative amino acid determination of Telomycin by the Moore and Stein procedure[16] (acid hydrolysate) and by mass spectrometry[17] (acid and basic hydrolysis) showed that with the exception of tryptophan all amino acids are present in equimolar amounts (Table I).

Table I

Amino acid	Moore and Stein (acid hydrolysis)	Mass spectrometry (acid hydrolysis)	Mass spectrometry (alkaline hydrolysis)
Gly	1.05	0.98	1.97[a]
Ala	1.00	1.00	1.00
Asp	0.97	0.92	0.38
Ser	0.98	0.95	0.18
Thr	2.16	2.09	
Hyleu	0.80	1.04	
trans-3Hyp	0.84	1.62	1.77[b]
cis-3Hyp	0.81		
Try			0.45
MeTry			0.80
Abu[c]			0.42

[a] Includes glycine produced by decomposition of, *e.g.*, the hydroxy amino acids. [b] Includes leucine, which has the same mass number. [c] α-Aminobutyric acid.

Treatment of the acidic and alkaline hydrolysates of Telomycin with a highly active preparation of D-amino acid oxidase[18] showed no evidence of any significant oxidation; this indicates (but does not establish definitely) that all amino acids in Telomycina re of the L configuration.

Lactone Structure

Evidence that Telomycin is a macrocyclic lactone was obtained as follows. The infrared spectrum of Telomycin possesses a peak at 1745 cm^{-1}, characteristic of an ester or lactone function. On treatment with 0.32 *N* barium hydroxide at ambient temperature, the infrared band at 1745 cm^{-1} disappeared, accompanied by a change in the optical rotation from $[\alpha]^{28}D$ −133° to $[\alpha]^{28}D$ −32° (*c* 1, methanol–water, 2:1). The change of the rotation is in the direction predicted by an extension of Witkop's application[19] of Hudson's lactone rule.[20]

Telomycic acid, which contains all the original amino acids, has the same ultraviolet spectrum as Telomycin. No second fragment containing a hydroxyl group could be detected. This is consistent with a lactone structure for Telomycin. Potentiometric titration of Telomycic acid disclosed an additional carboxylic acid function (pK_a = 3.6). As expected, Telomycin travels to the cathode. Chromic acid oxidation[21a] showed that the alcoholic OH group of threonine is protected in Telomycin but not in Telomycic acid. Consequently, threonine must be the O-terminus of the lactone.

Amino Acid Sequence

A. Alkaline Hydrolysis. Water-Soluble Fraction. Partial acidic and enzymatic hydrolysis of both Telo-

(7) Threonine was separated from *allo*-threonine by prolonged electrophoresis (4 hr, pH 1.9, 3 kv, Whatman No. 3MM) and by two-dimensional paper chromatography (BWA (4:5:1) and *n*-butyl alcohol saturated with water containing 10% dimethylamine v/v).

(8) J. C. Sheehan, K. Maeda, A. K. Sen, and J. A. Stock, *J. Am. Chem. Soc.*, **84**, 1303 (1962).

(9) J. D. Ofle, M. A. Logan, and R. B. Arlinghaus, *J. Biol. Chem.*, **237**, 3667 (1962).

(10) F. Irreverre, K. Morita, A. V. Robertson, and B. Witkop, *J. Am. Chem. Soc.*, **85**, 2824 (1963).

(11) J. C. Sheehan and J. G. Whitney, *ibid.*, **85**, 3863 (1963).

(12) R. Hill, *Advan. Protein Chem.*, **20**, 37 (1965).

(13) Th. Wieland and L. Wirth, *Ber.*, **82**, 468 (1949).

(14) K. Biemann, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 275.

(15) H. R. Snyder and D. Matteson, *J. Am. Chem. Soc.*, **79**, 2217 (1957).

(16) We are grateful to Dr. Stanford Moore for determining the amino acid content of Telomycin. The determination was later repeated in our laboratory, since the color values for the *cis*- and *trans*-3-hydroxyprolines and the *erythro*-β-hydroxyleucine were unknown at the early stage of the investigation.

(17) Professor K. Biemann and his associates performed the mass spectrometric measurements.

(18) Experiments were carried out by Dr. S. C. J. Fu, Children's Cancer Research Foundation, Boston, Mass.

(19) B. Witkop, *Experientia*, **12**, 376 (1956).

(20) C. S. Hudson, *J. Am. Chem. Soc.*, **22**, 338 (1910); **61**, 1525 (1939).

(21) (a) J. C. Sheehan, H. G. Zachau, and W. B. Lawson, *J. Am. Chem. Soc.*, **80**, 3349 (1958); (b) K. Heyns, G. Anders, and E. Becker, *Z. Physiol. Chem.*, **287**, 120 (1951); (c) S. Nakamura and H. Umezawa, *Chem. Pharm. Bull. Japan*, **14** (9), 981 (1966); (d) see also ref 12.

mycin and Telomycic acid were unsuccessful. However, partial basic hydrolysis[21b-d] led to well-defined cleavage into peptide fragments.

Telomycin was treated with 4 *N* sodium hydroxide at 0–3° for 17–21 days. The basic solution was then neutralized on an ice-cooled IRC-50 ion-exchange column. The water eluate was lyophilized and separated by electrophoresis at pH 6.4 or 1.9 into five peptides. The peptides contain, after hydrolysis with constant-boiling hydrochloric acid and subsequent electrophoresis at pH 1.9, the amino acids given in Table II.

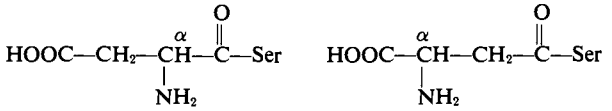
Table II

Pentapeptide	Asp, Ser, Thr, *a*Thr, Ala
Tetrapeptide	Asp, Ser, Thr, *a*Thr
Tripeptide I	Asp, Ser, Thr
Tripeptide II	Thr, *a*Thr, Ala
Dipeptide	Asp, Ser

Determination of the N-terminal group (Sanger[22,23]) showed that aspartic acid is the only N-terminal amino acid in Telomycin, Telomycic acid, and the penta-, the tetra-, the tri- (I), and the dipeptide. Threonine is the N-terminal amino acid of the tripeptide II. Hydrazinolysis of the pentapeptide[24] revealed alanine as C-terminal.

With the experimental data of the partial basic hydrolysis, and with the C- and N-terminal groups determined, one can propose for the pentapeptide the following structure: Asp-Ser-Thr-*a*Thr-Ala.

The foregoing results do not indicate whether the amino group of the aspartic acid is in the α or β position. The pK value of the amino function in the pentapeptide was 8.8; this value corresponds well to a β-aspartyl residue. For example, the amino function of asparagine has a pK of 8.80 and that of isoasparagine has a pK of 8.02.[25] In addition to the pK studies, two peptides with a sequence Asp-Ser were synthesized with the free carboxyl group in the α and β positions, respectively. Both compounds were compared with the Asp-Ser pro-



duced on alkali degradation of Telomycin. It was shown by electrophoresis and ninhydrin coloring (brownish color for the β-peptide, the usual blue-violet color for the α-peptide) that the degradation product was in every respect identical with the synthetic β-dipeptide.

B. Purification and Degradation of the Water-Insoluble Fraction. The water-insoluble part of the basic hydrolysate of Telomycin which originally adhered to the IRC-50 column was eluted with aqueous methanol. The compound eluted appeared homogeneous by electrophoresis at pH 1.9. The electrophoretic mobility is somewhat greater than that of Telomycin. The compact spot shows ultraviolet fluorescence and is ninhydrin positive (initially yellow-green, changing to blue).

The ultraviolet spectrum of the compound is essentially the same as that of Telomycin and Telomycic acid. All three show tryptophan absorption and an additional maximum at 339 mμ. The ratio of the optical density at 339 mμ to the optical density at 280 mμ is 1.3 in the compound as compared to 1.6 in Telomycin. This indicates that some degradation of the chromophore has occurred and the electrophoretic pattern is therefore misleading. The crude hexapeptide mixture can be separated by thin layer chromatography (isopropyl alcohol–3% ammonia) in silica gel G into one main and three minor zones. The ratio of the optical densities of 339 mμ/280 mμ of the main component is the same as in Telomycin. Acid hydrolysis of the purified component gave glycine, *erythro*-β-hydroxyleucine, and *cis*- and *trans*-3-hydroxyproline. Alkaline hydrolysis gave glycine, tryptophan, β-methyltryptophan, *cis*- and *trans*-3-hydroxyproline, and a trace of ninhydrin-positive substance with the electrophoretic behavior of leucine.

The determination of the N-terminal amino acid of the purified hexapeptide according to Sanger[22] and Edman[26–28] gave DNP-glycine and PTH-glycine, respectively. For the Edman degradation an improved procedure, described by Wieland,[29] was applied. The PTH derivatives were compared with authentic samples on thin layer plates. After spraying with fluorescein, the PTH-amino acids were visible as dark spots in the ultraviolet light. The second step of the Edman degradation revealed PTH-*trans*-3-hydroxyproline.[30]

A tripeptide containing both tryptophans and *erythro*-β-hydroxyleucine was isolated both from the acidic and the basic partial hydrolysates of the hexapeptide. Edman degradation of the latter tripeptide showed *erythro*-β-hydroxyleucine to be N-terminal and hydrazinolysis produced only tryptophan. Since the C-terminal amino acid of Telomycin acid is *cis*-3-hydroxyproline (hydrazinolysis at 90° for 7 hr), this must also be the position occupied in the hexapeptide. All of the foregoing observations (excluding the ultraviolet spectrum) together with pK_a studies (the pK_a of 3.6 in Telomycic acid is attributed to the carboxyl group of the *cis*-3-hydroxyproline) are compatible with the representation of Telomycin as structure I.

The Chromophore of Telomycin

A dehydrotryptophan is suggested for the chromophore of Telomycin. The following lines of evidence led to this conclusion.

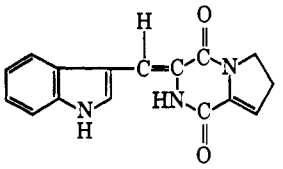
1. The ultraviolet spectrum of Telomycin, Telomycic acid, and the hexapeptide have, in addition to the tryptophan absorption, a band at 339 mμ. Repeated attempts to isolate the chromophore from hydrolysates failed. At elevated temperatures rapid decomposition occurred.

2. The molecular weight determination of Telomycin gave a value of 1280. The sum of the molecular weights of the 11 amino acids (less 10 moles of water

(22) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
(23) S. Blackburn and A. G. Lowther, *ibid.*, **48**, 126 (1951).
(24) S. Akabori, K. Ohno, and K. Nanta, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).
(25) J. C. Sheehan and S. Nakamura, in preparation.
(26) P. Edman, *Acta Chem. Scand.*, **4**, 277 (1950).
(27) H. Fraenkel-Conrat, H. J. I. Harris, and A. L. Levy, "Methods of Biochemical Analysis," Vol. III, Interscience Publishers, Inc., New York, N. Y., 1955, p 359.
(28) P. Edman, *Acta Chem. Scand.*, **10**, 766 (1956).
(29) Th. Wieland and H. W. Schnabel, *Ann.*, **657**, 226 (1962).
(30) A reference sample was provided by J. C. Whitney.

and a lactone bond) is 1273. On this basis an additional conjugated system can be excluded.

3. From an alkaline hydrolysate of Telomycin, indole-3-aldehyde and a "yellow compound" were isolated. The "yellow compound" gave on basic hydrolysis tryptophan. Hydrogenation of the "yellow compound" with rhodium on charcoal followed by acid hydrolysis gave proline and octahydrotryptophan. Analysis, molecular weight determination, and ultraviolet spectrum support structure II for this "yellow compound."



II

4. Since indole-3-aldehyde may be formed by a retro-aldol condensation, a derivative of 2-amino-3-(indol-3-yl)acrylic acid could be responsible for the ultraviolet absorption of Telomycin.

In order to record the ultraviolet spectra of derivatives of the dehydrotryptophan, N-benzyl- and N-propyl-2-benzamido-3-(indol-3-yl)acrylamide, 1-[2-benzamido-3-(indol-3-yl)acryloyl]piperidine, and methyl 2-benzamido-3-(indol-3-yl)acrylate were prepared *via* an azlactone synthesis.[31,32]

Methyl 2-benzamido-3-(indol-3-yl)acrylate showed an ultraviolet absorption maximum at 341 mμ (ε 21,000), a spectrum indeed very similar to the chromophore of Telomycin, 339 mμ (ε 22,000).

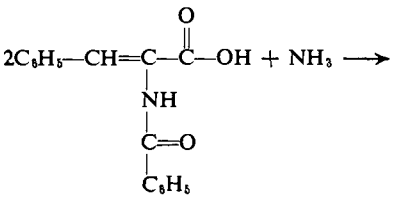
Summation of the ultraviolet spectra of methyl 2-benzamido-3-(indol-3-yl)acrylate and β-methyltryptophan minus benzamide gives an ultraviolet spectrum which is nearly identical with the ultraviolet spectrum of Telomycin (Table III).

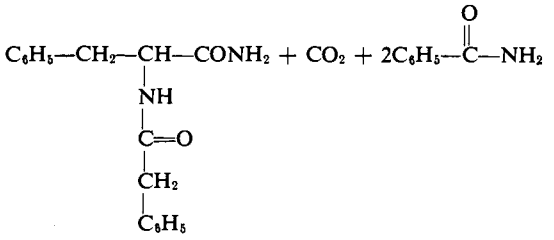
Table III

λ, mμ (ε)	λ, mμ (ε)
Methyl 2-benzamido-3-(indol-3-yl)acrylate	β-Methyltryptophan
227 (37,376)	220 (35,062)
275 (10,752)	274 (5426)
341 (20,908)	280 (4898)
	288 (5695)
Benzamide	
275 (676)	
225 (10,000)	
Telomycin[a]	Chromophore
222 (63,732)	220 + 227 (62,432)
277 (13,746)	274 + 275 (15,496)
290 (11,890)	288
339 (22,058)	341 (20,908)

[a] The ε values were based on a molecular weight of 1271.33 for Telomycin.

5. To justify the postulation of a dehydrotryptophan system, it is necessary to account for the formation of tryptophan during the basic hydrolysis. At first it is surprising to see the double bond reduced under basic conditions. An experiment of Erlenmeyer[33–35] provides a plausible explanation. Treatment of α-N-benzamidocinnamic acid with a 10% ammonia solution gave 1 mole of β-phenyl-α-phenylacetamidopropionamide, 2 moles of benzamide, and 1 mole of carbon dioxide.[33–34] Since the reaction of phenylpyruvic acid





with ammonia gives also β-phenyl-α-phenylacetamidopropionic acid, Erlenmeyer deduced that phenylpyruvic acid is formed in a first step by the hydrolysis of α-benzamido-β-phenylacrylic acid with ammonia. Phenylpyruvic acid (2 moles) reacts with ammonia to give a ketone–ammonia adduct. By elimination of water a double bond in the β,γ position to the carboxyl group will be formed.[36–38] Decarboxylation and tautomerization give β-phenyl-α-phenylacetamidopropionic acid.[39] Subsequent acid hydrolysis of β-phenyl-α-phenylacetamidopropionamide gave 0.48 equiv of phenylalanine and phenylacetic acid.

In analogy, we obtained on basic hydrolysis of Telomycin 0.46 mole of DL-tryptophan. In addition, thin layer chromatography in two solvent systems showed indolyl-3-acetic acid, in accord with the proposed mechanism given in Scheme I.

6. Basic hydrolysis of equimolar amounts of methyl 2-benzamido-3-(indol-3-yl)acrylate and threonine with barium hydroxide gives tryptophan.

Basic hydrolysis of Telomycin releases ammonia by decomposition of the hydroxyamino acids. Dehydrotryptophan presumably hydrolyses to indolylpyruvic acid. Indolylpyruvic acid (2 moles) reacts with ammonia to give a ketone–ammonia adduct. After dehydration, decarboxylation, and hydrolysis, tryptophan is formed in 50% maximum yield.

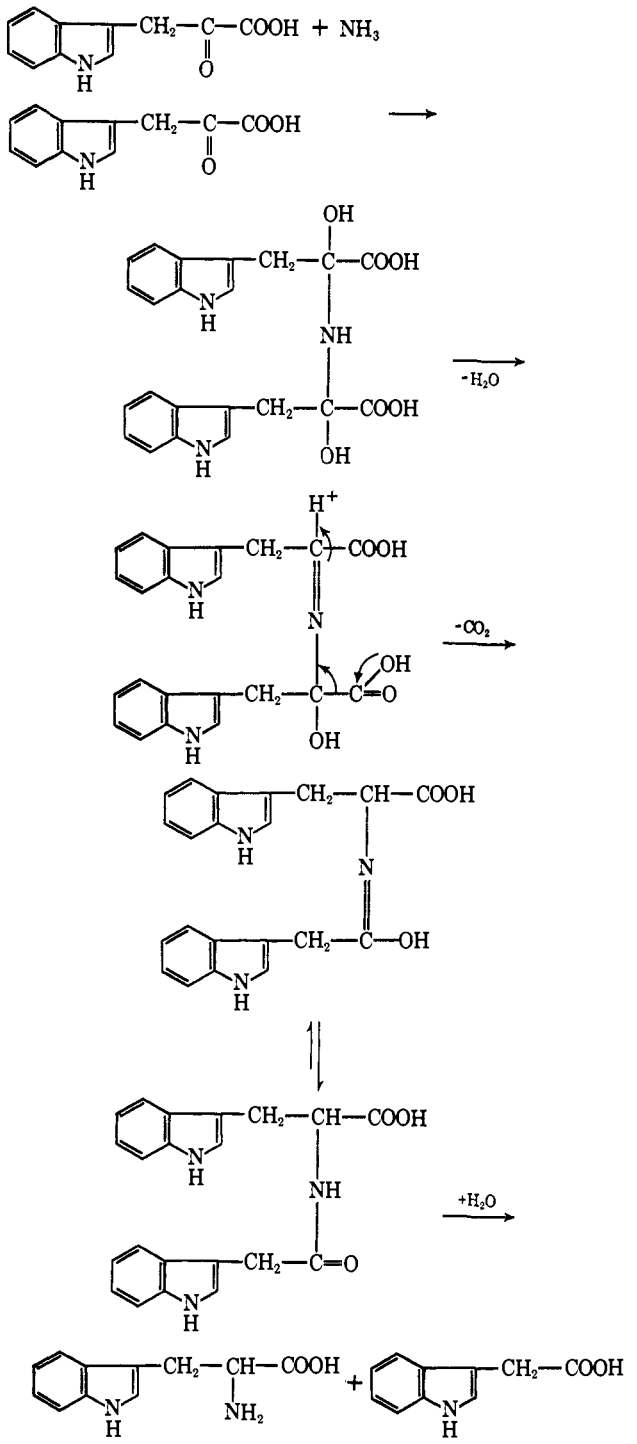
Experimental Section

Microanalyses were performed by Dr. S. M. Nagy and associates at M.I.T. and by Midwest Microlaboratories, Inc., Indianapolis, Ind. Melting points were determined on a Kofler hot-stage microscope and are uncorrected. The infrared spectra were measured on a Perkin-Elmer Model 237 recording spectrophotometer. Ultraviolet spectra were measured on a Cary Model 14 recording spectrophotometer.

Telomycin was supplied as Batch 60F 176 from Bristol Laboratories (Division of Bristol-Meyers, Inc.), Syracuse, N. Y. A small

(31) A. Ellinger and C. Flamand, *Ber.*, **40**, 3029 (1907).
(32) J. Stiller, *J. Chem. Soc.*, 473 (1937).
(33) E. Erlenmeyer, *Ber.*, **30**, 2976 (1897); **31**, 2238 (1898).
(34) E. Erlenmeyer, *Ann.*, **307**, 71,146 (1899).
(35) A. Galat, *J. Am. Chem. Soc.*, **72**, 4436 (1950).
(36) E. Dane, O. Balcke, H. Hammel, and F. Müller, *Ann.*, **607**, 92 (1957).
(37) A. Schönberg, R. Moubasher, and A. Mustafa, *J. Chem. Soc.*, 176 (1948).
(38) A. Schönberg and R. Moubasher, *Chem. Rev.*, **50**, 261 (1952).
(39) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Henry Holt and Co., New York, N. Y., 1959, pp 349–350.

Scheme I



sample of Telomycin was dried over phosphorus pentoxide at 110° (0.1 mm) for 10 hr.

Anal. Calcd for $C_{59}H_{77}O_{19}N_{13}$: C, 55.70; H, 6.05; N, 14.32. Found: C, 54.07; H, 6.48; N, 14.25; ash, 0.33.

Electrophoresis was carried out on Whatman No. 3 MM chromatography paper at 3 kv by the hanging-strip technique. Buffers of the following pH values were used (v/v): pH 1.9, acetic acid 20, formic acid 2, water to 100, this solution made 2 *M* with respect to cadmium acetate; pH 6.2, pyridine 150, acetic acid 30, water to 3000; pH 9.6, trimethylamine 90, *n*-butyl alcohol 150, water 2750, acetic acid 30.

Equivalent Weight of Telomycin. Formol titration[6] under nitrogen against 0.1 *N* sodium hydroxide in neutral diluted (3:1) aqueous 35% formalin with phenolphthalein as indicator gave equiv wt 1270, 1250.

Molecular Weight of Telomycin. The Signer isothermal distillation method[5] with azobenzene as a reference substance, pyridine as solvent, and a temperature of 70–80° with an equilibrium time of 14 days gave mol wt 1280.

Titration of Telomycin. Telomycin (139 mg) was dissolved in a mixture of 10 ml of water and 1 ml of 1 *N* hydrochloric acid. The solution was titrated with 1 *N* sodium hydroxide (using a microburet with 0.002-ml divisions, and pH-Stat Radiometer Model TTT1). A blank solution was titrated by the same procedure. The corrected titration curve of Telomycin was obtained from the difference of both titration curves: mol wt of Telomycin = 139 mg of Telomycin/0.107 (ml of 1 *N* sodium hydroxide) = 1300 (pK_{COOH} = 2.1, pK_{NH_3} = 8.6). The homogeneity of Telomycin was established by paper electrophoresis. Two batches, one amorphous (No. CoF17c) and one crystalline (No. 2773-23-3), gave exactly the same pattern with migration toward the cathode, at pH 1.9. The application of 125 mg of each sample to the starting line in a 1.5-cm streak followed by a 3.5-hr run at 2.5 kv and treatment of the dried electropherogram with *t*-butyl hypochlorite and starch revealed one major component (15.8 cm from origin) and a faint, evidently very minor spot of greater mobility (19.8 cm). Electrophoresis at pH 6.2 caused Telomycin to migrate 16.5 cm toward the cathode, as a single spot.

Thin layer chromatography of Telomycin on silica gel G in isopropyl alcohol–3% ammonia (10:2) gives one spot with R_f 0.20.

Amino Acid Composition of Telomycin. A. Acid Hydrolysis. In one experiment 5 mg of Telomycin was heated for 15 hr at 110° in a sealed ampoule with 0.2 ml of constant-boiling hydrochloric acid and 0.05 ml of glacial acetic acid. The dark solution was evaporated in a vacuum desiccator over potassium hydroxide and the residue dissolved in 0.1 ml of water containing a little ethanol to assist solution. Part of the solution was subjected to two-dimensional chromatography in (1) *n*-butyl alcohol–water–acetic acid (4:5:1) and (2) phenol saturated with water (NH_3 atmosphere). The dried chromatogram was washed in ether to remove an excess of phenol and treated with ninhydrin (1% in acetone containing 5% pyridine). Spots due to Asp, Ser, Thr, Ala, 3Hyp, and β-Hyleu were observed. A corresponding pattern checked against a known amino acid mixture was obtained by electrophoresis (3.5 hr at 3 kv and pH 1.9). The *cis*- and *trans*-3-hydroxyprolines split into a slower moving *trans* and a faster moving *cis* component (both spots gave yellow ninhydrin color). To cite one example, the relative mobilities (taking Gly as unity) were: Gly, 1.00; Ala, 0.90; Ser, 0.74; Hyleu, 0.70; *a*Thr, 0.67; Thr, 0.63; *cis*-3Hyp, 0.60; Asp, 0.53; *trans*-3Hyp, 0.43.

B. Alkaline Hydrolysis of Telomycin. In one experiment, 2 mg of Telomycin and 0.5 ml of 10% barium hydroxide solution were heated in a sealed tube for 14 hr at 110°. The yellow solution was neutralized with Dry Ice and centrifuged. The supernatant layer was evaporated to dryness and part of the residue examined by paper electrophoresis (3 hr at 3 kv and pH 1.9). The relative mobilities (Gly as unity) were Gly, 1.00; Ala, 0.92; *cis*-3Hyp, 0.65; Asp, 0.62; Try, 0.57; MeTry, 0.54; *trans*-3Hyp, 0.49.

Quantitative Amino Acid Analysis. A sample of Telomycin, dried for 24 hr at 0.1 mm, was weighed into a 16 × 120 mm heavy-walled Pyrex tube. The peptide was dissolved in 0.5 ml of constant-boiling (6 *N*) glass-distilled hydrochloric acid. The tube was sealed under nitrogen. Hydrolysis was conducted for 7 hr in an oven at 110 ± 2°. The cooled tube was opened and the contents were evaporated to dryness over potassium hydroxide pellets in a vacuum desiccator at room temperature for 20 hr. The black residue was dissolved in 0.5 ml of water–isopropyl alcohol (1:1) and applied to a Dowex 50-X12 column. The results of the analysis are shown in Table I.

Isolation of Hydroxyprolines from Telomycin. A. Acid Hydrolysis. An acid hydrolysate of Telomycin (1.5 g) was transferred to a column (70 × 2 cm) of Dowex AG-50W-X8 (100–200 mesh) cation exchange resin and eluted with pyridine (0.2 *M*)–formic acid buffer of pH 3.25 containing 40% ethanol. Fractions (10 ml) were collected. Ninhydrin maxima occurred around fractions 45 (OD 0.9), 55 (4.0), 65 (6.9), 85 (2.0). The fractions were grouped as shown in Table IV, the groups were lyophilized, and the residues were examined by paper electrophoresis at pH 1.9 and by paper chromatography with BWA.

The group I fractions were rechromatographed. Evaporation of fractions 9–14 gave 81 mg of residue shown by electrophoresis to be essentially *trans*-3Hyp contaminated with some of the *cis* form. The product was dissolved in 3 ml of water, the clarified solution was lyophilized, and the residue was crystallized from water–ethanol as colorless plates (51 mg); mp 350° dec; $[\alpha]^{29}D$ −19.5° (*c* 1, water).

Anal. Calcd for $C_5H_9NO_3$: C, 45.80; H, 6.87; N, 10.68. Found: C, 45.79; H, 6.88; N, 11.12.

Table IV

Group	Fractions	Wt, mg	Amino acids present[a]
I	39–45	110	*trans*-3Hyp (Ser, Thr, Asp, *cis*-3Hyp)
II	46–60	450	Ser, Thr, Asp, *cis*-3Hyp
III	61–75	235	Ser, Thr, Asp *e*3Hyleu
IV	76–95	150	Ser, *e*3Hyleu (Thr, Asp, Ala)

[a] Minor components shown in parentheses.

B. Alkaline Hydrolysis. A barium hydroxide hydrolysate of Telomycin (3 g) was transferred to a column of Dowex 50-X8 (ammonium form); the column was eluted with 5 *N* ammonium hydroxide. The eluate was evaporated to dryness giving 2.4 g of a pale yellow syrup which was transferred to a column (38 × 4.25 cm) of Dowex 50-X8 (200–400 mesh) cation exchange resin and eluted with pH 4.25 pyridine (0.4 *M*)–formic acid buffer in 15-ml fractions. Fractions 45–59, comprising the first ninhydrin peak, were combined and evaporated. The residue, which contained *trans*- and *cis*-3Hyp, together with Asp, Gly, Ala, Abu, as shown by electrophoresis and chromatography, was twice rechromatographed on a Dowex 50-X3 (200–400 mesh) cation exchange column with a pH 3.1 pyridine (0.4 *M*)–formic acid buffer. The ninhydrin peaks were grouped in the usual way. Group 1 gave 190 mg of nearly pure *cis*-hydroxyproline, which was dissolved in water, clarified with charcoal, and lyophylized giving 61 mg of colorless solid. Two successive crystallizations (ethanol–water) gave 31 mg of the electrophoretically pure *cis*-3Hyp: mp 241–242° dec; $[\alpha]^{31}D$ −17° (*c* 2, water).

Anal. Calcd for $C_5H_9NO_3 \cdot {}^1/_2H_2O$: C, 42.85; H, 7.14. Found: C, 43.1; H, 6.78.

Reduction of the Hydroxyprolines with Red Phosphorus and Hydrogen Iodide. Reduction of the *trans* and *cis* forms of 3-hydroxyproline by the method used for hydroxyleucine[8] gave proline in both cases, as demonstrated by electrophoresis and by the ninhydrin color reaction.

Isolation of Serine and Determination of Configuration. The residue from the evaporation of fractions 76–95 (group IV) obtained as described above in the ion exchange chromatography of the acid hydrolysate of Telomycin was dissolved in 2 ml of water and 5 ml of ethanol was added. The crystalline material (35 mg) was pure serine: $[\alpha]^{26}D$ −8.5° (*c* 1.6, water); $[\alpha]^{28}D$ 13° (*c* 1.5, 1 *N* hydrochloric acid). These values correspond to L-serine.[40]

Isolation of β-Methyltryptophan from Telomycin. A mixture of 1 g of Telomycin in 50 ml of 10% barium hydroxide was refluxed (ascarite guard tube) for 40 hr. The cooled mixture was diluted with water, carefully neutralized with 2 *N* sulfuric acid, concentrated, and transferred to a column (57 × 2.2 cm) of Dowex AG-50-X8 (100–200 mesh) cation exchange resin equilibrated with pyridine (0.4 *M*)–formic acid buffer of pH 4.25, which was used also as eluting solvent. Ninhydrin-positive fractions were collected. Group II (fraction 97–117) gave, upon lyophylization, 42 mg of a colorless solid. Two successive crystallizations from water yielded colorless needles: mp 250°; ultraviolet, 289 mμ (ε for mol wt 218, 4950), 280 (4180), 273 (4690), 200.5 (32,600).

The mass spectrum showed a main peak at *m/e* 144 (ethyl indolylium ion) and another strong peak at *m/e* 117.

β-Methyltryptophan obtained from Telomycin was compared with isomers A and B of the β-methyltryptophan of Snyder[15] by paper chromatography and paper electrophoresis and identified as isomer A of β-methyltryptophan (Table V).

Table V

Solvent system	MeTry	Isomer A (Snyder[15])
2,4,6-Collidine–2,6-lutidine–water (2:2:1)	0.29	0.29
BuOH–AcOH–H_2O (4:2:1)	0.53	0.53
BuOH–EtOH–H_2O (4:2:1)	0.27	0.27
Water-saturated BuOH	0.50	0.50

(40) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1961, p 2202; see also E. Fischer and W. A. Jacobs, *Ber.*, **39**, 2942 (1906).

Isolation of DL-Tryptophan. The combined fractions of group IV from the β-methyltryptophan isolation gave, on lyophilization and charcoal treatment, 98 mg of a colorless solid. Crystallization from aqueous methanol gave tryptophan as colorless plates. The infrared spectra of the product and its DNP derivative were identical with those of authentic DL-tryptophan and DNP-DL-Try, respectively.

Isolation of α-Aminobutyric Acid (Abu) from Alkaline Hydrolysate of Telomycin. A 24-hr hydrolysate of 1 g of Telomycin was applied to five sheets of Whatman No. 31 chromatography paper. The papers were developed with butanol–acetic acid–water (BAW) (4:1:5). The ninhydrin-positive region of R_f 0.35 was cut from the sheets and the strips were eluted with water. Lyophilization gave 75 mg of an amorphous solid. The latter was rechromatographed in the BAW system. The lyophilized product was crystallized from ethanol–water (13 mg). The R_f value in BAW was the same as that of authentic α-aminobutyric acid (0.36). The DNP derivative had the same melting point as an authentic sample, 143–144°; the mixture melting point was underpressed.

Telomycin Acid. Telomycin (0.55 g) was suspended and stirred in 5% barium hydroxide. The suspension became clear after 10 min. The solution was neutralized by the addition of 1 *N* sulfuric acid until pH 5.4–5.6. Barium sulfate was removed by centrifugation and the supernatant was freeze-dried to give a colorless powder of Telomycic acid. Telomycic acid is homogeneous by paper electrophoresis and thin layer chromatography: paper electrophoresis (pH 1.8, 3 kv, 3 hr), Telomycin, 14 cm toward the cathode, Telomycic acid, 14 cm toward the anode; (pH 6.2, 3 kv, 3 hr) Telomycin, 7 cm toward the cathode, Telomycic acid, 5 cm toward the anode; thin layer chromatography on silica gel G (BuOH–AcOH–H_2O, 15:1:14), Telomycin R_f 0.15, $[\alpha]^{28}D$ −125°, Telomycic acid R_f 0.11, $[\alpha]^{28}D$ −32° (*c* 1, MeOH–H_2O 2:1). The ultraviolet spectrum of Telomycic acid is the same as that of Telomycin. The 1745-cm^{-1} band which is present in the infrared spectrum of Telomycin is missing in Telomycic acid.

Partial Base Hydrolysis of Telomycin. Telomycin (6 g) was treated with 4 *N* sodium hydroxide at 0–3° for 20 days. The basic solution was neutralized on an ice-cooled IRC-50 column (50 cm × 2.5 cm) and the water-soluble portion was washed from the column with distilled water until the eluate was ninhydrin negative. The eluate was freeze-dried giving 2.35 g of dry product which was separated in 50-mg portions on Whatman No. 3 MM paper (80 × 46 cm) (pH 6.2, 3 kv, 3.5 hr). Under these conditions the aspartyl peptides with aspartic acid are traveling toward the anode, whereas the neutral peptides and the neutral amino acids are still traveling toward the cathode. Guide strips were cut off and sprayed with ninhydrin. To cite one example, the relative mobilities (Asp taken as unity) were: peptide A, 0.53; B, 0.57; C, 0.67; D, 0.77. Strips containing peptides were eluted with 90% aqueous methanol. The methanol solution was evaporated, and the residue was treated several times with a few milliliters of ether, dissolved in a few drops of water, centrifuged from traces of insoluble material, and precipitated with an excess of acetone. This procedure was repeated twice; a colorless powder remained. Hydrolysis with constant-boiling hydrochloric acid at 105° for 8 hr and subsequent electrophoresis at pH 1.9, 3 kv, 3.5 hr, and two-dimensional paper chromatography on Whatman No. 1 (I, BWA, 53 hr; II, *n*-butyl alcohol saturated with water and 10% dimethylamine v/v, 130 hr) showed, when checked against known samples, the following amino acid composition: pentapeptide, Asp, Ser, Thr, *a*Thr, Ala; tetrapeptide, Asp, Ser, Thr, *a*Thr (trace of Ala); tripeptide I, Asp, Ser, Thr (trace of Ala and *a*Thr); dipeptide, Asp, Ser. Quantitative amino acid analyses as described gave the following ratio (Asp taken as unity): pentapeptide, Asp = 1, Ser = 0.9, Thr + *a*Thr = 1.8, Ala = 1.1; tetrapeptide, Asp = 1, Ser = 0.9, Thr + *a*Thr = 1.8, Ala = 0.01; tripeptide I, Asp = 1, Ser = 0.9, Thr = 0.9; dipeptide, Asp = 1, Ser = 0.9. The neutral tripeptide II was separated by electrophoresis (pH 1.9, 3 kv, 3 hr). The relative mobility was 0.75 for Ala taken as unity. Isolation was accomplished as described for the aspartyl peptides. Acid hydrolysis and subsequent electrophoresis (pH 1.9, 3 kv, 3.5 hr) and two-dimensional paper chromatography on Whatman No. 1 (I, BWA (4:5:1); II, *n*-butyl alcohol saturated with water and 10% dimethylamine v/v) gave Thr, *a*Thr, and Ala. Quantitative amino acid analysis gave the following: Ala = 1, Thr + *a*Thr = 1.8 (Ala taken as unity).

Determination of the N-terminal Group (Sanger Method). In a typical experiment the peptide was eluted from three electropherograms (Whatman No. 3MM), *ca.* 0.2–1 μ*M* was dissolved in 0.1 ml of 1% trimethylamine, and to this solution 10 μl of FDNB in 0.2

ml of ethanol was added. After standing for 2 hr, a few drops of water and trimethylamine solution were added and the excess of FDNB was removed by extraction with three portions of peroxide-free ether. After evaporation of the aqueous solution to dryness, the residue was taken up in 5 drops of constant-boiling (5.7 *N*) hydrochloric acid, placed in capillary tube, sealed, and heated for 6 hr at 105°. The hydrolysate was transferred to a test tube, diluted to 1 *N* hydrochloric acid, and extracted with four 5-ml portions of peroxide-free ether. The combined ether extract was evaporated *in vacuo*, and the residue was dissolved in a few drops of acetone and transferred to a paper chromatogram.

The *t*-amyl alcohol system of Blackburn and Lowther[23] was chosen. The paper (Whatman No. 4) was dipped in a pH 6 phthalate buffer (50 ml of 0.1 *N* phthalate and 45.5 ml of 0.1 *N* sodium hydroxide made up to 100 ml with water) and allowed to dry. The DNP-amino acid of the acid hydrolysate was applied to Whatman No. 4 paper and developed with *t*-amyl alcohol saturated with pH 6 phthalate buffer.

The penta-, the tetra-, the tri- (I), and the dipeptide showed only DNP-aspartic acid, R_f 0.01 (besides traces of DNP-phenol).

Determination of the N-terminal Group (Edman). The pentapeptide (9 mg) was dissolved in 0.6 ml of water and applied to six paper strips (1 × 7 cm). Only half of each strip was moistened and afterward dried for 5 min at 90°. The peptide-carrying part of the strip was treated with 0.05 ml of 20% solution of phenyl isothiocyanate in peroxide-free dioxane. The wet strip was hung on a little hook of a glass rod in an air-tight 1-l. flask for 2 hr at 40°. The flask contained 5 ml of dioxane, pyridine, and water. Excess phenyl isothiocyanate was removed from each air-dried strip by washing with benzene.

To split the phenylthiohydantoin, the air-dried strips were kept in a desiccator for 16 hr at 100-mm pressure. The desiccator contained 5 ml of constant-boiling hydrochloric acid. The PTH derivatives were extracted four times with 10 ml of water-free acetone. After evaporation of the acetone layer the residue was applied to a thin layer plate (silica gel G, Merck) and chromatographed together with known PTH samples in chloroform–formic acid (20:1).[29,41] Before checking in the ultraviolet light, the plate was sprayed with a 1% fluorescein solution. The pentapeptide did not show PTH-aspartic acid. The same result was obtained with Telomycin.

Hydrazinolysis of the Pentapeptide (Akabori). A solution of 5 mg of the pentapeptide in 0.3 ml of anhydrous hydrazine (freshly distilled from barium hydroxide) was treated in a sealed tube at 105° for 12 hr. After evaporation of the hydrazine in a desiccator over sulfuric acid, the residue was dissolved in 1 ml of water and shaken for 2 hr with 0.2 ml of benzaldehyde. The aqueous layer was separated, extracted with 2–3-ml portions of ether, and subjected to electrophoresis (pH 1.9, 3 kv, 3 hr). The only strong amino acid spot was due to alanine.

Purification of the Ultraviolet-Active Hexapeptide. The water-insoluble portion—the hexapeptide which adhered to the IRC-50 column—was eluted with 90% methanol. The methanolic layer was evaporated under reduced pressure, giving 2.58 g (43% overall yield) of an impure hexapeptide. The hexapeptide was applied in 50-mg portions on thin layer plates (silica gel G, Merck) 20 × 20 cm and developed with isopropyl alcohol–3% ammonia (8:2) for 4–5 hr. The hexapeptide separates into one main (R_f 0.33) and three minor zones (R_f 0.38, 0.45, 0.64). All zones gave on acid and basic hydrolysis: glycine, tryptophan, β-methyltryptophan, *erythro*-3-hydroxyleucine, and *cis*- and *trans*-3-hydroxyproline. The minor zone (R_f 0.38) gave alanine in addition. The purified hexapeptide shows ultraviolet maxima (measured in ethanol–water 2:1) at 339, 290, 277, and 222.5 mμ. Quantitative amino acid analysis gave (Gly taken as unity): Gly = 1, *erythro*-β-Hyleu = 0.8, *cis*-3Hyp = 0.8, *trans*-3Hyp = 0.8.

Determination of the N-Terminal Hexapeptide Group (Sanger). The only visible spot besides a trace of DNP-phenol was DNP-glycine.

Edman Degradation of the Purified Hexapeptide. Under the condition described, PTH-glycine was split first, followed by PTH-*trans*-3-hydroxyproline. Qualitative amino acid analysis of the remaining peptide after the first Edman degradation gave *erythro*-3-hydroxyleucine, *cis*-3-hydroxyproline, and only a trace of glycine. After the second Edman degradation glycine and *trans*-3-hydroxyproline were missing.

Partial Acid Hydrolysis of the Hexapeptide. The hexapeptide (1.5 g) was hydrolyzed with 1.25 *N* 50% ethanolic hydrochloric acid under reflux for 6 hr. The ultraviolet absorption ratio 340 mμ/280 mμ decreased rapidly during the hydrolysis, as shown in Table VI. The ethanol was removed under reduced pressure. A

Table VI

Hours	0	0.5	1	2	4	6
$E_{340m\mu}/E_{280m\mu}$	1.60	0.75	0.62	0.38	0.33	0.30

precipitate formed on storage at 0–5° which was collected (850 mg). Glycine, *cis*- and *trans*-3-hydroxyprolines, and a trace of *erythro*-β-hydroxyleucine were detected in the filtrate by paper electrophoresis.

The precipitate was dissolved in water and filtered from insoluble material. The filtrate was acidified with 5 *N* hydrochloric acid. The resulting precipitate (700 mg) was applied to a chromatographic column of silica gel G (100 mesh, 1.8 × 75 cm) using BuOH–AcOH–H_2O (15:1:4) as solvent. The column was eluted with BuOH–AcOH–H_2O (4:2:1). Fractions were applied to thin layer chromatography plates (silica gel G) and developed with the latter solvent. The fractions which showed a characteristic spot (R_f 0.45, ultraviolet and ninhydrin positive) were combined (162 mg). The fraction was purified further by preparative thin layer chromatography on silica gel G using the same solvent; the ultraviolet-positive portion was removed and eluted.

Barium Hydroxide Hydrolysis of the Tripeptide. The tripeptide (3 mg) was hydrolyzed in 0.5 ml of a 5% barium hydroxide solution in a sealed tube under nitrogen at 105° for 7 hr and the hydrolysate was neutralized by the addition of Dry Ice. Paper electrophoresis (pH 1.9, 3 kv, 3 hr, Whatman No. 3 MM paper) revealed glycine (1), leucine (0.74), tryptophan (0.56), and β-methyltryptophan (0.53). The relative mobilities are given in parentheses; glycine is taken as unity. Paper chromatography (BuOH–AcOH–H_2O 4:1:2, Whatman No. 1 filter paper) revealed glycine (R_f 0.23), leucine(R_f 0.65), tryptophan (R_f 0.51), and β-methyltryptophan (R_f 0.58).

Hydrolysis of the Tripeptide with Hydrochloric Acid. The peptide was hydrolyzed with constant-boiling hydrochloric acid (0.5 ml) in a sealed tube under nitrogen at 105° for 7 hr. The hydrolysate was dried under reduced pressure over sodium hydroxide, diluted with water, neutralized on an IR-4B (OH-type) column, eluted with water, and evaporated to a small volume. Glycine and *erythro*-β-hydroxyleucine were observed in the hydrolysate, but the amount of glycine was rather small compared with that of *erythro*-β-hydroxyleucine.

The N-terminal amino acid of the tripeptide was identified as *erythro*-β-hydroxyleucine by Edman degradation. As expected, the *erythro*-β-hydroxyleucine is dehydrated during the Edman degradation.

Edman Degradation of the Tripeptide. The peptide (20 mg) was dissolved in water (1 ml) and pyridine (2 ml). Phenyl isothiocyanate (0.2 ml) was added and the mixture kept for 4 hr (25°) at pH 8.7–9 by addition of 0.1 *N* sodium hydroxide. The mixture was then extracted several times with benzene followed by cyclohexanone to remove the excess of phenyl isothiocyanate and then adjusted to pH 3.5 by the addition of 1 *N* hydrochloric acid.

The extracts were combined and evaporated giving 12 mg of PTC-peptide. The PTC-peptide (3 mg) was cyclized in a mixture of 0.5 *N* hydrochloric acid (0.5 ml) and 0.5 ml of dioxane for 15 hr at room temperature. The N-terminal amino acid was extracted with ethyl acetate at pH 7.2 and chromatographed on Whatman No. 1 (previously treated with 1% starch solution) with *n*-heptane–pyridine (7:3). Three spots due to PTH-amino acids with R_f values of 0.24 (trace) (PTH-glycine), 0.34 (presumably PTH-*erythro*-β-hydroxyleucine), and 0.61 (PTH-dehydro-*erythro*-β-hydroxyleucine) were revealed by the azide method. The C-terminal amino acid of the tripeptide was revealed as tryptophan by the Akabori technique.

Isolation of the "Yellow Compound" (Structure II). The hexapeptide (2 g) was hydrolyzed with 1 *N* sodium hydroxide (80 ml) in a sealed tube under nitrogen for 7 days at 55°. The ultraviolet absorption ratio of the hexapeptide at 339 mμ/280 mμ decreased as follows (hours are given in parentheses): $E_{339m\mu}/E_{280m\mu}$ 1.24 (4), 1.05 (10), 0.81 (24), 0.30 (72), 0.22 (96). The mixture was extracted three times wth 80-ml portions of ethyl acetate and the combined extracts were evaporated. The residue crystallized from methanol to yield 35 mg of yellow monoclinic prisms, decomposing at 280–285°.

Anal. Calcd for $C_{16}H_{13}O_2N_3$: C, 68.80; H, 4.69; N, 15.05; mol wt, 279.29. Found: C, 68.71; H, 4.81; N, 15.02; mol wt,

(41) M. Brenner, A. Niedewieser, and G. Pataki, *Experientia*, 17, 145 (1961).

279 (by mass spectrometry). Barium hydroxide hydrolysis of the "yellow compound" gave tryptophan as the sole ninhydrin-positive product.

The "yellow compound" (10 mg) was hydrogenated in 20 ml of aqueous methanol (water–methanol (1:2)) over 30% rhodium on charcoal (30 mg) at room temperature. About 8 cc of hydrogen was consumed in 24 hr. After removal of catalyst and solvent a 3-mg portion was hydrolyzed with 1 ml of constant-boiling hydrochloric acid in a sealed tube under nitrogen at 100–105° for 7 hr. After removal of the acid by ion exchange chromatography, proline and octahydrotryptophan were identified by paper chromatography (Whatman No. 4; BWA (4:1:2); BuOH–H_2O (4:2:1)) and electrophoresis (pH 1.9, 3 kv, 3 hr).

Isolation of Indole-3-aldehyde. After the extraction of the "yellow compound" with ethyl acetate, 60 mg of indole-3-aldehyde was recovered from the mother liquor. The indole-3-aldehyde was crystallized from benzene and identified by mass spectroscopy, comparison of infrared spectra, and mixture melting point with an authentic sample.

Hydrazine Decomposition of Telomycic Acid. Telomycic acid (15 mg) and hydrazine (0.7 ml) were heated in a sealed tube at 95–100° for 8 hr.

The hydrazine was removed over sulfuric acid, and the residue was dissolved in 1 ml of water and shaken with 0.5 ml of benzaldehyde for 2 hr. The mixture was extracted several times with ethyl acetate and the water phase was evaporated. *trans*-3Hyp was detected as the C-terminal amino acid by paper electrophoresis (pH 1.8) and paper chromatography (Whatman No. 1): BuOH–AcOH–H_2O 4:1:2; *trans*-3Hyp 23.5 cm, *cis*-3Hyp 23.5 cm (24 hr); lutidine–collidine–water 2:2:1, *trans*-3Hyp 2.5 cm, *cis* 5.0 cm (36 hr); BuOH–H_2O–NH_4OH–$(CH_3)_2O$ 40:30:5:5, *trans*-3Hyp 22.5 cm, *cis*-3Hyp 20 cm (5 days).

Synthesis of 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one.[31] Finely powdered indole-3-aldehyde (14.5 g, 0.1 mole), 17.9 g (0.1 mole) of hippuric acid, 17 g (1.7 moles) of acetic acid anhydride, and 10 g (0.1 mole) of potassium bicarbonate were stirred together at room temperature and heated on a steam bath for 10 min. The mixture set into a crystalline brown-yellow mass. It was allowed to stand overnight at room temperature. The crude azlactone was treated with 150 ml of hot water, filtered, and washed with 100 ml of 3% acetic acid, followed by 100 ml of water. The dry material weighed 22.8 g (79% of the theory).

Crude azlactone (3 g) was dissolved in *ca.* 50 ml of chloroform and pressed with nitrogen into a silica gel G column (4 × 23 cm). The absorbed crude azlactone split into two zones when it was eluted under pressure with *ca.* 300 ml of chloroform. The fast-moving yellow zone gave 0.503 g of crystalline 4-(1-acetylindol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one, mp 208°. The slow-moving orange zone gave 0.989 g of crystalline 4-(indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one, mp 228 (lit.[31] mp 202–220°).

Synthesis of 2-Benzamido-3-(indol-3-yl)acrylic Acid. 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one (288 mg, 1 mmole) was dissolved in 25 ml of hot 1% sodium hydroxide. The insoluble residue was filtered and treated in the same manner until all was dissolved. The combined alkali filtrates were acidified with hot 0.5 *N* hydrochloric acid to pH 5.5. The acid precipitated immediately. Recrystallization from 70% ethanol gave 210 mg of 2-benzamido-3-(indol-3-yl)acrylic acid: mp 232°; ultraviolet, 227 mμ (ε 38,104), 276 (11,142), 336 (18,293).

Synthesis of 2-Benzamido-3-(indol-3-yl)acrylate. 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one (288 mg, 1 mmole) was heated in 40 ml of anhydrous methanol containing 10 drops of trimethylamine for 10 min on a steam bath. After cooling the ester crystallized. Recrystallization from methanol gave 205 mg of methyl 2-benzamido-3-(indol-3-yl)acrylate: mp 243–245° dec; ultraviolet, 227 mμ (ε 37,376), 275 (10,752), 341 (20,908).

Synthesis of 1-[2-Benzamido-3-(indol-3-yl)acryloyl]piperidine. 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one (288 mg, 1 mmole) and 100.8 mg (1.2 mmoles) of piperidine in 0 ml of peroxide-free dioxane were heated for 45 min on a steam bath. The mixture was lyophilized. Crystallization of the residue from a few milliliters of methanol gave 220 mg of 1-[2-benzamido-3-(indol-3-yl)-acryloyl]piperidine; mp 137–138° dec; ultraviolet, 248 mμ (ε 27,768); 282 (13,727), 313 (16,775).

Anal. Calcd for $C_{23}H_{23}N_3O_2 \cdot CH_3OH$: C, 71.11; H, 6.66; N, 10.37. Found C, 71.69; H, 6.48; N, 10.75.

Synthesis of N-Propyl-2-benzamido-3-(indol-3-yl)acrylamide. 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one (288 mg, 1 mmole) and 69.8 mg (1.2 mmoles) of propylamine in 5 ml of peroxide-free dioxane were heated on a steam bath for 45 min. The mixture was lyophilized. Crystallization of the residue from a few milliliters of dioxane gave 250 mg of N-propyl-2-benzamido-3-(indol-3-yl)-acrylamide, mp 228°; ultraviolet, 280 mμ (ε 9341), 320 (12,863).

Anal. Calcd for $C_{21}H_{21}N_3O_2 \cdot {}^1/_2C_4H_8O_2$: C, 70.85; H, 6.54; N, 10.74. Found: C, 70.58; H, 6.39; N, 10.74.

Synthesis of N-Benzyl-2-benzamido-3-(indol-3-yl)acrylamide. 4-(Indol-3-ylmethylene)-2-phenyl-2-oxazolin-5-one (288 mg, 1 mmole) and 107 mg (1.2 mmoles) of benzylamine in 5 ml of peroxide-free dioxane were heated on a steam bath for 45 min. The mixture was lyophilized. Crystallization of the residue from a few milliliters of methanol gave 212 mg of N-benzyl-2-benzamido-3-(indol-3-yl)acrylamide: mp 245°; ultraviolet, 217 mμ (ε 42,616), 276 (11,705), 333 (21,125).

Anal. Calcd for $C_{25}H_{21}N_3O_2$: C, 75.34; H, 5.31; N, 10.60. Found: C, 75.94; H, 5.25; N, 10.63.

Basic Hydrolysis of Methyl 2-Benzamido-3-(indol-3-yl)acrylate. A mixture of 15 mg (0.25 mmole) of methyl 2-benzamido-3-(indol-3-yl)acrylate and 6 mg (0.25 mmole) of threonine in 1 ml of 10% aqueous barium hydroxide was heated in a sealed ampoule for 8 hr at 110°. The hydrolysate was diluted with few milliliters of water and the solution neutralized with Dry Ice. The barium carbonate precipitate was washed with water and the combined water solutions were evaporated in a desiccator over phosphorus pentoxide. The residue was dissolved in a few drops of water and compared (a) by electrophoresis (Whatman No. 3MM, pH 1.9, 3 kv, 3 hr), (b) thin layer chromatography (silica gel G, *n*-butyl alcohol–acetic acid–water 4:1:5), and (c) paper chromatography (Whatman No. 1, *n*-butyl alcohol–acetic acid–water 4:1:5) with a sample of tryptophan. Spraying with ninhydrin revealed only tryptophan besides a trace of glycine.

Identification of Indolyl-3-acetic Acid in the Alkaline Hydrolysis of Telomycin. To ten ampoules, each containing 100 mg of Telomycin, was added hot concentrated aqueous barium hydroxide (5 ml per ampoule). After sealing, the ampoules were heated in an oven for 12 hr at 105°, then opened and the contents pooled. The aqueous solution was neutralized to pH 2 with 1 *N* sulfuric acid. After filtration of the barium sulfate, the water solution was extracted three times with ether. The combined ether solutions were then washed with water. The dried ether layer (magnesium sulfate) was evaporated under reduced pressure. A red-brown tar (40 mg) remained. The tar was dissolved in a few drops of methanol, applied to silica gel G plates, and chromatographed with chloroform–96% acetic acid (95:5) and methyl acetate–isopropyl alcohol–25% ammonium hydroxide (45:35:20), respectively.[42] After spraying with Ehrlich's reagent, a main spot was visible due to indolyl-3-acetic acid as shown by comparison with an authentic sample. Indole and skatole were present in traces. Spraying with 2,4-dinitrophenylhydrazine reagent[43] revealed much indole-3-aldehyde.

Benzyl-N-carbobenzoxy-β-L-aspartyl-O-*t*-butyl-L-serine *t*-Butyl Ester. To a solution of O-*t*-butyl-L-serine *t*-butyl ester[44] (4.34 g, 20 mmoles) and α-benzyl-N-carbobenzoxy-L-asparate[45] (7.14 g, 20 mmoles) in 50 ml of methylene chloride was added $EtN{=}C{=}N(CH_2)_3N(CH_3)_2 \cdot HCl$ (4.22 g, 22 mmoles). The solution was stored overnight at room temperature and then evaporated. The residue was partitioned between ethyl acetate and water. The ethyl acetate layer was successively washed by dilute citric acid, water, 5% sodium bicarbonate, and water. The dried ethyl acetate solution (over magnesium sulfate) was filtered and evaporated. An oil remained (10.35 g, 91%). Thin layer chromatography on silica gel G with *n*-propyl alcohol–water (70:30 v/v) showed one spot, R_4 0.84.

Benzyl-N-carbobenzoxy-β-L-aspartyl-L-serine. Benzyl-N-carbobenzoxy-β-L-aspartyl-O-*t*-butyl-L-serine *t*-butyl ester (10.25 g) was dissolved in 50 ml of dry trifluoracetic acid. After 45 min, the trifluoroacetic acid was evaporated under reduced pressure at 40°. The residual oil solidified on trituration with ether (200 ml), yield 4.56 g (56%). A sample was recrystallized from ethyl acetate–petroleum ether (bp, 30–60°) mp 154–155°. Thin layer chromatography on silica gel G with *n*-propyl alcohol–water (970:30 v/v) showed one spot, R_f 0.77.

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Anal. Calcd for $C_{22}H_{24}N_2O_8$: C, 59.46; H, 5.40; N, 6.30. Found: C, 59.44; H, 5.58; N, 6.43.

β-N-Aspartyl-L-serine. A solution of benzyl-N-carbobenzoxy-L-aspartyl-L-serine (2 g) in aqueous methanol (70:30 v/v) was hydrogenated at room temperature over 10% palladium on charcoal (1 g) for 7 hr. The catalyst was removed by filtration, the solution was evaporated under reduced pressure, and the residue (940 mg) recrystallized from aqueous methanol, mp 89–91°. Thin layer chromatography on silica gel G with *n*-propyl alcohol–water (70:30 v/v) gives a brownish spot, R_f 0.15, with ninhydrin (1% in acetone).

Anal. Calcd for $C_7H_{12}N_2O_6$: C, 38.18; H, 5.45; N, 12.72. Found: C, 37.80; H, 5.36; N, 12.56.

***t*-Butyl-N-carbobenzoxy-α-L-aspartyl-O-*t*-butyl-L-serine *t*-Butyl Ester.** To a solution of O-*t*-butyl-L-serine *t*-butyl ester (4.34 g, 20 mmoles) and β-*t*-butyl N-carbobenzoxyaspartate (6.46 g, 20 mmoles) in 50 ml of methylene chloride was added $EtN{=}C{=}N(CH_2)_3N(CH_3)_2{\cdot}HCl$ (4.22 g, 22 mmoles). After storage overnight at room temperature the solution was concentrated. The residual oil was dissolved in ethyl acetate. The solution was washed successively with citric acid, water, 5% sodium bicarbonate, and water. The dried solution was filtered and evaporated to an oil (9.95 g, 95%). Thin layer chromatography on silica gel G with *n*-propyl alcohol–water (70:30 v/v) gives one spot, R_f 0.74.

N-Carbobenzoxy-α-L-aspartyl-L-serine. *t*-Butyl-N-carbobenzoxy-α-L-aspartyl-O-*t*-butyl-L-serine *t*-butyl ester (9.85 g) was dissolved in 50 ml of dry trifluoroacetic acid. After 45 min the trifluoroacetic acid was evaporated under reduced pressure at 40°. The remaining oil solidified upon addition of ether (350 ml): yield, 3.64 g (54%); mp 155° after recrystallization from ethyl acetate–petroleum ether. Thin layer chromatography of silica gel G with *n*-propyl alcohol–water (70:30 v/v) gave one spot.

Anal. Calcd for $C_{15}H_{18}N_2O_8$: C, 50.84; H, 5.08; N, 7.90. Found: C, 50.58; H, 5.29; N, 7.52.

α-L-Aspartyl-L-serine. A solution of N-carbobenzoxy-α-L-aspartyl-L-serine (2 g) in aqueous methanol (70:30) was hydrogenated over palladium on charcoal (1 g) for 7 hr at room temperature (1 atm). The catalyst was removed and the solution evaporated: yield, 1.12 g (90.3%); mp 175° dec after one recrystallization from aqueous methanol. Thin layer chromatography on silica gel G with *n*-propyl alcohol–water (70:30 v/v) gave one spot, R_f 0.17. Spraying with ninhydrin gave a blue-violet color.

Anal. Calcd for $C_7H_{12}N_2O_6$: C, 38.18; H, 5.45; N, 12.72. Found: C, 38.32; H, 5.82; N, 12.51.

Comparison of the Dipeptide (Asp-Ser) Degradation Product from Telomycin with Synthetic Samples of α-L-Aspartyl-L-serine and β-L-Aspartyl-L-serine. The respective samples were applied to Whatman No. 3MM paper (pH 6.2, 3 kv, 5 hr). The paper was dried and sprayed with ninhydrin (1% solution in acetone). β-L-Aspartyl-L-serine was in ninhydrin color (brown) and in mobility on electrophoresis (R_f 0.77; Asp = 1 taken as unity) identical with dipeptide Asp-Ser isolated from a partial basic hydrolysate of Telomycin.

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The Crystal and Molecular Structure of Dihydrothymine

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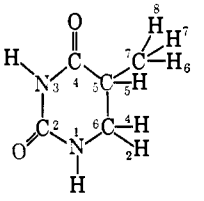
Abstract: The crystal structure of dihydrothymine has been solved from three-dimensional X-ray diffraction data collected by counter and photographic measurements and refined to $R = 0.048$. The crystals are orthorhombic with unit cell parameters $a = 7.336$ Å, $b = 23.474$ Å, $c = 7.034$ Å. The hydrogenated carbon atoms are out of the plane of the other ring atoms by about 0.35 Å and the methyl group is equatorial. Hydrogenation also causes significant changes in the nonhydrogenated part of the molecule. The structure is disordered, the two enantiomorphs occupying all sites in the space group Pbca in the ratio 3:2.

The dihydropyrimidines are interesting compounds both from a biochemical and a structural point of view. They are implicated in the catabolism of pyrimidine bases, and dihydrouracil occurs in alanine-RNA, possibly playing the role of "insulating" the coding triplet from neighboring nucleotides.[2] Structurally, the molecules consist of a π-electron system closed by two saturated carbon atoms, thus containing features favoring both a planar and a partly nonplanar ring. Little appears to be known about the chemistry of dihydropyrimidines, but the observation has been made that when a pyrimidine nucleoside is hydrogenated at the 5,6 double bond, the sugar can be removed from it under hydrolytic conditions much gentler than normally possible.[3]



dihydrothymine

No dihydrogenated π-electron system of this type appears to have been subjected to detailed structure analysis. The present paper describes the crystal and molecular structure of dihydrothymine.

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