TALLYSOMYCIN, A NEW ANTITUMOR ANTIBIOTIC COMPLEX RELATED TO BLEOMYCIN
II. STRUCTURE DETERMINATION OF TALLYSOMYCINS A AND B

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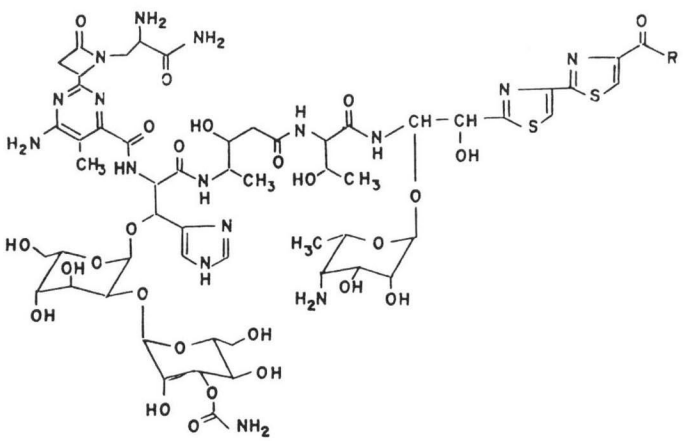
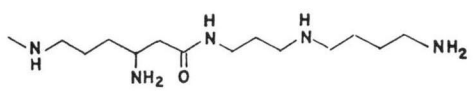
The structures of tallysomycins A and B, two major components of a new antitumor antibiotic complex, have been determined. They are glycopeptide antibiotics structurally related to bleomycin: four amino acid moieties and a disaccharide fragment which are the constituents of bleomycin molecule are also present in the tallysomycins. Tallysomycins A and B contain two new amino acids and a unique amino sugar, 4-amino-4,6-dideoxy-L-talose, which have not been hitherto found in the phleomycin-bleomycin group of antibiotics. In addition tallysomycin A has an additional amino acid, L-β-lysine, and thus a longer peptide chain than bleomycin or tallysomycin B. Tallysomycins A and B have the same terminal amine moiety, spermidine.

Tallysomycin is a new antitumor antibiotic complex produced by the unidentified actinomycetes strain No. E465–94. The production, isolation and properties of tallysomycins have been reported in the preceding paper.[1] Two major components A and B of tallysomycin complex were isolated as a copper-chelated form and shown to belong to the bleomycin-group of antibiotics. This paper presents evidence to show that tallysomycins A and B possess the following structures:



Tallysomycin A R =



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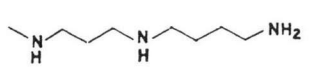


General Structural Characteristics and Preliminary Degradation Studies

Tallysomycins A and B gave positive reactions with ninhydrin and anthrone reagents, and showed UV spectra similar to those of the bleomycin group of antibiotics.[1] The molecular formulae of $C_{68}H_{107}N_{21}O_{27}S_2$ and $C_{62}H_{95}N_{19}O_{26}S_2$ were assigned to tallysomycins A and B, respectively, by analyses and were confirmed by the degradation studies described below and also by the ^{13}C NMR spectra.[2] The proton NMR spectrum of tallysomycin A indicated the presence of four C-methyl groups, 10 high-field methylene protons (δ 1.5~1.8 ppm), seven protons in the anomeric proton region (δ 4.8~5.6 ppm), four aromatic protons (δ 7.48, 8.02 (2H), 8.69 ppm) and 40~50 protons at around δ 2.2~4.5 ppm. The PMR spectrum of tallysomycin B was similar to that of component A in the aromatic and anomeric proton regions with fewer protons in the methylene and methine regions. Thus tallysomycins are clearly differentiated by PMR from bleomycins which show signals for four protons in the anomeric proton region.

As described below the peptide chain skeleton of tallysomycin A is composed of seven amino acids (**I**, **II**, ***III***, **IV**, **V**, **VI** and **VII**)* and one amine (**VIII**), of which amino acids **I**, **II**, **IV** and **V** are the same as those found in the bleomycin structure (compounds, **I**, **II**, **IV** and **V** described in the bleomycin papers[3,4,5]).

Tallysomycin B contains amino acids **I**, **II**, **III**, **IV**, **V** and **VI** and amine **VIII** but does not have amino acid **VII**.

Tallysomycin A was hydrolyzed with 6 N HCl at 115°C for 21 hours in a sealed tube. The resultant precipitate was separated and the filtrate was extracted with ethyl acetate. The separated aqueous layer was found to contain at least seven ninhydrin-positive compounds, each of which was isolated by ion-exchange chromatography (Dowex 50W×4) developed with an increasing concentration of aqueous HCl solution. Six amino acids (**I**, **II**, **III**, **IV**, **V** and **VII**) and an amine component (**VIII**) were thus isolated from the eluates and crystallized. Amino acid **VI** was not contained in the aqueous fraction of the hydrolyzate.

Amino acid **I** ($C_4H_9NO_3$) was identified as L-threonine by TLC, IR, PMR, melting point and optical rotation.

Amino acid **II** ($C_9H_{12}N_4O_4$) showed UV absorption maxima at 235 and 274 nm. It was identified as β-amino-β-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)-propionic acid[4] by comparison with an authentic sample prepared from bleomycin.

Amino acid **IV** ($C_6H_9N_3O_3$) was identified as L-*erythro*-β-hydroxyhistidine[5] by direct comparison with an authentic sample obtained from bleomycin.

Amino acid **V** ($C_3H_8N_2O_2$) was isolated in relatively low yield from the hydrolyzate. It was identified as L-β-aminoalanine.

Amino acid **VII** ($C_6H_{14}N_2O_2$) was characterized as crystalline hydrochloride and picrate. It was identified as L-β-lysine by comparison with an authentic sample prepared from capreomycin.[6] This amino acid was not present in the hydrolyzate of tallysomycin B.

Amine component **VIII** ($C_7H_{19}N_3$) was isolated as its crystalline trihydrochloride and identified

* Designations adopted for the constitutive amino acids (**I**, **II**, **III**, **IV**, **V**, and **VI**) of tallysomycin in this paper correspond to those used by TAKITA *et al.* for bleomycin[3].

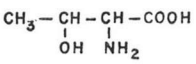
as spermidine.

A mild acid hydrolysis of tallysomycins was attempted to isolate the sugar moiety presumed to be present in the tallysomycin structure. Tallysomycin A was treated with 0.3 N H_2SO_4 at 80°C for 20 hours, and the reaction mixture was passed over an Amberlite CG–50 column to remove basic fragments in the hydrolyzate. The effluent was chromatographed on a column of Avicel-SF to obtain compound **IX** which was characterized as the trimethylsilyl and peracetyl derivatives. Compound **IX** was identified as 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulopyranose[7], the disaccharide moiety present in bleomycins.

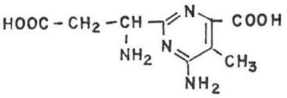
Similar hydrolysis of tallysomycin **B** also gave the same sugar fragment **IX**.

The structures for the above-described amino acids (**I**, **II**, **IV**, **V** and **VII**), amine (**VIII**) and disaccharide fragment (**IX**) are shown at right:

I



II



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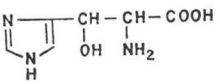
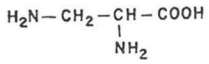
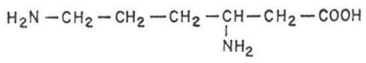


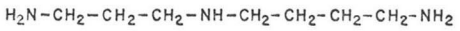
V



VII



VIII



IX

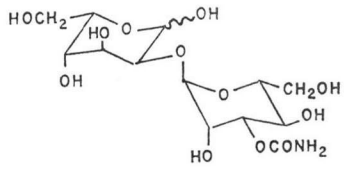
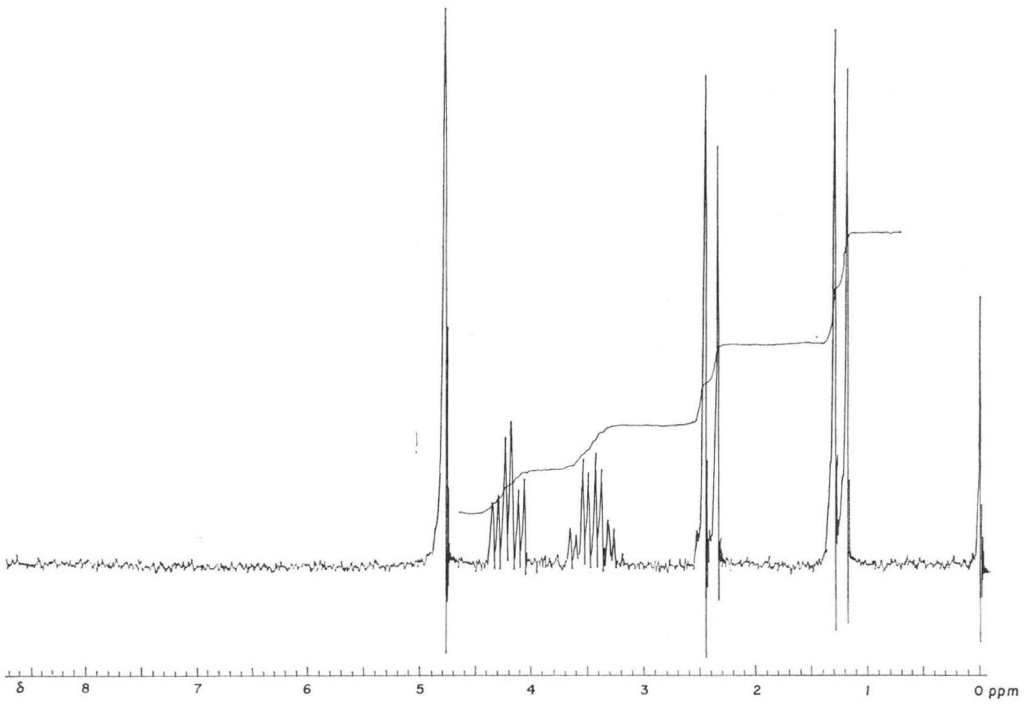
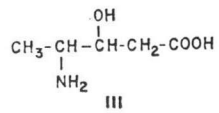


Fig. 1. PMR spectrum of **III** (60 MHz in D_2O)



Structure of Amino Acid III

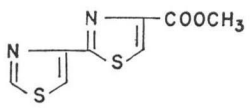
Amino acid **III** was crystallized from aqueous ethanol and analyzed as $C_5H_{11}NO_3 \cdot \frac{1}{2}H_2O$. The PMR spectrum of **III** (Fig. 1) indicated the presence of one methyl (δ 1.23 ppm, *d*, J=6.7 Hz), one methylene (δ 2.39 ppm, *d*, J=7.1 Hz) and two methine groups (δ 3.44 ppm, *d-q*, J=6.7 & 3.1 Hz; δ 4.18 ppm, *d-t*, J=7.1 & 3.1 Hz). These PMR data along with the IR spectrum and analytical results suggested **III** to be 4-amino-3-hydroxy-*n*-valeric acid, which was confirmed by synthesis of the racemate according to published method.[8] As described later, **III** was also isolated as a γ-lactam form (**X**) after methanolysis of a peptide fragment. The PMR spectrum of **X** indicated that the C-4 methyl and the C-3 hydroxyl groups were oriented in the *trans*-configuration, and hence **III** is *erythro*-(3S, 4R or 3R, 4S)-4-amino-3-hydroxy-*n*-valeric acid.



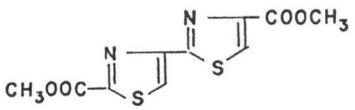
III

Partial Structure for Compound VI

All of the above-characterized compounds were isolated from the aqueous part of the acid hydrolyzate of tallysomycins A and B but none of these compounds showed the strong UV absorption at 290 nm which was exhibited by tallysomycins A or B. As described earlier, a resinous precipitate was formed after 6 N HCl hydrolysis and there was a solvent-extractable acidic material in the filtrate, both of which showed a UV absorption at 290 nm. The acidic compound in the solvent extract was treated with diazomethane to give the methyl ester **XI** ($C_8H_6N_2O_2S_2$) which was identified as methyl 2,4′-bithiazolyl-4-carboxylate[9] by PMR and mass spectra.



XI



XII

The resinous precipitate separated from the acid hydrolyzate resisted further purification because of its extremely poor solubility in most organic solvents. However, it was treated with alkaline permanganate solution and the oxidation product was converted to methyl ester **XII** ($C_{10}H_8N_2O_4S_2$) which was identified as dimethyl 2,4′-bithiazolyl-2′,4-dicarboxylate[10] by PMR and mass spectra.

Thus compound **VI** should be a 2′-substituted-2,4′-bithiazolyl-4-carboxylic acid with an unstable side chain at the 2′ position, which is lost under drastic hydrolytic condition. Attempts were therefore made to obtain small peptide fragments which might contain compound **VI** in its intact form.

Peptide Fragments Obtained by Mild Hydrolysis

When kept in 6 N HCl at 30°C for 3 days, tallysomycin A was gradually cleaved into several peptide fragments and a sugar moiety, which were separated by CM-Sephadex C–25 chromatography to isolate the disaccharide component **IX** along with three peptide fragments (designated as peptides L, M and P). The amino acid composition for each of the peptide fragments as shown below was deduced from the results of total acid hydrolysis and UV and PMR spectral data.

	Constitutive amino acids or amine
Peptide L	**II, III, IV, V**
Peptide M	**II, IV, V**
Peptide P	**I, VI, VII, VIII**

Peptide M was identified as "pseudotripeptide" of bleomycin[11] by direct comparison with an authentic specimen isolated from the mild acid hydrolyzate of bleomycin. On treatment with conc.HCl at 37°C, peptide L underwent an N→O acyl migration to afford a new peptide N which showed an ester band at 1,730 cm^{-1} in the IR spectrum. Further hydrolysis of peptide N in conc.HCl gradually liberated amino acid **III** and yielded peptide M. Thus the structures of peptides L, M and N as well as the reaction sequence involved were determined as shown below:

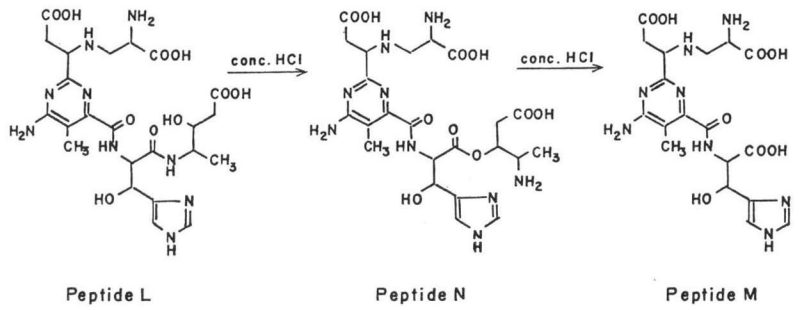
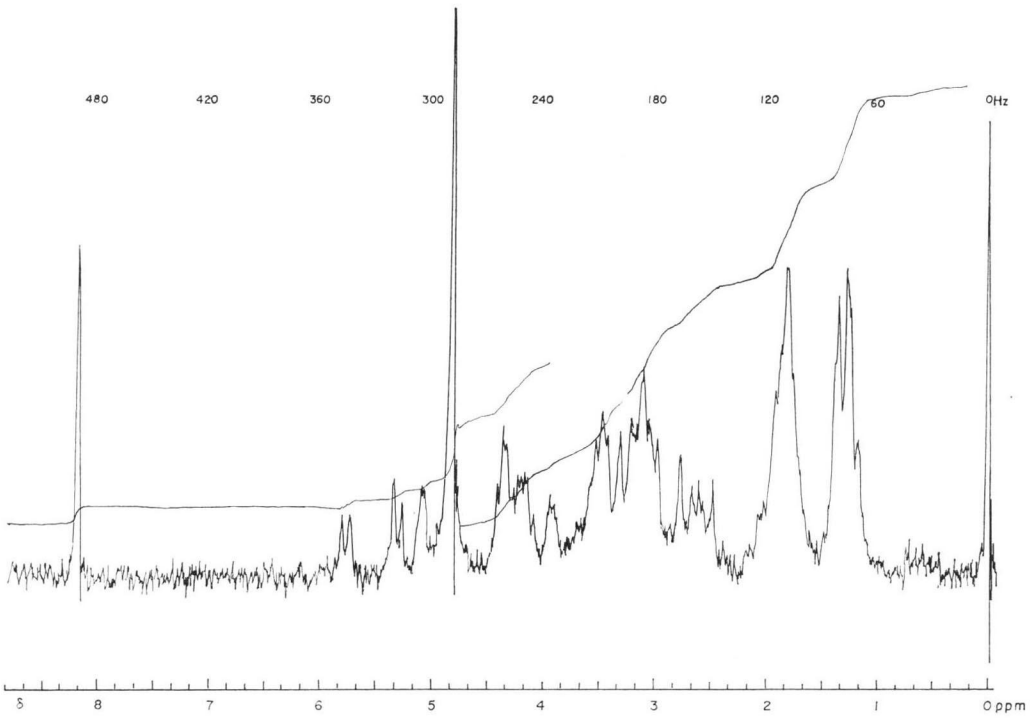


Fig. 2. PMR spectrum of peptide W·HCl (60 MHz in D_2O).



Peptide P showed a UV absorption maximum at 290 nm, indicating the presence of the bithiazole carboxylic acid moiety in this fragment. The PMR spectrum of peptide P showed two aromatic protons (δ 8.07 ppm) and three protons in the low-field anomeric proton region (δ 4.9~5.7 ppm), suggesting that compound **VI** might retain its intact structure in peptide P. In fact, the ^{13}C NMR spectra of peptides L and P together with that of disaccharide fragment **IX** contained all the carbon signals demonstrated by the CMR of tallysomycin A.[2]

Similarly, mild hydrolysis of tallysomycin B yielded a new peptide fragment P_b along with the above-described peptides L and M and disaccharide fragment **IX**. Peptide P_b differed from peptide **P** in the lack of L-β-lysine moiety (**VII**).

The selective cleavage of a histidyl peptide bond[19] by N-bromosuccinimide (NBS) was successfully applied in the structure elucidation of bleomycin.[12] When tallysomycin A was treated with NBS in the cold and the reaction product hydrolyzed, a new UV-absorbing fragment (designated peptide W) was isolated after chromatography on an Amberlite XAD-2 column. Peptide W was shown to consist of compounds, **I**, **III**, **VI**, **VII** and **VIII** by hydrolysis, microanalysis and PMR spectrum (Fig. 2). The CMR spectrum of peptide W[2] was also consistent with the assigned composition. Similar treatment of tallysomycin B with NBS yielded peptide W_b which contained compounds **I**, **III**, **VI** and **VIII**.

The isolation of peptides L, M, P and W afforded the additional information that peptide L should be linked to peptide P through amino acid **III** in the peptide chain of tallysomycin A.

Structure of Compound VI

Although the bithiazole-containing moiety, compound **VI**, could not be isolated as a single entity that retained the complete structure, peptide fragments P and W were considered to contain compound **VI** in its intact form on the basis of analytical and spectral data. The PMR spectra of peptides P and W indicated the presence of two aromatic protons assignable to the bithiazole ring protons along with three protons in the low-field anomeric proton region, the latter being the signals characteristic of the intact compound **VI**. The ^{13}C NMR spectra of peptides P and W indicated 32 and 37 carbon signals, respectively, and substraction of the signals assignable to the established constituents (**I**, **III**, **VII** and **VIII**) left 15 carbon signals to be assigned to compound **VI**. Seven of the 15 signals were assigned to 4-carbonyl-2,4′-bithiazole unit based on the CMR data reported for bleomycin components[13]. Off-resonance CMR spectra indicated the remaining eight carbon signals of compound **VI** should be for one methyl and seven methine carbons. These CMR data for compound **VI** are summarized in Table 1. The 220 MHz PMR spectrum of pep-

Table 1. Carbon signals assigned to compound **VI** from the CMR spectra of peptides P and W

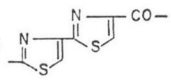
Signals assigned to compound **VI** (δ_C^{TMS})		Type of carbon	Assignment
Peptide W	Peptide P		
ppm 16.9	ppm 16.7	$-CH_3$	
55.2	55.1	$-CH<$	
63.8	63.7	〃	
64.9	65.2	〃	
68.4	68.5	〃	
72.0	72.0	〃	
81.6	82.1	〃	
100.9	101.1	〃	
120.2	120.5	〃	
125.8	125.8	〃	
148.2	148.4	$-\overset{\vert}{C}-$	
150.0	150.1	〃	
163.4	163.4	〃	
163.7	164.0	〃	
172.5	173.1	$-\overset{\Vert}{C}-$ O	

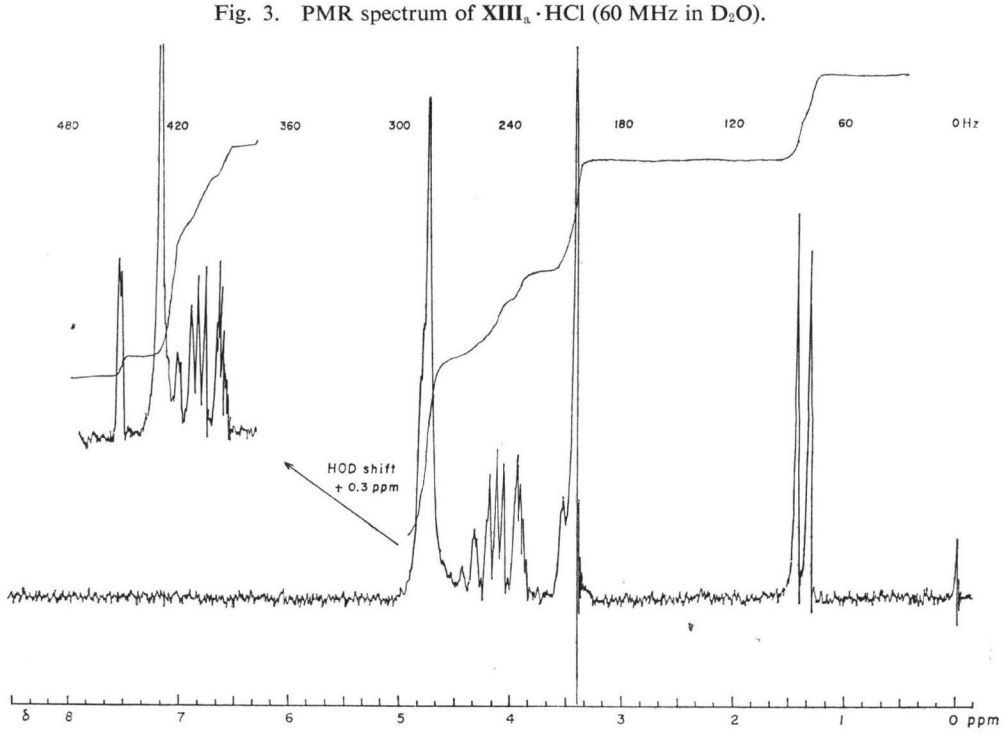
Table 2. Protons assigned to compound **VI** from the 220 MHz PMR spectrum of peptide P

Chemical shift	Coupling	Protons	Assignment
δ: 1.39 ppm	d (J=6.8 Hz)	3H	$-CH_3$
3.58	m	1H	>CH–N
3.96	m	1H	>CH–O
4.18	t	1H	>CH–O
4.46	m	1H	>CH–O
5.15	d (J=2.0 Hz)	1H	anomeric H
5.37	d (J=4.5 Hz)	1H	>CH–OH
5.82	d (J=4.5 Hz)	1H	>CH–O
8.28	s	1H	thiazole protons
8.30	s	1H	thiazole protons

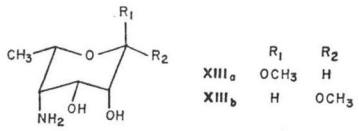
tide P indicated that one methyl, seven methine and two aromatic protons could well be allocated to compound **VI** moiety as shown in Table 2, which also supported the above CMR results. These spectral data together with the analytical results on peptides P and W indicated a formula of $C_{15}H_{18}N_4O_6S_2$ for the residue of compound **VI** present in the peptide fragments.

Peptides P and W gave positive reactions with ninhydrin, anthrone and RIMINI reagents but were negative to FEHLING reaction. This and the above NMR data suggested the presence of a sugar moiety in compound **VI**. Peptide W was refluxed in methanolic hydrogen chloride and the reaction mixture was chromatographed over an Amberlite XAD–2 column to give three major degradation products: methyl glycoside **XIII**, compound **X** which was identified as the γ-lactam of amino acid **III** described earlier, and a new peptide fragment designated peptide Y. The methyl glycoside **XIII** was further

Fig. 3. PMR spectrum of $\mathbf{XIII}_a \cdot HCl$ (60 MHz in D_2O).

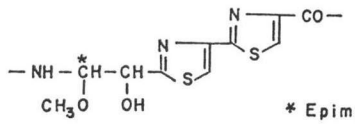


separated into α- and β-anomers (**XIII**$_a$ and **XIII**$_b$). The PMR spectrum of **XIII**$_a$ is shown in Fig. 3. The analytical and mass spectral data along with PMR decoupling experiments indicated **XIII** to have a 4-amino-4,6-dideoxyhexose structure. Furthermore, small coupling constants observed for the anomeric protons ($J_{1ax,2}=2.0$ Hz; $J_{1eq,2}=<1.0$ Hz) and for the C_4–C_5 protons ($J=ca.$ 2.0 Hz) suggested a *talo* configuration for the amino sugar. **XIII**$_a$ afforded crystalline N,O-triacetate (**XIV**) which showed the same melting point and PMR spectrum as those reported for methyl 4-acetamido-2,3-di-O-acetyl-4,6-dideoxy-α-D-talopyranoside.[14] Since the optical rotations of **XIII**$_a$ and **XIII**$_b$ showed an opposite sign to that reported for the D-sugar anomers but were identical with those of the L-forms,[15] **XIII** was determined to be methyl 4-amino-4,6-dideoxy-L-talopyranoside.

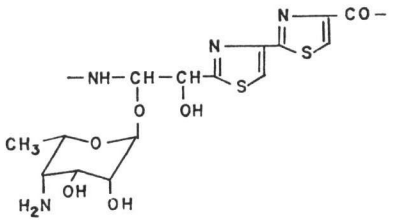


	R_1	R_2
XIII$_a$	OCH_3	H
XIII$_b$	H	OCH_3

The PMR spectrum and total hydrolysis of the new peptide fragment Y indicated that amino acid **III** and the amino sugar unit were cleaved from peptide W on methanolysis and instead one O-methyl group was introduced. Two low-field protons at around δ 5.1 ~ 5.6 ppm were still present in the PMR spectrum of peptide Y but appeared as more complicated signals than those observed in peptide W, suggesting that epimerization might have occurred when the amino sugar moiety was eliminated from peptide W. The separation of epimers was accomplished on peptide Y$_b$ which was obtained by a similar methanolysis of peptide W$_b$. In the PMR spectra of both epimers Y$_{b1}$ and Y$_{b2}$, the two low-field protons were observed as two distinct doublets at δ 5.15 and 5.50 ppm ($J=5.8$ Hz) in Y$_{b1}$ and at δ 5.16 and 5.40 ppm ($J=5.0$ Hz) in Y$_{b2}$. A PMR decoupling experiment indicated that the two low-field protons were located at vicinal position. O-Acetylation of peptide Y$_{b1}$ caused a downfield shift (*ca.* 0.9 ppm) of the δ 5.15 ppm proton which was thus determined to be on a carbon bearing a hydroxyl group. The molecular formula of peptide Y$_{b1}$ was unambiguously established by the field desorption mass spectrometry of Y$_{b1}$ and its N,O-pentaacetate to be $C_{21}H_{35}N_7O_5S_2$ (intense $M^+ + 1$ peak at *m/e* 530; M^+ peak for pentaacetate at *m/e* 739), thus leaving a $C_3H_7NO_2$ moiety for the side chain structure of unknown part in peptide Y or Y$_b$. Putting the above-described analytical and spectral information together, the partial structure for the bithiazole carboxylic acid moiety in peptide Y or Y$_b$ was deduced as shown below:



The above structure satisfactorily explains the characteristic low-field chemical shifts observed for the two vicinal protons in peptides Y$_{b1}$ and Y$_{b2}$. It is apparent that 4-amino-4,6-dideoxy-L-talose moiety (**XIII**) should have been linked to the epimeric carbon shown above in peptide W or P as well as in tallysomycins. The anomeric proton of the amino sugar observed in the PMR spectra of peptides W and P appeared as a doublet at δ 5.1 ppm with a coupling constant of $J=2.0$ Hz. These PMR data were consistent with those found for the anomeric proton of α-anomer of the methyl glycoside (**XIII**$_a$), indicating an α-glycosidic linkage for the amino sugar moiety in peptide W or P. Thus the structure of compound **VI** residue is as shown below:

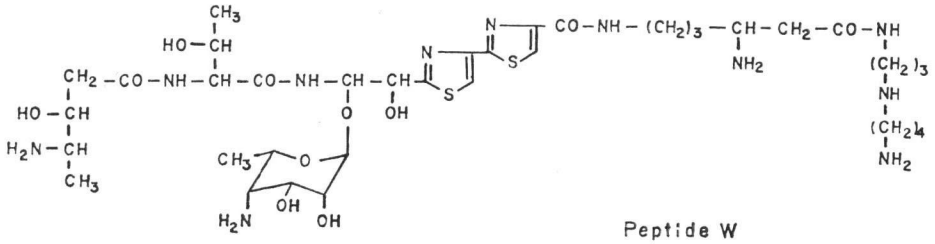


Structure of Peptides W and P

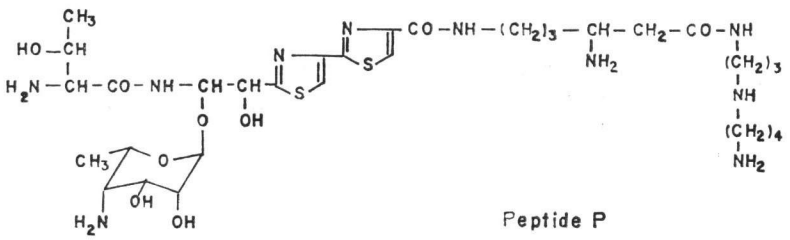
In the methanolysis experiment of peptide W stated above, a small fragment (**XV**) was isolated which was shown to consist of L-β-lysine (**VII**) and spermidine (**VIII**). The constitutive amino acid and amine compositions elucidated earlier for peptide fragments **P** and **W** as well as the established structure of bleomycin suggested the amino acid sequence of **III**→**I**→**VI**→(**VII**, **VIII**) in peptide W, which was unequivocally determined as described below.

The terminal structure of peptide W (and W_b) was determined by microbiological and chemical means: strains of *Serratia marcescens* are known to produce spermidine oxidase[16] which cleaves the substrate into 1,3-diaminopropane and γ-aminobutyraldehyde (then to Δ^1-pyrroline), and the enzyme was used in the biotransformation of bleomycin A_5[17]. As will be published elsewhere in detail, when treated with an enzymatic preparation obtained from *S. marcescens* IAM-1223, tallysomycins A and B afforded bioactive transformation products designated tallysomycins E_{1a} and E_{1b}, respectively, which were shown upon hydrolysis to contain 1,3-diaminopropane instead of spermidine, indicating that spermidine (**VIII**) is the terminal amine in the peptide structure of both tallysomycins A and B.

Alternatively, peptide W was dinitrophenylated in the usual manner and the product hydrolyzed in refluxing 6 N HCl. The reaction mixture was purified by chromatography to afford DNP-derivatives of compounds **III**, **VII** and **VIII** along with free amino acid **I**. The DNP derivative of **VII** was identified as β-DNP-β-lysine by comparison with an authentic sample prepared by a published method[18]. The UV, PMR and mass spectra of DNP-**VIII** indicated the presence of two DNP groups in the derivative, one located on the secondary amino group and the other on one of the two primary amino groups of



Peptide W



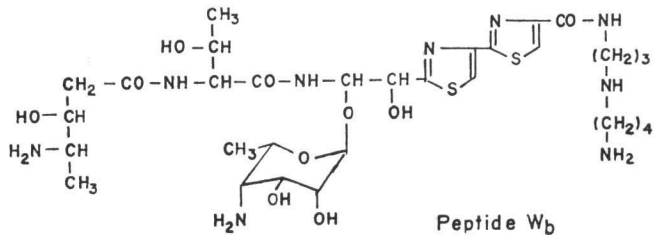
Peptide P

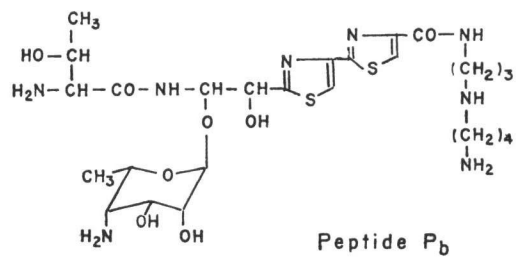
spermidine. Although the spectral data did not define which of the two primary amino groups was dinitrophenylated, the results of the spermidine oxidase experiments described above indicated that the two amino groups in the diaminobutane part of spermidine should have been dinitrophenylated.

Similarly dinitrophenylation of peptide P and subsequent hydrolysis yielded DNP derivatives of compounds, **I**, **VII** and **VIII**, affording additional evidence that L-threonine (**I**) was the N-terminal of peptides P and I was acylated with amino acid **III** in peptide W.

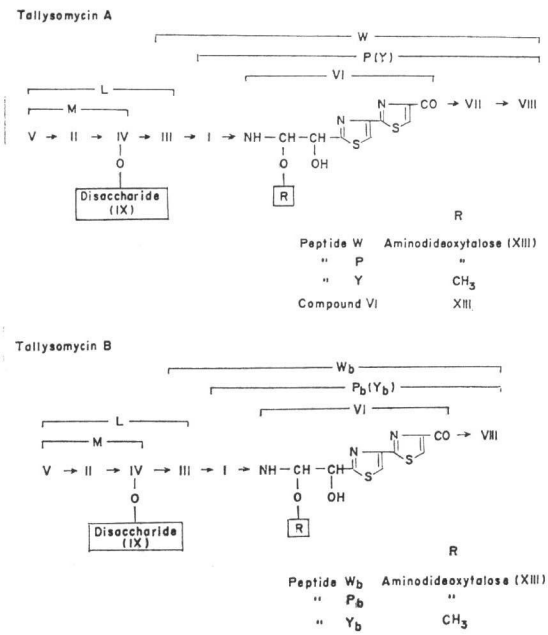
The amino acid sequence in peptide W was thus decided to be **III**→**I**→**VI**→**VII**→**VIII** and the structures shown ahead were given to peptides W and P.

Tallysomycin B was shown to haves permidine as the terminal amine moiety, and the peptide fragments obtained from tallysomycin B (W_b and P_b) did notc ontain L-β-lysine. Therefore the structures of peptides W_b and P_b are as shown at right:





Scheme 1. Peptide fragments of tallysomycins A and B



As described earlier, peptide P must be linked to peptide L through amino acid **III** in the peptide chain of tallysomycin A, and the ^{13}C NMR spectra of peptides L and P and disaccharide moiety **IX** accounted for all the carbons of tallysomycin A. Furthermore, the carbon signals assigned to amino acid **IV** and disaccharide **IX** in the CMR spectra of tallysomycins A and B and peptide L[2] were very close to those reported for bleomycin,[18] suggesting that the disaccharide moiety is glycosidically linked to the β-hydroxyl group of amino acid **IV** as in the case of bleomycin. Therefore the structures shown before are assigned to tallysomycins A and B. The peptide fragments discussed in the above structural studies are schematically shown in Scheme 1.

Discussion

Tallysomycins A and B are closely related to bleomycin in that the chromophores present in tallysomycins are the same as those of bleomycin, and four amino acid moieties and a disaccharide fragment of bleomycin are also found in tallysomycins A and B. Tallysomycins A and B are different from bleomycin in two amino acid components, **III** and **VI**, the latter amino acid having glycosidically linked to 4-amino-4,6-dideoxy-L-talose, the amino sugar not previously reported in nature. Amino acid **VI** was shown to have a substituted carbinolamine structure in the side chain. It is interesting to note that maytansine,[20] a potent antitumor substance isolated from *Maytenus serrata,* and the pyrrolobenzodiazepine antitumor antibiotics such as anthramycin[21], tomaymycin[22] and sibiromycin[23] contain a similar carbinolamine functional group, which is considered to be essential for the antitumor activity of these natural products.[24] Amino acid **III** was determined to be 4-amino-3-hydroxy-*erythro*-*n*-valeric acid which is a des-methyl analogue of the amino acid contained in bleomycin. Another significant feature of tallysomycin A is the presence of an additional amino acid moiety, L-β-lysine, resulting in a longer peptide chain in tallysomycin A than bleomycin or tallysomycin B. Furthermore, in contrast to bleomycin components, the major fermentation products of tallysomycin contain spermidine as the sole terminal amine moiety.

Experimental

TLC was performed on silica gel (Kieselgel $60F_{254}$, Merck) and cellulose (Avicel SF, Funakoshi) plates using the solvent systems shown below.

System No.	Plates	Solvent
S–101	Silica gel	*n*-PrOH - Pyridine - AcOH - H_2O (15: 10: 3: 12)
S–102	〃	MeOH - 10% $AcONH_4$ (1: 1)
S–121	〃	Acetone - 10% $AcONH_4$ (10: 9)
S–123	〃	MeOH - 10% $AcONH_4$ - 10% NH_4OH (10: 9: 1)
PL–111	〃	$CHCl_3$ - MeOH (7: 3)
SD–102	〃	*n*-BuOH saturated with 1 N NH_4OH
SD–103	Cellulose	*n*-BuOH - EtOH - H_2O (5: 3: 2)
SD–104	Silica gel	$CHCl_3$ - MeOH (97: 3)

Ordinary PMR spectra were obtained on a JEOL C60HL and 220 MHz PMR was determined by a Varian 220 HR spectrometer. CMR spectra were recorded on Varian XL–100 (25.2 MHz) and CFT–20 (20.0 MHz) spectrometers operated in the FOURIER transform system. Electron impact mass spectra were measured on a Hitachi RMU–6MG mass spectrometer using the direct inlet probe and field desorption mass spectra on a JEOL OIS–FI/FD mass spectrometer.

Total acid hydrolysis of tallysomycin A

A solution of tallysomycin A (3.0 g) in 100 ml of 6 N HCl was heated at 115°C for 21 hours in a sealed vessel. Insoluble material (504 mg) was separated by decantation, the solution diluted with water and extracted with EtOAc. Evaporation of the extract afforded 82 mg of oily residue. The aqueous layer was concentrated *in vacuo* and passed over a column of Dowex 50W × 4 (H^+, 400 ml). The column was developed successively with water (1.5 liters), 0.1 N HCl (1 liter), 0.2 N HCl (1.7 liters), 1.2 N HCl (3.1 liters) and 3 N HCl (1.4 liters). The elution was monitored by ninhydrin reaction and absorption at 254 nm to collect fractions which were concentrated under reduced pressure. Amino acids **I** (288 mg) and **III** (300 mg) were eluted with 0.2 N HCl, **V** (75 mg), **IV** (282 mg), **VII** (290 mg) and **II** (223 mg) with 1.2 N HCl and amine **VIII** (328 mg) with 3 N HCl. Crude preparations were purified by ion-exchange chromatography and then crystallized.

Amino acid **I**: Colorless rods from aqueous EtOH, m.p. 245°C (dec.). $[\alpha]_D^{22}$ −18° (*c* 0.5, H_2O). TLC: Rf 0.54 (S–101) and 0.62 (S–123). Anal. Calc'd for $C_4H_9NO_3$: C 40.33, H 7.62, N 11.76. Found: C 40.25, H 7.77, N 11.70. IR and PMR spectra identical with those of L-threonine.

Amino acid **II**: Crystalline monohydrochloride from aqueous EtOH, m.p. >210°C (dec.). TLC: Rf 0.42 (S–101) and 0.59 (S–123). $\lambda_{max}^{H_2O}$ 235 nm (ε 9,600) and 274 nm (ε 6,400). The IR and PMR spec-

tra were consistent with those of β-amino-β-(4-amino-6-carboxy-5-methylpyrimidin-2-yl)propionic acid prepared from bleomycin[4].

Amino acid **III**: Colorless needles from aqueous EtOH, m.p. 217.5~218.5°C. $[\alpha]_D^{27}$ +11.3° (*c* 0.4, H_2O). TLC: Rf 0.52 (S–101) and 0.57 (S–123). IR: ν_{max}^{KBr} 3,430, 3,000~2,800, 1,650, 1,570, 1,400, 1,400, 1,160. MS: *m/e* 134 (M^++1), 115, 100, 74, 44. Anal. Calc'd for $C_5H_{11}NO_3 \cdot \frac{1}{2}H_2O$: C 42.25, H 8.51, N 9.85. Found: C 42.54, H 8.61, N 9.85.

Amino acid **IV**: Colorless prisms of monohydrochloride from aqueous EtOH containing HCl, m.p. 210°C (dec.). $[\alpha]_D^{27}$ +35° (*c* 0.5, H_2O). TLC: Rf 0.28, (S–101) and 0.53 (S–123). Anal. Calc'd for $C_6H_9N_3O_3 \cdot HCl$: C 34.71, H 4.85, N 20.24, Cl 17.08. Found: C 34.57, H 4.78, N 20.18, Cl 16.97. PMR spectrum identical with that of L-*erythro*-β-hydroxyhistidine.

Amino acid **V**: Colorless monohydrochloride crystallized from aqueous EtOH containing HCl, m.p. >235°C (dec.). $[\alpha]_D^{24}$ +4° (*c* 0.25, H_2O). TLC: Rf 0.21 (S–101) and 0.43 (S–123). Anal. Calc'd for $C_3H_8N_2O_2$ HCl: C 25.63, H 6.45, N 19.93. Found: C 25.53, H 6.47, N 19.64. The spectral data were in agreement with those of L-β-aminoalanine.

Amino acid **VII**: Amorphous powder of monohydrochloride. $[\alpha]_D^{21.5}$ +19.5° (*c* 0.75, H_2O). TLC: Rf 0.26 (S–101) and 0.27 (S–123). The PMR spectrum was consistent with that for L-β-lysine. The picrate was crystallized from aqueous MeOH, m.p. >240°C (dec.). Anal. Calc'd for $C_6H_{14}N_2O_2 \cdot (C_6H_3N_3O_7)_2$: C 35.57, H 3.34, N 18.54. Found: C 35.81, H 3.08, N 18.40.

Amine **VIII**: Fine needles of trihydrochloride by crystallization from aqueous EtOH, m.p. 214~215°C. TLC: Rf 0.08 (S–101) and 0.03 (S–123). Anal. Calc'd for $C_7H_{19}N_3 \cdot 3HCl$: C 33.02, H 8.71, N 16.50, Cl 41.77. Found: C 32.94, H 8.76, N 16.31, Cl 42.08. PMR and IR spectra identical with those of spermidine hydrochloride.

The oily residue (55 mg) obtained from the EtOAc extract was treated with an ethereal solution of diazomethane. The resulting methyl ester was chromatographed on a silica gel column, developed with $CHCl_3$ and the elution monitored by UV absorption at 280 nm. The UV-absorbing fractions were collected and evaporated to afford 11 mg of **XI**, which was purified by sublimation at 120°C/10 mm. M.p. 174~178°C, λ_{max}^{MeOH} 287 nm (ε 12,200). MS: *m/e* 226 (M^+), 195, 178, 128, 112, 71, 59. PMR $\delta_{TMS}^{CDCl_3}$ in ppm: 3.98 (3H, *s*), 8.30 (1H, *s*), 8.32 (1H, *d*, J=1.7 Hz) and 8.95 (1H, *d*, J=1.7 Hz).

The insoluble material (130 mg) isolated from the above acid hydrolyzate was dissolved in 0.05 N NaOH (50 ml). The solution was treated portionwise with 200 mg of $KMnO_4$ at room temperature and stirred for 3 hours. The reaction mixture was filtered and the filtrate was extracted with EtOAc at pH 2.0. The EtOAc layer was concentrated and treated with excess diazomethane solution. The methyl ester thus obtained was purified by silica gel chromatography to yield 5 mg of **XII**. λ_{max}^{MeOH} 286 nm (ε 10,600). MS: *m/e* 284 (M^+), 253, 226, 201, 193, 186, 168, *etc.* PMR $\delta_{TMS}^{CDCl_3}$ in ppm: 3.93 (3H, *s*), 4.01 (3H, *s*), 8.16 (1H, *s*) and 8.36 (1H, *s*).

Preparation of 4-amino-3-hydroxy-*n*-valeric acid

Levulinic acid (30 g) was brominated in conc.HCl solution yielding 13.7 g of 3-bromolevulinic acid, m.p. 50~52°C. An aqueous solution of the bromo acid (6.0 g) was treated with an equimolar amount of sodium carbonate at 65°C for one hour and then with an excess of hydroxylamine at room temperature. The resulting 3-hydroxylevulinic acid oxime was crystallized from ether to afford white leaflets (2.5 g), m.p. 144~145°C, $\nu_{c=o}^{KBr}$ 1,725 cm^{-1}. An aqueous EtOH solution of the oxime (820 mg) was hydrogenated over 10% palladium on charcoal at room temperature for 16 hours. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to leave a colorless oil, which was crystallized from aqueous EtOH to give a mixture of diastereoisomers. Yield: 354 mg, m.p. 198~200°C. The mixture was recrystallized carefully from aqueous EtOH. The colorless needles deposited first (38 mg) gave an identical PMR spectrum with that of amino acid **III** obtained above, m.p. 224~226°C. TLC: Rf 0.52 (S–101) and 0.57 (S–123). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.23 (3H, *d*, J=6.6 Hz), 2.39 (2H, *d*, J=7.0 Hz), 3.47 (1H, *d–q*, J=6.6 & 3.1 Hz) and 4.22 (1H, *d–t*, J=7.0 & 3.1 Hz). Anal. Calc'd for $C_5H_{11}NO_3$: C 45.10, H 8.33, N 10.52. Found: C 44.73, H 8.23, N 10.27.

Isolation of disaccharide fragment **IX**

A solution of tallysomycin A (500 mg) in 25 ml of 0.3 N H_2SO_4 was stirred for 21 hours at 80°C.

The solution was neutralized with barium hydroxide, centrifuged to separate the precipitated barium sulfate and concentrated *in vacuo*. The residue was chromatographed on a column of Amberlite CG-50 (H^+, 45 ml), developed successively with water (800 ml), 0.1 N HCl (1.4 liters) and 1.0 N HCl (1.4 liters). The course of the elution was followed by anthrone and ninhydrin reactions. The early fractions which were positive to only anthrone reagent were concentrated to afford crude **IX** (173 mg). Several ninhydrin-positive materials were obtained from the subsequent 0.1 N and 1 N HCl eluates. The crude disaccharide fragment (**IX**, 170 mg) was rechromatographed on a column of Avicel SF (40 ml) using a mixture of *n*-BuOH - EtOH - H_2O (5: 3: 2) as a developer. The anthrone-positive fractions were combined and evaporated to yield a homogeneous solid of **IX** (24 mg). $[\alpha]_D^{27.5}+52.1°$ (*c* 0.35, H_2O). TLC: Rf 0.74 (S-123). IR $\nu_{c=o}^{KBr}$ 1,705 cm^{-1}.

IX (10 mg) was treated with trimethylchlorosilane and hexamethyldisilazane in a usual way to yield trimethylsilyl-**IX**. GLC (OV-17, 3%, temperature programming of 5°C/min from 150°C to 300°C) Rt 18.7 and 19.5 (major) min. MS: *m/e* 889 (M^+), 799, 451, 422.

IX (27 mg) was acetylated in pyridine at room temperature and the product was purified by silica gel column chromatography with $CHCl_3$ - MeOH (100: 1) to yield 14 mg of peracetyl-**IX** as white solid. TLC: Rf 0.12 (SD-104). GLC (SE-30, 1%, temperature programming of 5°C/min from 150°C to 290°C) Rt 26.5 min. MS: *m/e* 619 (M^+-60), 605, 576, 455, 322, 317, 289, 169. The PMR and IR spectra were identical with those of heptaacetyl-2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-glucopyranose prepared from bleomycin.

Mild acid hydrolysis of tallysomycin A—Isolation of peptides L, M and P

A solution of tallysomycin A (3 g) in 500 ml of 6 N HCl was kept standing at 30°C for 3 days. The solution was evaporated at 30°C under reduced pressure to a sticky residue, which was applied to a column of CM-Sephadex C-25 (350 ml) preequilibrated with 1% $HCOONH_4$. Elution was with an increasing concentration (1% to 6%) of $HCOONH_4$ solution, and was monitored by anthrone and ninhydrin reactions and UV absorption. The disaccharide fragment **IX** (500 mg) eluted first with 1% $HCOONH_4$ solution, followed by peptides M (408 mg) and L (153 mg) with the same solvent. Peptide P (349 mg) was eluted with 6% $HCOONH_4$ solution. These peptide fragments were purified by a combination of CM-Sephadex and Sephadex LH-20 chromatography and isolated as amorphous formates. The hydrochloride salts were prepared by dissolving the formates in MeOH, adding methanolic HCl solution to pH 2.0 and precipitating with addition of Et_2O.

Peptide L: TLC: Rf 0.48 (S-102) and 0.48 (S-123). $\lambda_{max}^{H_2O}$ 236 nm (ε 10,600) and 285 nm (ε 4,400). $\nu_{c=o}^{KBr}$ 1,635 cm^{-1}. PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.16 (3H, *d*, J=6.4 Hz), 2.07 (3H, *s*), 2.2~2.5 (2H, *m*), 2.88 (2H, *d*, J=6.0 Hz), 3.43 (2H, *d*, J=6.7 Hz), 3.7~4.4 (4H, *m*), 4.83 (1H, *d*, J=7.2 Hz), 5.28 (1H, *d*, J=7.2 Hz), 7.40 (1H, *s*) and 8.49 (1H, *s*).

Peptide M: TLC: Rf 0.36 (S-102) and 0.32 (S-123). $\lambda_{max}^{H_2O}$ 237 nm (ε 9,800) and 285 nm (ε 4,100). PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.12 (3H, *s*), 2.87 (2H, *d*, J=6.0 Hz), 3.42 (2H, *d*, J=6.7 Hz), 3.9~4.3 (2H, *m*), 4.78 (1H, *d*, J=6.0 Hz), 5.32 (1H, *d*, J=6.0 Hz), 7.34 (1H, *s*), 8.54 (1H, *s*). The PMR and IR spectra and TLC behavior were identical with those of the "pseudotripeptide" of bleomycin.

Peptide P: TLC: Rf 0.08 (S-102) and 0.32 (S-121). $\lambda_{max}^{H_2O}$ 290 nm (ε 14,200). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.22 (6H, *d*), 1.6~2.0 (10 H, *m*), 2.62 (2H, *d*), 2.9~4.3 (17H, *m*), 4.99 (1H, *d*, J=1.8 Hz), 5.17 (1H, *d*, J=4.5 Hz), 5.68 (1H, *d*, J=4.5 Hz) and 8.07 (2H, *s*). Anal. Calc'd for $C_{32}H_{56}N_{10}O_9S_2 \cdot 5HCOOH$: C 43.61, H 6.53, N 13.74, S 6.29. Found: C 43.52, H 6.72, N 13.61, S 6.24.

Mild hydrolysis of tallysomycin B—Isolation of peptide P_b

Tallysomycin B (900 mg) was hydrolyzed with 6 N HCl at 30°C for 3 days and the hydrolyzate was fractionated by a method similar to the above yielding peptide P_b (54 mg) together with disaccharide **IX** and peptides L and M.

Peptide P_b: TLC: Rf 0.22 (S-102) and 0.46 (S-121). $\lambda_{max}^{H_2O}$ 290 nm (ε 12,800). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.26 (6H, broad-*d*), 1.6~2.2 (6H, *m*), 2.9~4.4 (14H, *m*), 4.99 (1H, *d*, J=1.8 Hz), 5.18 (1H, *d*, J=4.4 Hz), 5.69 (1H, *d*, J=4.4 Hz) and 8.11 (2H, *s*). Anal. Calc'd for $C_{26}H_{44}N_8O_8S_2 \cdot 4HCOOH$: C 42.65, H 6.20, N 13.26, S 7.59. Found: C 41.84, H 6.20, N 13.35, S 8.22.

Conversion of peptide L to peptides N and M

A solution of peptide L (100 mg) in conc.HCl (5 ml) was kept at 37°C for 18 hours, and then evaporated below 30°C under reduced pressure. The resulting residue was chromatographed on a column of Sephadex LH-20 with 50% MeOH to afford peptide N (81 mg) as an amorphous white powder. TLC: Rf 0.40 (S-102) and 0.44 (S-123). $\lambda_{max}^{H_2O}$ 236 nm (ε 8,300) and 286 nm (ε 3,900). $\nu_{c=o}^{KBr}$ 1,730 & 1,640cm^{-1}.

Peptide N (107 mg) was further treated with conc.HCl at 37°C for 3 days and the hydrolyzate was charged on a column of CM-Sephadex C-25 which was developed with 1% $HCOONH_4$. The eluate positive to both UV and ninhydrin reaction was concentrated *in vacuo* and desalted by passing through a column of Sephadex LH-20. Evaporation of the appropriate fractions afforded 23 mg of white solid, whose physicochemical and spectral data were consistent with those of peptide M.

Isolation of peptides W and W_b

Tallysomycin A (9.83 g) was dissolved in a solvent mixture (3 liters) of pyridine - AcOH - H_2O (1: 10: 19), treated portionwise with N-bromosuccinimide (NBS, 0.23 g) at 0~2°C and stirred for one hour at the same temperature. Sodium thiosulfate (5.46 g) was added to the reaction mixture to destroy excess NBS and the mixture was heated under reflux for one hour. The solution was then concentrated under reduced pressure to an oily residue which was dissolved in water, adjusted to pH 9.0 and charged on a column of Amberlite XAD-2 (900 ml). The column was washed with water and eluted with 0.01 N HCl. The fractions giving positive ninhydrin reaction and absorption at 280 nm were further examined by TLC (S-123) and concentrated *in vacuo* to yield 1.47 g of peptide W hydrochloride. M.p. 193~195°C (dec.). TLC: Rf 0.10 (S-102) and 0.16 (S-121). pKa' 8.0 (1 eq.), 8.9 (2) and 10.2 (2), titration equivalent: 1,043. $\lambda_{max}^{H_2O}$ 290 nm (ε 12,700). Anal. Calc'd for $C_{37}H_{65}N_{11}O_{11}S_2 \cdot 5HCl \cdot 2H_2O$: C 39.59, H 6.64, N 13.73, S 5.71, Cl 15.79. Found: C 39.38, H 6.43, N 13.87, S 5.80, Cl 15.70.

Tallysomycin B (10.0 g) was worked up by a procedure analogous to the above to afford 1.34 g of peptide W_b as a white solid. M.p. 194~196°C (dec.). TLC: Rf 0.24 (S-102) and 0.32 (S-121). pKa': 8.3 (1 eq.), 9.2 (1) and 10.2 (2), titration equivalent: 912. $\lambda_{max}^{H_2O}$ 290 nm (ε 13,100). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.0~1.5 (9H, *m*), 1.5~2.3 (6H, *m*), 2.5 (2H, *d*), 2.7~4.5 (16H, *m*), 5.01 (1H, *d*, J=1.7 Hz), 5.21 (1H, *d*, J=4.5 Hz), 5.60 (1H, *d*, J=4.5 Hz), 8.05 (2H, *s*). Anal. Calc'd for $C_{31}H_{53}N_9O_{10}S_2 \cdot 4HCl \cdot H_2O$: C 39.62, H 6.33, N 13.41, S 6.82, Cl 15.09. Found: C 39.74, H 6.46, N 13.56, S 7.29, Cl 14.43.

Methanolysis of peptide W—Isolation of compound **X**, **XIII** and peptide Y

Peptide W (600 mg) in 500 ml of methanolic hydrogen chloride (1.5 N) was refluxed for 3 hours. The reaction mixture was evaporated at 50°C under reduced pressure to leave an oily solid which was chromatographed on a column of Amberlite XAD-2 (100 ml). Development of the column with water gave a mixture (125 mg) of **X** and **XIII**, and subsequent development with 0.01 N HCl yielded peptide Y-containing fractions. The mixture of **X** and **XIII** (103 mg) was rechromatographed with Amberlite CG-50 (NH_4^+, 20 ml) to isolate 20 mg of **X** and 54 mg of **XIII** (anomeric mixture). The hydrochloride of **XIII** (124 mg) was chromatographed on a column (φ 2.0×65 cm) of Amberlite XT-2, which was developed with water. The β-anomer (**$XIII_b$**, 20 mg) eluted first, followed by a mixture (29 mg) and then the α-anomer (**$XIII_a$**, 59 mg). **$XIII_a$** was acetylated in a usual manner to give **XIV** which was crystallized from acetone and *n*-hexane. **$XIII_a$**: m.p. 175~176°C. $[\alpha]_D^{21.5}$ −91° (*c* 1.0, H_2O). TLC: Rf 0.44 (SD-103) and 0.76 (S-102). MS: *m/e* 178 (M^++1), 146, 128, 116, 73, 59. Anal. Calc'd for $C_7H_{15}NO_4 \cdot HCl$: C 39.35, H 7.55, N 6.56. Found: C 39.28, H 7.85, N 6.42. Triacetate of **$XIII_a$** (**XIV**): Colorless prisms. M.p. 137.5~138.5°C, $[\alpha]_D^{24.5}$ −89.2° (*c* 0.37, $CHCl_3$). MS: *m/e* 304 (M^++1), 272, 243, 199, 184. Anal. Calc'd for $C_{13}H_{21}NO_7$: C 51.48, H 6.98, N 4.62. Found: C 51.54, H 7.20, N 4.51.

$XIII_b$: $[\alpha]_D^{21.5}$ +35° (*c* 0.4, H_2O). TLC: Rf 0.36 (SD-103) and 0.68 (S-102). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.38 (3H, d, J=6.8 Hz), 3.41 (1H, quintet), 3.52 (3H, s), 3.5~4.3 (3H, m), 4.50 (1H, broad-s).

Compound **X**: TLC: Rf 0.77 (S-102). MS: *m/e* 115 (M^+), 100, 87, 72, 58, 44, *etc.* PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.21 (3H, *d*, J=5.7 Hz), 2.23 (1H, *d-d*, J=17.2 & 3.5 Hz), 2.85 (1H, *d-d*, J=17.2 & 6.2 Hz), 3.62 (1H, *d-q*, J=5.7 & 2.6 Hz), 4.14 (1H, *d-d-d*, 6.2, 3.5 & 2.6 Hz). $\nu_{c=o}^{KBr}$ 1,690 cm^{-1}.

The crude peptide Y preparation obtained above (200 mg) was chromatographed on a column of CM-Sephadex C-25 (φ 1.0×30 cm) which was developed with increasing concentrations of aqueous ammonium formate. A small amount of **XV** (6 mg) was eluted first, followed by peptide Y (110 mg).

Peptide Y (epimeric mixture) was converted to the hydrochloride and chromatographed over a column of Diaion HP-20 but further separation into epimers was unsuccessful.

Peptide Y: TLC: Rf 0.22 (S-102) and 0.40 (S-121). $\lambda_{max}^{H_2O}$ 291 nm (ε 13,600). Anal. Calc'd for $C_{27}H_{47}N_9O_6S_2 \cdot 4HCl \cdot H_2O$: C 39.47, H 6.50, N 15.34, S 7.79, Cl 17.26. Found: C 39.48, H 6.67, N 15.28, S 7.58, Cl 17.00.

XV: TLC: Rf 0.07 (S-102) and 0.06 (S-121). MS: *m/e* 274 ($M^+ + 1$), 273 (M^+), 257, 229, 215, 159, 129. *etc.*

Methanolysis of peptide W_b

Analogous methanolysis of peptide W_b (410 mg) and subsequent chromatographic separation gave a mixture (107 mg) of **XIII** and **X**, and crude peptide Y_b (182 mg). The latter (a mixture of Y_{b1} and Y_{b2} hydrochloride, 110 mg) was charged on a column of Amberlite XT-2 (160 ml) which was carefully developed with water. The course of the elution was monitored by ninhydrin reaction and absorption at 280 nm. Evaporation of appropriate fractions gave 54 mg of peptide Y_{b1} and 26 mg of Y_{b2}. The two peptide fragments showed identical mobilities in several TLC systems but different PMR spectra. Peptide Y_{b1} was acetylated in pyridine to give the pentaacetate which was purified by silica gel chromatography.

Peptide Y_{b1}: TLC: Rf 0.40 (S-102) and 0.56 (S-121). $\lambda_{max}^{H_2O}$ 290 nm (ε 13,000). Field desorption MS: *m/e* 530 ($M^+ + 1$), 512, 498 and 486. PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.15 (3H, d, J = 6.2 Hz), 1.5 ~ 2.5 (6H, *m*), 2.7 ~ 3.4 (8H, *m*), 3.40 (3H, *s*), 3.4 ~ 4.3 (2H, *m*), 5.15 (1H, *d*, J = 5.8 Hz), 5.50 (1H, *d*, J = 5.8 Hz), 8.15 (2H, *s*). Anal. Calc'd for $C_{21}H_{35}N_7O_5S_2 \cdot 3HCl$: C 39.48, H 6.00, N 15.35, S 10.02. Found: C 39.23, H 6.12, N 15.13, S 9.64. Pentaacetate of Y_{b1}: Field desorption MS: *m/e* 739 (M^+).

Peptide Y_{b2}: PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.32 (3H, d, J = 6.1 Hz), 5.16 (1H, d, J = 5.0 Hz), 5.40 (1H, d, J = 5.0 Hz), signals for other 21 protons essentially the same as those of peptide Y_{b1}.

Dinitrophenylation of peptide W

To a stirred solution of peptide W (805 mg) and sodium carbonate (1.4 g) in water (35 ml) was added an ethanolic solution of 2,4-dinitrofluorobenzene (1.4 g). Stirring was continued for one hour at ambient temperature and the yellow precipitate deposited was collected by filtration. The crude product was chromatographed on a silica gel column (φ 1.7 × 40 cm) developed by $CHCl_3$ - MeOH (85: 15). The desired fractions were evaporated under reduced pressure to leave 910 mg of yellow solid (DNP-peptide W). $\lambda_{max}^{10\% \text{ dioxane in MeOH}}$: 354 nm ($E_{1cm}^{1\%}$ 395). The DNP-peptide (880 mg) was hydrolyzed by refluxing with a mixture of 6 N HCl (50 ml) and dioxane (110 ml) for 10 hours. The insoluble material formed was removed by decantation and the clear hydrolyzate was washed with $CHCl_3$. The aqueous layer was concentrated *in vacuo* and chromatographed on a column of XAD-2 (20 ml). The column was developed with water to elute a mixture of a ninhydrin-positive material and inorganic salt, which was separated by chromatography over Amberlite IRC-50 (H^+, 20 ml) developed with 0.2 N HCl to isolate L-threonine (**I**). The above XAD-2 column was further eluted with aqueous MeOH to afford 22 mg of DNP-amino acid **VII**. TLC: Rf 0.01 (PL-111) and 0.06 (SD-102). λ_{max}^{AcOH} 261.5 nm (ε 7,100) and 345 nm (ε 13,400). MS: *m/e* 313 ($M^+ + 1$), 294, 236, 183, 167. PMR $\delta_{DSS}^{D_2O+DCl}$ in ppm: 1.75 (4H, *m*), 2.71 (2H, *d*, J = 5.6 Hz), 2.7 ~ 3.1 (2H, *m*), 4.21 (1H, *m*), 7.03 (1H, *d*, J = 10.0 Hz), 8.02 (1H, *d-d*, J = 10.0 & 2.7 Hz), 8.70 (1H, *d*, J = 2.7 Hz).

The above insoluble material and $CHCl_3$ extract were combined (664 mg) and applied to a silica gel column (φ 1.5 × 40 cm). The early yellow fractions eluted with $CHCl_3$ - MeOH (9: 1) were evaporated to afford a yellow solid which was purified by preparative TLC (Kieselgel, solvent PL-111) to obtain DNP-amino acid **III** (42 mg). The later yellow fractions eluted with $CHCl_3$ - MeOH (8: 2) were worked up similarly to afford DNP-amine **VIII** (46 mg).

DNP-**III**: TLC: Rf 0.14 (PL-111) and 0.25 (SD-102). λ_{max}^{AcOH} 261 nm (ε 6,800) and 346 nm (ε 13,200). PMR $\delta_{DSS}^{D_2O+K_2CO_3}$ in ppm: 1.24 (3H, *d*, J = 6.7 Hz), 2.41 (2H, *d*, J = 7.1 Hz), 3.9 ~ 4.3 (2H, *m*), 6.98 (1H, *d*, J = 10.1 Hz), 8.03 (1H, *d-d*, J = 10.1 & 2.7 Hz), 8.77 (1H, *d*, J = 2.7 Hz). MS: *m/e* 299 (M^+), 281, 240, 210, 193, 164.

DNP-**VIII**: TLC: Rf 0.07 (PL-111) and 0.16 (SD-102). λ_{max}^{AcOH} 352 nm (ε 22,800). MS: *m/e* 478 ($M^+ + 1$), 460, 447, 293, 267, 235, 190, 167. PMR $\delta_{TMS}^{d_6\text{-DMSO}}$ in ppm: 1.5 ~ 2.2 (6H, *m*), 2.87 (2H, *m*),

3.3~3.6 (6H, *m*), 6.98 (1H, *d*, J=9.5 Hz), 7.29 (1H, *d*, J=8.8 Hz), 8.08 (2H, broad *d-d*, J=9.1 & 2.7 Hz), 8.34 (1H, *d*, J=2.7 Hz), 8.75 (1H, *d*, J=2.7 Hz).

Dinitrophenylation of peptide P

An aqueous solution of peptide P (120 mg) was treated with 2,4-dinitrofluorobenzene (300 mg) in the same manner as above. The precipitate formed (150 mg) was hydrolyzed with a mixture of dioxane and 6 N HCl and the hydrolyzate was separated into water-soluble and water-insoluble fractions. The latter was chromatographed on a silica gel column developed with $CHCl_3$-MeOH (9: 1) to isolate DNP-amino acid **I** (25 mg) followed by DNP-**VIII** (39 mg). Further purification of DNP-**I** was made by preparative TLC.

DNP-**I**: TLC: Rf 0.14 (PL-111), 0.28 (SD-102). PMR $\delta_{DSS}^{D_2O+K_2CO_3}$ in ppm: 1.32 (3H, *d*, J=6.0 Hz), 4.0~4.5 (2H, *m*), 6.79 (1H, *d*, J=9.4 Hz), 8.10 (1H, *d-d*, J=9.4 & 2.7 Hz), 8.92 (1H, *d*, J=2.7 Hz).

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