TOTAL STRUCTURE OF THE HIGHLY MODIFIED PEPTIDE ANTIBIOTIC COMPONENTS OF THIOPEPTIN

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(Received for publication January 20, 1983)

On the basis of ^{1}H and ^{13}C NMR evidence, the structures of two series of the highly modified sulfur-containing peptide antibiotic thiopeptin were elucidated.

The thiopeptins produced by *Streptomyces tateyamensis* belong to a group of highly modified sulfur containing peptide antibiotics[1], the structures of several having only recently been determined by X-ray crystallography or physical methods[2~8]. They inhibit primarily Gram-positive bacteria[9] and exhibit no significant differences in their inhibition of protein synthesis in cell-free *Escherichia coli*[10]. Thiopeptin B, the major component is valuable as a feed-additive because of its marked growth promoting action in swine and chickens[11] and was recently shown to be effective as a lactic-acidosis preventive in sheep and cattle[12].

Thiopeptin B and four minor components A_1, A_2, A_3 and A_4 were characterized by MIYAIRI *et al.*[9,13] and shown primarily by acid hydrolysis experiments[14] to be closely related to the antibiotic thiostrepton, whose structure, with the exception of the side chain, was determined by X-ray crystallography by ANDERSON and coworkers[2]. TORI *et al.*,[8] from a ^{13}C NMR study of the antibiotic, proposed a sequence consisting of two dehydroalanine (Deala) residues for the side chain which was subsequently confirmed by our own ^{1}H NMR study at 300 MHz (see preceding paper). Of the spectroscopic techniques available to us it soon became apparent that only ^{1}H and ^{13}C NMR spectroscopy were to be potentially useful in the structural elucidation of the thiopeptins. The molecules were not sufficiently volatile to yield a molecular ion even under field-desorption conditions and only a fragment involving the dihydroquinoline moiety and adjacent peptide was identifiable (see below). Moreover, despite the fact that some of the **a** components were obtained in crystalline form, they were found not to be suitable for X-ray crystallography. A strategy, involving ^{1}H NMR comparison with thiostrepton, was therefore adopted in order to delineate their structural differences. Pertinent details of confirmatory ^{13}C NMR evidence are also discussed[15] whereas a full discussion of the ^{13}C NMR assignments is the subject of a companion paper.

Results and Discussion

Thiopeptin components were separated by silica gel chromatography similar to that previously described[9] and found to be homogeneous by TLC. ^{1}H NMR analysis of the components from various batches however, indicated a two compound system in the majority of cases which was confirmed by HPLC. Consistent differences in the ^{1}H NMR spectra allowed them to be grouped into two distinct series arbitrarily designated by the subscripts **a** and **b** and some of their physical and chemical properties are summarized in Table 1. In some batches all components from the **a** series and only A_{1b} from the **b**

Table 1. Physical and chemical data for thiopeptin components.

Component	Rf[a]	Rf[b]	**a** Series	**b** Series	λ_{max}^{MeOH} (E%) nm
Acid	0.11	0.30	B_a: $C_{71}H_{84}N_{18}O_{16}S_6$	B_b: $C_{71}H_{82}N_{18}O_{15}S_6$	B_a: 250 (327)
Methyl ester	0.80	0.78	A_{1a}: $C_{72}H_{86}N_{18}O_{16}S_6$	A_{1b}: $C_{72}H_{84}N_{18}O_{15}S_6$	A_{1a}: 250 (331) A_{1b}: 250 (335)
De-Deala (Amide)	0.54	0.52	A_{4a}: $C_{68}H_{82}N_{18}O_{16}S_6$	A_{4b}: $C_{68}H_{80}N_{18}O_{16}S_6$	A_{4a}: 250 sh (281)
Bis-De-Deala (Amide)	0.63	0.60	A_{3a}: $C_{65}H_{79}N_{17}O_{15}S_6$	A_{3b}: $C_{65}H_{77}N_{17}O_{15}S_6$	A_{3a}: 250 sh (287)

[a] In methanol - methylene chloride (1: 19; +1% NH_3) on silica gel GF TLC plates.
[b] As in [a] but without ammonia.

series were found to be admixed with the corresponding components from the other series. Thiopeptin B_b was separated from B_a by multiple preparative TLC on silica gel and thiopeptins A_{3b} and A_{4b} were analyzed as mixtures. Insufficient sample in the case of thiopeptin A_2 prevented MIYAIRI *et al.*[9] from establishing its uniqueness whereas in our fermentations it was not produced.

^{1}H NMR Spectra—General Features

Good chemical shift dispersion of all components was obtained under a variety of conditions of solvent and temperature to allow complete analysis of all spin patterns from double resonance experiments. Sharp spectra were obtained for the A components of both series in $CDCl_3$ whereas sharpening of those for B_a and B_b could be achieved by the addition of a few drops of DMSO-d_6 or CD_3OD. As ^{2}H-exchange of active hydrogens accompanies the addition of CD_3OD, the data for thiopeptin B_a in Table 2 were obtained in $CDCl_3$ - DMSO-d_6 (16 :1) which apparently causes minimal shifts of non-active hydrogens. Correlations between thiostrepton and thiopeptin spectra were not straightforward due in part to variable positions of the active hydrogens. Also ^{2}H-exchange of the OH signals in the presence of CD_3OD was initially only of limited use as the addition is accompanied by appreciable solvent shifts of certain resonances as well as slow exchange of peptide NH protons resulting in concomitant loss of multiplet structure of coupled resonances. Under fully ^{2}H-exchanged conditions in CD_3OD - $CDCl_3$ (1: 4) however, (the components have only limited solubility in CD_3OD) requiring at least three days at room temperature, these differences were effectively minimized. An accurate count of peptide NH protons could thus be made whereas the count for the OH signals was less reliable even though their temperature dependence and reduction in intensity on irradiation of the water peak as previously demonstrated for thiostrepton, allows them to be readily distinguished from the peptide resonances. After the hydroxyl resonances were identified, correlation of the remainder of proton resonances between thiostrepton and the thiopeptins could be made to within 0.04 ppm but with several exceptions. These were interpreted in terms of four structural changes which will now be discussed.

Nature of Amino Acids

Amino acid analyses of the acid hydrolysates of the various thiopeptin components (see Table 3) gave threonine, valine and alanine in the molar ratio of 1: 1: 2 as well as the minor products cystine, thiostreptine and 2-aminomethylthiazole-4-carboxylic acid, the latter three products having been observed previously for thiostrepton[16]. Reduction of thiopeptin A_{1b} with sodium borohydride prior to hydrolysis increased the value for alanine and showed a peak corresponding to butyrine (see Table 3) suggesting the presence of Deala and dehydrobutyrine residues[17]. That the Ile residue in thiostrepton is replaced by Val in the thiopeptins and attached to the dihydroquinoline ring was confirmed by mass spectra of the TMS derivatives of all thiopeptins. The highest peak at *m/z* 521.2687

Table 2. Comparison of ^{1}H NMR assignments of thiostrepton A_1 with thiopeptins in $CDCl_3$ at 25°C.[a]

Assignment[b]	b Series			a Series		
	Thiostrepton A_1	Thiopeptin A_{1b}	Thiopeptin A_{1a}	Thiopeptin B_a[c]	Thiopeptin A_{4a}	Thiopeptin A_{3a}
Ile 3-CH_3	0.88 d (7)	—	—	—	—	—
Val CH_3	—	0.99 d (7)	0.98 d (7)	0.97 d (7)	0.97 d (7)	0.97 d (7)
Ile 5-CH_3	0.94 t (7)	—	—	—	—	—
Val CH_3	—	0.99 d (7)	0.98 d (7)	0.96 d (7)	0.97 d (7)	0.97 d (7)
Thr CH_3(1)	0.98 d (7)	0.99 d (7)	0.98 d (7)	0.95 d (7)	0.97 d (7)	0.97 d (7)
Thr H-3(1)	1.06 m	~1.10 obsc	~1.15 obsc	~1.15 obsc	obsc	obsc
Ala CH_3(1)	1.17 d (7)	1.20 d (7)	1.22 d (7)	1.20 d (7)	1.20 d (7)	1.19 d (7)
Thstn 3-CH_3	1.19 s	1.21 s	1.22 s	1.21 s	1.21 s	1.20 s
Q CH_3	1.35 d (7)	1.35 d (7)	1.35 d (7)	1.34 d (7)	1.34 d (7)	1.35 d (7)
Thstn 5-CH_3	1.36 d (7)	1.37 d (7)	1.37 d (7)	1.36 d (7)	1.37 d (7)	1.37 d (7)
Ala CH_3(2)	1.46 d (7)	1.47 d (7)	1.55 d (7)	1.53 d (7)	1.53 d (7)	1.51 d (7)
But CH_3	1.62 d (7)	1.63 d (7)	1.64 d (7)	1.63 d (7)	1.63 d (7)	1.63 d (7)
Thr CH_3(2)	1.75 d (7)	1.76 d (7)	1.79 d (7)	1.78 d (7)	1.79 d (7)	1.78 d (7)
Ile H-3	~1.75 obsc	—	—	—	—	—
Val H-3	—	1.88 h (~6, 7)	1.92 h (~6, 7)	1.91 h (~6, 7)	1.90 h (~6, 7)	1.90 h (~6, 7)
Pip H-4β	2.28 dt (6, 12, 12)	2.27 dt (6, 13, 13)	2.13 m	2.13 m	2.11 m	2.13 m
Pip H-3α	~3.00 obsc	~3.04 obsc	2.13 m	2.13 m	2.11 m	2.13 m
Ile H-2	3.01 d (6)	—	—	—	—	—
Val H-2	—	2.88 d (5.5)	2.88 d (6)	2.88 d (6)	2.88 d (6)	2.88 d (6)
Cys H-5α	3.15 dd (11.5, 13)	3.13 dd (11.5, 13)	3.14 dd (11, 13)	3.16 dd (11.5, 13)	3.12 dd (12, 13)	3.12 dd (12, 13)
Pip H-3β	3.50 dd (6, 19)	3.51 dd (6, 19)	2.37 m	2.34 m	2.35 m	2.34 m
Q H-7β	3.65 dd (1.5, 6)	3.62 dd (1.5, 6)	3.64 dd (1.5, 6)	3.62 dd (1.5, 5.5)	3.62 dd (1.5, 6)	3.63 dd (1.5, 6)
Cys H-5β	3.73 dd (9, 11.5)	3.72 dd (9, 11.5)	3.72 dd (8.5, 11)	3.68 dd (9, 11.5)	3.72 dd (9, 12)	3.72 dd (9, 11)
Thstn H-4	3.84 dq (5, 7)	3.84 dq (5, 7)	3.84 dq (~5, 7)	3.83 dq (~5, 7)	3.83 dq (~5, 7)	3.83 dq (~5, 7)
Ala H-2(1)	3.89 dq (6, 7)	3.86 dq (6, 7)	3.88 dq (6, 7)	3.87 dq (~6, 7)	3.88 dq (6, 7)	3.88 dq (6, 7)
CO_2CH_3	—	3.93 s	3.93 s	—	—	—
Thstn C4-OH	4.09 d (5)	4.06 d (5)	4.08 br.s	4.14 obsc	4.08 d (5)	4.06 d (5)

Table 2. (Continued)

Assignment[b]	b Series			a Series		
	Thiostrepton A_1	Thiopeptin A_{1b}	Thiopeptin A_{1a}	Thiopeptin B_a[c]	Thiopeptin A_{4a}	Thiopeptin A_{2a}
Pip H-4α	~4.11 obsc	4.12 dd (6, 13)	4.16 dd (3, 8)	4.14 dd (3, 8)	4.14 dd (3, 8)	4.18 dd (2.5, 8)
OH/NH	4.12 br.s	3.96 br.s	3.96 br.s	4.00 br.s	3.96 br.s	3.96 br.s
Pip H-2β	—	—	~4.46 obsc	~4.44 obsc	~4.46 obsc	4.38 dd (3.5, 10)
Thr H-2(1)	4.48 dd (3, 8)	4.46 dd (3, 7)	4.46 dd (3, 8)	4.44 dd (3, 8)	4.46 dd (3, 8)	4.46 dd (3, 8)
Deala NH_2(3)	4.54 br.s	—	—	—	—	7.17 br.s[d]
Q H-8α	4.70 dd (1.5, 8)	4.67 dd (1.0, 8)	4.68 dd (1.5, 8)	4.68 dd (1.5, 8)	4.67 dd (1.5, 8)	4.68 dd (1.0, 8)
Ala H-2(2)	4.79 dq (7, 7)	4.78 dq (7, 7)	4.94 dq (7, 7)	4.93 dq (7, 7)	4.92 dq (7, 7)	4.92 dq (7, 7)
Cys H-4β	4.98 dd (9, 13)	4.96 dd (9, 13)	4.97 dd (9, 13)	4.98 dd (9, 13)	4.97 dd (9, 13)	4.97 dd (8.5, 13)
Deala H-3_c(1)	5.12 br.s	5.11 br.s	5.16 br.s	5.20 br.s	5.15 br.s	5.14 br.s
Pip H-6β	5.23 br.s	5.26 br.s	4.47 s	4.50 s	4.48 s	4.48 s
Q H-11	~5.34 q (7)	5.33 q (7)	5.35 q (7)	5.34 q (7)	5.35 q (7)	5.35 q (7)
OH/NH	5.36 s	5.37 s	5.40 s	5.38 s	5.39 s	5.38 s
Deala H-3_c(3)	5.49 br.s	5.53 br.s	5.52 br.s	5.52 br.s	5.42 br.s	—
Deala H-3_c(2)	5.59 br.s	6.08 s	6.05 s	6.05 s	—	—
'H_2O'[e]	5.74 br.s[f]	—	—	—	5.74 br.s[f]	5.69 br.s
Thstn H-2	5.79 d (10)	5.78 d (10)	5.80 d (11)	5.83 d (10)	5.79 d (11)	5.80 d (10)
Deala H-3_t(1)	5.80 d (~2.5)	5.80 d (2.5)	5.82 d (2.5)	5.82 d (2.5)	5.82 d (2)	5.82 d (2)
Thr H-2(2)	5.87 d (10)	6.87 d (9.5)	6.85 d (10)	6.84 d (10)	6.85 d (10)	6.85 d (10)
But H-3	6.22 q (7)	6.21 q (7)	6.23 q (7)	6.22 q (7)	6.22 q (7)	6.22 q (7)
Q H-6	6.33 dd (6, 10)	6.34 dd (6, 10)	6.36 dd (6, 10)	6.38 dd (6, 10)	6.35 dd (6, 10)	6.35 dd (6, 10)
Ala NH(2)	6.41 d (7)	6.35 d (7)	6.49 d (7)	6.42 d (7)	6.45 d (7)	6.44 d (7)
Thr H-3(2)	6.41 q (7)	6.46 q (7)	6.57 q (7)	6.56 q (7)	6.57 q (7)	6.55 q (7)
Deala H-3_t(3)	6.73 d (2.5)	6.75 s	6.74 s	6.65 s	—	—
Deala H-3_t(2)	6.83 d (2.5)	6.81 d (2)	6.80 d (~2.5)	6.76 d (2)	6.73 d (2)	—
Q C8-OH	6.86 d (8)	6.82 d (8)	6.84 d (8)	6.65 d (8)	6.81 d (8)	6.80 d (8)
Q H-5	6.92 d (10)	6.91 d (10.5)	6.94 d (10)	6.94 d (10)	6.88 d (10)	6.87 d (10)
Thr NH(1)	6.93 d (8)	6.96 d (7.5)	6.90 d (8)	6.90 d (8)	6.93 d (8)	6.93 d (8)

Table 2. (Continued)

Assignment[b]	b Series			a Series		
	Thiostrepton A_1	Thiopeptin A_{1b}	Thiopeptin A_{1a}	Thiopeptin B_a[c]	Thiopeptin A_{4a}	Thiopeptin A_{3a}
Q H-3	7.34 s	7.31 s	7.35 s	7.33 s	7.34 s	7.34 s
Thz H-5(3)	7.49 s	7.49 s	7.44 s	7.44 s	7.43 s	7.43 s
Thstn C3-OH	7.54 s	7.64 s	7.74 s	7.60 s	7.65 s	7.63 s
Ala NH(1)	7.60 d (6)	7.53 d (6)	7.59 d (6)	7.58 d (6)	7.55 d (6)	7.55 d (6)
Thstn NH	7.61 d (10)	7.57 d (10)	7.60 d (10.5)	7.59 d (10)	7.58 d (10)	7.58 d (10)
Deala NH(1)	7.84 br.s	7.82 br.s	7.87 br.s	7.90 br.s	7.86 br.s	7.86 br.s
Thz H-5(1)	8.15 s	8.15 s	8.15 s	8.15 s	8.14 s	8.14 s
Thz H-5(4)	8.30 s	8.28 s	8.20 s	8.18 s	8.16 s	8.18 s
Thz H-5(2)	8.31 s	8.59 s	8.58 s	8.57 s	8.58 s	8.59 s
Thr NH(2)	8.32 d (10)	9.72 d (9.5)	9.79 d (10)	9.79 d (10)	9.75 d (~10)	9.77 d (10)
But NH	8.55 br.s	8.49 br.s	8.50 br.s	8.52 br.s	8.49 br.s	8.51 br.s
Deala NH(3)	9.03 br.s	8.59 br.s	8.59 br.s	8.75 br.s	—	—
Pip C5-NH	9.90 s	9.92 s	9.72 s	9.68 s	9.73 s	9.71 s
Deala NH(2)	10.00 br.s	9.99 br.s	9.93 br.s	9.96 br.s	9.93 br.s	—

[a] Chemical shifts in ppm downfield of internal TMS; coupling constants in Hz (±0.5) are given in brackets.
Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, dt=doublet of triplets, q=quartet, dq=doublet of quartets, h=hextet, m=multiplet, br=broad, obsc=obscured (overlapping signals).

[b] See Fig. 1 for subunits.

[c] In $CDCl_3$ - DMSO-d_6 (16: 1).

[d] The assignment is for Thz(4) $CONH_2$ in the case of thiopeptin A_{3a}.

[e] Two-proton H_2O signal.

[f] Observed at 65°C.

Fig. 1. Structure of thiopeptins.

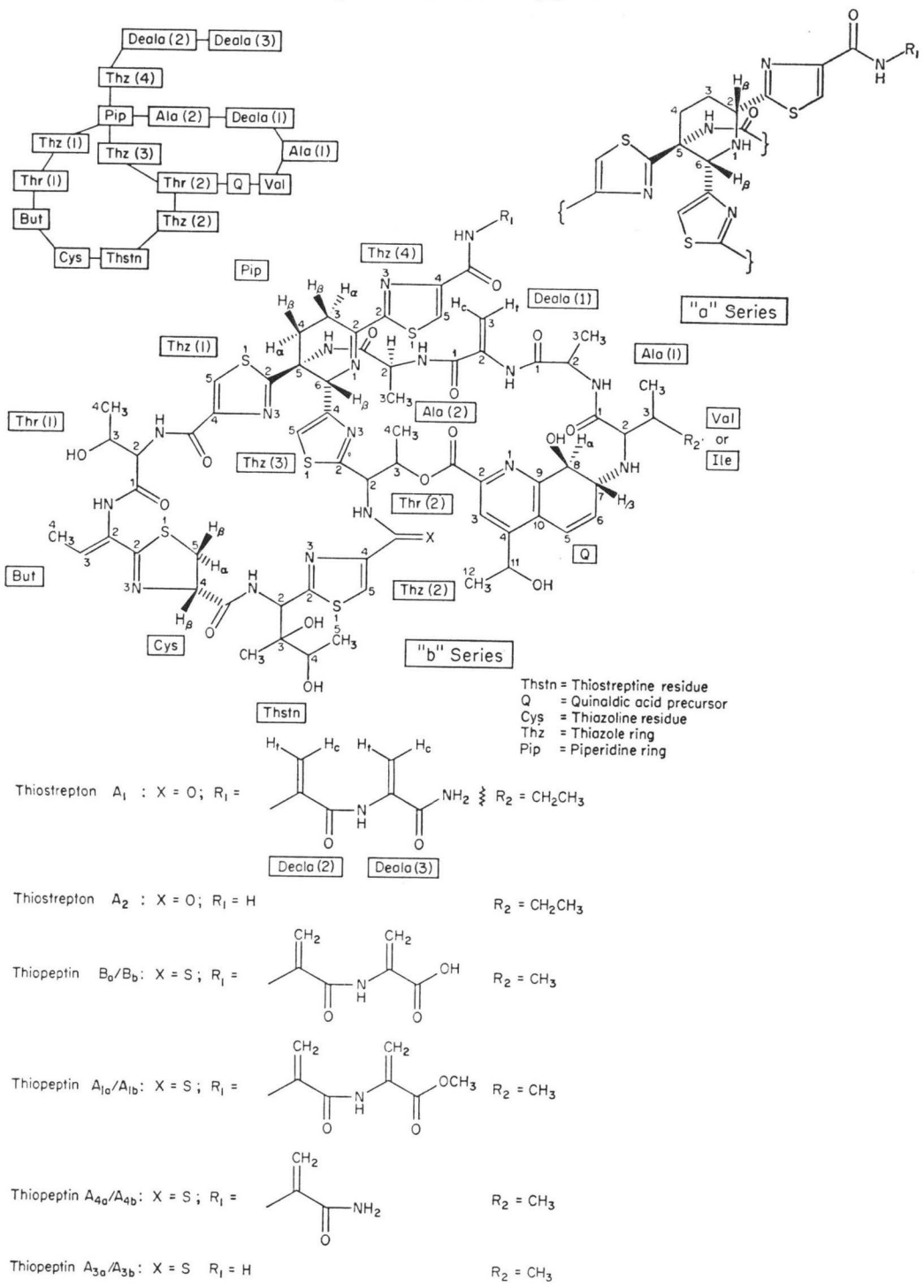


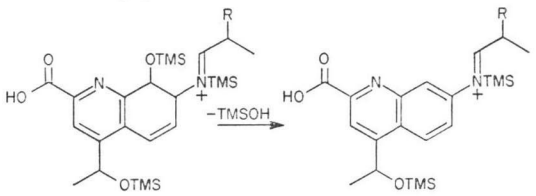
Table 3. Amino acid analysis of acid hydrolysates of thiopeptins.[a]

Amino acid	A_{1b}	A_{1b}[c]	A_{1a}	B_a	A_{4a}	A_{8a}
Threonine	0.58	0.52	0.52	0.57	0.53	0.56
Alanine	0.99	1.30	0.95	0.98	0.96	0.91
Valine	0.50	0.43	0.52	0.53	0.51	0.55
Cystine	0.25	0.30	Small	Small	Small	Small
Thiostreptine	0.11	0.11	0.12	0.13	0.11	0.14
ATC[b]	0.13	0.13	0.08	0.09	0.08	0.09
Butyrine	—	0.08	—	—	—	—

[a] Values in μmol/mg of thiopeptin.
[b] 2-Aminomethylthiazole-4-carboxylic acid.
[c] Reduced with $NaBH_4$ prior to hydrolysis.

which corresponds to fragment **a** (calcd. 521.2686) (Fig. 2) can readily aromatize with loss of TMSOH to fragment **b** (*m/z* 431.2187, calcd. 431.2185), whereas the corresponding peaks in thiostrepton occur at 14 mass units higher. Subsequent measurements on the deuterated silyl derivatives confirmed the presence of two and three silyl groups in the fragments **b** and **a** respectively.

Fig. 2. Mass spectral fragmentation of thiostrepton and thiopeptins.



R=CH_2CH_3 (Thiostrepton) *m/z* 535 *m/z* 445
R=CH_3 (Thiopeptins) *m/z* 521 *m/z* 431

Examination of the methyl region of the ^1H NMR spectrum of thiostrepton reveals eight doublets, one triplet and a singlet whereas spectra of the thiopeptins have one extra doublet replacing the triplet. This doublet which occurs at δ 0.98 in A_{1a} in $CDCl_3$ (see Table 4) was shown to be coupled to a resonance at δ 1.92 (Val H-3) which appears as a hextet (two overlapping heptets with outer satellites not observed) which in turn is coupled to a second methyl group at δ 0.98 and a doublet at 2.88 (Val H-2). Irradiation of this doublet collapses the hextet to a quintet thus confirming the presence of a Val residue. No coupling between Val H-2 and the adjacent amine NH proton as in thiostrepton was noted presumably due to an unfavorable rate of exchange between bulk water and the NH proton. Analogously, the threonine and two alanine resonances were identified but in these cases coupling of the amide NH to the adjacent methine proton readily supports their identification. The chemical shift of the highly shielded resonance for Thr H-3(1) has been discussed for thiostrepton (see companion paper) and reflects a similar proximity of the proton to the ring currents of the dihydroquinoline ring. This resonance is not obscured in thiopeptin A_{1b} in $CDCl_3$ and irradiation at this position (δ 1.10) causes one of the three methyl doublets at δ 0.99 to collapse to a singlet and the doublet of doublets at δ 4.46 to a doublet ($J_{H2,H3}$ = 3 Hz). Irradiation of the latter resulted in collapse of the doublet at δ 6.96 to a singlet and the resonance at δ 1.10 to a quartet thereby confirming the presence of the Thr (1) residue.

Nature of Thiazoline (Cys), Dihydroquinoline (Q) and Esterified Threonine (Thr (2)) Rings

It is evident from Table 5 that the thiazoline (Cys) and dihydroquinoline (Q) ring systems of thiostrepton are retained in both series of thiopeptins. Besides chemical shift, the various coupling constants in both series were found to be identical to that in thiostrepton implying similar conformations of the envelope and flattened half-chair forms of the thiazoline and dihydroquinoline rings respectively.

Assignment of the protons of the modified threonine residue attached to C6 of the piperidine ring

Table 4. Comparison of ^1H NMR assignments of amino acid residues in thiostrepton A_1 and thiopeptins A_{1a} and A_{1b} in $CDCl_3$ and CD_3OD - $CDCl_3$ (1 : 4) at 25°C.[a]

Assignment	Thiostrepton A_1		Thiopeptin A_{1b}		Thiopeptin A_{1a}	
	$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)
Ile						
H-2	3.01 d (6)	3.02 d (4)	—	—	—	—
H-3	~1.75 obsc	1.98 m	—	—	—	—
3-CH_3	0.88 d (7)	0.82 d (7)	—	—	—	—
4-CH_2	~1.20 obsc	obsc	—	—	—	—
5-CH_2	0.94 t (7)	0.90 t (7)	—	—	—	—
Val						
H-2	—	—	2.88 d (5.5)	2.97 d (4)	2.88 d (6)	2.94 d (4)
H-3	—	—	1.88 h (~6, 7)	~2.33 m	1.92 h (~6, 7)	2.23 m
4-CH_3	—	—	0.99 d (7)	0.78 d (7)	0.98 d (7)	0.82 d (7)
4-CH_3	—	—	0.99 d (7)	0.79 d (7)	0.98 d (7)	0.82 d (7)
Thr(1)						
NH	6.93 d (8)	7.16 (8) → o	6.96 d (7.5)	—	6.90 d (8)	—
H-2	4.48 dd (3, 8)	4.48 dd → d (3)	4.46 dd (3, 7)	4.45 dd → d (3.5)	4.46 dd (3, 8)	4.42 obsc
H-3	1.06 m	~1.63 obsc	~1.10 m	obsc	~1.15 obsc	obsc
4-CH_3	0.98 d (7)	1.04 d (7)	0.99 d (7)	1.09 d (7)	0.98 d (7)	1.06 d (7)
Ala(1)						
NH	7.60 d (6)	7.86 d (6) → o	7.53 d (6)	—	7.59 d (6)	—
H-2	3.89 dq (6, 7)	3.85 dq → q (7)	3.86 dq (6, 7)	3.84 dq → q (7)	3.88 dq (6, 7)	3.84 dq → q (7)
3-CH_3	1.17 d (7)	1.20 d (7)	1.20 d (7)	1.18 d (7)	1.22 d (7)	1.20 d (7)
Ala(2)						
NH	6.41 d (7)	7.16 d (7) → o	6.35 d (7)	—	6.49 d (7)	7.12 d → o
H-2	4.79 dq (7, 7)	4.78 dq → q (7)	4.78 dq (7, 7)	4.78 dq → q (7)	4.94 dq (7, 7)	4.92 dq → q (7)
3-CH_3	1.46 d (7)	1.47 d (7)	1.47 d (7)	1.47 d (7)	1.55 d (7)	1.52 d (7)

[a] Multiplicity changes on ^2H-exchange are indicated by →; complete disappearance of an active hydrogen is designated by s → o or d → o.

Table 5. Comparison of ^{1}H NMR assignments of thiazoline (Cys) and dihydroquinoline (Q) rings in thiostrepton A_1 and thiopeptins A_{1a} and A_{1b} in $CDCl_3$ and CD_3OD - $CDCl_3$ (1 : 4) at 25°C.

Assignment		Thiostrepton A_1		Thiopeptin A_{1b}		Thiopeptin A_{1a}	
		$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$(1 : 4)
Cys	H-4β	4.98 dd (9, 13)	5.02 dd (9, 13)	4.96 dd (9, 13)	5.01 dd (9, 13)	4.97 dd (9, 13)	5.00 dd (9, 13)
	H-5β	3.73 dd (9, 11.5)	3.69 dd (9, 11)	3.72 dd (9, 11.5)	3.69 dd (9, 11)	3.72 dd (8.5, 11)	3.68 dd (9, 11)
	H-5α	3.15 dd (11.5, 13)	3.21 dd (11, 13)	3.13 dd (11.5, 13)	3.21 dd (11, 13)	3.14 dd (11, 13)	3.17 dd (11, 13)
Q	H-5	6.92 d (10)	6.95 d (10)	6.91 d (10.5)	6.96 d (10)	6.94 d (10)	6.96 d (10)
	H-6	6.33 dd (6, 10)	6.46 dd (6, 10)	6.34 dd (6, 10)	6.46 dd, obsc	6.36 dd (6, 10)	6.47 dd (6, 10)
	H-7	3.65 dd (1.5, 6)	3.67 dd (1.5, 6)	3.62 dd (1.5, 6)	3.64 dd (1.5, 6)	3.64 dd (1.5, 6)	3.62 dd (2, 6)
	H-8	4.70 dd (1.5, 8)	4.43 dd → d (1.5)	4.67 dd (1.0, 8)	4.39 dd → br.s	4.68 dd (1.5, 8)	4.44 dd → br.s
	C8-OH	6.86 d (8)	—	6.82 d (8)	—	6.84 d (8)	—
	H-11	~5.34 q (7)	~5.36 obsc	5.33 q (7)	~5.37 q, obsc.	5.35 q (7)	5.37 q (7)
	12-CH_3	1.35 d (7)	1.42 d (7)	1.35 d (7)	1.42 d (7)	1.35 d (7)	1.40 d (7)

Table 6. ^{1}H NMR assignments of thiostreptine (Thstn) and threonine residues (Thr (2)) in thiostrepton A_1 and thiopeptins A_{1a} and A_{1b} in $CDCl_3$ and CD_3OD - $CDCl_3$ (1 : 4) at 25°C.

Assignment	Thiostrepton A_1		Thiopeptin A_{1b}		Thiopeptin A_{1a}	
	$CDCl_3$	CD_3OD - $CDCl_3$ (1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$ (1 : 4)	$CDCl_3$	CD_3OD - $CDCl_3$ (1 : 4)
Thr Me (2)	1.75 d (7)	1.76 d (7)	1.76 d (7)	1.74 d (7)	1.79 d (7)	1.76 d (7)
Thr H-3 (2)	6.41 q (7)	6.41 q (7)	6.46 q (7)	6.45 q (7)	6.57 q (7)	6.47 q (7)
Thr H-2 (2)	5.87 d (10)	5.84 br.s	6.87 d (9.5)	6.86 br.s	6.85 d (10)	6.82 br.s
Thr NH (2)	8.32 d (10)	—	9.72 d (9.5)	—	9.79 d (10)	—
Thstn H-2	5.79 d (10)	5.80 s	5.78 d (10)	5.78 s	5.80 d (11)	5.78 s
Thstn NH	7.61 d (10)	—	7.57 d (10)	—	7.60 d (10.5)	—

through Thz (3) in thiostrepton and thiopeptins was complicated by the almost zero coupling between H-2 and H-3, making the CHNH proton doublets difficult to distinguish from the pair for the thiostreptine (Thstn) residue (see Table 6). In the case of thiostrepton A_1 (see companion paper) this problem was solved by double resonance experiments in CD_3OD - $CDCl_3$ (1 : 4) where, even though not resolved, the broader singlet at δ 5.84 was shown to be coupled to the quartet at δ 6.41 which in turn was coupled to the most downfield methyl doublet at δ 1.76, thus defining the CH_3CHCH sequence in Thr (2). Whereas Thstn H-2 and Thr H-2 (2) have similar chemical shifts in thiostrepton A_1, one of the doublets as well as the corresponding NH doublet, occur significantly to lower field in both series of the thiopeptins. That the downfield shifted resonances are in fact due to the CHNH protons of Thr (2) was similarly confirmed for A_{1a} in CD_3OD - $CDCl_3$ (1 : 4) at 25°C, but unequivocally at 65°C where now the small coupling of *ca* 1.0 Hz between H-2 and H-3 resolved giving a doublet of quartets (J=1.0, 7.0 Hz) for H-3.

The reason for the appreciable downfield shifts of the CHNH protons in Thr (2) became clear from a ^{13}C NMR analysis of the thiopeptins and thiostrepton A_1. In both series of thiopeptins a peak appears at 189.9 ppm in CD_3OH - $CDCl_3$ (1 : 4), downfield of all other resonances and giving a broadened singlet on gated decoupling. When measured in the fully deuterated solvent mixture a split peak is observed suggesting a carbonyl group flanked by a slowly exchanging peptide NH group[18]. Earlier microanalytical data on the thiopeptins[9] as well as our own, indicate a higher sulfur content compared to thiostrepton and taken together with the unusual low field position of the amide carbon, a thiocarbonyl group was entertained which readily accounts for the yellow color of all thiopeptins. Thiocarbonyl groups have been shown by KALINOWSKI and KESSLER[19] to appear downfield of their oxygen analogues in a predictable manner by the relationship $\delta_{C=S}=1.45\ \delta_{C=O}-46.5$ ppm; this relationship also holds true for various aromatic amides (see companion paper). On calculation, this gives $\delta_{C=O}=163.0$ ppm for $\delta_{C=S}=189.9$ ppm in very good agreement with that assigned to the corresponding amide carbonyl in thiostrepton (161.1 ppm) and that observed in the model compound thiazole-4-carboxamide in DMSO-d_6 (162.8 ppm). The thiocarbonyl resonance 189.7 ppm in the thio-analogue of the thiazole-4-carboxamide moreover supports the proposed modification. The downfield shifts of the CHNH proton doublets can now be readily understood in terms of the diamagnetic anisotropy of the thioamide group[20]. In particular the shifts observed for the methine proton of the model amide *N*-methyl-*N*-isopropyl acetamide compared to the corresponding thio-analogue are 1.10 and 0.47 ppm for the *Z* and *E* isomers respectively[21] thus favoring the *Z* configuration as found in thiostrepton.

Nature of Piperidine (Pip) Ring

The only difference that distinguishes the two series of thiopeptins, originates in the piperidine ring

which will be discussed separately for each series.

b Series

Table 7 summarizes the ^1H NMR data of the piperidine ring protons of thiostrepton A_1 and thiopeptin A_{1b} at two different temperatures in $CDCl_3$. The doublet of doublets for Pip H-4α, obscured by the residual water peak at 25°C is clearly visible at higher temperatures. It is abundantly clear from the Table that both compounds have the same Δ^1-piperidine ring system. As was pointed out previously from a first order analysis of the four spin CH_2CH_2 system in thiostrepton, the chemical shifts are anomalous and are attributed to the neighboring ring currents of the thiazole substituents at Pip C2, C5 and C6. The chemical shifts therefore depend critically on the overall conformation of this moiety and provides strong evidence not only for the same relative, but also absolute configuration of the thiazole rings at these centers. In addition to the geminal and vicinal coupling constants which characterize the first order spin pattern in thiopeptin A_{1b}, the homoallylic couplings of 1.5 and 3.5 Hz involving H-6β with H-3 and H-3α respectively, were confirmed. These couplings were removed by addition of trifluoroacetic acid-*d* (TFA-*d*) because of ^2H-exchange of the allylic hydrogens at C3 *via* an imine-enamine equilibrium as observed in the case of thiostrepton.

a Series

The first order four spin CH_2CH_2 pattern in the **b** series is replaced by a more complex second order five spin system for members of the **a** series. The spin system defied analysis in $CDCl_3$ or CD_3OD - $CDCl_3$ (1 : 4) because of poor resolution of the two upfield multiplets. Upon addition of TFA-*d* to B_a, A_{1a} or A_{3a} in CD_3OD - $CDCl_3$ (1 : 9) however, all five resonances move to lower field including the singlet H_F at δ 4.53 in A_{1a} (see Table 8), reminiscent of the protonation of an amine. For all three components, the protonation shifts for H_E and H_F were always observed to be the same and the largest which suggested an amine function flanked by two methine groups. The results therefore suggested the

Table 7. Comparison of ^1H NMR assignments of Δ^1-piperidine ring in thiostrepton A_1 with thiopeptin A_{1b} in $CDCl_3$ at 25°C and 45°C.

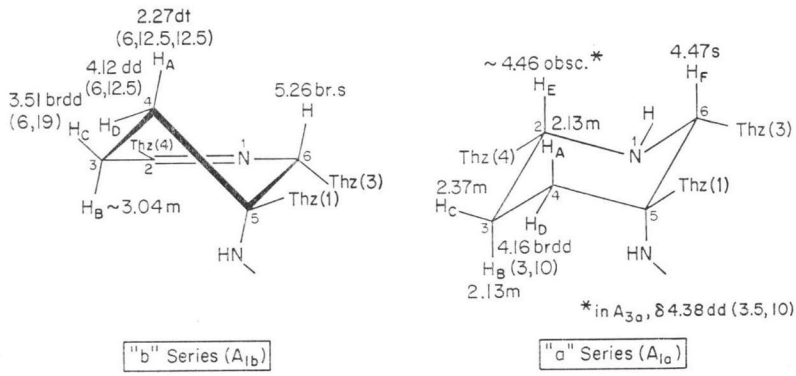
Assignment	Thiostrepton A_1		Thiopeptin A_{1b}	
	25°C	45°C	25°C	45°C
Pip H-3β	3.50 dd (6, 19)	3.50 dd (6, 19)	3.51 dd (6, 19)	3.50 dd (6, 19)
Pip H-3α	~3.00 obsc.	~3.00 m	~3.04 obsc.	~3.03 m
Pip H-4β	2.28 dt (6, 12, 12)	2.29 dt (6, 12, 12)	2.27 dt (6, 12.5, 12.5)	2.27 dt (6, 12.5, 12.5)
Pip H-4α	~4.11 obsc.	4.14 dd (6, 12)	4.12 dd (6, 12.5)	4.14 dd (6, 12.5)
Pip H-6β	5.23 br.s	5.24 br.s.	5.26 br.s.	5.27 br.s.

Table 8. ^1H NMR assignments and protonation shifts of piperidine ring protons in thiopeptin A_{1a} with TFA-*d* in CD_3OD - $CDCl_3$ (1 : 9) at 45°C.

Symbol[a]	Assignment	CD_3OD - $CDCl_3$ (1 : 9)		$\Delta\delta$ (ppm)
		No TFA-*d*	+10% TFA-*d*	
A	Pip H-4β	2.13 m	2.37 dt (3.5, 13, 13)	0.24
B	Pip H-3α	2.13 m	~2.56 m	0.43
C	Pip H-3β	2.37 m	~2.49 m	0.12
D	Pip H-4α	4.16 br.dd (~4, 10)	4.32 br.d (12.5)	0.16
E	Pip H-2β	4.43 dd (3.5, 10)	5.07 dd (4, 10)	0.64
F	Pip H-6β	4.53 s	5.17 s	0.64

[a] See Fig. 3.

Fig. 3. Conformation and ^1H NMR assignments of piperidine ring in thiopeptins A_{1a} and A_{1b} in $CDCl_3$ at 25°C.



corresponding saturated piperidine ring system as characteristic of the **a** series. Moreover, by careful adjustment of temperature and concentration of TFA-*d*, the five spin system became approximately first order (see Table 8) which enabled the sequence of ring protons to be determined by double resonance. The results for the parallel assignments in $CDCl_3$ are shown in Fig. 3. H_E was chosen as the point of departure because of its unequivocal assignment to Pip H-2 on the basis of its multiplicity and protonation shift. H_E on irradiation was shown to be coupled to H_C and either H_A or H_B which overlap. The experiment was repeated in CD_3OD - $CDCl_3$ (1 : 9) containing 10% TFA-*d* at 45°C (Table 8) in which one of the resonances, arbitrarily designated as H_B, was shifted to lower field of H_A, leaving H_A as a doublet of triplets. This is reminiscent of the multiplet structure for the high field resonance assigned to Pip H-4β in thiostrepton A_1 and A_2 and the **b** series of thiopeptins. Neither H_A and H_D were significantly affected which were thus assigned to the protons at C4 and H_B and H_C at C3. The coupling of H_B and H_C to H_E is first order and hence the splittings of the doublet of doublets for H_E (3.5 and 10 Hz) reflect the true coupling constants. For a normal chair conformation of the piperidine ring, H_E is therefore axial and the thiazole ring at C2 equatorial. Irradiation of H_E also established that H_B is associated with the larger coupling constant and must therefore be axial. Irradiation of H_D in the protonated medium collapsed H_A from a doublet of triplets to a doublet of doublets where the splittings of 3.5 and 12.5 Hz only approximate the true vicinal coupling constants. H_E remained unchanged whereas both H_B and H_C sharpened slightly as expected if H_D is equatorial. The anomalous chemical shifts for H_A and H_D at C4 for corresponding members of both series are very similar as shown for thiopeptins A_{1a} and A_{1b} in Fig. 3 and strongly argues for the same orientation of the protons to the thiazole and acylamino substituents at C5. Moreover the almost identical chemical shifts of H_E and H_F in A_{1a} is consistent with the same orientation of the thiazole substituent at C2 and C6 which therefore completely defines the conformation and relative stereochemistry of the piperidine ring as in Fig. 3. The chair conformation maximizes the number of equatorial substituents and has the same relative stereochemistry at C5 and C6 as found in thiostrepton and the **b** series. Moreover the same absolute configuration at these centers would be expected on biogenetic grounds.

The question of absolute stereochemistry can be argued from a different viewpoint. It is evident from a comparison of the ^1H NMR data for both series of thiopeptins with that of thiostrepton that the overall conformation is very similar in solution which places strong conformational constraints on the orientation as well as configuration of ring substituents. Besides the same relative configuration at C5

Table 9. ^{13}C NMR assignments of piperidine ring carbons in thiostrepton A_1 and thiopeptins A_{1a}, A_{1b} and B_a in CD_3OD - $CDCl_3$ (1 : 4) at 40°C and on protonation.[a]

Assignment	Thiostrepton A_1	Thiopeptin			
		A_{1b}	A_{1a}	B_a	B_a+6% TFA-*d*
Pip C2	161.0	160.9 s	56.8 d	56.9	56.4 v.br.
Pip C3	23.4	23.4 t	27.1 t	27.2	26.1 v.br.
Pip C4	27.9	28.1 t	32.4 t	32.5	32.3 br.
Pip C5	56.3	56.4 s	57.5 s	57.6	57.7 br.
Pip C6	62.9	62.9 d	60.7 d	60.8	60.3 v.br.

[a] In ppm downfield of TMS.

and C6 of the piperidine ring, the orientation of the substituents at these centers was found to be very similar in both series as shown in Fig. 3. The absolute configuration of all amino acid res'dues from both series was found to be the same, *i.e.* L- for Ala, Thr and Val, and D- for Cys which provides convincing evidence for the same absolute configuration at C5 and C6 of the piperidine ring in both series which is thus completely defined as 2*R*, 5*R*, 6*R*.

Support for the saturated piperidine ring structure in the **a** series comes from ^{13}C NMR evidence. Comparison of the spectrum of thiopeptin A_{1a} with A_{1b} (see Table 9) shows the presence of one additional resonance at 56.8 ppm in the 45 ~ 80 ppm region whereas the resonance assigned to the imine carbon at 160.9 ppm in A_{1b} is missing. Addition of TFA-*d* to B_a resulted in exchange broadening of the piperidine resonances but the same partial protonation shifts observed for the resonances at 56.9 and 60.8 ppm readily confirms their assignment as Pip C2 and C6 respectively.

The backbone of the bicyclic structure incorporates C5 and C6 of the piperidine ring and saturation of the N1-C2 double bond is expected to cause little change in the overall conformation of the molecule because of a minimal change in the relative orientations of the two thiazole and peptide substituents at these centers on proceeding from a half-chair to a chair conformation of the Pip ring (see Fig. 3). Only small chemical shift changes are observed for the neighboring Pip C5-NH and Ala (2) resonances between corresponding members of the **a** and **b** series (Table 2) which allows unequivocal assignment of the two Ala residues. This supports the assignment made on the basis of the thiostrepton crystal structure[2] where Ala H-2 (2) lies in the deshielding cone of the dihydroquinoline ester carbonyl. The latter is therefore assigned to the resonance at lower field and assignments for the corresponding methyl and NH protons readily follow.

Nature of Side Chain

On the basis of our ^1H NMR study (see companion paper) it was proposed that the side chain of thiostrepton consists of two Deala residues confirming the ^{13}C NMR results of TORI *et al.*[8]. Instead of the six doublets (J=2.5 Hz) characterizing the methylene protons of the three Deala residues in thiostrepton, of which one is located in the bicyclic portion of the molecule, thiopeptins $B_{a/b}$ and $A_{1a/b}$ have four doublets coupled in pairs, in addition to two sharp singlets in the same region of the spectrum when examined in CD_3OD - $CDCl_3$ (1 : 4). In $CDCl_3$ (Table 2), the upfield member of the two pairs, as for the three pairs in thiostrepton, occurs as a broad singlet which sharpens to a doublet on removal of the allylic coupling with the NH proton by either ^2H-exchange or irradiation of the corresponding NH signal. It is known[22] that J_{gem} of vinyl methylene protons is small and that the sign can be positive or negative depending largely on the electro-negativity of the substituent. Moreover, both sharp singlets and the NH singlet at δ 8.59 in A_{1a} and A_{1b} and δ 8.75 in B_a, are missing in the spectrum of A_{4a} which

implicates the terminal Deala residue having a modified amide group. Thiopeptin B_a forms a sodium salt having characteristic bands in the infrared spectrum at 1610 and 1400 cm^{-1} and is therefore a carboxylic acid which readily accounts for its different chromatographic behavior from that of thiostrepton and the other thiopeptins. Besides its small Rf, thiopeptin B_a 'tails' on silica gel TLC in methanol - methylene chloride (1 : 9) and is severely retarded on addition of ammonia to the solvent system. This is in sharp contrast to the considerably more mobile character of the other components (see Table 1) which form discrete spots and whose Rf's are unaffected by ammonia. The end groups of A_{1a} and A_{1b}, because of the similar zero coupling between the methylene protons, must be of similar electro-negativity to that of the carboxyl group. Both compounds have a three proton singlet at δ 3.93 and are therefore assigned as the methyl esters. Whereas thiopeptin A_{4a} has one Deala residue missing, thiopeptin A_{3a} lacks two of the residues. As Deala residues are readily susceptible to hydrolysis[17,23], it is not surprising therefore to find that both thiopeptin B_a and A_{1a} give rise to thiopeptins A_{4a} and A_{3a} on acid hydrolysis with TFA and supports the proposal that thiopeptins B_a and A_{1a} differ only in the nature of the side chain.

Confirmation of the proposed differences in the nature of the side chains comes from ^{13}C NMR evidence. Thiopeptin B_a has 71 carbons whereas A_{1a} and A_{1b} each have 72, the extra resonance yielding a quartet at 51.5 ppm on gated decoupling with $^1J_{^{13}C-H}=148$ Hz characteristic of a methyl ester. Thiopeptins A_{4a} and A_{3a} each have 68 and 65 carbons respectively, the missing carbons being readily assigned to two Deala residues on the basis of the assignments for the model compound 2-acetamido acrylic acid (see companion paper).

Conclusion

The total structure of all thiopeptin components proposed on the basis of ^1H and ^{13}C NMR evidence is shown in Fig. 1. They are closely related to thiostrepton and differ at four sites of the molecule. The presence of the thioamide group, which is responsible for the yellow color of the thiopeptins, is unique for a naturally occurring compound.[24] Moreover, as for the corresponding amide group in thiostrepton, the thioamide has the *Z* configuration. The mode of biosynthesis of the thiopeptide group is of interest as the structurally related antibiotic, nosiheptide[4] (=multhiomycin)[3] is also modified at this point having cysteine instead of threonine involved in a thioester linkage. The origin of the difference between the two series of thiopeptins lies solely in the nature of the piperidine ring, the **b** series retaining the Δ^1-piperidine moiety as in thiostrepton, while the **a** series are characterized by a fully saturated piperidine ring system. The evidence strongly supports the D configuration at the new asymmetric center Pip C2 in the **a** series, the same as at C4 and C6 in thiostrepton and the **b** series. A simple biosynthetic scheme incorporating α-aminoadipic acid (Aad) for the formation of the pyridine ring in micrococcin P as proposed by WALKER *et al.*[25] can also account for the piperidine ring in thiostrepton and the thiopeptins but with subsequent addition of a peptide amide at C5. Formally, the intermediacy of 2,5-diamino adipic acid is suggested but its participation in secondary metabolism has never been shown. More recently BYCROFT[26] has suggested the intriguing possibility that its origin may be the result of the interaction of two Deala units in single peptide chain, but evidence for any of these proposed schemes has yet to be forthcoming. If in fact Aad is the biosynthetic precursor it will be of interest whether the D or L isomer is incorporated in light of the fact that the D configuration at Pip C2 is found, reminiscent of the presence of the D Aad side chain in the cephalosporins even though the L isomer is incorporated[27]. Biosynthetically, all four thiopeptin components $B_{a/b}$ and $A_{1a/1b}$ are simply related by an oxidation or reduction and hydrolysis or esterification sequence whereas the other components $A_{4a/4b}$ and $A_{3a/3b}$ were shown not to be true fermentation products but de-deala and bis-de-deala acid artifacts of thiopeptin $B_{a/b}$ (and $A_{1a/1b}$) respectively.

Experimental

Infrared spectra were recorded in chloroform solution on a Perkin-Elmer 137 spectrometer and ultraviolet spectra in methanol on a Cary 15 instrument. ^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz were obtained using a Varian SC 300 instrument as previously described[15]. Low resolution mass spectra were obtained on a LKB 9000 and high resolution spectra on a Varian MAT-731 spectrometer. Analytical HPLC was performed on a Varian Model 4100 instrument.

Isolation and Purification

Fermentation broths were worked up similarly to the procedure of MIYAIRI *et al.*[9]. Dried mycelia from Merck and Fujisawa, obtained under a variety of fermentation conditions gave thiopeptin of variable composition and were extracted as follows:

Dried mycelia was extracted with 70% aqueous acetone overnight at room temperature and celite added to facilitate in filtration. After filtration, the mycelia was further washed with the same solvent and the filtrate concentrated. Prior to extraction with chloroform or ethyl acetate, the aqueous solution was saturated with NaCl to preven tformation of an emulsion. The chloroform layer was removed, concentrated to a small volume and added to five volumes of *n*-hexane to precipitate the crude thiopeptin. After sitting overnight at 0°C the precipitate was filtered off and dried. A typical procedure for the chromatographic separation of the thiopeptin components follows:

Thiopeptin (8.5 g) was dissolved in methanol - methylene chloride (1: 19, +0.5% NH_3, 170 ml) and applied to the top of a column of silica gel 60 (E. Merck, 230~400 mesh, 270 g, 51×3.8 cm). The column was eluted with the same solvent until the eluate became colored at which point 100 ml fractions were collected and monitored by TLC on silica gel GF plates using the same solvent system. Only a trace of thiopeptin A_{1a} was eluted in the first few fractions (3×100 ml) from this batch, after which thiopeptins A_{3a} and A_{4a} began to emerge. Fraction 4 gave almost pure A_{3a} (0.75 g) and subsequent fractions (5× 100 ml) of chromatographically pure thiopeptin A_{4a} (1.42 g) as pale yellow solids. Elution with methanol - methylene chloride (1: 4, +2% NH_3, 10×100 ml) gave thiopeptin B_a (1.66 g) which was rechromatographed on silica gel 60 to give pure thiopeptin B_a as a pale yellow solid on evaporation of the solvent. UV and ^{1}H NMR (see text); IR ($CHCl_3$) μ 3.0 vs, 3.4 s, 5.75 s, 5.9 sh, 6.05 vs, 6.3 w, 6.55 vs, 6.70 vs, 6.90 s, 7.25 m, 7.65 w, 7.8 m, 8.3 w, 8.6 m, 8.8 m, 8.95 m, 9.1 m, 9.35 m, 9.75 w, 9.9 w, 10.45 w, 10.7 w, 10.85 w, 11.1 sh and 11.25 s.

Thiopeptin A_{3a} (0.74 g) was rechromatographed on a silica gel 60 (60 g, 36×2.2 cm) column and eluted with methanol - methylene chloride (1: 19, +0.5% NH_3, 6×8 ml) to give chromatographically pure thiopeptin A_{3a} (0.40 g). The yellow solid was dissolved in $CHCl_3$, centrifuged and the solid obtained after evaporation of the supernatant, recrystallized from tetrahydrofuran - ethyl acetate (1: 3), giving clusters of fine needles at −10°C. The needles were collected and dried at 50°C for 24 hours for microanalysis resulting in a weight loss of 3%. UV and ^{1}H NMR (see text). IR (see below).

Anal. Calcd. for $C_{65}H_{79}N_{17}O_{15}S_6 \cdot H_2O$: C 50.41, H 5.27, N 15.37, S 12.42.
Found: C 50.84, H 5.40, N 14.89, S 12.11.

Various thiopeptin A_{1b} fractions from different batches were pooled (0.22 g), dissolved in methanol - methylene chloride (1: 99) (6 ml) and filtered onto a column of silica gel 60 (14×1 cm). The yellow band at the top of the column began to move and was eluted with methanol - methylene chloride (3: 97, 7×3 ml). Chromatographically pure thiopeptin A_{1b} (0.11 g) was obtained which crystallized from chloroform - ether (2: 1) as yellow plates. A sample for microanalysis was dried at 50°C for 24 hours under high vacuum resulting in a 5.3% loss in weight. IR (see below).

Anal. Calcd. for $C_{72}H_{84}N_{18}O_{18}S_6 \cdot 4H_2O$: C 49.30, H 5.29, N 14.37, S 10.97.
Found: C 49.93, H 5.13, N 14.79, S 10.40.

Similarly another batch yielded pure thiopeptin A_{1a} which recrystallized from ethyl acetate at 0°C as yellow plates.

Sodium Salt of Thiopeptin B_a

Thiopeptin B_a (20 mg) was dissolved in tetrahydrofuran - methanol (1: 1, 20 ml) to which water (1 ml) was added. The solution was chilled, acidified with a few drops of 1 N HCl and quickly neutralized with 5% aqueous sodium bicarbonate with the addition of water or methanol to prevent the bicar-

Table 10. Infrared differences for thiopeptin components (μ) in $CHCl_3$[a].

B_a	A_{1a}	A_{1b}	A_{4a}	A_{8a}
—	—	—	7.1 w	—
—	7.4 s	—	—	—
10.45 w	10.45 w	10.1 w	10.5 w	10.5 w
10.7 w	—	10.65 w	—	—
10.85 w	10.85 w	10.85 w	10.95 m	10.9 w
11.1 sh	11.1 sh	11.1 sh	—	11.1sh

a) Intensities are abbreviated as w=weak, m=medium, s=strong, vs=very strong.

bonate or thiopeptin from precipitating. The final solution of 15 ml was extracted with ethyl acetate three times and on evaporation yielded the sodium salt as a pale yellow solid. ^1H NMR: almost identical to that of thiopeptin B_a. IR ($CHCl_3$) same as for thiopeptin B_a but with extra bands at 1620 sh and 1400 cm^{-1}.

The IR of other thiopeptin components where different from that recorded for thiopeptin B_a are summarized in Table 10.

Separation of Thiopeptin B_a and B_b

^1H NMR analysis of thiopeptin B in CD_3OD - $CDCl_3$ (1: 4) from various batches showed a mixture of the **a** and **b** components which was confirmed by HPLC on a μPorasil column (Waters) using the solvent system methanol - methylene chloride (1: 9, +1% NH_3). A typical sample (75 mg) having the ratio $B_a/B_b=1:2$ was applied to 8 prewashed silicagel HF plates (Analtech 20×20×0.025 cm) and developed (5×) with methanol - methylene chloride (3: 17, +1% NH_3). The band was divided into three sections and shown after elution to have the approximate B_a/B_b ratios of 2: 1, 1: 2 and 1: 4 (by ^1H NMR) in decreasing order of Rf. The mixture of lowest Rf having $B_a/B_b=1:4$ was reapplied on 2 plates and multiply developed as before to give predominantly pure thiopeptin B_b as a pale yellow solid whose ^1H NMR is almost identical to that of thiostrepton A_{1b} but with the absence of the CH_3O singlet at δ 3.93 (see Table 2).

Acid Hydrolysis of Thiopeptin B_a

Thiopeptin was dissolved in trifluoroacetic acid and the time course of the hydrolysis followed by TLC on silica gel. The production of thiopeptins A_{8a} and A_{4a} was optimal between 3 to 4 hours at room temperature after which time several substances of lower Rf were formed. The products after separation by preparative TLC were shown by Rf and ^1H NMR to be identical with thiopeptin A_{8a} and A_{4a}.

Similarly thiopeptin A_{1a} was shown to give rise to A_{8a} and A_{4a}.

$NaBH_4$ Reduction of Thiopeptin A_{1b}

Thiopeptin A_{1b} (3.3 mg) was dissolved in methanol - methylene chloride (1: 1, 1 ml), chilled and $NaBH_4$ (10.5 mg) added. The mixture was gently stirred for 3.5 hours, more $NaBH_4$ (5 mg) added and allowed to react for a further 2.5 hours at room temperature. The pH was adjusted to 4.0 with concentrated HCl using pH paper, the mixture evaporated to dryness and the residue hydrolyzed with conc. HCl and subjected to amino acid analysis (see Table 1).

Amino Acid Analysis

Analyses on hydrolysates (6 N HCl for 20 hours) were performed on a Beckman Model 121 MH amino acid analyzer. The amino acids Thr, Val and Ala obtained on hydrolysis of the thiopeptin components were all shown to have the L-configuration and Cys the D-configuration by the MANNING-MOORE procedure[28].

Acknowledgments

We wish to thank the Fujisawa Pharmaceutical Co., Japan and the Department of Developmental Microbiology (Merck) for samples of thiopeptin mixture, Mr. CARL HOMNICK for performing the amino acid analyses and Mr. JACK SMITH and Dr. JERROLD LIESCH for mass spectral data.

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