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# TOTAL STRUCTURE OF THE PEPTIDE ANTIBIOTIC COMPONENTS OF THIOPEPTIN BY $^{1}H$ AND $^{13}C$ NMR SPECTROSCOPY

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Thiopeptin, produced by *Streptomyces tateyamensis* and belonging to a group of highly modified sulfur-containing peptide antibiotics is a valuable growth promotant in swine and chickens[1] and was shown to be effective as a lactic-acidosis preventive in sheep and cattle[2]. The antibiotic complex was characterized by Miyairi *et al*[3] and shown to consist of a major component B and four minor components $A_1$, $A_2$, $A_3$ and $A_4$. No structures for any of the components have been reported although acid hydrolysis experiments[4], primarily on thiopeptin B, have demonstrated a close relationship to thiostrepton, the structure of which was largely determined by X-ray crystallography[5] and its side-chain by $^{13}C$ NMR spectroscopy[6]. We wish to report here on the structure determination of the various thiopeptin components primarily by $^{1}H$ and $^{13}C$ NMR techniques.

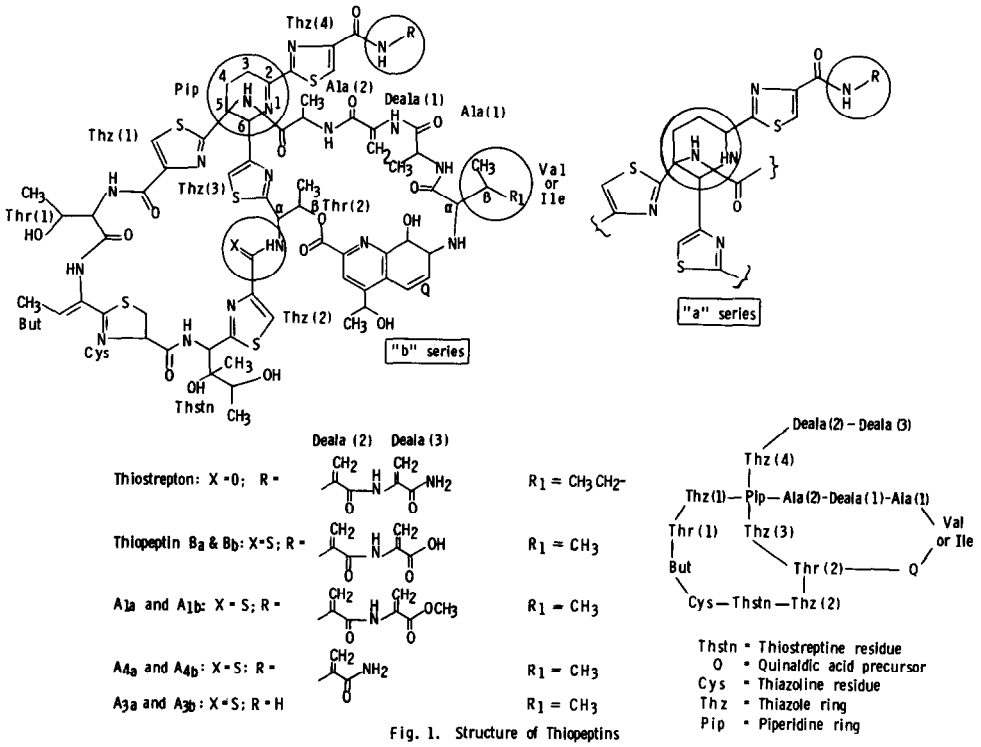
Thiopeptin components were separated by silicagel chromatography similar to that previously described[3]. Subsequent analysis of the components by $^{1}H$ NMR at 300 MHz and HPLC of various batches indicated in the majority of cases a two compound system. Consistent differences in their $^{1}H$ NMR spectra allowed them to be grouped into two distinct series arbitrarily designated by the subscripts *a* and *b* as summarized in Table 1. Pure components $A1_a$, $A1_b$, $A3_a$, $A4_a$ and $B_a$ were isolated from some batches whereas thiopeptin $B_b$ was separated from $B_a$ by multiple preparative tlc on silicagel. Thiopeptins $A3_b$ and $A4_b$ were not separated from the "*a*" components, but were analyzed as mixtures. Thiopeptin $A_2$ was not produced in our fermentation.

Table 1: Molecular formulae for thiopeptin components*

| | "*a*" Series | "*b*" Series |
|---|---|---|
| Acid | $B_a$ - $C_{71}H_{84}N_{18}O_{18}S_6$ | $B_b$ - $C_{71}H_{82}N_{18}O_{18}S_6$ |
| Methyl ester | $A1_a$- $C_{72}H_{86}N_{18}O_{18}S_6$ | $A1_b$- $C_{72}H_{84}N_{18}O_{18}S_6$ |
| Des-Deala (Amide) | $A4_a$- $C_{68}H_{82}N_{18}O_{16}S_6$ | $A4_b$- $C_{68}H_{80}N_{18}O_{16}S_6$ |
| Bis-Des-Deala (Amide) | $A3_a$- $C_{65}H_{79}N_{17}O_{15}S_6$ | $A3_b$- $C_{65}H_{77}N_{17}O_{15}S_6$ |

*from microanalytical and $^{1}H$ and $^{13}C$ NMR data.

Correlations between the majority of resonances in the $^{1}H$ NMR spectra of thiostrepton and the thiopeptins were made to within 0.04 ppm and differences were noted at four different sites of the molecule which are circled in Figure 1 and which shall now be discussed.




Thiostrepton: X = O; R = $R_1 = CH_3CH_2-$

Thiopeptin $B_a$ & $B_b$: X = S; R = $R_1 = CH_3$

$A_{1a}$ and $A_{1b}$: X = S; R = $R_1 = CH_3$

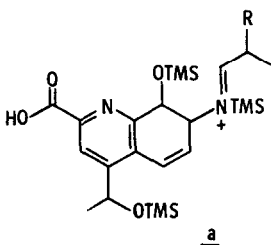
$A_{4a}$ and $A_{4b}$: X = S: R = $R_1 = CH_3$

$A_{3a}$ and $A_{3b}$: X = S; R = H $R_1 = CH_3$

Fig. 1. Structure of Thiopeptins

*Nature of Amino Acids*

Amino acid analysis of the acid hydrolysate of the thiopeptin components gave Thr, Val and Ala in the molar ratio of 1:1:2 indicating that the Ile residue in thiostrepton is replaced by Val. That this residue is attached to the quinaldic acid precursor portion was confirmed by high-resolution mass spectral data of the TMS derivatives of all components. The highest peak at m/e 521.2687 corresponds to fragment *a* (calc. 521.2686) (Fig. 2) which can readily aromatize by losing TMSiOH (m/e 431.2187, calc. 431.2185). The $^1H$ NMR spectrum[7] of thiostrepton in $CDCl_3$ reveals eight methyl doublets, one triplet and one singlet in the region 0-2 ppm whereas the thiopeptins, instead of the triplet, have an extra doublet at δ0.98 (Val $CH_3$, J = 7 Hz). This doublet is coupled to a hextet (outer satellites not observed) at δ1.92 (Val β-C<u>H</u>, J = 6 Hz) which in turn is coupled to a doublet at δ2.88 (Val α-C<u>H</u>, J = 6 Hz). Moreover, the $^{13}C$ NMR spectrum of thiostrepton[7] has 15 resonances in the 0-35 ppm region whereas all thiopeptins have only 14, comprising 10 x $CH_3$, 3 x $CH_2$ and 1 x CH carbons. One $CH_2$ resonance is missing which occurs at 23.0 ppm for thiostrepton. The presence of Val is thus firmly established.




a

Thiostrepton (R = $CH_2CH_3$) m/e 535

Thiopeptins (R = $CH_3$) m/e 521

Fig. 2

*Nature of Piperidine Ring*

*"b" Series:* Comparison of the chemical shifts and coupling constants for the piperidine protons in thiostrepton and thiopeptin $A_{1b}$ indicate that both compounds have the same $\Delta^1$-piperidine ring structure. The chemical shifts of the $-CH_2CH_2-$ protons are anomalous due to the neighbouring ring currents of the thiazole rings and analyze as a first order four spin system characterized by the following coupling constants: $J^{gem}_{4\alpha,4\beta}$ = 12.5 Hz; $J^{gem}_{3\alpha,3\beta}$ = 19 Hz; $J^{vic}_{3\beta,4\beta}$ = $J^{vic}_{3\alpha,4\alpha}$ = 6 Hz; $J^{vic}_{3\alpha,4\beta}$ = 12.5 Hz; $J^{vic}_{3\beta,4\alpha}$ = $J_{3\beta,6\beta}$ = 1.5 Hz; $J_{3\alpha,6\beta}$ = 3.5 Hz. On the basis of its homoallylic coupling to the protons at C3, H-6 was assigned to the unresolved broad singlet at $\delta$5.26 in $A_{1b}$ and $\delta$5.23 in thiostrepton. Addition of d-TFA exchanges both the allylic protons at C3 via an amine $\rightleftharpoons$ enamine equilibrium giving two doublets for H-4$\alpha$ and H-4$\beta$ ($J^{gem}$ = 12.5 Hz) and removes the homoallylic coupling to H-6$\beta$ which now appears as a very sharp singlet.

*"a" Series:* The piperidine ring protons of the "*a*" series are characterized by a complex second order spin system which becomes almost first order on protonation with d-TFA suggesting a $-CH_2CH_2CH-$ fragment. All five resonances move to lower field on addition of the acid including the singlet for H-6 ($\delta$4.47 in $A_{1a}$) which undergoes the same shift as the resonance for H-2 ($\delta$4.44, dd, J = 3.5, 10 Hz, axial)[8] suggesting an amine flanked by two CH groups. It is proposed therefore that the saturated piperidine ring system characterizes the "*a*" series. Confirmation comes from comparison of the $^{13}C$ NMR spectra of $A_{1a}$ and $A_{1b}$. $A_{1b}$ has one extra carbon at 160.9 ppm (161.0 ppm in thiostrepton) in the 140-190 ppm region attributed to the imine carbon C2 whereas $A_{1a}$ has one extra carbon at 56.8 ppm (C2) in the 45-80 ppm region, similar to that assigned to C6 at 60.7 ppm, both of which undergo the same upfield protonation shift with d-TFA.

*Nature of Modified Threonine Residue, Thr(2)*

The -CHNH- protons of Thr(2) in the thiopeptins were found appreciably downfield of those in thiostrepton, e.g. the $\alpha$-CH and NH doublets in the latter appear at $\delta$5.87 and $\delta$8.32 (J = 10 Hz) whereas in thiopeptin $A_{1a}$ they occur at $\delta$6.85 and $\delta$9.79 respectively. The reason for these shifts was not immediately clear until the $^{13}C$ NMR evidence was carefully analyzed. In both series of thiopeptins, a peak at 189.9 ppm appears in 20% $CD_3OH/CDCl_3$ downfield of all other resonances. It remains a singlet on gated decoupling but is observed as a split peak when measured in the fully deuterated solvent mixture and is therefore assigned to a carbon adjacent to a slowly exchanging peptide NH group[7]. Microanalytical data on the thiopeptins support a higher sulfur content compared to thiostrepton (Table 1) and hence a thioamide group was entertained which would readily account for the low field shifts observed for the -CHNH- protons[9]. Further support was obtained as follows. Thiocarbonyl groups appear downfield of their oxygen analogues in a predictable manner by the relationship $\delta_{C=S} = 1.45\ \delta_{C=O} - 46.5$ ppm[10]. 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 thiazole-4-carboxamide in DMSO-$d_6$ (162.8 ppm). Moreover, the thiocarbonyl resonance at 189.7 ppm in the thioanalogue of the thiazole-4-carboxamide provides further strong support.

*Nature of Side Chain*

Thiopeptins $B_{a/b}$ and $A_{1a/1b}$ have two Deala residues in their side chain as in thiostrepton which can be readily distinguished from the other Deala residue in the bicyclic portion.

Thiopeptin $B_a$ forms a sodium salt and was assigned a carboxylic acid; $A_{1a/1b}$ have a three proton singlet at δ3.93 and are therefore methyl esters. The $^1H$ and $^{13}C$ NMR resonances for both Deala residues are missing in $A_{3a/b}$ and for only one in $A_{4a/b}$ and are identical to the products obtained from facile acid hydrolysis of $B_{a/b}$ and $A_{1a/1b}$.

This therefore completely defines the thiopeptins structurally. The two series of thiopeptins are close structural analogues of thiostrepton. However, they are unique in being, as far as we are aware, the first reported examples of naturally occurring thioamides. Biosynthetically, the components $B_{a/b}$ and $A_{1a/1b}$ can be envisaged as being simply related by an oxidation or reduction and hydrolysis or esterification sequence, the other components $A_{4a/4b}$ and $A_{3a/3b}$ being des-Deala and bis-des-Deala acid artefacts of thiopeptin $B_{a/b}$ (and $A_{1a/1b}$) respectively.

A full report of this work will be published elsewhere.

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7. $^1H$ NMR and $^{13}C$ NMR spectra were obtained at 300 MHz and 75 MHz respectively using a Varian SC-300 MHz NMR spectrometer in the FT mode. $^1H$ NMR spectra were measured using acquisition times (AT) of 1 and 2 sec with sweep width (SW) of 4000 Hz and concentrations in the range 3.5-5.0 mg/0.4 ml. $^{13}C$ NMR spectra were obtained with AT 0.4-0.6 sec, SW 15000 Hz and pulse flipping angle 50° on samples of ca. 60 mg/0.4 ml in 20% $CD_3OH/CDCl_3$ and 20% $CD_3OD/CDCl_3$ in 5 mm tubes at 45°C. Slow exchange of peptide NH protons in the latter solvent allows identification of carbons adjacent to the NH groups because of $^2H$-isotope induced upfield shifts of ca. 0.1 ppm.

8. The absolute configuration at this new asymmetric center was tentatively assigned as *R* and will be discussed in the full paper.

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