STRUCTURAL STUDIES ON THIOCILLINS I, II AND III

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XXIX)[1]

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Thiocillins I, II and III were compared with micrococcin P_1 by analysis of acid hydrolyzates of the native and the reduced antibiotics as well as by means of ^1H and ^{13}C NMR spectroscopies. As a result of these studies, the differences of these antibiotics were clarified in their structural units, and the structures of thiocillins I, II and III were assigned on the basis of the proposed structure of micrococcin P_1.

A number of antibiotics possessing a high sulfur content and thiazole rings and are of a polypeptidic nature have been isolated from *Actinomycetes* and other microorganisms. Earlier, structural studies by chemical degradation procedures had been made on micrococcin P[2,3], thiostrepton[4,5,6] and siomycin A[7,8]. However, these attempts had succeeded only in part, probably because the high reactivity of 2-substituted thiazole-4-carboxylic acid residues resulted in the formation of more complicated degradation products. In 1970, the major portion of the structure of thiostrepton was elucidated by X-ray crystallographic analysis[9]. The total structures of thiostrepton and siomycin A have been proposed on the basis of this X-ray analysis and by comparison of the ^{13}C NMR spectra and the degradation products of both antibiotics[10]. The structural relationship of the two antibiotics has been further confirmed by ^{15}N and ^1H NMR spectroscopies[11,12]. Recently, the structures of thiopeptins have been proposed in a similar way[13]. In 1977, the structure of nosiheptide was determined by X-ray analysis[14].

The structure of micrococcin P has been proposed on the basis of its ^{13}C NMR spectra and the results of the earlier degradative studies and also on the basis of the following assumption; *i.e.*, the sequence of the structural units in micrococcin P is similar to those of thiostrepton and nosiheptide, by WALKER *et al.* in 1977[15]. Somewhat later, BYCROFT and GOWLAND made minor revisions of the structures of micrococcin P_1 and P_2 by the ^{13}C and ^1H NMR spectroscopies and also the analysis of hydrolyzates of the native and reduced antibiotics[16]. The proposed structure of micrococcin P_1 is shown in Scheme 1.

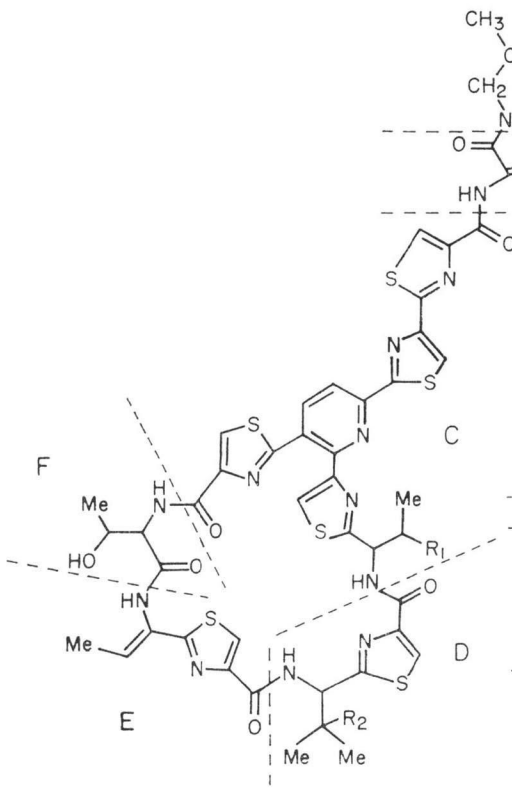
We have reported the isolation of thiocillins I, II and III from *Bacillus* strains and the close similarity of these antibiotics to micrococcin P. The liberation of micrococcinic acid and one mole of threonine by acid hydrolysis from thiocillins I, II and III has already been noted[17]. The results reported here clarify the differences in the structural units of these related antibiotics.

The hydrolyzates of thiocillins I, II, III and micrococcin P_1* were examined according to the method previously used for micrococcin P by WALKER *et al.*[2,3] (illustrated in Fig. 1) in parallel experiments. First the hydrolyzates obtained by the usual acid hydrolysis conditions were examined.

From the acid insoluble fractions of all antibiotics, micrococcinic acid was identified[17]. The ether

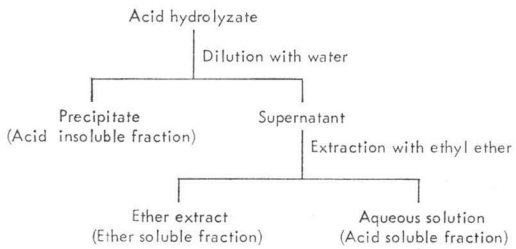
* Micrococcin P_1 used in this experiment was isolated by TLC from a sample of micrococcin P kindly supplied from Dr. WALKER[17].

Scheme 1.

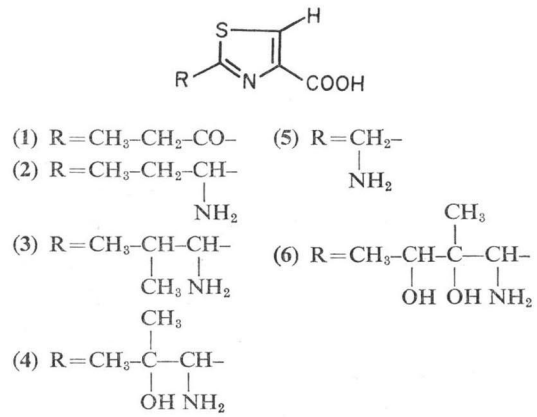


	R_1	R_2
Micrococcin P_1	OH	H
Thiocillin I	OH	OH
Thiocillin II	OCH_3	OH
Thiocillin III	OCH_3	H

Fig. 1. Fractionation procedure of acid hydrolyzates.

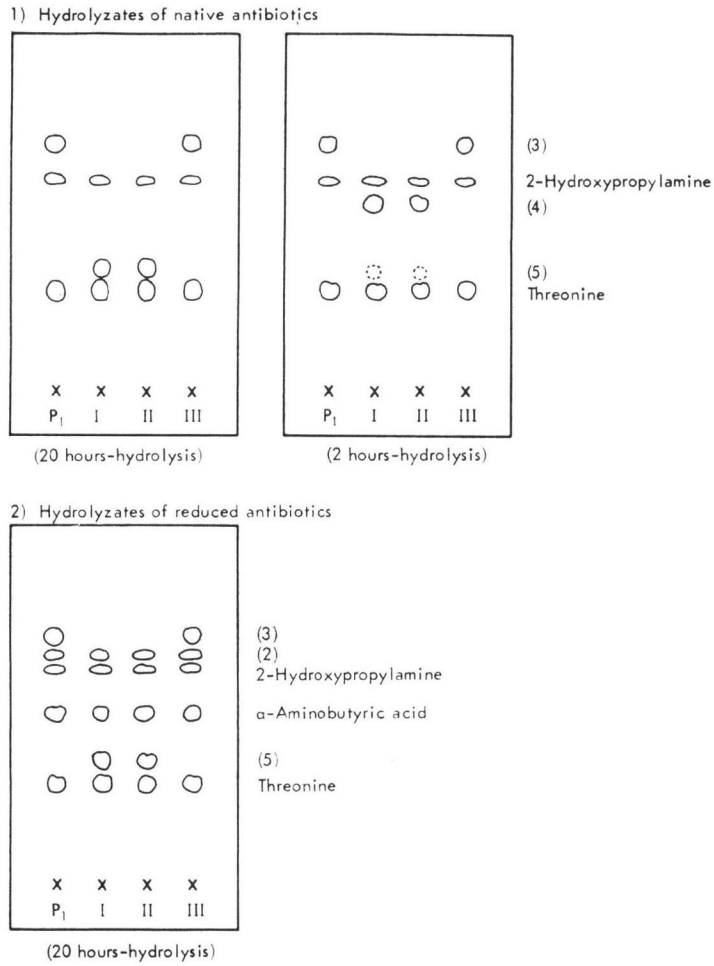


Scheme 2.



soluble fractions from thiocillins I, II and III were compared by TLC with that from micrococcin P_1, which was considered to be 2-propionylthiazole-4-carboxylic acid [(**1**) in Scheme 2] derived from structural unit E (Scheme 1)[16]. Compound (**1**) was found in all ether soluble fractions. The acid soluble fractions from the four antibiotics were then examined by TLC and also by automatic amino acid analysis (Fig. 2 and Table 1). 2-Hydroxypropylamine and approximately one mole of threonine were found in

Fig. 2. Thin-layer chromatograms of acid soluble fractions.
Precoated cellulose plate (Eastman Chromatogram Sheet), *n*-butanol - acetic acid - water (3: 1: 1).
P_1: Micrococcin P_1, I: Thiocillin I, II: Thiocillin II, III: Thiocillin III.



all acid soluble fractions. However, a substance assumed to be 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid [(**3**) in Scheme 2] derived from structural unit D of micrococcin P_1[16], was found only in the sample from thiocillin III. Alternatively, another ninhydrin positive and UV absorbing substance was found in the samples from thiocillins I and II (Fig. 2–1). This substance was isolated and identified as 2-aminomethylthiazole-4-carboxylic acid [(**5**) in Scheme 2] from the UV and ^{1}H NMR spectra. Previously this compound had been isolated as a hydrolysis product from thiostrepton[4]. This is not a

Table 1. Retention times of compounds contained in acid soluble fractions in automatic amino acid analysis.

Compound	For basic amino acids*	For acid and neutral amino acids
(**4**)	30(min)	221(min)
(**5**)	35	—
(**2**)	39	—
(**3**)	41	—
Ammonia	119	—
2-Hydroxypropylamine	127	—
Threonine	—	74
α-Aminobutyric acid	—	131

* Experimental conditions were slightly modified. The details are described in Experimental section.

structural unit of thiostrepton and when the antibiotic had been hydrolyzed for a shorter period, thiostreptin [(**6**) in Scheme 2] had been isolated[5]. Similarly when thiocillins I and II were hydrolyzed for a shorter period, another substance was detected (Fig. 2–1). This substance was isolated and its structure determined to be 2-(1-amino-2-hydroxy-2-methylpropyl)thiazole-4-carboxylic acid [(**4**) in Scheme 2] by its UV, ^{1}H NMR and ^{13}C NMR spectra. Conversion of (**4**) to (**5**) by further acid hydrolysis was confirmed.

Thiocillins I, II, III and micrococcin P_1 were reduced with $NaBH_4$. The reduced products were hydrolyzed and examined as above. Micrococcinic acid was found in all the acid insoluble fractions. In the ether soluble fractions, only a trace amount of compound (**1**) was found. By analysis of the acid soluble fractions, approximately one mole of α-aminobutyric acid and 2-(1-aminopropyl)thiazole-4-carboxylic acid [(**2**) in Scheme 2], produced from structural units B and E by hydrogenation[16], were found in all the specimens in addition to the products from the native antibiotics (Fig. 2–2). All the above thiazole compounds were isolated and the correspondence of these findings on the TLC to the results of amino acid analyses was confirmed. The retention times of these compounds in the amino acid analysis are

Fig. 3. 100-MHz ^{1}H NMR spectra in $CDCl_3$.
a) Thiocillin I, b) Thiocillin II, c) Thiocillin III, d) Micrococcin P_1.

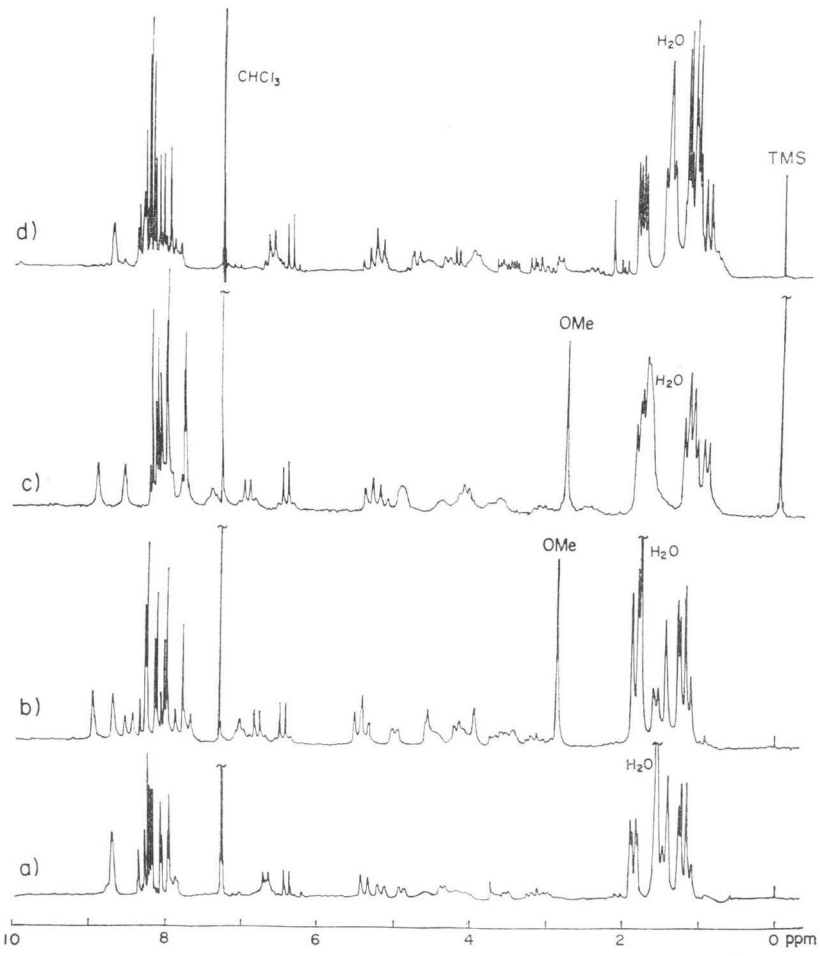


Table 2. ^{13}C NMR data of thiocillins I, II and III compared with micrococcin P_1, and tentative assignments.

Tentative assignment	Micrococcin P_1 Observed	(Reference 16)	Thiocillin I	II	III
CONH	171.0s	(170.8)	170.9s	171.2s	171.6s
Thiazole	170.0s	(169.9)	167.7s	168.2s	170.2s
$-C\begin{matrix}S\\ \diagup\\ \diagdown\\ N\end{matrix}$	168.8s	(168.8)	168.7s	168.2s	168.1s
	168.7s	(168.5)	168.7s	168.6s	168.6s
	166.6s	(166.4)	167.0s	167.2s	166.9s
	166.1s	(165.8)	166.2s	166.2s	166.1s
	165.6s	(165.4)	165.2s	164.7s	164.7s
	162.9s	(162.7)	162.9s	162.9s	162.9s
	161.9s	(161.6)	161.7s	161.8s	161.7s
	161.3s	(161.0)	161.3s	160.8s	160.9s
	161.1s	(160.8)	161.1s	161.1s	160.9s
	160.7s	(160.3)	160.7s	160.8s	160.7s
Thiazole	153.9s	(153.8)	154.3s	154.9s	154.7s
$>C=N$	151.3s	(151.2)	151.5s	152.1s	152.3s
	150.6s	(150.4)	150.5s	150.4s	150.4s
	149.9s	(149.9)	149.9s	150.4s	150.4s
Pyridine	149.7s	(149.7)	149.7s	149.7s	149.8s
$>\overset{\alpha}{C}=N$	149.7s	(149.5)	149.7s	149.7s	149.6s
	149.5s	(149.0)	149.7s	149.6s	149.5s
	148.6s	(148.6)	148.5s	148.8s	148.8s
Pyridine-γ=CH	140.4d	(140.2)	140.1d	139.6d	139.5d
$-C=\overset{\beta}{CH}-$ (B, E)	131.3d	(128.7)*	131.3d	131.6d	131.6d
$-\overset{|}{N}H$	128.8d	(128.3)*	129.1d	129.0d	128.7d
$-\overset{\alpha}{C}=CH-$ (B, E)	129.5s	(130.2)*	129.5s	129.6s	129.6s
$\overset{|}{N}H$	129.2s	(129.6)	129.1s	129.0s	129.0s
Pyridine-β=$\overset{|}{C}$–	128.3s	(129.3)*	128.5s	129.0s	129.0s
Thiazole	125.6d	(125.3)	125.4d	125.7d	125.4d
$S\diagup\!\!\diagup CH$	125.1d	(124.9)	125.4d	125.7d	124.8d
	124.7d	(124.4)	125.4d	124.6d	124.2d
	124.2d	(123.9)	124.5d	124.6d	124.2d
	121.4d	(121.6)	121.4d	121.8d	121.8d
	121.4d	(121.0)	120.9d	119.8d	119.8d
Pyridine-β=CH	118.8d	(118.6)	118.9d	119.7d	119.5d
$-\overset{|}{CH}-CH_3$ (C)	68.0d	(68.0)	67.9d	77.0d	76.5d
O (F)	67.6d	(67.5)	67.9d	67.9d	67.3d
(A)	66.6d	(66.5)	66.6d	66.7d	66.5d
(C)	58.1d	(58.2)	57.5d	57.3d	57.8d
$-\overset{\alpha}{O}H-$ (F)	56.2d	(56.2)	56.2d	56.1d	56.2d
$\overset{|}{N}$ (D)	55.8d	(55.8)	57.5d	57.3d	56.2d

Table 2. (continued)

Tentative assignment		Micrococcin P_1 Observed	(Reference 16)	Thiocillin I	II	III
OCH_3	(C)	—	—	—	56.7q	56.6q
$-CH_2-NH$	(A)	47.5t	(47.4)	47.5t	47.6t	47.8t
$>CR_2-$	(D)	33.6d	(33.5)	72.8s	72.8s	33.4d
CH_3-C	(A)	20.4q	(20.4)	20.4q	20.5q	20.5q
	(C)	19.9q	(19.8)	20.1q	15.5q	15.5q
	(D)	19.6q	(19.5)	27.4q	27.4q	20.1q
	(D)	19.3q	(19.2)	26.9q	26.8q	19.4q
	(F)	19.0q	(18.9)	19.1q	18.7q	18.6q
$CH_3-C=$	(E)	14.4q	(14.4)	14.4q	14.6q	14.6q
	(B)	13.9q	(13.8)	13.8q	13.8q	13.9q

The experimental conditions are described in Experimental section. Abbreviations, s, d, t, and q represent singlet, doublet, triplet, and quartet, respectively, observed in the ^1H single-frequency off-resonance decoupled spectra.

* Assignments should be interchanged in Ref. 16).

shown in Table 1.

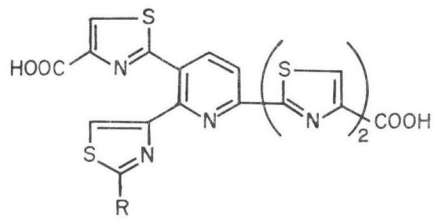
From the above results, the structural units A, B, E and F of micrococcin P_1 are commonly present in thiocillins I, II, and III, and the structural unit D of micrococcin P_1 is present in a modified form in thiocillins I and II. However, the difference between micrococcin P_1 and thiocillin III and also between thiocillins I and II could not be clarified by these degradative studies. The differences were assumed to be present in structural unit C.

100 MHz ^1H NMR spectra of thiocillins I, II and III were measured in $CDCl_3$ and compared with that of micrococcin P_1 (Fig. 3). In a survey of these spectra, characteristic differences were shown in the region of methyl signals. A quite clear difference from others was shown in the spectra of thiocillins II and III, in which singlet signals due to OCH_3 were observed at δ_H 2.84 and 2.78, respectively.

25 MHz ^{13}C NMR spectra of thiocillins I, II, III and micrococcin P_1 were measured in a mixture of $CDCl_3$ - CD_3OH (4: 1) and compared with the literature data[16] of micrococcin P_1 (Table 2). The signals of micrococcin P_1 are substantially the same for the observed and the previously reported data. Most signals of thiocillins I, II and III agreed well with those of micrococcin P_1, but some apparently different signals (marked by underline in Table 2) were observed in higher fields. The OCH_3 signals were apparently observed in the spectra of thiocillins II and III. The ^{13}C signals were assigned on taking account of the findings obtained by the above degradation experiments. By assuming that a modification in structural unit D occurs in thiocillins I and II (–CH< converted to –C(OH)<), which was already proved, and that a modification in unit C occurs in thiocillins II and III (NH–CH–CH(OH)–CH_3 converted to NH–CH–CH(OCH_3)–CH_3), the differences in ^{13}C signals of these antibiotics were adequately explained. Furthermore, these considerations made it possible to assign the ^{13}C signals more precisely (see Scheme 1 and Table 2).

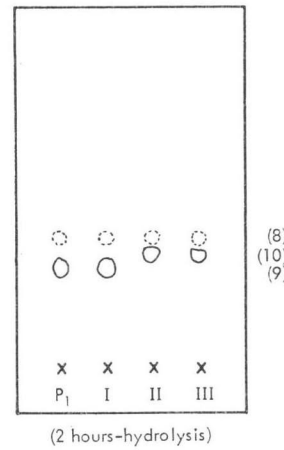
Micrococcinic acid [(**8**) in Scheme 3] is considered to be derived from structural unit C through elimination reaction of H_2O or CH_3OH during acid hydrolysis under the usual hydrolysis conditions. The constituent compounds (**9**) and (**10**) in Scheme 3 may be isolated by milder acid hydrolysis, but isolation

Scheme 3.



(8) R=–CO–CH_2–CH_3 (Micrococcinic acid)
(9) R=–CH(NH_2)–CH(OH)–CH_3
(10) R=–CH(NH_2)–CH(OCH_3)–CH_3

Fig. 4. Thin-layer chromatograms of acid insoluble fractions.
Precoated silica gel plate (Merck silica gel F_{254}), chroloform - ethanol - 14% ammoniacal water (4: 7: 2). P_1: Micrococcin P_1, I: Thiocillin I, II: Thiocillin II, III: Thiocillin III.



of these compounds has not yet been reported. We attempted the isolation of these compounds. When micrococcin P_1 and thiocillins I, II and III were hydrolyzed for a shorter period and the acid insoluble fractions were examined by TLC, beside micrococcinic acid two additional UV absorbing substances were detected by TLC (Fig. 4); one was found in the samples from micrococcin P_1 and thiocillin I and the other from thiocillins II and III. These were predicted to be compounds (**9**) and (**10**), respectively. On isolation the structures were confirmed by ^{1}H NMR spectra.

From these studies, the structures of thiocillins I, II and III are assigned as in Scheme 1 on the basis of the proposed structure of micrococcin P_1.

Experimental

Amino acid analysis was carried out with an amino acid analyzer Hitachi KLA-5. For acid and neutral amino acids, the normal conditions directed for the instrument were used, but for basic amino acids slight modification was made; *i.e.*, a column, 0.7×25 cm, packed with Hitachi Custom Ion-Exchange Resin 2613, was used with 0.35 M citrate buffer, pH 6.0.

Measurements of ^{1}H and ^{13}C NMR Spectra of Thiocillins I, II and III and Micrococcin P_1

^{1}H NMR spectra were taken with a Varian XL-100-12A NMR spectrometer at 100.058 MHz using $CDCl_3$ solutions containing tetramethylsilane (TMS) as an internal standard in 5-mm spinning tubes at ordinary probe temperatures. Usual FT NMR measurement conditions were as follows: spectral width, 1500 Hz; acquisition time, 2 s; pulse width, 10 μs (pulse flipping angle, 33°); number of data points, 6144; number of transients, *ca.* 1000.

^{13}C NMR spectra were recorded on a Varian XL-100-12A NMR spectrometer at 25.16 MHz using $CDCl_3$ - CD_3OH (4: 1) solutions containing TMS as an internal standard in 5-mm spinning tubes at 70°C. Usual FT NMR measurement conditions were as follows: spectral width, 6038 Hz; acquisition time, 0.6 s; pulse width, 18 μs (pulse flipping angle, 43°); number of data points, 7,419; numbers of transients, 40,000~100,000. The ^{13}C NMR signals were assigned by using known chemical-shift rules and ^{1}H single-frequency off-resonance decoupling techniques. Chemical shifts are expressed by δ values (ppm downfield from TMS). Accuracies of δ_H and δ_C are within 0.01 and 0.1 ppm, respectively. The ^{13}C chemical shift data are listed in Table 2.

Acid Hydrolysis of Thiocillins I, II and III and Micrococcin P_1

A few milligrams each of thiocillins I, II, III and micrococcin P_1 were hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzates were diluted with water and the resulted precipitates (acid insoluble fraction) were collected by centrifugation. The supernatants were extracted with ethyl ether to give ether soluble fractions and acid soluble fractions as illustrated in Fig. 1.

The acid insoluble fractions were dissolved in dilute aqueous ammonia and examined with TLC on a precoated silica gel plate (Merck silica gel F_{254}) with chloroform - ethanol - 14% ammoniacal water (4: 7: 2). A UV absorbing spot with Rf value of *ca.* 0.40 was shown in all samples and identified with the authentic micrococcinic acid as already has been reported[17]. However, when the hydrolysis time was shortened (2 hours), two additional UV absorbing spots were shown; one in the samples from micrococcin P_1 and thiocillin I and the other in the samples from thiocillins II and III (Fig. 4). These were determined to be compounds (**9**) and (**10**), respectively, as described later.

The four samples of ether soluble fraction were also examined by TLC as above. All the samples gave a UV absorbing spot with Rf value of *ca.* 0.48. It was presumed to be 2-propionylthiazole-4-carboxylic acid (**1**) based on the results of previous studies on micrococcin P_1[2,3,16].

The acid soluble fractions were examined with TLC on a precoated cellulose plate (Eastman Chromatogram Sheet) with *n*-butanol - acetic acid - water (3: 1: 1) and with an automatic amino acid analyzer. Identical results were shown with the samples of micrococcin P_1 and thiocillin III. The three ninhydrin positive spots (left side of Fig. 2–1) were identified or presumed to be threonine (no UV absorption), 2-hydroxypropylamine[16] (no UV absorption) and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (**3**)[2,16], respectively. In the samples of thiocillins I and II, an unknown ninhydrin positive and UV absorbing spot was detected instead of (**3**). This compound was identified as 2-aminomethylthiazole-4-carboxylic acid (**5**) as described later. When the hydrolysis time was shortened (1 ~ 5 hours), an additional ninhydrin positive and UV absorbing spot was shown accompanied with decreased amounts of (**5**) in thiocillins I and II (the right side of Fig. 2–1). This compound was proved to be 2-(1-amino-2-hydroxy-2-methylpropyl)thiazole-4-carboxylic acid (**4**) as described later. In the amino acid analyses of these samples, good correspondence to the results in TLC experiments was observed and the amount of threonine was determined to be *ca.* 0.80 μmoles per mg of all antibiotics. The retention times of these compounds (Table 1) were determined using the preparations isolated from the TLC except for threonine.

Acid Hydrolysis of Reduced Thiocillins I, II and III and Micrococcin P_1

Some 4 mg each of the above antibiotics were dissolved in 400 μl of a mixture of tetrahydrofuran and methanol (6: 1). Some 4 mg of sodium borohydride was added and the solution was stirred at room temperature for 7 hours. Then a few mg of acetic acid was added to the solution to degrade excess reagent. The reaction mixture was diluted with water, and extracted with *n*-butanol. The extract was washed with water and concentrated to dryness to give the reduced products of the four antibiotics.

The reduced antibiotics were hydrolyzed for 20 hours and the hydrolyzates were examined in the same manner as above. Micrococcinic acid was found in all the acid insoluble fractions. A trace amount of (**1**) was found in all the ether soluble fractions.

The acid soluble fractions of the four samples gave two additional ninhydrin positive spots in comparison with that in the case of the native antibiotics (Fig. 2–2). One was no UV absorbing substance and identified with α-aminobutyric acid by direct comparison with the authentic specimen in amino acid analysis and the amount was estimated to be *ca.* 0.70 μmoles per mg of the reduced antibiotics. The other showed UV absorption ($\lambda_{max}^{0.01N\ HCl}$: 247 nm) and was presumed to be 2-(1-aminopropyl)thiazole-4-carboxylic acid (**2**)[16]. Good correspondence in amino acid analysis to the results in TLC was also observed.

Isolation of 2-Aminomethylthiazole-4-carboxylic Acid (**5**)

Thiocillin II (150 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was evaporated and then diluted with water. The resulted precipitate was removed by centrifugation and the supernatant was concentrated to dryness. The residue was subjected to TLC on a precoated silica gel plate (Merck silica gel F_{254}) with *n*-butanol - acetic acid - water (4: 1: 2) by continuous flow development for 16 hours. The zone of compound (**5**) was cut out and extracted with

acidified 50% aqueous methanol. This was further purified by TLC on a precoated cellulose plate (Eastman Chromatogram Sheet) with *n*-butanol - acetic acid - water (4: 1: 2). The zone of compound (**5**) (Rf 0.37) was extracted with acidified 50% aqueous methanol. The extract was evaporated and then adsorbed on a small column of Dowex 50×8 (NH_4^+) at pH 2.0. The column, after washing with water, was eluted with 0.3 N ammoniacal water. Ninhydrin positive fractions were collected and concentrated to dryness. The resultant residue was dissolved in dilute hydrochloric acid and again concentrated to a residue, which was then crystallized from water - ethanol. The hydrochloride of compound (**5**) was obtained as a colorless crystalline powder (3 mg).

It showed a UV maximum at 247 nm in 0.01 N HCl. In the 100-MHz ^1H NMR spectrum measured in D_2O, only two peaks, δ_H 4.57 (2H, s, H_2NCH_2–) and 8.46 (1H, s, a proton of a thiazole ring), were shown.

Some 2 mg of the above compound was dissolved in pyridine - water (1: 1). Acetic anhydride was added to the solution, which was stirred for 2 hours at 0°C. The reaction mixture was evaporated under reduced pressure, and the residue was dissolved in methanol. Etherial solution of diazomethane was added to the solution and stood for 1 hour at 0°C. The reaction mixture was evaporated to dryness and separated by TLC on the precoated silica gel plate with chloroform - methanol (9: 1). A zone detected by UV absorption (Rf 0.44) was extracted with chloroform - methanol (1: 1). The extract was evaporated to a residue, which was again extracted with a small volume of chloroform. Evaporation of the solvent gave the *N*-acetyl methyl ester derivative of compound (**5**) as a colorless oil. It gave a molecular ion peak at *m/z* 214 in the mass spectrum.

From the above results, the structure of compound (**5**) was confirmed.

Isolation of 2-(1-Amino-2-hydroxy-2-methylpropyl)thiazole-4-carboxylic Acid (**4**)

Thiocillin II (100 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 2 hours. The hydrolyzate was evaporated and then diluted with water. By centrifugation, the precipitate was removed. The supernatant was concentrated to a residue, which was subjected to TLC on a precoated cellulose plate with *n*-butanol - acetic acid - water (4: 1: 2). The zone of compound (**4**) (Rf 0.56), being overlapped with 2-hydroxypropylamine, was cut out and extracted with acidified 50% aqueous methanol. The extract was concentrated to a residue, which was dissolved in 0.01 N HCl and adsorbed on a small column of Dowex 50×8 (NH_4^+). When the column was eluted with 0.3 N ammoniacal water, compound (**4**) was eluted, faster than 2-hydroxypropylamine. Evaporation and lyophilization of the eluate gave a colorless powder (7 mg) of compound (**4**).

In the UV spectrum measured in 0.01 N HCl, a maximum at 247 nm was shown. In the 100-MHz ^1H NMR spectrum measured in D_2O, signals at δ_H 1.25 (3H, s, $-CH_3$), 1.40 (3H, s, $-CH_3$), 4.72 (1H, s, $-CH(NH_2)-$) and 8.09 (a proton of a thiazole ring) were shown. In the 25-MHz ^{13}C NMR spectrum measured in D_2O, the following signals were observed: δ_C 169.4, 163.4 (–COOH and thiazole –C<S, N>), 153.2 (thiazole >C=N), 127.1 (thiazole CH<S>), 71.7 (>C(OH)–), 61.1 ($-CH(NH_2)-$), 26.5 and 25.6 ($-CH_3$).

Some 2 mg of the above compound was converted to the *N*-acetyl methyl ester derivative in the same manner as the above section. A mass spectral ion peak at *m/z* 273 (M+1) was observed.

From these results, the structure of compound (**4**) was confirmed.

Conversion of Compound (**4**) to Compound (**5**)

Some 1 mg of compound (**4**) was processed by hydrolysis procedure with constant boiling hydrochloric acid at 110°C for 20 hours. When the product was examined by TLC on the cellulose plate with *n*-butanol - acetic acid - water (4: 1: 2), a predominant amount of compound (**5**) was found by ninhydrin reaction, beside a small amount of compound (**4**). By automatic amino acid analysis, the ratio of compounds (**5**) and (**4**) was estimated to be approximately 4: 1 from the peak areas.

Isolation of Compound (**9**)

Thiocillin I (53 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 2 hours. The hydrolyzate was separated into acid soluble and insoluble fractions in the same manner as in the above section. The acid insoluble fraction was dissolved in a dilute NH_4OH and subjected to TLC on a

precoated silica gel plate with chloroform - ethanol - 14% ammoniacal water (4: 7: 2). A UV absorbing zone (Rf 0.30) was extracted with chloroform - ethanol - 14% ammoniacal water (1: 2: 1). The residue obtained by evaporation of the solvent was dissolved in a dilute ammoniacal water and charged on a small column of Amberlite XAD-2. After washing with water, the column was eluted with a mixture of methanol and 3% ammoniacal - water (1: 1). The eluate fractions showing UV absorption were collected and concentrated under reduced pressure. Finally lyophilization gave a colorless powder (10 mg) of compound (**9**). Disodium salt of compound (**9**) was obtained by dissolving in an equivalent amount of sodium hydroxide solution and then lyophilization.

The 100-MHz ^{1}H NMR spectrum was measured in D_2O. The signals at δ_H 0.96 (3H, d, J=6.0, $-CH_3$), 3.77 (1H, m, -CH(OH)-), 4.12 (1H, d, J=6.7, $-CH(NH_2)-$), accounts for the partial structure $-CH(NH_2)-CH(OH)-CH_3$. Two ^{1}H signals for the pyridine ring were observed at δ_H 7.62 and 7.88 (2H, ABq, J=8.2) and four ^{1}H signals for thiazole rings were found as singlets at δ_H 7.66, 7.79, 7.96 and 8.00. Similar signals observed in the spectrum of dimethyl micrococcinate measured in CD_3OD were used as a reference for the above assignment.

Isolation of Compound (**10**)

Thiocillin II (100 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 2 hours. The hydrolyzate was separated into acid soluble and insoluble fractions in the same manner as above.

The acid insoluble fraction was purified by TLC followed by adsorption and elution on an Amberlite XAD-2 column in essentially the same manner as in the preceding section. The free form of compound (**10**) was obtained as a colorless amorphous powder (9.5 mg). The disodium salt of compound (**10**) was prepared by the same manner as above.

The 100-MHz ^{1}H NMR spectrum was measured in D_2O. In this case, resolution of the signals were not so fine, but the following were observed: δ_H 0.84 (3H, d), ~3.3 (1H, m), 3.35 (3H, s), 4.37 (1H, d), these imply the partial structure $-CH(NH_2)-CH(OCH_3)-CH_3$, two pyridine-ring proton signals at δ_H 7.29 and 7.60, and four thiazole-ring proton signals at δ_H 7.56, 7.65, 7.84 and 8.51.

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