CHICAMYCIN*, A NEW ANTITUMOR ANTIBIOTIC

II. STRUCTURE DETERMINATION OF CHICAMYCINS A AND B

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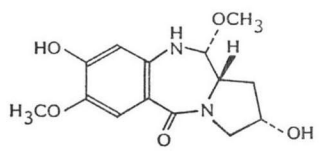
Structures of chicamycins A and B have been determined from a series of chemical degradation studies coupled with spectroscopic analysis. Chicamycin A is 2(*S*),11(*R*),11a(*S*)-1,2,3,10,11,11a-hexahydro-2,8-dihydroxy-7,11-dimethoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one, and chicamycin B is 2(*S*),11a(*S*)-1,2,3,11a-tetrahydro-2,8-dihydroxy-7-methoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one which is the demethanol form of chicamycin A. The structure of chicamycin B is closely related to that of neothramycin, differing only in the position of a hydroxyl substituent on the pyrrolidine ring.

Chicamycin is a new member of the pyrrolobenzodiazepine family of antibiotics elaborated by a strain of *Streptomyces* sp. (J576-99). It was obtained in two active forms, chicamycins A and B, depending upon the isolation procedure used. Both forms of the antibiotic exhibit an antitumor effect on murine leukemia along with weak antimicrobial activity against some Gram-positive bacteria. The taxonomy of strain J576-99, production, isolation and chemical and biological properties of chicamycin have been reported in the preceding paper[1]. This paper presents evidence that chicamycins A and B have the structures shown in Fig. 1.

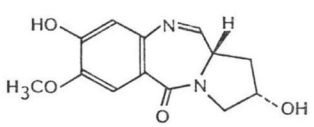
General Structural Characteristics

The UV absorption maxima of chicamycins A (**Ia**) and B (**Ib**) were observed at 223, 237, 262 and 317 nm in methanol which resembled those of neothramycin[2] and tomaymycin[3] but differed from those of sibiromycin[4], anthramycin[5] and mazethramycin[6] (Fig. 2). Thus, the chromophore part of chicamycin was assumed to be similar to that of neothramycin or tomaymycin. The mass spectrum of **Ia** showed the molecular ion peak at *m/z* 294 and the base peak ion at *m/z* 262, corresponding to a loss of a methanol unit from the molecule. The molecular ion of **Ib** appeared at *m/z* 262 and the fragment ions of **Ib** were almost consistent with those of **Ia** suggesting that **Ib** should be a demethanol form of **Ia**. This was evidenced by comparison of their ^{1}H NMR spectra: the ^{1}H NMR spectrum of **Ib** lacked one of the two OCH_3 (δ 3.30 ppm) groups and the NH proton (δ 7.94 ppm) observed with **Ia**, while an ad-

Fig. 1. Structures of chicamycins A and B.



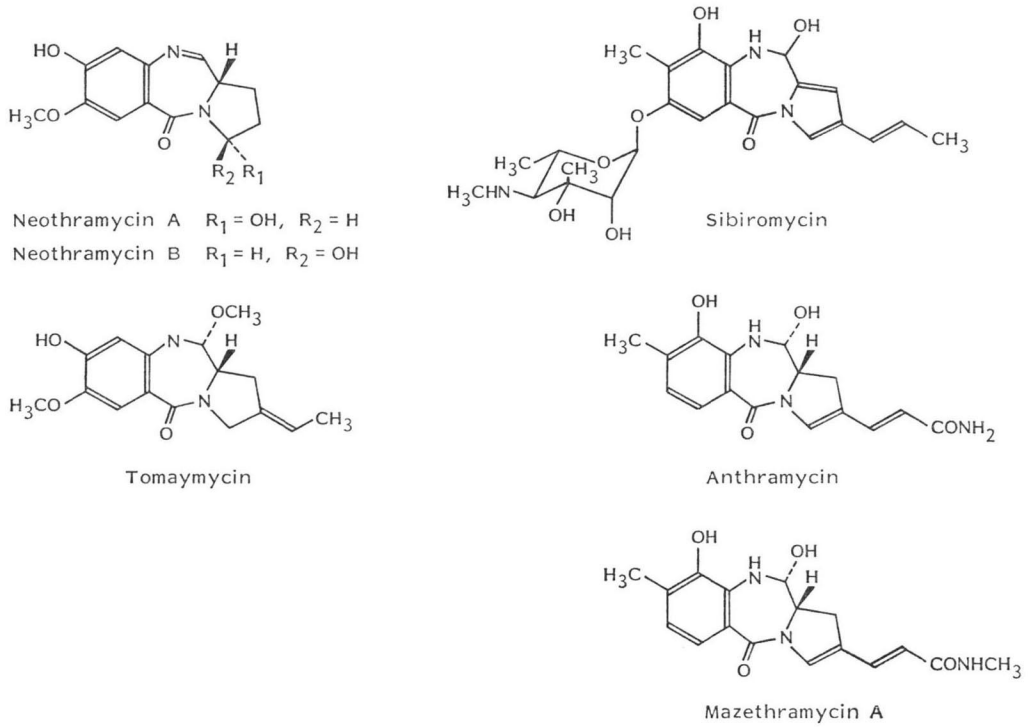
Chicamycin A (**Ia**)



Chicamycin B (**Ib**)

* This antibiotic was originally designated as BBM-2040.

Fig. 2. Structures of pyrrolo-[1,4]-benzodiazepine antibiotics.



ditional double bond proton (δ 8.24 ppm) was present in the spectrum of **Ib**. **Ib** was prepared from **Ia** in a good yield when **Ia** was treated with pyridine at room temperature, while **Ib** was converted to **Ia** by heating with methanol. The present structure study was performed mostly with crystalline chicamycin A (**Ia**).

^{1}H NMR and ^{13}C NMR Spectra of Chicamycin A

The 360 MHz NMR of **Ia** was determined in pyridine-d_5. The observed signals and their assignments are given in Table 1. The signals assignable to two aromatic protons (6.88 and 8.17 ppm), one NH (7.94), one phenolic OH (11.68) and two OCH_3 (3.30 and 3.75) are very similar to the corresponding signals of tomaymycin determined under the same conditions. The proton on the carbinolamine carbon (H_{11}, 4.77) resonated as a doublet which collapsed into a singlet upon irradiation of the NH proton. The lack of coupling between H_{11} and H_{11a} is commonly observed in the anthramycin-tomaymycin group of antibiotics which have 11-*R* and 11a-*S* configurations[11]. Thus it seems that the 1,4-benzodiazepine moiety of chicamycin A is identical with that of tomaymycin. In the ^{1}H NMR spectrum, the alcoholic proton appeared as a doublet at 6.34 ppm. The decoupling experiment revealed that the alcoholic proton coupled with a methine proton at 4.53 ppm (H_2) which, in turn, coupled with the high-field methylene protons (δ 2.39, H_{1A} and 2.57, H_{1B}) and also a proton at 4.14 ppm (H_{3A}). Irradiation of either of the non-equivalent methylene protons (H_{1A} and H_{1B}) converted a triplet proton at 4.08 ppm (H_{11a}) into a doublet, accompanied by a significant change in the coupling pattern of H_2 proton. Therefore, it was concluded that the secondary hydroxyl group of chicamycin A was located at C-2 of the pyrrolidine ring.

The ^{13}C NMR spectrum of chicamycin A supported the structure as determined by the ^{1}H NMR analysis. The presence of 14 carbon signals was indicated by the ^{13}C NMR spectrum as shown in Table

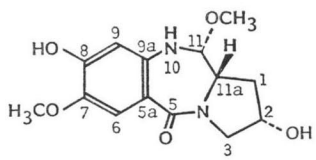
Table 1. ^{1}H NMR (360 MHz) of chicamycin A (in pyridine-d_5).

Chemical shift δ (ppm)	Proton	Coupling multiplicity (J: Hz)	Assignment
2.39	1H	m	H_{1A}
2.57	1H	m	H_{1B}
3.30	3H	s	C_{11}-OCH_3
3.75	3H	s	C_7-OCH_3
4.08	1H	t (8.1)	H_{11a}
4.14	1H	dd (12.0 & 5.8)	H_{3A}
4.48	1H	dd (12.0 & 6.0)	H_{3B}
4.53	1H	m	H_2
4.77	1H	d (J=6.4)	H_{11}
6.34	1H	d (J=7.4)	C_2-OH
6.88	1H	s	H_9
7.94	1H	d (J=6.4)	N_{10}-H
8.17	1H	s	H_8
11.68	1H	s	C_3-OH

Table 2. ^{13}C NMR of chicamycin A (in pyridine-d_5).

Carbon	Chemical shift (δ: ppm)	Multiplicity on off-resonance
1	24.4	t
2	53.3	d
3	41.9	t
5	151.7***	s
5a	126.8*	s
6	90.0	d
7	137.5	s
8	150.1***	s
9	101.6	d
9a	125.4*	s
11	73.4	d
11a	43.4	d
7-OCH_3	41.4**	q
11-OCH_3	38.9**	q

*, **, ***: Assignments may be interchangeable.



2. Although most of the signals appeared at considerably lower field than the corresponding carbons of the related antibiotics determined in deuterodioxane or DMSO-d_6, the carbon signals of chicamycin A were assigned as shown in Table 2 on the basis of off-resonance decoupling and comparison with the literature data of neothramycin[7] and pretomaymycin[8].

Acid Hydrolysis of Chicamycin A (**Ia**)

In order to confirm the structure assigned by the ^{1}H NMR and to determine the configuration of C_2-OH, cleavage of **Ia** was carried out by two routes. On acetylation in pyridine (Fig. 3), **Ia** afforded the di-*O*-acetyl-demethanol derivative **II**, (M^+ m/z 346), indicating the presence of two acetylable hydroxyl groups in **Ia**. The removal of a methanol unit from **Ia** occurred during acetylation as was indicated by the ^{1}H NMR spectrum. This was verified by the fact that the same acetyl derivative was obtained by acetylation of **Ib**. **II** was treated with *m*-chloroperbenzoic acid at -20°C for 3 hours[9]. After removal of the acidic by-products by washing with alkali, the reaction mixture was chromatographed on silica gel to give the pure oxo-compound **III** (M^+ m/z 362). The ^{1}H NMR spectrum of **III** indicated the absence of N=CH (7.83 ppm, doublet) observed for **II** and the appearance of NH-CO (9.07 ppm, singlet). The IR spectrum in KBr showed a new amide carbonyl band at around 1690~1700 cm^{-1}.

Fig. 3. Acid degradation of chicamycin A.

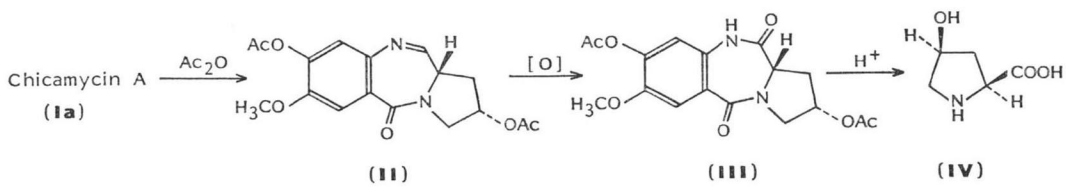


Table 3. Physico-chemical properties of compound **IV**, *cis*-4-hydroxy-L-proline and *trans*-4-hydroxy-L-proline.

	Compound **IV**	*cis*-4-OH-L-proline	*trans*-4-OH-L-proline
Mp	258~259°C (dec)	257~258°C (dec)	273~275°C (dec)
$[\alpha]_D^{24}$ (*c* 1.0, H_2O)	−51.5°	−56.5°	−75.3°
TLC Solvent A*1 Rf	0.30	0.30	0.35
B*2	0.46	0.46	0.60
Amino acid analysis (minutes) Rt	43	43	34
Anal	Calcd for $C_5H_9NO_3$: C 45.80 H 6.92 N 10.68	Found: 45.68 6.71 10.34	

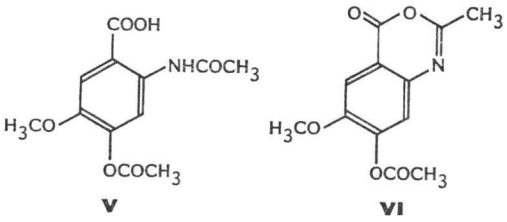
*1 Solvent A: Phenol - H_2O (4: 1)
*2 Solvent B: 10% $AcONH_4$ - acetone - conc NH_4OH (9: 10: 1)

III was heated under reflux with 6 N HCl for 20 hours. The hydrolysate was charged on a column of Dowex 50WX4 which was developed with 0.02 N HCl. The eluted amino acid (**IV**) was identified as *cis*-4-hydroxy-L-proline by physico-chemical data and co-chromatography. It was differentiated from the *trans* isomer (Table 3). The IR and ^1H NMR spectra of **IV** were superimposable with those of an authentic sample.

Alkaline Hydrolysis of Chicamycin A (**Ia**)

The aromatic ring moiety of chicamycin A was not isolated in the above described acid hydrolysis, presumably due to acid instability of the fragment. When **Ia** was hydrolyzed in 1.0 N NaOH under a nitrogen atmosphere, the reaction mixture contained 4-hydroxy-5-methoxyanthranilic acid[7] as indicated by TLC, though the acid was unstable and could not be isolated as a pure solid. The reaction mixture was, therefore, acetylated *in situ* in pyridine and the product purified by silica gel chromatography to yield two acetyl derivatives, **V** (minor) and **VI** (major). The ^1H NMR and MS (M^+ *m/z* 267) revealed that **V** was 4-acetoxy-5-methoxy-*N*-acetylanthranilic acid. The major product **VI** was crystallized as colorless needles from methanol. The molecular formula of $C_{12}H_{11}NO_5$ was assigned to **VI** by MS spectrum (M^+ *m/z* 249) and microanalysis. The IR spectrum of **VI** lacked the amide band at 1690 cm^{-1} which was present in **V**. **VI** is, therefore, 7-acetoxy-6-methoxy-2-methyl-4*H*-3,1-benzoxazin-4-one formed by cyclization of diacetate **V** (Fig. 4).

Fig. 4. Alkaline degradation products from chicamycin A.



Structures of Chicamycins A (**Ia**) and B (**Ib**)

The above hydrolytic results provided confirmation of the structure of **Ia** as deduced by the ^1H NMR analysis. The stereochemistry at 2 and 11a were established both as *S*-configuration since *cis*-4-hydroxy-L-proline was isolated by the acid hydrolysis of **Ia**. As stated before, the lack of splitting between H_{11} and H_{11a} in the ^1H NMR of **Ia** supported an *R*-configuration for C_{11}. Thus, the structure of **Ia** was assigned as 2(*S*),11(*R*),11a(*S*)-1,2,3,10,11,11a-hexahydro-2,8-dihydroxy-7,11-dimethoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one. **Ib** has been determined as a demethanol derivative of **Ia**. The ^1H NMR

of **Ib** indicated an azomethine structure (–N=CH–) leading to the assignment that **Ib** is 2(*S*),11a(*S*)-1,2,3,11a-tetrahydro-2,8-dihydroxy-7-methoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one.

Discussion

Chicamycin is a new member of the pyrrolobenzodiazepine family of antibiotics. As often observed in this group of antibiotics, chicamycin was isolated as either the natural azomethine form (chicamycin B) or its methanol adduct form (chicamycin A). The 1,4-benzodiazepine group of antibiotics may be further divided into 3 subgroups by the substitution pattern on the benzene ring: namely (1) the anthramycin-mazethramycin group, (2) the tomaymycin-neothramycin group and (3) the sibiromycin group. Chicamycin is closely related to neothramycin differing only in the position of a hydroxyl group on the pyrrolidine ring. Neothramycin has a hydroxyl group at the C-3 forming a carbinol amine structure there, whereas chicamycin possesses an α-hydroxyl group at C-2. The greater stability of chicamycin than neothramycin might partly be attributed to the absence of a carbinol amine structure in the pyrrolidine ring. The C_2-β-hydroxyl analog of chicamycin has been synthesized by scientists of Fujisawa[10]. Recently, KANEKO *et al.* of Bristol-Myers prepared the α- and β-hydroxyl isomers of chicamycin and compared their activity (personal communication: A new process for the synthesis of pyrrolo[1,4]-benzodiazepine antitumor antibiotics). It is interesting that the natural α-analog showed higher activity than the corresponding β-form.

Experimental

Acetylation of Chicamycins A (**Ia**) and B (**Ib**) to **II**

A solution of **Ia** (500 mg) in acetic anhydride (2 ml) and pyridine (3 ml) was stirred at room temperature for 4 hours and the mixture was concentrated *in vacuo* to dryness. The residue was dissolved in 1 ml of ethyl acetate and applied on a column of silica gel (ϕ 1.0×50 cm) which was developed with ethyl acetate. Upon monitoring by TLC with solvent system of EtOAc - MeOH (4: 1), the appropriate fractions were pooled and evaporated *in vacuo* to afford 508 mg of diacetyldemethanolchicamycin A (**II**) as white powder. Mp 110~112°C. TLC (EtOAc - MeOH, 4: 1) Rf 0.52. UV λ_{max}^{MeOH} 220 nm (ε 27,000) 243 (sh, 16,200), 320 (3,800). IR ν_{max}^{KBr} 1765, 1738, 1628 cm^{-1}. ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm 2.10 (3H, s), 2.32 (3H, s), 2.4~2.6 (2H, m), 3.5~4.2 (2H, m), 3.88 (3H, s), 5.3~5.5 (2H, m), 7.01 (1H, s), 7.55 (1H, s), 7.82 (1H, d). MS *m/z* 346 (M^+), 304, 286, 244 *etc.*

Anal Calcd for $C_{17}H_{18}N_2O_6 \cdot H_2O$: C 56.04, H 5.53, N 7.69.
Found: C 56.87, H 5.33, N 7.31.

Chicamycin B (**Ib**, 450 mg) was acetylated by an analogous procedure to yield 313 mg of acetate which was identical with **II** by physico-chemical properties.

Oxidation of Diacetyldemethanolchicamycin A (**II**)

A solution of *m*-chloroperbenzoic acid (900 mg) in 5 ml of CH_2Cl_2 was added dropwise into a solution of **II** (1.39 g) in 5 ml of CH_2Cl_2 at −20°C under vigorous stirring. The mixture was stirred for 3 hours at −20°C, then warmed up to room temperature and filtered. After being washed with saturated $NaHCO_3$ solution to remove acidic products, the solution was evaporated *in vacuo* to a sticky solid which showed a major spot at Rf 0.54 on TLC (EtOAc - MeOH, 4: 1, UV irradiation). The solid was charged on a column of silica gel (ϕ 3.0×40 cm) which was developed with EtOAc. The appropriate fractions were pooled and concentrated *in vacuo* to afford 470 mg of oxo-compound **III**. Mp 130~132°C. TLC (EtOAc - MeOH, 4: 1) Rf 0.54. UV λ_{max}^{MeOH} 228 nm (ε 23,500), 257 (sh, 10,100), 307 (3,600). IR ν_{max}^{KBr} 1770, 1740, 1700, 1635, 1615 cm^{-1}. ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm 1.95 (3H, s), 2.0~2.5 (2H, m), 2.32 (3H, s), 3.85 (3H, s), 3.7~4.2 (2H, m), 5.25 (2H, m), 6.75 (1H, s), 7.48 (1H, s), 9.07 (1H, s). MS *m/z* 362 (M^+), 320, 302, 260, 242 *etc.*

Anal Calcd for $C_{17}H_{18}N_2O_7 \cdot \frac{1}{2}H_2O$: C 54.97, H 5.16, N 7.54.
Found: C 54.50, H 4.91, N 7.20.

Acid Hydrolysis of Oxo-compound (III)

Compound **III** (470 mg) was heated with 2 ml of 6 N HCl at 105°C for 20 hours in a sealed tube. The hydrolysate was diluted with water and passed through a column of Diaion HP-20 (ϕ 1.0×6 cm) to remove lipophilic products. The spent solution and water washes were combined, decolorized with activated charcoal and concentrated to dryness. The residue was chromatographed on a column of Sephadex G-15 (ϕ 1.0×55 cm) developing with water. The ninhydrin-positive fractions were pooled and concentrated *in vacuo* to give white solid of **IV** which was crystallized from aqueous ethanol solution, 26.5 mg. Mp 258~259°C (dec). $[\alpha]_D^{24}$ −51.5° (*c* 1.0, H_2O). IR ν_{max}^{KBr} 3200, 2940, 1630, 1570, 1438, 1390 cm^{-1}. ^1H NMR $\delta_{DSS}^{D_2O}$ ppm 2.2~2.6 (2H, m), 3.42 (2H, dd), 4.20 (2H, dd), 4.62 (1H, m). Identified as *cis*-4-hydroxy-L-proline by TLC, ^1H NMR and optical rotation value.

Alkaline Hydrolysis of Chicamycin A (Ia)

A solution of **Ia** (213 mg) in 20 ml of 1 N NaOH was refluxed for 1 hour under nitrogen atmosphere. The solution was cooled in an ice bath, diluted with 50 ml of water and extracted with two 50 ml-portions of 1-BuOH at pH 5.0. Evaporation of the 1-BuOH extract afforded a sticky solid which contained 4-hydroxy-5-methoxyanthranilic acid by TLC. This solid was acetylated with acetic anhydride (1 ml) and pyridine (3 ml) at room temperature. After addition of 30 ml of water, the reaction mixture was extracted twice with 30 ml of $CHCl_3$. The extracts were combined, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a sticky residue which was chromatographed on a column of silica gel (ϕ 1.0×40 cm). Elution of the column with *n*-hexane - acetone (95: 5) gave the major acetate **VI** which, upon crystallization from MeOH, afforded 44 mg of colorless needles of **VI**. Subsequent elution with *n*-hexane - acetone - MeOH (9: 9: 2) afforded the minor acetate **V** which contained impurities by TLC. This acetate was further chromatographed on a column of silica gel (ϕ 1.0×35 cm) with $CHCl_3$ - AcOH (100: 1) elution to give white powder of **V** (7.7 mg).

Compound **V**: Mp 126~127°C. IR ν_{max}^{KBr} 2920, 1760, 1690, 1640, 1520 cm^{-1} *etc.* UV λ_{max}^{MeOH} 226 nm (ε 19,700), 259 (12,000), 316 (4,100). MS *m/z* 267 (M^+), 249, 225, 207, 192, 183 *etc.* ^1H NMR $\delta_{TMS}^{pyridine\text{-}d_5}$ ppm 2.00 (3H, s), 2.18 (3H, s), 3.58 (3H, s), 7.78, (1H, s), 7.86 (NH), 8.65 (1H, s), 12.57 (OH).

Compound **VI**: Mp 176~178°C. IR ν_{max}^{KBr} 3040, 1775, 1745, 1640, 1500 cm^{-1} *etc.* UV λ_{max}^{MeOH} 231 nm (ε 20,300), 261 (5,500), 277 (sh, 3,000), 318 (2,900), 331 (sh, 2,500). MS *m/z* 249 (M^+), 208, 207, 192 *etc.* ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm 2.34 (3H, s), 2.43 (3H, s), 3.90 (3H, s), 7.13 (1H, s), 7.55 (1H, s).

Anal Calcd for $C_{12}H_{11}NO_5$: C 57.83, H 4.45, N 5.62.
Found: C 57.65, H 4.41, N 5.53.

Acknowledgment

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