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Chetracin A and Chaetocins B and C, Three New Epipolythiodioxopiperazines from *Chaetomium* spp.¹⁾

TAKAO SAITO,^{a,2a)} YASUSHI SUZUKI,^{a,2b)} KIYOTAKA KOYAMA,^{a)}
SHINSAKU NATORI,^{*,a)} YOICHI IITAKA,^{b)}
and TAKESHI KINOSHITA^{c)}

Meiji College of Pharmacy,^{a)} Yato-cho, Tanashi-shi, Tokyo 188, Japan, Faculty of Pharmaceutical Sciences, University of Tokyo,^{b)} Hongo, Bunkyo-ku, Tokyo 113, Japan, and Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,^{c)} Hiro-machi, Shinagawa-ku, Tokyo 140, Japan

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A new dimeric epipolythiodioxopiperazine named chetracin A with two tetrasulfide bridges was isolated from *Chaetomium nigricolor* and *C. retardatum*, and the structure (3) proposed chiefly from proton and carbon-13 nuclear magnetic resonance (¹H- and ¹³C-NMR) data was established by X-ray analysis. Two congeners of chaetocin (2) named chaetocins B and C were isolated from *C. virescens* var. *thielavioideum*, and were proved to be homologs with one disulfide and one trisulfide bridge (9) and with two trisulfide bridges (10), respectively, from spectral data, chiefly NMR and fast atom bombardment mass spectrometry/mass spectrometry (FAB MS/MS). The structure-activity relationships observed for the antibacterial activity and cytotoxicity of the related compounds are discussed.

Keywords—chetracin A; chaetocin B; chaetocin C; epipolythiodioxopiperazine; mycotoxin; cytotoxicity; antibacterial activity; *Chaetomium* spp.; FAB MS/MS; X-ray analysis

Epipolythiodioxopiperazines such as gliotoxin and sporidesmins occur widely as mold secondary metabolites with antiviral and antimicrobial properties as well as high mammalian toxicities.³⁻⁵⁾ The genus *Chaetomium* produces the group of compounds such as chetomin (1) and chaetocin (2).³⁻⁵⁾ In the course of our studies on mycotoxin production by fungi belonging to this genus and related fungi, four strains of *C. thielavioideum* CHEN, and one each of *C. cellulolyticum* CHAHAL et D. HAWKSW., *Farrowia* sp., and *Achaetomiella virescens* v. ARX, the two former species of which have been proved to be synonymous with *C. virescens* (v. ARX) UDAGAWA,⁶⁾ were found to produce a set of mycotoxins, chaetocin (2), chaetochromin, sterigmatocystin, and *O*-methylsterigmatocystin,^{7,8)} while *C. subglobosum* SERG. (now included in *C. globosum*) was found to metabolize chetomin (1).⁸⁾ During this work, extracts of cultures on rice of *C. abuense* LODHA (now assigned to *C. nigricolor* AMES),⁷⁾ *C. tenuissimum* SERGEJEVA,⁷⁾ and *Chaetomium* sp. TRTC 66.1778b (identified as *C. retardatum* CARTER et KHAN)⁸⁾ attracted our attention because of the production of mycotoxins exhibiting a remarkable cytotoxicity to HeLa cells and showing positive spots on thin-layer chromatograms with silver nitrate⁹⁾ and Ehrlich's reagents. It was known that strains of *C. virescens* produce homologous compounds of chaetocin (2) as congeners. This paper describes the characterization and structural elucidation of such compounds belonging to the epipolythiodioxopiperazines.

The dichloromethane extract of the culture on rice of *C. tenuissimum* revealed a strong cytotoxicity and a positive spot on the thin-layer chromatograms. The principle was isolated by silica gel chromatography and identified as chetomin¹⁰⁻¹²⁾ (1), which had been isolated previously from *C. cochliodes* PALLISER, *C. globosum* KUNZE ex FRIES and *C. subglobosum*.⁸⁾

The ethyl acetate extract of the culture on rice of *C. abuense* (*C. nigricolor*) showed the presence of a group of compounds. The extract was washed with hexane and dichloromethane successively and the remaining precipitate gave an epipolythiodioxopiperazine (3), which was identified with that obtained from *C. retardatum* (*vide infra*). Chromatography of the soluble parts of the extracts afforded cochliodinol (4), a bis(prenylindolyl)benzoquinone isolated previously from *C. cochliodes*, *C. globosum*, and *C. elatum* KUNZE ex FRIES.^{8,13)}

The dichloromethane and ethyl acetate extracts of moldy rice with *C. retardatum* also exhibited a noticeable cytotoxicity. The precipitate formed by concentration of the dichloromethane extract was composed chiefly of an epipolythiodioxopiperazine (5). Physical data for the compound (proton and carbon-13 nuclear magnetic resonance (NMR) data, see Tables I and II) indicated that the compound must be 11 α ,11' α -dihydroxychaetocin¹⁴⁾ (melinacidin IV¹⁵⁾) (5) as characterized previously from *Verticillium tenerum* (anamorph of *Nectria inverta*, now placed in *Verticillium lateritium*) and *Acrostalagmus cinnabarinus* var. *melinacidinus* (now placed in *Verticillium lateritium*), and the identity was confirmed by direct comparison with an authentic sample.

The colorless precipitate formed by concentration of the ethyl acetate extract of the moldy rice with *C. retardatum* was composed mainly of a new epipolythiodioxopiperazine named chetracin A (3), identical with that obtained from *C. abuense* (*C. nigricolor*). Due to its poor solubility and instability, the compound was converted to the acetate (3a), which was purified by high-performance liquid chromatography (HPLC) using a Develosil 60-3 column. Treatment of the acetate (3a) with ammonia gave the original compound (3). Reacetylation of 3 yielded the triacetate (3a). Elemental analyses suggested the molecular formula, C₃₆H₃₄N₆O₁₁S₈, for the triacetate (3a). The physical properties, especially the ¹H- and ¹³C-NMR data, of the triacetate (3a) revealed similarities with those of the triacetate (5a) of 11 α ,11' α -dihydroxychaetocin¹⁴⁾ as shown in Tables I and II. These findings suggested that chetracin A (3) might be a tetrathio homolog of dihydroxychaetocin (5). To confirm this, the following reactions were carried out. Treatment of chetracin A (3) and the triacetate (3a) with boiling potassium hydroxide solution gave bi-indol-3-yl (6),¹⁶⁾ while desulfurization of 3 with triphenylphosphine and ammonia (a modified version of the method reported for sporidesmins (7))^{16,17)} gave 11 α ,11' α -dihydroxychaetocin (5). The circular dichroism (CD) spectrum of 3a showed the same sign as 5a, indicating the same absolute configuration (Fig. 1). These facts indicated that chetracin A (3) must have the same nucleus as 11 α ,11' α -dihydroxychaetocin (5) but carry different numbers of sulfur atoms, *i.e.*, four sulfur atoms, for each component.

To confirm the configuration and to elucidate the conformation of the molecule, X-ray analysis of triacetylchetracin A (3a) was performed using a single crystal obtained by the vapor diffusion method. A single crystal of approximate dimensions 0.1 $\times$ 0.08 $\times$ 0.12 mm was cut out from an aggregate and sealed in a thin-walled glass capillary tube to prevent loss of water of crystallization. The diffraction data were obtained using CuK α radiation monochro-

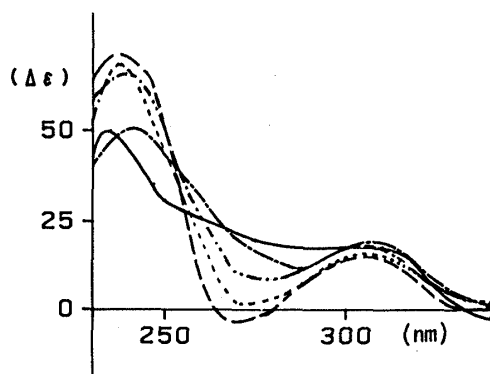


Fig. 1. CD Spectra of Chaetocin Derivatives (in MeOH)

-----, chaetocin diacetate (2a); —, chetracin A triacetate (3a); ·····, dihydroxychaetocin triacetate (5a); — · — ·, chaetocin B diacetate (9a); — — — —, chaetocin C diacetate (10a).

TABLE I. ¹H-NMR Data for Chaetocin Derivatives

Proton at	11 α ,11' α - Dihydroxy- chaetocin (5) ^{a)}	Dihydroxy- chaetocin triacetate (5a)	Chetracin A triacetate (3a)	Chaetocin diacetate (2a)	Chaetocin B diacetate (9a) ^{b)}	Chaetocin C diacetate (10a) ^{b)}	Chaetocin monosulfide diacetate (11a)	Dethiomethyl- thiochaetocin diacetate (12a)	Dethioacetyl- thiochaetocin diacetate (13a)
5a	5.48 (d, $J=4.1$)	5.17, 5.18	5.43, 5.45	5.33	5.46, 5.41 (5.69)	5.48 (5.78)	5.46	5.40	5.46
6	6.49 (d, $J=4.1$)	5.25, 5.28	5.05, 5.01	5.33	<i>ca.</i> 5.3	<i>ca.</i> 5.3	4.90	4.99	4.92
7, 8, 9, and 10	6.54—7.71	6.62—7.92	6.53—7.80	6.72—7.42	6.66—7.42	6.66—7.42	6.73—7.35	6.62—7.37	6.66—7.27
11	4.89	6.97, 5.06	6.69, 4.82	2.75, 3.81 (d, $J=15.1$)	2.56, 3.71 (d, $J=15.4$)	2.69, 3.60 (2.63, 3.30) (d, $J=15.0$)	2.47, 3.63 (d, $J=15.0$)	2.77, 2.86 (d, $J=13.3$)	2.92, 3.67 (d, $J=14.7$)
11-CH ₂ OH or 11-CH ₂ OCOCCH ₃	6.70	5.51, 2.47	4.09, 2.33	—	—	—	—	—	—
17	3.01	3.05, 2.98	2.98, 2.99	3.05	3.06, 3.14 (2.94)	3.15 (2.94)	2.90	3.00	2.98
18	4.21, 4.06 (d, $J=12.7$)	4.60, 4.62 (d, $J=12.7$) 4.85, 4.86 (d, $J=12.7$)	4.55, 4.61 (d, $J=11.5$)	4.61, 4.98 (d, $J=12.7$)	4.63, 4.91 (d, $J=12.7$) 4.30, 4.80 (4.33, 4.92) (d, $J=12.5$)	4.33, 4.80 (4.38, 4.91) (d, $J=12.5$)	4.59, 4.62 (d, $J=15.7$)	4.38, 4.71 (d, $J=11.6$)	4.71, 4.84 (d, $J=11.5$)
18-CH ₂ OH or 18-CH ₂ OCOCCH ₃	5.82	2.11	2.11, 2.14	2.13	2.14, 2.09	2.13	2.02	2.03	2.08
S-CH ₃ or S-COCH ₃	—	—	—	—	—	—	—	1.85, 2.24	1.82, 2.31

a) Measured in DMSO-*d*₆ for 5. b) The chemical shift values in parentheses are those of the minor conformer.

TABLE II. ^{13}C -NMR Data for Chaetocin Derivatives

Carbon at	11 α ,11' α - Dihydroxy- chaetocin (5) ^{a)}	Dihydroxy- chaetocin triacetate (5a)	Chetracin A triacetate (3a)	Chaetocin diacetate (2a)	Chaetocin diacetate (9a) ^{b)}	Chaetocin C diacetate (10a) ^{b)}	Chaetocin monosulfide diacetate (11a)	Dethiomethyl- thiochaetocin diacetate (12a)	Dethioacetyl- thiochaetocin diacetate (13a)
1 ^{c)}	165.0	165.3, 162.6	166.1, 167.2	165.2	165.2, 168.5 (167.1)	168.4 (167.1)	173.2	165.9	165.3
3	77.5 ^{e)}	75.2, 75.0 ^{h)}	76.8	74.7 ^{j)}	74.6, ^{k)} 78.9 ^{j)} (74.7 ^{m)}	79.0 ^{m)} (74.7 ^{o)}	79.8 ^{p)}	68.9 ^{q)}	74.4 ^{r)}
4 ^{c)}	160.7	160.3, 160.1	165.0	160.6	160.9, 163.3 (162.3)	163.5 (162.5)	172.6	162.6	161.9
5a	81.0 ^{f)}	83.0, 82.4	83.2	80.7	81.0, 80.1 (82.1)	80.2 (82.1)	78.5	80.5	81.2
6a	149.9	148.8, 148.3	149.2, 149.5	149.3	149.2, 150.9 (149.3)	150.6 (149.3)	149.2	150.2	150.6
7 ^{d)}	109.2	111.0, 110.5	109.5, 110.0	110.6	110.1, 110.9 (110.1)	110.9 (110.8)	110.7	109.6	110.2
8 ^{d)}	127.5	125.8	125.2	125.2	125.5, 126.5 (125.9)	126.2 (125.9)	124.8	124.4	126.1
9 ^{d)}	129.2	130.5, 130.4	130.7, 130.8	130.2	130.2, 130.9 (130.1)	130.9 (130.1)	130.2	129.6	129.9
10 ^{d)}	118.2	120.4, 120.3	120.1, 119.6	119.9	119.6, 119.5 (120.0)	119.4	120.2	118.5	119.1
10a	129.4	128.19, 128.17	128.6, 128.7	127.4	125.5, 126.2 (127.4)	126.2 (126.9)	128.1	128.3	127.5
10b	66.1	65.8, 65.0	64.4, 64.1	59.8	59.9, 58.5 (60.0)	58.4 (59.6)	63.6	58.8	58.8
11	81.5 ^{f)}	81.5, 79.7	82.6, 77.2	39.5	39.6, 45.3 (42.9)	45.3 (42.9)	31.6	40.3	41.7
12	77.7 ^{e)}	75.2, 74.6 ^{h)}	79.5, 80.6	74.0 ^{j)}	73.8, ^{k)} 74.0 ^{j)} (74.1 ^{m)}	73.9 ^{m)} (74.1 ^{o)}	78.8 ^{p)}	68.3 ^{q)}	72.4 ^{r)}
17	27.2	27.7, 28.0	29.9, 29.1	28.0	28.1, 28.0 (29.1)	28.0 (29.1)	28.1	28.6	30.8 ^{s)}
18	58.3	59.3, 59.2	61.7, 62.0	59.6	59.7, 61.0 (61.9)	61.0 (61.9)	58.1	64.0	63.8
18-O-COCH ₃	—	169.4, 169.3 ^{h)}	168.8, 169.2 ^{j)}	169.4	169.5, 169.6 (169.0)	169.6 (169.1)	169.7	170.0	169.6
18-O-CO-CH ₃	—	20.5	20.4, 20.5	20.5	20.6, 20.7	20.8	20.4	20.5	20.6
11-O-COCH ₃	—	168.2, ^{h)} 22.0	168.3, ^{j)} 22.0	—	—	—	—	—	—
S-CH ₃ or S-CO-CH ₃	—	—	—	—	—	—	—	13.7, 15.6	190.6, 192.7 30.7, 30.4 ^{s)}

a) Measured in DMSO- d_6 for 5. b) The chemical shift values in parentheses are those of the minor conformer. c—s) May be interchanged.

TABLE III. Crystal Data for Triacetylchetracin A (3a)

Triacetylchetracin A, $C_{36}H_{34}N_6O_{11}S_8 \cdot 2H_2O$
FW = 1019.2, MW = 983.2
Tetragonal space group $P4_3$, $Z=4$
Unit cell dimensions $a=b=15.337(8)$, $c=19.992(10)$ Å
$U=4686$ Å ³ , $D_{calc}=1.445$ g cm ⁻³ , $D_m=1.414$ g cm ⁻³
μ for $CuK_\alpha=40.4$ cm ⁻¹

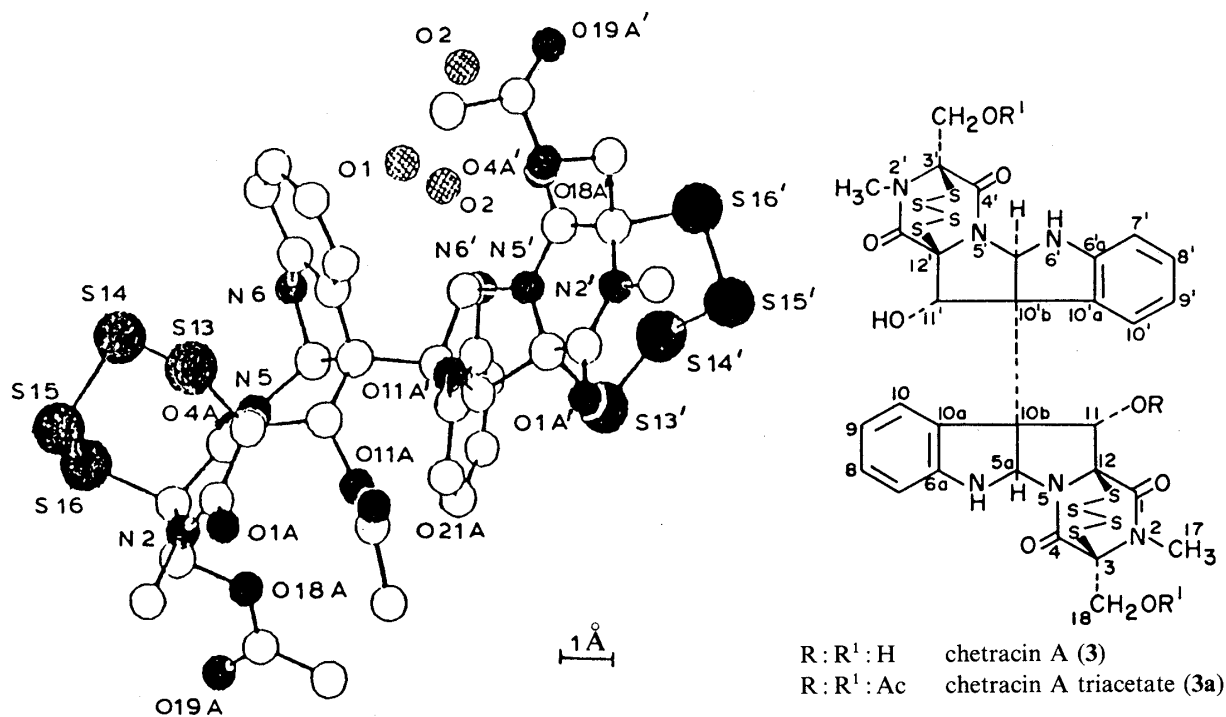


Fig. 2. Absolute Configuration of Chetracin A Triacetate (3a) Determined by X-Ray Crystallography

mated by a graphite plate. The crystal data are listed in Table III.

The intensities of 3062 reflections were measured in the 2θ range of 6° through 178° . The structure was solved by the direct method using MULTAN¹⁸⁾ and refined by the block-diagonal least-squares method to an R value of 0.084. The difference map indicated three water oxygen atoms. The occupancy factor of these oxygen atoms was estimated from the difference electron-density map. The absolute configuration was determined by the anomalous dispersion method. $|F|^2$ values were calculated for 392 Friedel pairs introducing the dispersion corrections for atomic scattering factors of C, O, N and S atoms for CuK_α radiation and compared with the observed values. Of the total of 64 pairs for which the difference in structure factors between h, k, l and $\bar{h}, \bar{k}, \bar{l}$ exceeds $2\sigma(F_o)$, 55 pairs demonstrated clearly the absolute configuration shown in Fig. 2.

Final refinement was carried out by introducing the dispersion corrections for C, N, O and S atoms and anisotropic thermal parameters for all atoms. Hydrogen atoms were not included. The R values were reduced to 0.081 for the 3062 observed structure factors. Figure 2 illustrates the molecular structure of chetracin A triacetate (3a), and the chemical structure of chetracin A was established as shown in the formula (3). The absolute configuration confirmed by the X-ray analysis was same as that of $11\alpha, 11'\alpha$ -dihydroxychaetocin¹⁴⁾ (5).

The dioxopiperazine rings are in boat conformations and the sulfide bridges link the bows

and the sterns. Four possible conformations exist in the tetrasulfide bridge.⁴⁾ The NMR spectra of the acetate (**3a**) indicated the presence of only one conformer in the solutions. X-ray analysis revealed that the central pairs of the sulfur atoms are aligned along the line connecting the two nitrogen atoms of the piperazine ring (Fig. 3a). The interatomic distances between the central sulfur atoms and the nitrogen atoms (3.143, 3.169, 3.190, 3.206 Å) were shorter than the sum of their van der Waals radii (3.35 Å). This might arise from donor-accepter interactions between nitrogen and sulfur atoms and may be the reason for the stability of the conformation.^{19,20)}

The dioxopiperazine rings are in boat forms but the rings are more planar than in the case of a disulfide bridge,¹⁹⁾ i.e., the angles formed between the two planes of atoms about the amide groups of the acetate (**3a**) are 160.8° and 159.9° (Fig. 3b), which are larger than those in chaetocin (**2**), which have been reported as 130°. The four terminal S-S bonds (1.996, 2.005, 2.002, 2.011 Å) are shorter than the two central S-S bonds (2.066 and 2.096 Å), as in the case of the tetrasulfide bridge of sporidesmin G (**7**),²⁰⁾ indicating a 'conjugated diene' nature.

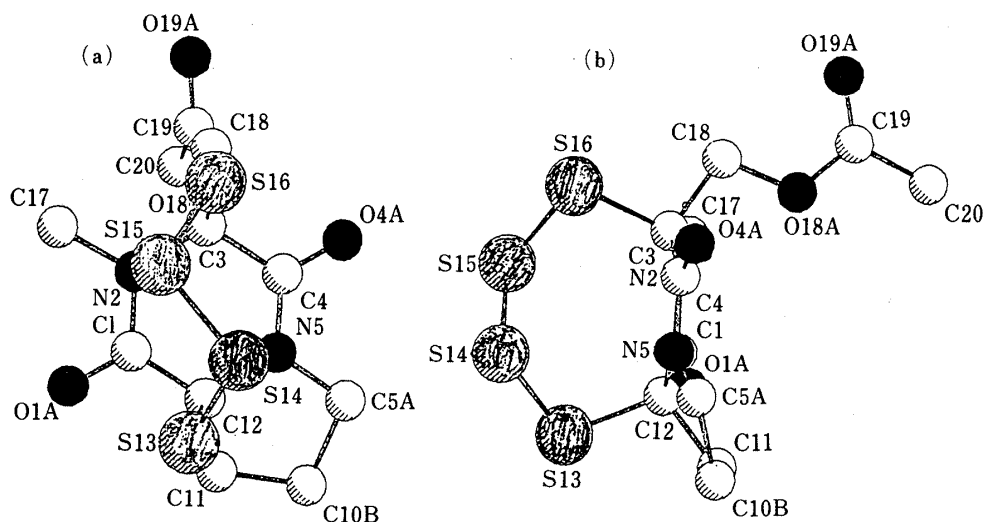


Fig. 3. Conformation of the Tetrasulfide Bridge of Chetracin A Triacetate (**3a**)

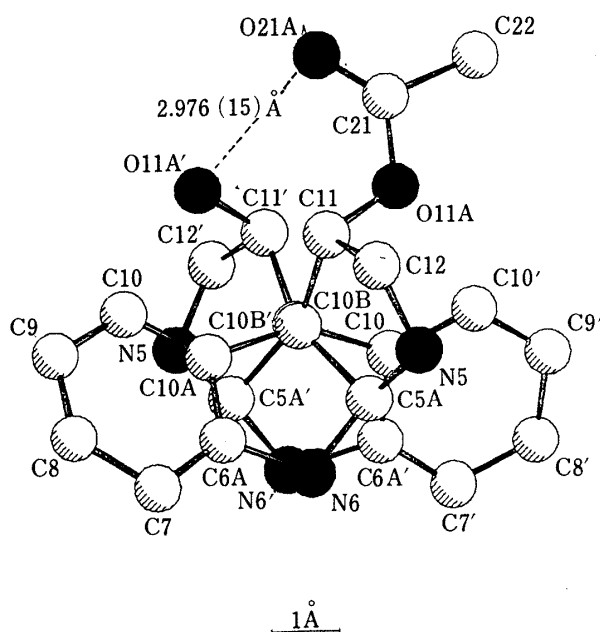


Fig. 4. Torsion of the C10B-C10B' Bond of Chetracin A Triacetate (**3a**)

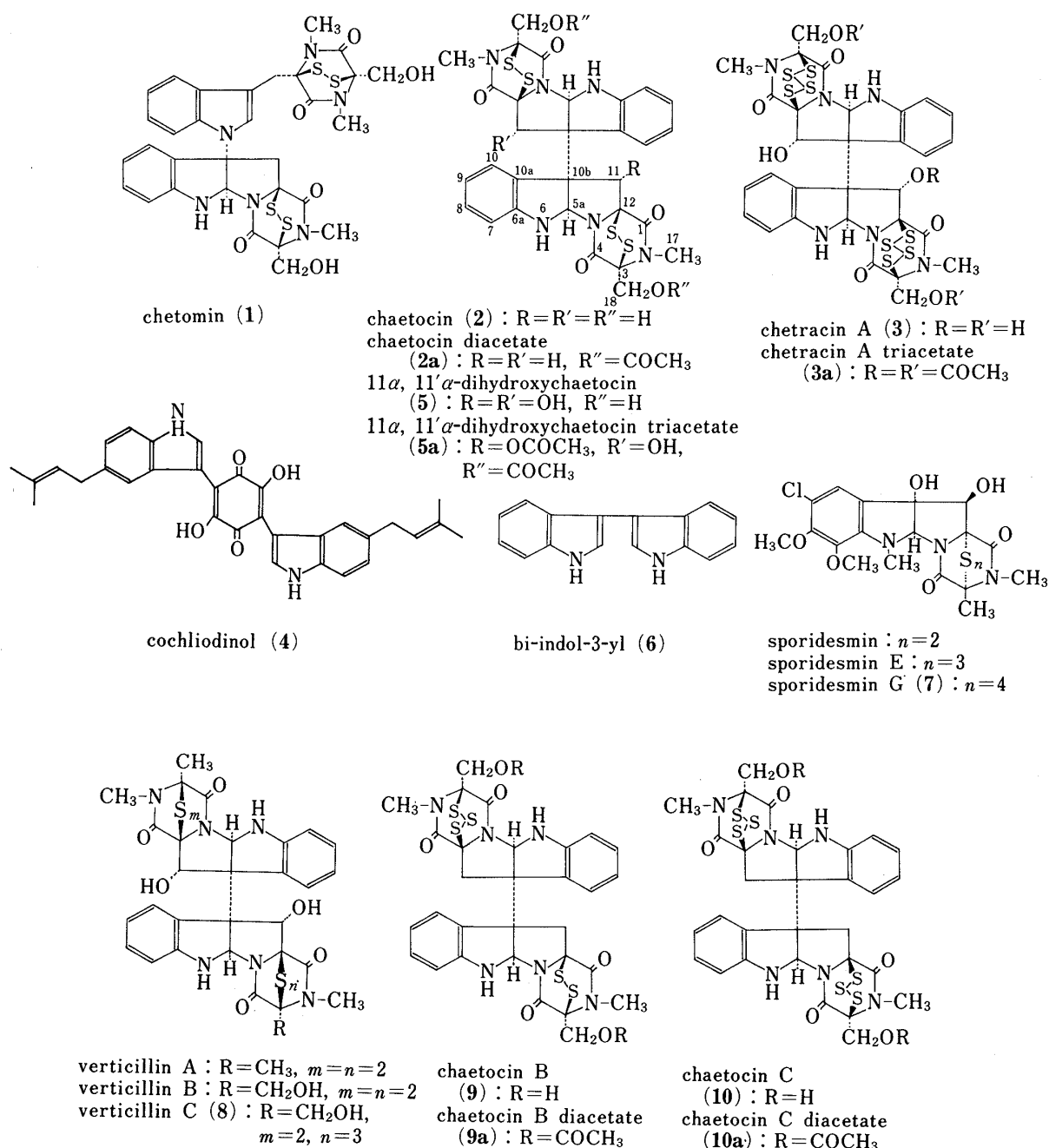


Chart 1

Chetracin A (3) is a symmetric dimer having two primary and two secondary alcohol groups but forms triacetate (3a) by acetylation with acetic anhydride in pyridine as reported in the case of dihydroxychaetocin¹⁴⁾ (5) and verticillins (8).¹⁶⁾ Examination of the interatomic distances of the non-bonded atoms around the oxygen atom of the 11' α -hydroxyl group which was not acetylated showed that the hydroxyl group (O11A'-H) is sterically hindered and forms a hydrogen bond of 2.976(15) Å with the carbonyl oxygen atom (O21 A) of the newly formed acetyl group at the 11 α -hydroxyl group (Fig. 4).²¹⁾

Although compounds with a tetrasulfide bridge such as gliotoxin G, sporidesmin G, and sirodesmin B have been found among monomeric epipolythiodioxopiperazines, chetracin A (3) is the first case in a dimeric compound.

The production of chaetocin¹⁹⁾ (2) by *C. thielavioideum* (*C. virescens* var. *thielavioideum*)

was reported in previous papers.^{7,8)} Reexamination of seven strains of species cultured on rice and wheat revealed that several of the strains produced two congeners other than the main epipolythiodioxopiperazine, chaetocin (**2**). The best producer of the congeners, *C. thielavioideum* NHL 2827, was selected and cultured on wheat. The dichloromethane extract was concentrated to give a precipitate which was composed chiefly of a mixture of epipolythiodioxopiperazines. The mixture was applied to HPLC using Nucleosil NO₂-5 and eluted with CHCl₃-MeOH (150:1) to give two new compounds named chaetocins B (**9**) and C (**10**). High-resolution fast atom bombardment mass spectrometry (FAB MS) of chaetocin B (**9**) showed the (M+H)⁺ ion at 729.0781, indicating the molecular formula C₃₀H₂₈N₆O₆S₅. Due to its poor solubility and instability, the compound (**9**) was derived to the diacetate (**9a**), C₃₄H₃₂N₆O₈S₅ ((M+H)⁺ 813.0959 by FAB MS), for spectral examination. The ultraviolet (UV) and infrared (IR) absorptions of **9a** were similar to those of chaetocin diacetate (**2a**). Desulfurization of **9** with triphenylphosphine^{16,17)} gave chaetocin (**2**), indicating an identical skeleton with **2**.

Since chaetocin (**2**) is a symmetric dimer, all corresponding signals in the ¹H- and ¹³C-NMR of the two halves of the molecule appear as one signal. On the other hand, most of the proton and carbon signals in the two halves of the molecule of chaetocin B diacetate (**9a**) were duplicated and suggested that one half of the molecule is the same as chaetocin diacetate (**2a**) but the other half is different from **2a** (Tables I and II). To confirm the structure and the numbers of sulfur atoms in each component, FAB MS/mass spectrometry (FAB MS/MS)^{22,23)} was performed on the acetate (**9a**). The spectra of chaetocin diacetate (**2a**) and chaetocin B diacetate (**9a**) are shown in Fig. 5, and the fragmentation patterns are assigned as shown in Chart 2. The results clearly indicated that chaetocin B (**9**) is the sulfur homolog of chaetocin (**2**), where one of the two disulfide bridges in **2** has been replaced by a trisulfide in **9**. Based on the proposed structure (**9**), the ¹H- and ¹³C-NMR spectra of the acetate (**9a**) were reexamined employing ¹H-¹H correlation spectroscopy (COSY) and ¹³C-¹H COSY (C-H

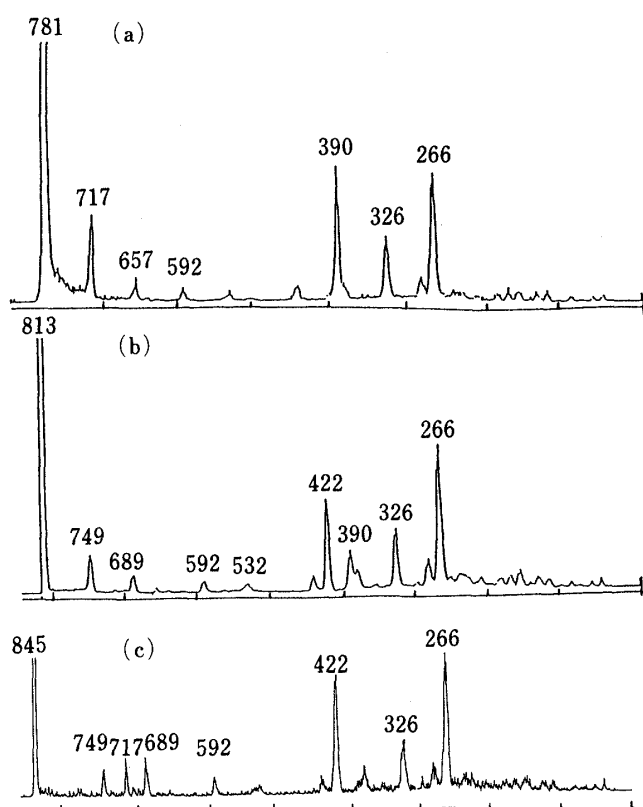


Fig. 5. FAB MS/MS Spectra of (a) Chaetocin Diacetate (**2a**), (b) Chaetocin B Diacetate (**9a**), and (c) Chaetocin C Diacetate (**10a**)

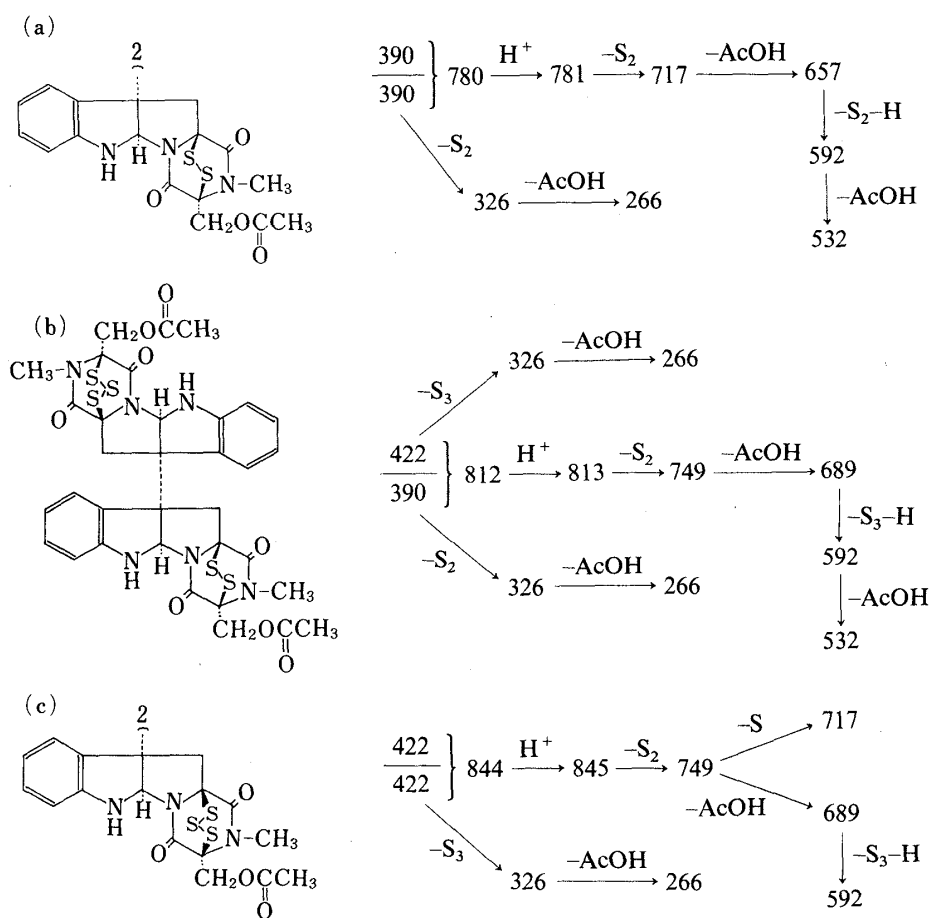


Chart 2. Fragmentation Patterns of (a) Chaetocin Diacetate (**2a**), (b) Chaetocin B Diacetate (**9a**), and (c) Chaetocin C Diacetate (**10a**)

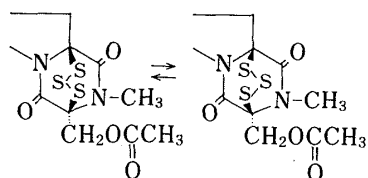


Chart 3. Two Conformers of the Trisulfide in Chaetocins B (**9**) and C (**10**)

COSY). Most signals corresponding to the trisulfide part in the molecule of **9a** appeared in two split bands and the sum of the intensities of the two was equal to the intensities of one corresponding band in the disulfide part. This phenomenon suggests the existence of two conformers of the trisulfide part of the molecule (Chart 3), as reported in the case of sporidesmin E (**7**).¹⁷⁾ Determination of the ¹H-NMR of **9a** in toluene-*d*₈ at -19, 24, and 92 °C showed equilibration of the two conformers in the solution and a decrease of the minor conformer at the higher temperature. The ¹H- and ¹³C-NMR data of **9a** thus unequivocally assigned are shown in Tables I and II.

The absolute configuration of chaetocin B (**9**) was proved to be identical to that of chaetocin¹⁹⁾ (**2**) by comparison of the CD spectra of the acetates (**2a** and **9a**) (Fig. 1).

Chaetocin C (**10**) was also converted to the acetate (**10a**) for spectral examination, and high-resolution MS (by FAB MS) of **10a** revealed the (M+H)⁺ ion at *m/z* 845.0643, indicating the molecular formula C₃₄H₃₂N₆O₈S₆. This formula corresponds to a dimer composed of two halves having the same trisulfide bridge.

Since the yield of chaetocin C (**10**) was quite poor, preparation of the trisulfide from chaetocin (**2**) was attempted by the previously reported method (with sulfur and phosphorus

pentasulfide)¹⁷⁾ for detailed spectral examinations. The main product, accompanied by the byproduct confirmed to be identical with chaetocin B (9), was found to be identical with the natural trisulfide (10). Desulfurization of chaetocin C (10) with triphenylphosphine gave chaetocin (2). Using the synthetic specimen, the acetate (10a) was prepared and detailed examinations of the spectra were performed for comparison with 2a and 9a (Tables I and II). The spectra of 10a coincided with those obtained by subtraction of the signals of 2a from those of 9a. FAB MS/MS of 10a (Fig. 5 and Chart 2) also demonstrated the correctness of the structure.

The CD spectrum (Fig. 1) of 10a showed the same sign as 2a, indicating the same absolute configuration. The structure of chaetocin C was thus established as 10.

Since the discovery of the bacteriostatic effect of gliotoxin against a wide range of gram-positive organisms,²⁴⁾ many reports have described antimicrobial and antiviral activities and cytotoxicity to mammalian cells of epipolythiodioxopiperazines, and the numbers of sulfur atoms were found to influence the activities.^{3,25,26)} In our own studies, compounds with the same nucleus but with different numbers of sulfur atoms were obtained. Thus, four derivatives of chaetocin (2) were prepared and the structure-activity relationships observed for the antimicrobial activities and cytotoxicity to HeLa cells of these compounds were examined. The chaetocin derivatives prepared were the monosulfide (11), dethiomethylthio derivative (12), dethioacetylthio derivative (13a), and dethiothiol derivative (13), and the structures of these compounds were confirmed from the ¹H- and ¹³C-NMR spectra (Tables I and II), (M+H)⁺ in FAB MS, and CD spectra.

The antimicrobial activities against *Staphylococcus aureus* FDA 209P, *Escherichia coli* NIHJ, and *Saccharomyces cerevisiae* ATCC 9763 were examined by the dilution method on agar plates.²⁷⁾ The results are summarized in Table IV. None of the compounds showed any activities towards the gram-negative bacteria or the fungus but they did exhibit a remarkable activity towards the gram-positive bacteria. Chaetocins B (9) and C (10), with trisulfide bridges, showed the strongest activities, followed by chaetocin (2), dihydroxychaetocin (5),

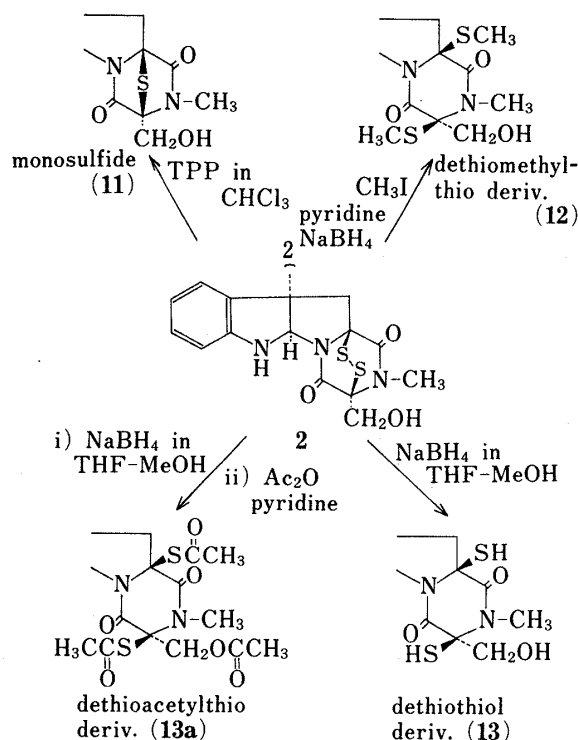


Chart 4

TABLE IV. Minimum Inhibitory Concentration of Chaetocin Derivatives²⁷⁾

Compound	$\mu\text{g/ml}^{\text{a)}$		
	<i>Staphylococcus aureus</i> FDA 209P	<i>Escherichia coli</i> NIHJ	<i>Saccharomyces cerevisiae</i> ATCC 9763
Chaetocin (2)	0.1 (0.78)	> 100 (> 100)	> 100 (> 100)
Chaetocin B (9)	0.05 (0.2)	> 100 (> 100)	> 100 (> 100)
Chaetocin C (10)	0.025 (0.05)	> 100 (> 100)	> 100 (> 100)
Monosulfide (11)	12.5 (50)	> 100 (> 100)	> 100 (> 100)
Dethiomethylthio derivative (12)	> 100 (> 100)	> 100 (> 100)	> 100 (> 100)
Dethioacetylthio derivative (13a)	50 (> 100)	> 100 (> 100)	> 100 (> 100)
Dethiothiol derivative (13)	0.025 (0.05)	50 (> 100)	> 100 (> 100)
Chetracin A (3)	0.39 (0.78)	> 100 (> 100)	> 100 (> 100)
11 α ,11' α -Dihydroxychaetocin (5)	0.2 (0.78)	> 100 (> 100)	> 100 (> 100)

a) At the inoculum size of 10^6 /ml. In parentheses, data obtained with the inoculum size of 10^8 /ml.

TABLE V. Cytotoxicity of Chaetocin Derivatives to HeLa Cells⁸⁾

Compound	$\mu\text{g/ml}^{\text{a)}$									IC ₅₀ $\mu\text{g/ml}$
	100	32	10	3.2	1.0	0.32	0.1	0.032	0.05	
Chaetocin (2)				4	4	4	3.5	1	0	0.04
Chaetocin B (9)				4	4	4	4	1	0	0.03
Chaetocin C (10)				4	4	4	4	2	0	0.02
Monosulfide (11)	4	4	1	0						12
Dethiomethylthio derivative (12)	4	0	0	0						64
Dethioacetylthio derivative (13a)	4	3.5	2	0						15
Dethiothiol derivative (13)				4	4	4	3	0	0	0.07
Chetracin A (3)				4	4	4	4	3	0	0.02
11 α ,11' α -Dihydroxychaetocin (5)				4	4	4	4	1	0	0.04
Chetomin (1)				4	4	4	3	2	0	0.02

a) The degree of cytotoxicity was estimated on a scale ranging from "0" (no cellular damage) through "4" (complete cytolysis) as described previously.²⁸⁾

and chaetracin A (3), with disulfide or tetrasulfide bridges, whereas the derivatives without bridges (11–13) did not show such activity (The thiol derivative (13) was assumed to be able to reform the disulfide bridge in the course of testing and to exhibit activity). Similar structure–activity relationships have been reported for dehydrogliotoxin derivatives.³⁾ The results indicated that the compounds are transformed to the thiolate ions in order to exhibit the activity, and instability of the episulfide bridge parallels the activity.³⁾

The cytotoxicity to HeLa cells of these compounds is shown in Table V. As reported previously,^{7,8)} the cytotoxicity to mammalian cells was quite remarkable and the IC₅₀ values of 1, 2, 3, 5, 9, and 10 were of the order of 0.02–0.07 $\mu\text{g/ml}$, while similar structure–activity relationships were observed to those recognized for the bacterial system.

A preliminary toxicity test of chaetocin (2) and its acetate (2a) was performed using ICR male mice. The drugs were dissolved in dimethyl sulfoxide and injected intraperitoneally at a dose level of 0.6 to 10 mg/kg. Two of three mice injected with 5.0 mg/kg of chaetocin (2) died 3 and 4 d after the injection, showing marked body weight loss. A postmortem examination revealed extensive fibrinous peritoneal adhesion and focal liver cell necrosis. The thymus and

spleen were small in size. All other mice, injected with 0.6 to 2.5 mg/kg of chaetocin, survived until sacrifice on day 10. Peritoneal adhesion was found in the mice given 1.2 mg/kg or more of the toxin, its extent and severity being proportional to the dose applied. In the liver, a few foci of necrosis and increased mitotic figures were noted. All mice receiving chaetocin acetate (**2a**) survived 10 d without showing noticeable symptoms. However, peritoneal adhesion was found in each one of two given 1.2 and 2.5 mg/kg and two given 5 or 10 mg/kg, again with dose-dependent severity. The liver was histologically unremarkable.²⁹⁾

Experimental

All melting points were determined with a Yanagimoto MP micromelting point apparatus and are uncorrected. The ¹H- and ¹³C-NMR spectra were recorded using a JEOL GX-400 (¹H 400 MHz and ¹³C 100 MHz) spectrometer in CDCl₃ with tetramethylsilane as internal standard. The chemical shifts are expressed in ppm (δ). UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a JASCO A-102 infrared spectrophotometer. The [α]_D values were determined with a JASCO DIP-140 digital polarimeter. CD spectra were recorded on a JASCO J-20 spectropolarimeter.

Kieselgel 60 F₂₅₄ (Merck) precoated plates were employed for thin-layer chromatography (TLC) and the spots were detected by UV illumination. Column chromatography was carried out on 70–230 mesh silica gel (Merck). HPLC was performed using a Waters M45J pump with an Oyo-Bunko Uvilog-5IIIA UV detector.

FAB MS and FAB MS/MS (CAD spectra; collisionally activated dissociation spectra²²⁾ were obtained with a JEOL JMS-HX100 tandem mass spectrometer, which consists of a conventional geometry high-resolution mass spectrometer followed by a second electrostatic analyzer. Xenon was employed as the source of the fast atom beam (6 keV). The liquid matrix used for FAB ionization was 3-nitrobenzyl alcohol instead of glycerol, which is generally used for FAB MS.

Isolation of Chetomin (1) from *C. tenuissimum*—*C. tenuissimum* QM 8187 (=ATCC 14532=CBS 151.60) was cultured on steamed rice (3 kg) at 29 °C for 30 d by stationary conditions. The culture was extracted with CH₂Cl₂ for 24 h twice at room temperature and the combined extract was concentrated and chromatographed through a silica gel column using a gradient mixture of CH₂Cl₂–EtOAc. The fraction eluted by CH₂Cl₂–EtOAc (4:1) was re-chromatographed on the same column employing CH₂Cl₂–MeOH (19:1) as the developer. Chetomin (**1**) (12.7 mg) was obtained as a colorless powder, mp 218–220 °C (dec.) (lit.⁸⁾ mp 219–221 °C (dec.)). [α]_D²⁰ + 332.7° (c=0.049, CHCl₃). UV λ_{max}^{EtOH} nm (log ε): 275 (3.92), 284 (4.01), 294 (3.92). IR ν_{max}^{KBr} cm⁻¹: 3400, 1680, 1672, 1605, 1450, 1347, 1230, 1065, 742. Identity with an authentic sample of chetomin (**1**) was established by TLC and IR.

Isolation of Cochliodinol (4) and Chetracin A (3) from *C. abuense*—*C. abuense* CBS 542.63 (ATCC 32394) (*C. nigricolor*) was cultured on rice (6 kg) at 25 °C for 30 d. The culture was extracted successively with CH₂Cl₂ and EtOAc at room temperature for 24 h. the EtOAc extract was triturated with hexane, the precipitate formed was collected and treated with CH₂Cl₂ to remove the insoluble part composed chiefly of chetracin A (**3**) (*vide infra*), and the CH₂Cl₂ soluble part was passed through a column of silica gel using a gradient mixture of CH₂Cl₂–MeOH. The violet band appearing on the column was eluted with CH₂Cl₂–MeOH (99:1) and recrystallized from acetone to give violet needles of cochliodinol (**4**) (63.2 mg), mp 213–215 °C (dec.) (lit.⁸⁾ mp 208 °C (dec.)). MS *m/z*: 506 (M⁺, C₃₂H₃₀N₂O₄); 130 (C₉H₈N). UV λ_{max}^{EtOH} nm (log ε): 282 (4.42), 470 (3.58). IR ν_{max}^{KBr} cm⁻¹: 3400, 3303, 1622, 1420, 1320, 1100, 982, 800. ¹H-NMR (in CDCl₃) δ: 1.93 (12 H), 3.5 (4H), 5.48 (2H), 7.15 (2H), 7.50 (2H), 7.91 (2H), 8.04 (2H). Identity with an authentic sample of cochliodinol (**4**)⁸⁾ was confirmed by TLC and IR.

Isolation of 11α,11′α-Dihydroxychaetocin (5) and Chetracin A (3) from *C. retardatum*—*C. retardatum* TRTC 66.1778b was cultured on rice (10 kg) at 29 °C for 30 d by a stationary conditions. The moldy rice was extracted successively with CH₂Cl₂ and EtOAc at room temperature for 24 h twice and both extracts were concentrated. The precipitate formed from the CH₂Cl₂ extract was filtered off, washed with acetone, and recrystallized from acetone to give 11α, 11′α-dihydroxychaetocin (**5**) (165 mg), an amorphous powder of mp 235–238 °C (dec.) (lit.¹⁴⁾ mp 232–234 °C (dec.)). [α]_D²⁰ + 728.1° (c=0.065, CHCl₃). MS *m/z*: 256 (S₀), 232 (base peak), 64 (S₂). UV λ_{max}^{EtOH} nm (log ε): 242 (4.24), 302 (3.68). IR ν_{max}^{KBr} cm⁻¹: 3380, 2910, 2840, 1688, 1660, 1602, 1480, 1470, 1350, 1300, 1240, 1200, 1060, 760, 740. ¹H-NMR (Table I), ¹³C-NMR (Table II).

Dihydroxychaetocin (**5**) was acetylated with Ac₂O in pyridine to give 11α,13,13′-triacetate (**5a**), an amorphous powder of mp 248–250 °C (dec.). [α]_D²⁰ + 818.2° (c=0.022, CHCl₃). CD Δε₂₀(nm): +69.0 (237), +1.6 (272), +15.9 (305). UV λ_{max}^{EtOH} nm (log ε): 242 (4.28), 302 (3.70). IR ν_{max}^{KBr} cm⁻¹: 3380, 1750, 1670, 1602, 1208, 1040, 750. ¹H-NMR (Table I), ¹³C-NMR (Table II). The triacetate in MeOH was hydrolyzed with 1.4% NH₄OH for 30 min at room temperature. The reaction mixture was adsorbed on Amberlite XAD-2 and, after washing with water, eluted with MeOH. Evaporation of the eluate gave dihydroxychaetocin (**5**) in a 64% yield.

The identity of **5** and **5a** was confirmed by comparison of the physical data with those reported previously.¹⁴⁾

The EtOAc extract of the moldy rice was concentrated and the precipitate formed (495 mg) was collected. The

crude chetracin A so obtained was acetylated with Ac_2O -pyridine, and the acetate formed was purified by HPLC using a Develosil 60-3 column and employing benzene-EtOAc-acetone (25:5:1) as the developing solvent. The product, triacetylchetracin A (**3a**) (203 mg), showed mp 260°C (dec.). $[\alpha]_D^{20} + 830.0^\circ$ ($c=0.018$, CHCl_3). CD $\Delta\epsilon^{20}$ (nm): +50.0 (235), +17.7 (310), 0 (330), -2.1 (345), 0 (380). Anal. Calcd for $\text{C}_{36}\text{H}_{34}\text{N}_6\text{O}_{11}\text{S}_8 \cdot 3/2 \text{H}_2\text{O}$: C, 42.80; H, 3.69; N, 8.32; S, 25.39. Found: C, 42.44; H, 3.37; N, 8.17; S, 25.71. UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ): 242 (4.38), 306 (3.72). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400, 1756, 1678, 1605, 1482, 1410, 1380, 1370, 1210, 1050, 750. ^1H -NMR (Table I), ^{13}C -NMR (Table II). A single crystal for X-ray analysis was obtained by the vapor diffusion method employing the acetate in benzene saturated with water diffused with xylene.

The triacetate (**3a**) in MeOH was treated with 1.4% NH_4OH at room temperature for 1 h to give chetracin A (**3**) by the same procedure as for dihydroxychaetocin (**5**). The products, chetracin A (**3**), a colorless powder, showed mp $248\text{--}251^\circ\text{C}$ (dec.). $[\alpha]_D^{20} + 723.5^\circ$ ($c=0.0012$, CHCl_3). UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ): 242 (4.33), 306 (3.70). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3410, 1660, 1635, 1605, 1480, 1415, 1380, 1310, 1205, 1140, 1095, 1065, 750.

Reacetylation of chetracin A (**3**) gave the same triacetate (**3a**).

Reactions of Chetracin A (3)—i) Chetracin A (**3**) (50 mg) was suspended in benzene (16 ml), and NH_4OH (28%, 0.2 ml) in MeOH (4 ml) was added. Then, after addition of triphenylphosphine (250 mg), the mixture was stirred for 3 min. The reaction mixture was adsorbed on Amberlite XAD-2 (100 ml) and the resin was washed with water and eluted with MeOH. The eluate was concentrated and the separated crystals were purified by HPLC on a column of Nucleosil 5 NO_2 using CH_2Cl_2 -MeOH (250:1) as the developer. $11\alpha, 11'\alpha$ -Dihydroxychaetocin (**5**) (14.2 mg) so obtained showed mp $235\text{--}238^\circ\text{C}$, $[\alpha]_D^{20} + 726.0^\circ$ ($c=0.088$, CHCl_3). Identity with an authentic sample was confirmed by IR, ^1H -NMR, and ^{13}C -NMR.

ii) Chetracin A (**3**) (200 mg) was refluxed in dioxane-water (4:1, 5 ml) containing KOH (200 mg) for 2.5 h. The reaction mixture, after addition of water, was extracted with EtOAc and the EtOAc layer was evaporated. The residue was chromatographed on a column of silica gel using hexane-acetone as the developing solvent to give bi-indol-3-yl (**6**) (68 mg), mp $285\text{--}288^\circ\text{C}$ (lit.¹⁶) mp $285\text{--}287^\circ\text{C}$). The identity of this product with the sample obtained from $11\alpha, 11'\alpha$ -dihydroxychaetocin (**5**) was confirmed by the same procedure. Anal. Calcd for $\text{C}_{16}\text{H}_{12}\text{N}_2$: C, 82.73; H, 5.21; N, 12.06. Found: C, 82.60; H, 4.89; N, 12.18. MS m/z : 230 (M^+), 204, 176, 116, 102, 88, 77. UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 231, 281, 288, 300. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3380, 1450, 1405, 1330, 1235, 1105, 1010, 910, 780, 735.

The aqueous layer after EtOAc extraction was acidified and extracted with EtOAc. The residue from the extract gave sulfur (5.2 mg), mp 119°C , MS m/z : 256 (M^+ , S_8), 224, 192, 160, 128, 96, 64.

Bi-indol-3-yl (**6**) (41 mg) was obtained from chetracin A triacetate (**3a**) (200 mg) by the same procedure.

Isolation of Chaetocins B (9) and C (10) from *C. virescens* var. *thielavioideum*—*C. thielavioideum* NHL 2827 (*C. virescens* var. *thielavioideum*) was cultured on wheat (7 kg) for 10 d at 30°C . The moldy wheat was extracted with CH_2Cl_2 at room temperature for 24 h twice and the combined extract was evaporated. The precipitate (600 mg) formed after concentration was collected and washed with benzene. The precipitate was then purified by HPLC on a Nucleosil 5 NO_2 column using CHCl_3 -MeOH (150:1) as the developing solvent (condition 1). Chaetocin (**2**) (10 mg), chaetocin B (**9**) (2.8 mg), and chaetocin C (**10**) (0.1 mg) were eluted in that order. They were converted to the respective acetates (**2a**, **9a**, and **10a**) by acetylation with Ac_2O -pyridine, and the products were purified by HPLC employing a Nucleosil 50-5 column with benzene-EtOAc-acetone (25:5:1) as the developer (condition 2).

Chaetocin diacetate (**2a**) showed mp 220°C (dec.) (lit.²⁸) mp $220\text{--}225^\circ\text{C}$ (dec.). $[\alpha]_D^{20} + 674.4^\circ$ ($c=0.078$, CHCl_3). CD (MeOH) $\Delta\epsilon^{20}$ (nm): +72.1 (237), 0 (264), -5.3 (270), 0 (282), +14.2 (303). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 205 (4.7), 238 (4.3), 301 (3.8). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3390, 1750, 1690, 1610, 1220, 1045, 750. MS (FAB) m/z : 781 ($\text{M} + \text{H}$)⁺, 717, 657, 592, 390, 326, 226. ^1H -NMR (Table I), ^{13}C -NMR (Table II).

Chaetocin B diacetate (**9a**) showed mp 225°C (dec.). $[\alpha]_D^{20} + 673.2^\circ$ ($c=0.079$, CHCl_3). CD (MeOH) $\Delta\epsilon^{20}$ (nm): +66.4 (240), +7.0 (278), +17.5 (305). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 209 (4.7), 237 (4.3), 299 (3.8). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3390, 1740, 1680, 1605, 1350, 1060, 750. MS(FAB) m/z : 813.0959 ($\text{M} + \text{H}$)⁺, Calcd for $\text{C}_{34}\text{H}_{33}\text{N}_6\text{O}_8\text{S}_5$, 813.0963, 749, 717, 688, 422, 390, 326, 266. ^1H -NMR (Table I), ^{13}C -NMR (Table II).

Chaetocin C diacetate (**10a**) showed mp 232°C (dec.). $[\alpha]_D^{20} + 658.3^\circ$ ($c=0.012$, CHCl_3). CD (MeOH) $\Delta\epsilon^{20}$ (nm): +50.5 (240), +10.5 (288), +17.1 (308). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 210 (4.5), 238 (4.1), 301 (3.7). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3450, 1750, 1680, 1605, 1220, 1050, 750. MS(FAB) m/z : 845.0643 ($\text{M} + \text{H}$)⁺, Calcd for $\text{C}_{34}\text{H}_{33}\text{N}_6\text{O}_8\text{S}_6$, 845.0666, 749, 717, 689, 422, 326, 266. ^1H -NMR (Table I), ^{13}C -NMR (Table II).

A solution of chaetocin B diacetate (**9a**) (10 mg) in MeOH (2 ml) was treated with 1.4% NH_4OH (2 ml) and the mixture was kept at room temperature for 40 min, then adsorbed on Amberlite XAD-2. The resin was washed well with water and eluted with MeOH. Crystals (5 mg) were obtained by evaporation of the solvent. The identity of the product with natural chaetocin B (**9**) was confirmed by TLC and IR. MS (FAB) m/z : 729.0781 ($\text{M} + \text{H}$)⁺, Calcd for $\text{C}_{30}\text{H}_{29}\text{N}_6\text{O}_6\text{S}_5$, 729.0752).

From chaetocin C diacetate (**10a**), chaetocin C (**19**) was obtained by the same procedure.

Formation of Chaetocin (2) from Chaetocin B (9) and from Chaetocin C (10)—Triphenylphosphine (3 mg) was added to a CHCl_3 solution (10 ml) of chaetocin B (16 mg), and the reaction mixture was kept standing at room temperature for 20 min. The mixture was purified by HPLC employing the same procedure as above (condition 1) to give chaetocin (**2**) (4 mg), which was identified by IR, ^1H -NMR, and TLC.

The same reaction with chaetocin C (10) gave chaetocin in a good yield.

Formation of Chaetocins B (9) and C (10) from Chaetocin (2)—Sulfur (100 mg) and P_2S_5 (100 mg) were added to chaetocin (30 mg) in CS_2 (5 ml), and the mixture was heated at $110^\circ C$ for 2 min. The reaction mixture was absorbed on Amberlite XAD-2 and the resin was washed with water and eluted with MeOH. The eluate was concentrated and the precipitate formed was collected and purified by HPLC (condition 1) to give chaetocin B (9) (3 mg) and chaetocin C (10) (5 mg). These were derived to the acetates (9a, 10a) respectively, and their identity with those from the natural products was confirmed by 1H -NMR and TLC.

Chaetocin Monosulfide (11)—Triphenylphosphine (50 mg) was added to chaetocin (50 mg) in $CHCl_3$ (10 ml). The solution was stirred for 1 h and the product was purified by HPLC (condition 1) to give the monosulfide (11) (38 mg). To characterize this compound, it was derived to the diacetate (11a) and purified by HPLC (condition 2). The diacetate 11a showed mp 189 – $190^\circ C$. $[\alpha]_D^{20} + 603.1^\circ$ ($c = 0.145$, $CHCl_3$). CD (MeOH) $\Delta\epsilon^{20}$ (nm): -17.7 (225), 0 (232), $+44.2$ (248), $+1.8$ (292), $+3.6$ (310). UV λ_{max}^{MeOH} (log ϵ): 212 (4.4), 249 (4.2), 299 (3.8). IR ν_{max}^{KBr} cm^{-1} : 3400, 1750, 1720, 1610, 1220, 1050, 750. MS (FAB) m/z : 717 ($(M+H)^+$, $C_{34}H_{33}N_6O_8S_2$), 358, 330.

Dethiomethylthiochaetocin (12)—Chaetocin (75 mg) was dissolved in CH_3I (6 ml), pyridine (0.6 ml) and MeOH (1 ml). $NaBH_4$ (30 mg) was added, and the mixture was stirred for 20 min at room temperature. The reaction mixture was then neutralized with $0.1 N$ HCl and extracted with ether. The solvent was evaporated off, the residue was purified by HPLC (condition 1) and the target compound (12) (54 mg) was obtained. This compound was acetylated and purified by HPLC (condition 2) to give the diacetate of dethiomethylthiochaetocin (12a), mp 190 – $194^\circ C$. $[\alpha]_D^{20} + 358.3^\circ$ ($c = 0.101$, $CHCl_3$). CD (MeOH) $\Delta\epsilon^{20}$ (nm): $+7.3$ (230), $+17.4$ (256), $+4.4$ (282), $+8.7$ (308). UV λ_{max}^{MeOH} nm (log ϵ): 217 (4.3), 241 (4.1), 304 (3.7). IR ν_{max}^{KBr} cm^{-1} : 3400, 1750, 1680, 1610, 1380, 1220. MS (FAB) m/z : 841 ($(M+H)^+$, $C_{38}H_{45}N_6O_8S_4$), 793, 745, 647, 420.

Dethiochaetocinthiol (13) and Dethioacetylthiochaetocin (13a)—A solution of chaetocin (2) (25 mg) in tetrahydrofuran–MeOH (1:1, 5 ml) was treated with $NaBH_4$ (3 mg), and the reaction mixture, after stirring for 10 min, was neutralized with $0.1 N$ HCl. The precipitate formed by concentration of the solution was collected and purified by HPLC (condition 1) to give 13.

The compound (13) (from 25 mg of 2) was acetylated by treatment with Ac_2O –pyridine to form the diacetate (13a) (14 mg) of the dethioacetylthio compound, mp 182 – $185^\circ C$. $[\alpha]_D^{20} + 265.8^\circ$ ($c = 0.125$, $CHCl_3$). CD (MeOH) $\Delta\epsilon^{20}$: 0 (219), -22.5 (226), $+46.6$ (246), $+1.5$ (296), $+3.0$ (308). UV λ_{max}^{MeOH} (log ϵ): 208 (4.7), 235 (4.3), 305 (3.5). IR ν_{max}^{KBr} cm^{-1} : 3400, 1750, 1680, 1610, 1220. MS (FAB) m/z : 953 ($(M+H)^+$, $C_{42}H_{45}N_6O_{12}S_4$), 877, 801, 725, 649, 476, 400, 324.

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- 2) Present address: a) *Takasago Research Institute, Inc., Kamata, Ohta-ku, Tokyo 144, Japan*; b) *Nagoya Branch, Daiichi Pharmaceutical Co., Ltd., Marunouchi, Naka-ku, Nagoya 460, Japan*.
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