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PHOTODIODE ARRAY DETECTION AND STRUCTURES OF GRISEOLIC ACID ANALOGUES FROM CULTURE BROTH

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ABSTRACT.—Investigation of a *Streptomyces* culture known to produce the cyclic adenosine 3′,5′-monophosphate phosphodiesterase inhibitor, griseolic acid, has led to the detection of three griseolic acid analogues. Hplc methodology coupled with photodiode array detection was developed to assist systematically in the search for griseolic acid analogues directly from culture broths. Purification of these components using reversed-phase and ion-exchange chromatographies resulted in the isolation of griseolic acid [**1**], deoxygriseolic acid [**2**], dihydrodeoxygriseolic acid [**3**], and a novel ring-opened griseolic acid analogue [**4**].

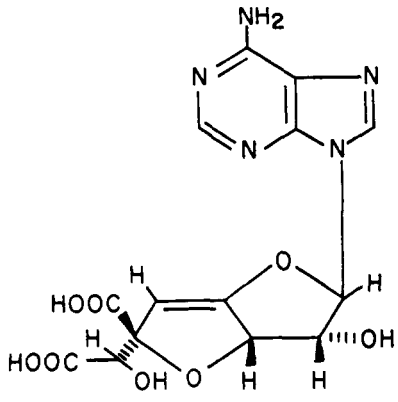
Griseolic acid [**1**], isolated from *Streptomyces griseoaurantiacus* by Nakagawa *et al.* (1) is a potent adenosine 3′,5′-cyclic monophosphate (cAMP) phosphodiesterase (PDE) inhibitor (2). Griseolic acid, which may be considered to be derived from adenosine and tartaric acid, has an adenine base, a bicyclic ring in the sugar moiety, and two carboxylic acid groups. The structure bears resemblance to cAMP. Compounds similar to griseolic acid that can inhibit cAMP PDE will increase the concentration of intracellular cAMP, a fundamental biochemical component widely distributed in mammalian tissues. Because cAMP mediates the effects of a large number of hormones and is involved with many important biochemical functions, chemical factors that inhibit its enzymatic degradation may be of considerable value, particularly as angiocardiokinetic agents (3,4). A study was undertaken to ascertain if novel griseolic acid analogues could be co-produced from *S. griseoaurantiacus*.

An efficient and reproducible hplc method was developed that detected griseolic acid directly from culture filtrates. When *S. griseoaurantiacus* NRRL-12314 (obtained from the Agricultural Research Service, Peoria, Illinois) was fermented in its production medium (1), griseolic acid was produced. Results from our laboratory of NRRL-12314 samples fermented in different media and analyzed by hplc revealed the existence of potential griseolic acid analogues. With the incorporation of photodiode array detector technology (5–7), three structural analogues of griseolic acid co-produced within a given fermentation could be readily detected by comparing online uv spectral scans with the known griseolic acid standard.[1] With the griseolic acid retention time markers in place, the hplc method was utilized to determine the optimum fermentation media conditions that would maximize production of the potential analogues. The information extrapolated from hplc ion-pair reagent studies simplified the isolation of griseolic acid and analogues from culture broth. The scheme, as outlined, is efficient and provides clear advantages over literature techniques (1). Compounds **1**, **2**, and **3** inhibit phosphodiesterase activity; compound **4** is inactive.[2]

Compounds **1** and **3** were isolated previously from culture broth (1,8) and **2** has been synthesized (9); however, this is the first report of detection and subsequent isolation of all four components from culture broth. Spectroscopic details leading to assignment of structure **4** are presented together with nmr data leading to the stereochemical considerations for component **3**. These hitherto unreported spectroscopic data support the assignment for the proposed structure **3** (8).

[1]A reference standard was kindly supplied by Dr. F. Nakagawa, Sankyo Co., Japan.

[2]Data supplied by Dr. R. Hart (Schering).



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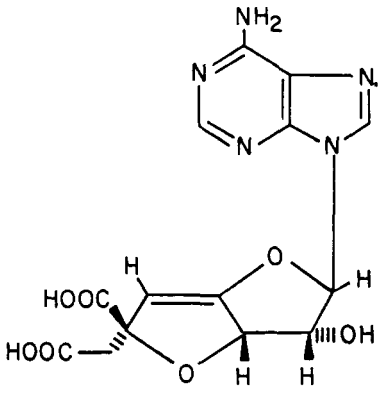

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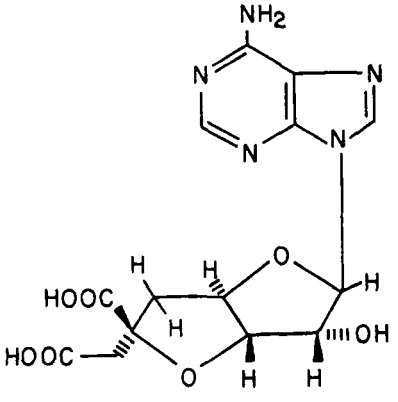

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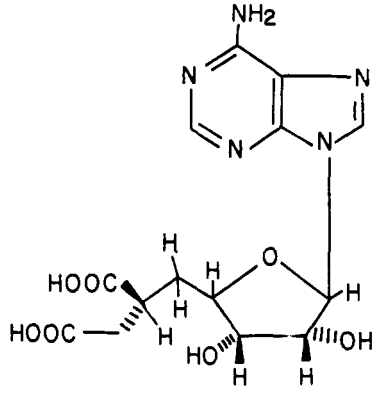

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RESULTS AND DISCUSSION

Hplc methodology developed for monitoring griseolic acid directly from fermentation broth, coupled with photodiode array spectral acquisition, led to a rapid screening approach for structural analogues of griseolic acid. Hplc conditions are given in the Experimental section. The elution characteristics of griseolic acid and its detection directly within the fermentation matrix are shown in Figures 1a and 1b. Confirmation of the presence of griseolic acid in the fermentation broth was obtained by uv spectral comparison of the chromatographic peak corresponding to the spiked retention time position with a griseolic acid reference spectrum (Figure 1c). The nucleoside base adenine is responsible for the characteristic uv chromophore with a λ max 263 nm.

Conditions for detection and hplc separation of the analogue are described in the Experimental and illustrated in Figure 2. It can be seen that there are similar uv spectra for the chromatographic peaks (22 and 25 min) on either side of griseolic acid, strongly suggesting the coexistence of griseolic acid analogues in the culture broth. These components were identified as **2** and **3**. Next, the uv spectra of all chromatographic peaks within the broth elution profile were compared with the griseolic acid spectrum; this comparison revealed the presence of an additional component eluting at 14 min (Figure 3) and displaying a uv spectrum quite similar to that of griseolic acid. Although the retention time for **4** was much shorter than those for **1–3**, the data suggested that the 14 min peak was indeed a related analogue. We were thus able to isolate components **1–4** from broth and to monitor the enriched fractions by hplc.

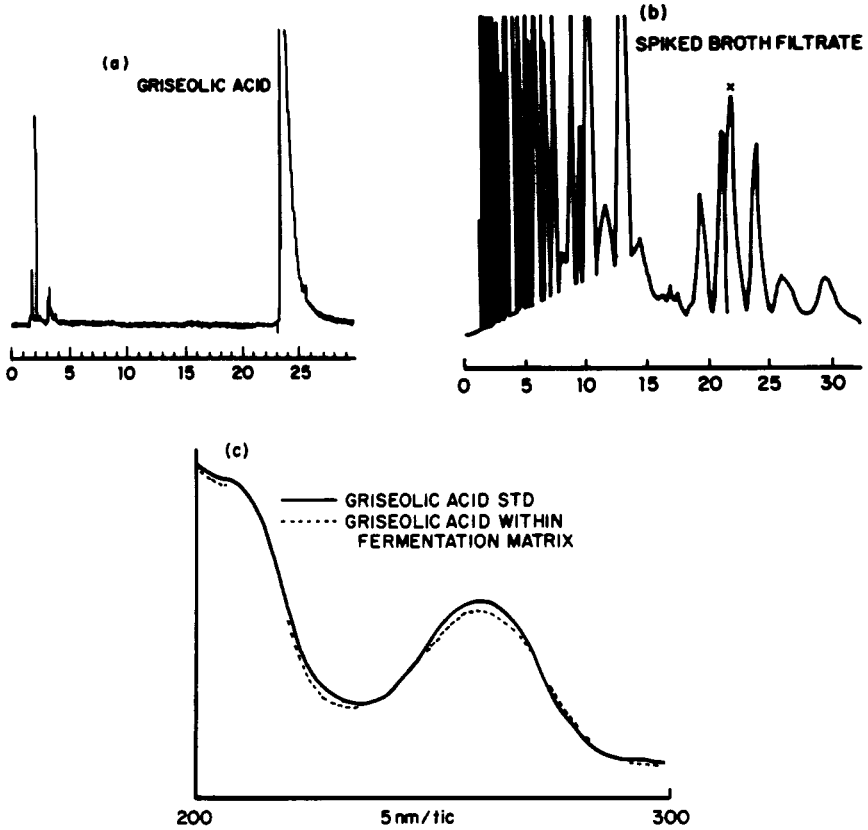


FIGURE 1. Elution profile of (a) griseolic acid [**1**] (1 μg) and (b) broth filtrate spiked with 0.22 μg griseolic acid. (c) Uv spectral comparison of griseolic acid within the broth elution profile with griseolic acid reference spectra.

The isolation procedure used to purify components **1–4** from filtered broth is shown in Scheme 1. This procedure is a modification of the literature procedure (1) for the isolation of griseolic acid relying on reversed-phase and ion-exchange chromatographies. First, pH-adjusted filtered broth was applied by a slow continuous feed onto an MCI CHP20P column. By adjusting the pH, we selectively eluted the components

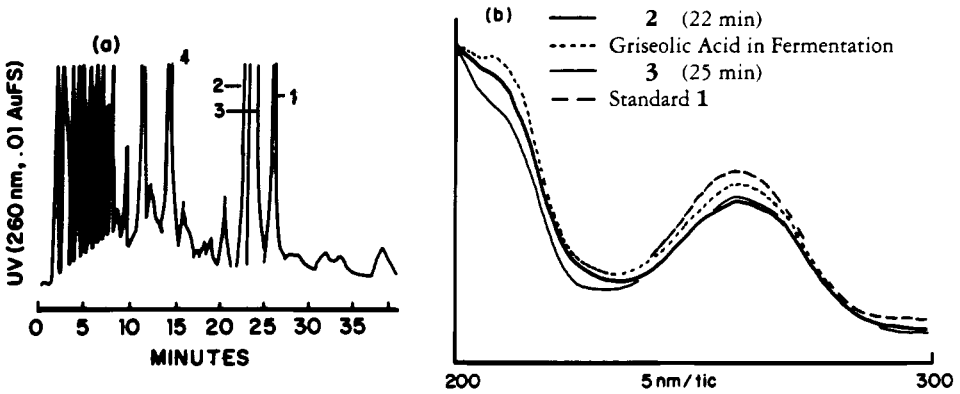


FIGURE 2. (a) Chromatographic profile of griseolic acid analogues. (b) Illustration of similar spectral characteristics of chromatographic peaks near the griseolic acid elution position.

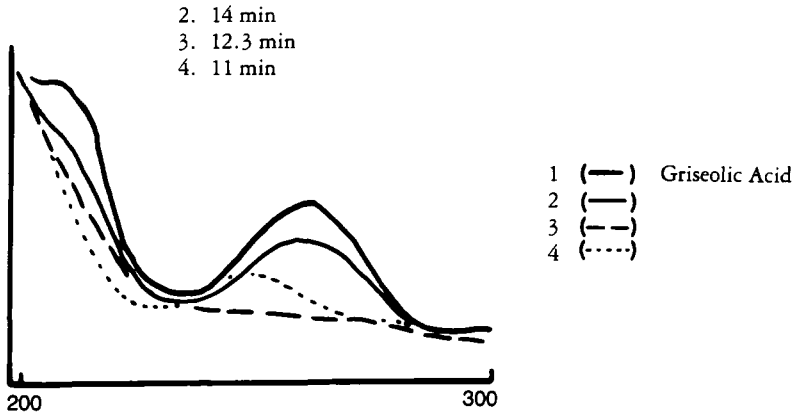
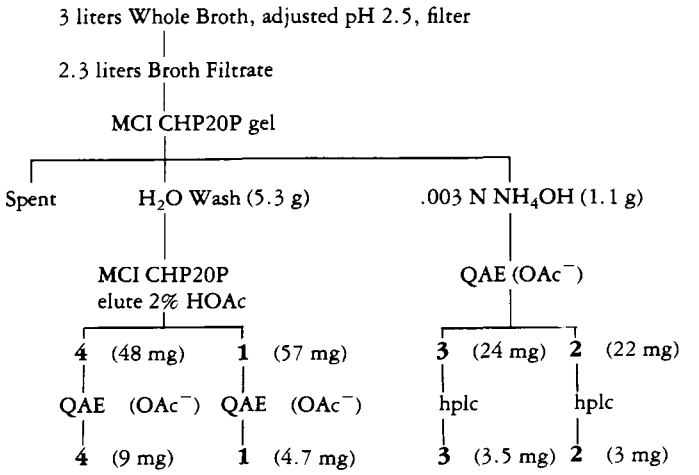


FIGURE 3. Uv spectral comparison of specific polar chromatographic peaks within the broth elution profile with griseolic acid standard spectra revealing similar spectral characteristics only for the 14 min peak.

of interest without addition of organic modifier. The fractions of interest were pooled and concentrated in vacuo by lyophilization. Enriched fractions were purified further by ion-exchange chromatography on QAE Sephadex as shown in Scheme 1. The components were selectively eluted by increasing the percentage of HOAc in a linear gradient. Compounds **2** and **3** required an hplc step followed by desalting to obtain pure compounds.

COMPOUND **4**.—Mass spectral data for **4** revealed a protonated molecular ion $[MH]^+$, *m/z* 368 (found 368.1206, calcd 368.1188), corresponding to a molecular formula of $C_{14}H_{18}N_5O_7$. Fragmentation of **4** gave *m/z* 136 [adenine, $[H]^+$ and a uv λ max 263 (H_2O) indicated the presence of adenine in the molecule.

The details and comparison of all the chemical shifts and coupling constants are given in Table 1. A COSY spectrum obtained in DMSO-d_6 revealed the chemical shifts and connectivities of the protons shown, and the assignment of H-3′ (δ 4.05) and H-4′ (δ 3.95) could be made unambiguously. Selective decoupling by irradiating at δ 4.65 (H-2′) collapsed the signal of H-3′ from a broad triplet to a doublet ($J = 6$ Hz). No change at H-4′ was observed. Two sets of geminal protons coupled to a single proton (H-6′) were observed. Furthermore, H-5′ and H-5′ were coupled to H-4′. In order to



SCHEME 1. Isolation of griseolic acid analogues **1–4**.

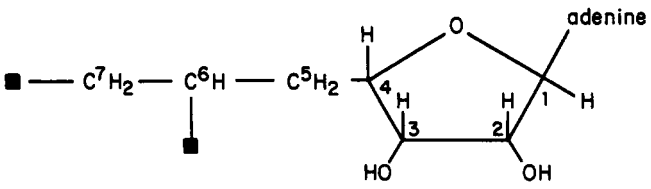


FIGURE 4. Partial structure of compound **4**.

accommodate this information, a partial structure was constructed as illustrated in Figure 4.

The ^{13}C-nmr spectrum of **4** showed the presence of 14 carbons. The chemical shift values for **4** and the comparison with chemical shifts for compounds **1–3** are given in Table 2. Assignments of the five adenine carbons were made by literature comparison (10). The chemical shift of the C-1 sugar (90.9 ppm) would indicate that there is substitution of adenine through nitrogen, and C-2 and C-3 are both substituted with OH groups (78.3, 78.5 ppm). This leaves substitution of the two carboxylic acids (C=O, 178.5, 179.5 ppm) at positions C-6 and C-7.

The size of the coupling between H-2′ and H-3′(J = 5 Hz) in **4** is comparable to the $J_{2,3}$ values in **1**, **2**, and **3**. This suggests that H-2′ and H-3′ are *cis* to each other. In this ring-opened compound, rigidity is lost when compared to the fused ring systems of **1**, **2**, and **3**. Because coupling constant data are unpredictable in the more flexible 5-membered ring systems, no further stereochemical assignments were made at this time for **4**.

DIHYDRODEOXYGRISEOLIC ACID [**3**].—The elucidation of structure **3** was made on the basis of spectroscopic data. Nmr data are given in Tables 1, 2, and 3, and fabms shows $[MH]^+$ *m/z* 366 and *m/z* 136. Further investigation of the stereochemistry of **3** was made by comparing chemical shifts and coupling constants for H-2′, H-3′, and H-4′ in **3** to the structure proposed. The sizes of the coupling constants for **3** are given in Table 3. The relative stereochemistry in **3** could be assigned based on coupling constants due to the rigidity of the fused-ring system and published X-ray data (11,12). We noted, however, that there was a paucity of nmr data for **3**. Since **3** is represented by an unusual and strained fused-ring system where H-3 and H-4 are located trans diaxial

TABLE. 1 ^{1}H-nmr Data for Compounds **1–4**.[a]

Compound (Solvent)	Proton						
	H-1′	H-2′	H-3′	H-4′	H-5′	H-6′	H-7′
1 D_2O	6.67 (s)	4.77 (d)	5.79 (q)	—	5.33 (d)	—	(4.7)
2 (DMSO-d_6)	6.48 (s)	4.55 (d) J = 5.1	5.90 (dd) J = 4.7,2.7	—	5.10 (d) J = 2.4	—	2.75 (d) J = 16.8 3.10 (d) J = 16.8
3 (DMSO-d_6)	6.20 (s)	4.39 (brd) J = 5	4.30 (dd) J = 10,5	4.25 (br tr) J = 11,10,6	1.85 (dd) J = 11,6 2.30 (tr) J = 11,11	—	2.82 (d) J = 16 3.00 (d) J = 16
4 (D_2O)	5.95 (d) J = 5	4.65 (dd) J = 5,6	4.05 (br tr) J = 5,6	3.95 (m)	1.92 (ddd) J = 15,10,5 2.25 (ddd) J = 15,8,4	2.84 (m)	2.48 (dd) J = 18,6 2.60 (dd) J = 18,8
4 (DMSO-d_6)	5.86 (d) J = 5	4.61 (dd) J = 5,4	4.01 (tr) J = 4,4	3.93 (d tr) J = 9,4,4	1.82 (m) 1.96 (m)	2.71 (m)	2.42 (dd) J = 16,5.5 2.48 (dd) J = 16,4

[a] ^{1}H nmr taken in solvent indicated on a Varian XL-300 instrument, δ in ppm, J(Hz). Adenine protons H-6 and H-8 were observed as singlets between δ 8.2 and δ 8.3.

TABLE 2. ^{13}C-nmr Data for Compounds **1–4**.[a]

Carbon	Compound (Solvent)			
	1 (D_2O) (8)	**2** (DMSO)	**3** (DMSO)	**4** (D_2O)
1	159.2	158.9	158.5	155.1
2	145.7	142.7	145.4	151.0
3	119.7	119.0	118.7	120.5
4	148.4	148.2	147.4	150.9
5	143.2	140.7		144.0
1′		97.4	99.2	90.9
2′		69.6	68.0	78.5
3′		83.4	85.5	78.3
4′		152.4	68.0	84.4
5′		98.8	42.8	39.5
6′		94.8	90.7	41.4
7′		42.9	45.2	37.7
−COOH . . .	171.5, 171.2	172.8, 170.6	175.7, 172.8	179.5, 178.5

[a] ^{13}C nmr obtained in solvent indicated on a Varian XL-300 instrument.

to each other, we performed detailed nmr studies that included COSY, NOESY, difference nOe, and homonuclear decoupling experiments. The size of the coupling between H-3′ and H-4′, $J = 10$ Hz, suggests a transdiaxial relationship, and the coupling constant $J_{2,3} = 5$ Hz between H-2′ and H-3′ indicates a *cis* relationship. Since there is virtually no coupling between H-1′ and H-2′, adenine is connected to the C-1 of the sugar in a *cis* orientation with respect to H-3′ and *trans* to H-4′. Furthermore, a difference nOe study revealed a through space interaction between H-3′ and H-5′ as expected. Component **3** can, therefore, be assigned the same structure (inclusive of stereochemistry) as previously proposed (8, 11, 12).

DEOXYGRISEOLIC ACID [**2**].—The structure of **2** was established on the basis of nmr and mass spectral data $[MH]^+$, *m/z* 364.0893 (calcd 364.0880) and *m/z* 136. Details are given in Tables 1 and 2. Comparison of spectroscopic data (11) to reported data indicates the same structure as previously proposed.

GRISEOLIC ACID [**1**].—Isolation of **1** from culture filtrate was followed by an hplc comparison with an authentic sample (8). Fabms ($[MH]^+$, *m/z* 380) confirmed the presence of griseolic acid.

In summary, the culture filtrate from *S. griseoaurantiacus* has been shown previously to yield griseolic acid and dihydrodeoxygriseolic acid. Hplc methodology was developed and coupled with photodiode array detection to screen rapidly for griseolic acid analogues directly from fermentation broths. Three griseolic acid analogues were detected and isolated from fermentation broth. Component **4** was detected in fermentation broth by hplc photodiode array acquisition due to the presence of the adenine chromophore. The compound does not inhibit cAMP PDE presumably because the

TABLE 3. Selective ^{1}H-nmr Data Indicating the Size of the Coupling Constants for Compound **3**.

Compound	*J* values in Hz				
	$J_{H\text{-}1',H\text{-}2'}$	$J_{H\text{-}2',H\text{-}3'}$	$J_{H\text{-}3',H\text{-}4'}$	$J_{H\text{-}4',H\text{-}5'}$	$J_{A,B}$ or J_{AX}
3	$J_{1,2} = 0$	$J_{2,3} = 5$	$J_{3,4} = 10$	$J_{4,5} = 6$	$J_{4,5} = 11$ $J_{5,5} = 11$

opened-ring form lacks the necessary rigidity and conformation effects of the two carboxylic acid groups present in the active components **1**, **2**, and **3** (13,14).

The hplc photodiode array detection approach has been used for rapid screening of culture broths (15,16) and provides a rapid approach to screening and detecting new structurally related entities in a complex matrix such as fermentation broth.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—A Varian XL 300 instrument was used to record ^{1}H-nmr and ^{13}C-nmr spectra in DMSO-d_6. The mass spectra were recorded on a Finnigan MAT-312 spectrometer. High-resolution data were obtained on a VG ZAB-SE mass spectrometer. The hplc was performed on a Waters Associates ALC 201 Liquid Chromotograph (Milford, Massachusetts) and a Hewlett-Packard HP 1040A diode array detector (Palo Alto, California).

FERMENTATION.—The inoculum was prepared in a medium containing beef extract 0.3%, tryptone 0.5%, yeast extract 0.5%, cerelose 0.1%, potato starch 2.4%, and $CaCO_3$ 0.2%. A 300-ml Erlenmeyer flask containing 70 ml of the above medium was sterilized, then inoculated with 3 ml of a stock suspension of the producing culture of *S. griseoaurantiacus* NRRL-12314 which had been maintained at −20°. The contents of the flask were incubated at 30° on a rotary shaker (300 rpm) for 48 h. Seed culture (2.5 ml) was transferred in to a 300-ml Erlenmeyer flask containing 70 ml of the same medium and incubated as above. This second stage seed culture (17.5 ml) was used as the inoculum to initiate the fermentation in a 2-liter Erlenmeyer flask containing 350 ml of a medium consisting of potato dextrin WPD-650 5%, soy grits 3.5%, cerelose 0.5%, and 1 mM $CoCl_2$ (10 ml). The fermentation was carried out at 30° on a rotary shaker (300 rpm) for 90 h.

INSTRUMENTATION.—*Hplc-Column.*—Shandon Hypersil ODS (5 μm) 15 cm × 4.6 mm, using a mobile phase consisting of an aqueous mixture of ion pair reagent (0.005 M tetrabutylammonium acetate and 0.005 M ammonium acetate) and MeOH (75:25), at 1 ml/min. Photodiode array signals were integrated at 235 and 260 nm.

ISOLATION.—At harvest, the whole broth (3 liters) was filtered through Whatman filter paper, and the broth filtrate (2.3 liters) was passed through a column (5 × 60 cm) of MCI CHP20P gel (Mitsubishi Chemical, Japan) equilibrated in dilute HOAc, pH 2. Upon completion of feed volume, the column was eluted with H_2O to give an enriched fraction (5.3 g) containing **1** and **4**. Elution with 0.003 N NH_4OH gave a mixture of **2** and **3** (1.1 g).

SEPARATION OF **1** AND **4**.—The fraction containing **1** and **4** was rechromatographed on MCI CHP2OP gel (1.5 × 30 cm), eluting with 2% aqueous HOAc. Fractions enriched with **4** were pooled to yield 48 mg. Further elution with HOAc gave **1** (57 mg). Compounds **1** and **4** were separately chromatographed on a QAE Sephadex (Pharmacia Fine Chemicals, Piscataway, New Jersey) column (1.1 × 20 cm) in the acetate form. Elution with a linear gradient of H_2O and increasing to 3% aqueous HOAc gave **4** (9 mg) after lyophilization. Elution of **1** from the column required 6% aqueous HOAc to yield 4.7 mg after lyophilization.

SEPARATION OF **2** AND **3**.—The fraction enriched in **2** and **3** was chromatographed on QAE Sephadex (OAc^-) eluting with a linear gradient of 0 to 6% HOAc. Compound **2** was eluted first (yield 24.3 mg) followed by **3** (23 mg). Each component was purified further by hplc using conditions described above followed by a desalting step on QAE Sephadex (OAc^-) and subsequent elution with 5% HOAc. In this manner **2** (3.5 mg) and **3** (1.7 mg) were obtained as white amorphous powders after lyophilization.

LITERATURE CITED

1. F. Nakagawa, T. Okazaki, A. Naito, Y. Iijima, and M. Yamazaki, *J. Antibiot.*, **38**, 823 (1985).
2. Y. Iijima, F. Nakagawa, S. Handa, T. Oda, A. Naito, and M. Yamazaki, *FEBS Lett.*, **192**, 179 (1985).
3. S.A. Waldman and F. Murad, *Pharmacol. Rev.*, **39**, 163 (1987).
4. V.C. Manganiello, *J. Mol. Cell. Cardiol.*, **19**, 1037 (1987).
5. H.P. Fiedler, *J. Chromatogr.*, **316**, 487 (1984).
6. D.N. Buchanan and J.G. Thoene, *J. Chromatogr.*, **344**, 23 (1985).
7. K. Zech, R. Huber, and H. Elgass, *J. Chromatogr.*, **282**, 161 (1983).
8. S. Takahaski, F. Nakagawa, K. Kawazoe, Y. Furukawa, S. Sato, C. Tamura, and A. Naito, *J. Antibiot.*, **38**, 830 (1985).
9. A. Naito, F. Nakagawa, T. Okazaki, A. Terahara, S. Iwado, and M. Yamazaki, U.S. Patent US 4,460,765, 1984; *Chem. Abstr.*, **102**, 4455z.

10. L.F. Johnson and W.E. Jankowski, "Carbon-13 NMR Spectra," John Wiley and Sons, New York, 1972, p. 276.
11. F. Nakagawa, Y. Tsujita, and M. Yamazaki, *Eur. Pat. Appl.*, EP 162,715, 1985; *Chem. Abstr.*, 104, 67509g.
12. S. Takahashi, F. Nakagawa, and S. Sato, *J. Antibiot.*, **41**, 705 (1988).
13. E.S. Severin, N.N. Gulyaev, T.V. Bulargina, and M.N. Kochetkava, *Adv. Enzyme Regul.*, **17**, 251 (1979).
14. Y. Murofushi, M. Kimura, Y. Iijima, M. Yamazaki, and M. Kaneko, *Chem. Pharm. Bull.*, **35**, 4442 (1987).
15. R. Mierzwa, R. Cooper, and B. Pramanik, *J. Chromatogr.*, **436**, 259 (1988).
16. R. Mierzwa, J. Marquez, M. Patel, and R. Cooper, in: "Natural Products Isolation." Ed. by G.H. Wagman and R. Cooper, Elsevier Holland J. Chromatography Library Series, Vol. 43, Ch. 2, in press.

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