EPIPOLYTHIOPIPERAZINEDIONE ANTIBIOTICS FROM *PENICILLIUM TURBATUM*

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A group of epipolythiopiperazinedione antibiotics was obtained from the fermentation broth of *Penicillium turbatum*. The fermentation, isolation, biological properties and structure elucidation of three metabolites of this group are discussed.

During our search for new antibiotics, *in vitro* antiviral activity was detected in the crude broths and its butanol extracts of a strain of *Penicillium turbatum* (WESTLING). *Penicillium turbatum* was isolated from a soil obtained from Mt. Ararat in Eastern Turkey and has been assigned the number NRRL 5630. The components responsible for the antiviral activity were found to be members of the epipolythiopiperazinedione antibiotics*.

Fermentation

The stock cultures of *Penicillium turbatum* were maintained as lyophilized pellets and under mineral oil. One lyophilized pellet was used to inoculate an agar slant composed of 20 g, glucose; 5 g, peptone (Difco); 0.5 g, KH_2PO_4; 0.02 g, $MgSO_4 \cdot 7H_2O$; 0.01 g, $FeSO_4$; 20 g, agar; 1,000 ml, H_2O. A vegetative culture was prepared by the inoculation of: 25 g, sucrose; 36 g, edible molasses; 6 g, corn steep solids; 2 g, K_2HPO_4; 10 g, NZ Case (Sheffield); 1,000 ml water with 1 ml spore suspension of a 7-day old culture to 100 ml of vegetative medium. Five percent of the resulting 48-hour culture was inoculated into 100 ml of fermentation medium which was composed of 20 g, edible molasses; 5 g, peptone (Difco); 2 g, $CaCO_3$; 10 g, glucose and 1,000 ml, H_2O. All growth conditions were maintained at 25°C. Fermentations in shaken culture were incubated in 100 ml/500 ml Erlenmeyer flasks for 72 hours at 25°C at 250 rev/min. on a rotary shaker with a 2″ (5.12 cm) stroke.

Several paper chromatographic solvent systems were used to distinguish this antiviral activity from other known antiviral antibiotics. Paper chromatograms were developed by descending chromatography on Whatman # 1 paper. The developed chromatograms were dried and plated by standard bioautographic techniques on glass plates of polio III virus infected BSC-I monkey kidney cells overlaid with agar. In the system of benzene saturated with water, the R_f value was 0.85 and in propanol-water (1 : 9) an R_f of 0.7 was obtained.

* After the work described in this paper was completed, and during the preparation of this manuscript, a report on a novel class of diketopiperazine from an unidentified fungus has appeared[1]. Two of the metabolites reported by DEVAULT and ROSENBROOK[1], seem to be the same as two of the metabolites isolated by us from *Penicillium turbatum*.

Isolation and Physical-Chemical Characterization

The filtered broth from 100 liters of fermentation medium was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was dried over sodium sulfate and concentrated under reduced pressure to yield 15.4 g of an oil. This oil was chromatographed over a 5.0×62.0 cm column of silica gel (grade 62; 60~200 mesh; Matheson, Coleman and Bell). The column was eluted with ethyl acetate-methanol (9 : 1), collecting 20 ml fractions at a rate of 3 ml/minute. During both this and subsequent chromatographies, the presence of two metabolites*, A26771A and A26771C, in column fractions was determined by thin-layer chromatography on silica gel (E. Merck, 20×20 cm, pre-coated, F-254, layer thickness 0.25 mm on glass, Brinkmann Instr., Inc.), developed in benzene-methanol (95 : 5). *Sarcina lutea* was used as a detecting organism on bioautographs. A26771A and A26771C could be detected with a silver nitrate spray reagent No. 224[2], and a third metabolite, A26771E, was detectable on thin-layer plates with phosphomolybdic acid spray. A26771A and A26771C appeared on the plates as yellow spots at room temperature, shortly after spraying and A26771E developed an intense blue color after spraying and heating at 100°C. A26771A and A26771C were biologically active. A26771E, however, was inactive against *Sarcina lutea*.

The column fractions, containing a mixture of the above-mentioned three factors, were combined and concentrated under reduced pressure to an oily residue of 1.8 g. This residue was re-chromatographed on a 4.5×39.0 cm silica gel column, eluting with chloroform-methanol (99 : 1) at a flow rate of 2 ml/minute. Fractions containing the active A26771A and the inactive A26771E were combined, concentrated under reduced pressure to a small valume and dissolved in 30 ml of acetone and 10 ml of diethyl ether. After standing at room temperature for 30 minutes, 400 mg of nearly colorless crystals of A26771E was obtained from this red-yellowish colored solution. A26771E was re-crystallized from warm acetone, mp 135°C. IR(KBr): 3350 cm^{-1} (OH), 1655, 1640, 1630 cm^{-1} (amide), 727, 694 cm^{-1} (mono-substituted phenyl). UV (EtOH): End absorption with a shoulder (ε 1000) at 255 nm (phenyl). $[\alpha]_D^{27}$ −47° (*c* 0.13, CH_3OH). NMR (DMSO-d_6): 7.20 (5H, s), 5.10 (1H, t, J=6.0), 3.82 (1H, d, J=15.0), 3.72 (1H, q, J=6.0, 12.0), 3.57 (1H, q, J=6.0, 12.0), 3.25 (1H, d, J=15.0 Hz), 3.04 (3H, s), 2.99 (3H, s), 2.25 (3H, s), 2.12 δ (3H, s). On shaking with D_2O the triplet at 5.10 disappeared and the quartets at 3.72 and 3.57 δ collapsed to doublets with J=12.0 Hz. Mass spectrum: M^++1 at 355 (weak), *m/e*=323.0878 ($C_{15}H_{19}N_2O_2S_2$), and *m/e*=307.1137 ($C_{15}H_{19}N_2O_3S$). Elemental analysis:

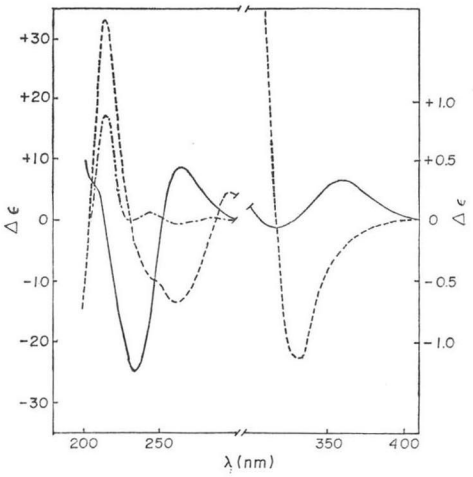
Calcd. for:	$C_{16}H_{22}N_2O_3S_2$:	C 54.21, H 6.26, N 7.90, O 13.54, S 18.09.
Found:		C 54.80, H 6.38, N 7.87, O 13.98, S 20.29.

The filtrate from the A26771E crystals and other biologically active fractions from the above silica gel column were combined and chromatographed on a 5.0×50.0 cm column of silica gel, Grade 62, eluting with chloroform at a flow rate of 0.5 ml/minute and collecting 5-ml fractions. A26771A was found in fractions 170~210. These fractions were combined, concentrated and re-chromatographed on a 2.0×110.0 cm column of silica gel, Grade 62, and were eluted with chloroform-methanol (98.5 : 1.5). The active fractions were combined and

* In addition to the three metabolites described in this paper, the fermentation produced two other factors. One of them, A26771B, is a new macrolide antibiotic and will be described in detail in a subsequent paper. The other factor, A26771D, has not been characterized.

concentrated; 90 mg of A26771A* crystallized in the form of yellow, prismatic crystals from acetone-diethylether-pentane, mp 105°C. IR (KBr): 3460 cm^{-1} (OH); 1680, 1665, 1640 cm^{-1} (amide), 734, 698 cm^{-1} (mono-substituted phenyl). $[\alpha]_D^{27}$ −88° (*c* 0.15, CH_3OH). NMR (DMSO-d_6): 7.34 (5H, s), 5.92 (1H, t, J=6.0), 4.41 (1H, q, J=6.0, 13.0), 4.23 (1H, q, J=6.0, 13.0), 3.99 (1H, d, J=16), 3.75 (1H, d, J=16 Hz), 3.15 (3H, s), 2.86 δ (3H, s). On shaking with D_2O the triplet at 5.92 disappeared and the quartets at 4.41 and 4.23 δ collapsed to doublets with J=12.0 Hz. Mass spectrum: M^+=324.0621 ($C_{14}H_{16}N_2O_3S_2$). Elemental analysis:

Fig. 1. CD spectra of A26771A (**II**), A26771C (**III**) and A26771E (**IV**) iu MeOH.



Calcd. for: $C_{14}H_{16}N_2O_3S_2$: C 51.80, H 4.95, N 8.05, O 14.80, S 19.80.
Found: C 52.10, H 5.05, N 8.10, O 15.10, S 19.40.

During the chromatography of A26771A, the presence of another biologically active compound was observed. The fractions containing this compound were combined, concentrated to an oil and purified on a 2.0×100.0 cm column of silica gel, Grade 62, eluting with ethyl acetate-hexane (1 : 2) at a flow rate of 2 ml/minute. The fractions containing the new active material were combined and concentrated to an oil, which crystallized from acetone-diethylether: pentane to give 12 mg of A26771C, mp 130°C. IR(KBr): 3480 cm^{-1} (OH), 1667, 1650, 1640 cm^{-1} (amide), 735, 702, 658 cm^{-1} (mono-substituted phenyl). $[\alpha]_D^{27}$ −187° (*c* 0.04, CH_3OH). NMR ($CHCl_3/D_2O$): 7.25 (5H, m), 4.26 (1H, d, J=12.5), 4.05 (1H, d, J=15.0), 3.85 (1H, d, J=12.5), 3.26 (1H, d, J=15.0 Hz), 3.13 δ (3H, s). Mass spectrum: M^+ at 388 (weak), *m/e*=260.1183 ($C_{14}H_{16}N_2O_3$). Elemental analysis:

Calcd. for: $C_{14}H_{16}N_2O_3S_4$: C 43.28, H 4.15, N 7.20, S 33.0.
Found: C 43.12, H 4.21, N 7.02, S 32.5.

The separation of A26771A and A26771C was monitored on silica gel tlc plates with chloroform-methanol (98 : 2).

Factor	Relative R_f values
A	0.48
C	0.37

Biological Properties

Antibiotics A26771A and A26771C have shown antiviral and antibacterial activity. A26771E was inactive in all these areas.

The activity of A26771A and A26771C against virus growth in tissue culture has been

* Comparison by paper bioautography of the epidithiopiperazinedione described by DEVAULT and ROSENBROOK[1], and A26771A showed they both had same R_f values. The gross structures of two novel diketopiperazines reported recently[1] are the same as A266771A and A26771E. The Abbott workers do not report optical properties. Consequently, we cannot compare the absolute configuration of A26771A with their metabolite.

Table 1. Antiviral activity of A26771A and A26771C

mcg/ml	Zone mm (microscopic examination)			
	Polio virus		Coxsackie virus	
	A26771A	A26771C	A26771A	A26771C
1000	50(4+)*	50(4+)	52(4+)	50(4+)
125	44(4+)	ND**	40(4+)	ND
100	ND	40(4+)	ND	40(4+)
15	34(4+)	ND	32(4+)	ND
1.5	16(4+)	ND	15(4+)	ND

* A grading of 4+ indicates lack of virus breakthrough.
** ND=Not done.

demonstrated against several viruses. The *in vitro* antiviral activity by the agar-diffusion test[3] is shown in Table 1.

The antibacterial activity of A26771A and A26771C determined by the agar dilution method, is summarized in Table 2. The antibiotics were dissolved and diluted in ethanol prior to incorporation into MUELLER-HINTON blood agar plates. The plates were inoculated according to the ICS agar dilution procedure and incubated 18 hours at 37°C.

Table 2. Antibacterial spectra of A26771A and A26771C

Organism	MIC in mcg/ml	
	A26771A	A26771C
Streptococcus pyogenes C203 (Group A)	< 0.25	<0.25
Streptococcus sp. 9960 (Group D)	16	8
Streptococcus sp. 9901 (Group D)	16	8
Diplococcus pneumoniae Park I	< 0.25	<0.25
Staphylococcus aureus 3055	8	8
Staphylococcus aureus 3074	16	8
Staphylococcus aureus 3130	16	2
Escherichia coli EC14	32	64
Enterobacter cloacae EB5	>64	64
Klebsiella pneumoniae KL3	16	32
Mima polymorpha MP1	>64	64
Proteus mirabilis PR6	64	64
Proteus rettgeri PR9	< 0.25	<0.2
Pseudomonas aeruginosa PS8	< 0.25	<0.2
Pseudomonas aeruginosa PS9	< 0.25	<0.5
Pseudomonas aeruginosa PI21 (Gentamicin resistant)	< 0.25	<0.5
Salmonella typhosa SA12	< 0.25	0.5
Shigella sonnei SH10	8	0.5
Serratia marcescens SE3	2	1.0

Structure Elucidation

A26771A is a neutral $C_{14}H_{16}N_2O_3S_2$ compound and belongs to the growing class of epidithiopiperazinedione antibiotics. This group includes gliotoxin[4] (**I**), sporidesmin[5]; aranotins[6,7] chaetocin[8], verticillin[9], melinacidins[10], oryzachlorin[11], and chaetomin[12]. Its mass spectrum shown an intense M^+-S_2 at 260 characteristic of these compounds[6]. The infrared spectrum shows the presence of hydroxy (3460 cm^{-1}) and mono-substituted phenyl (734, 698 cm^{-1}) groups. In the nmr spectrum (in DMSO-d_6), the exchangeable one proton triplet at 5.92 and a five proton singlet 7.34 δ confirm the presence of hydroxyl and mono-substituted phenyl groups. Further, the hydroxyl proton triplet at 5.92 δ (J=6 Hz) is coupled to the methylene protons centered at 4.41 and 4.23 δ with geminal coupling of 13 Hz, and coupling with the hydroxyl proton of 6 Hz. This assignment is confirmed by the disappearance of the triplet at 5.92 and collapse of the quartets at 4.41 and 4.23 to doublets with J=13.0 Hz upon shaking with D_2O, thereby establishing the presence of the CH_2OH moiety in A26771A. The two three-proton singlets at 3.15 and 2.86 are due to two NCH_3 groups, and the doublets at 3.99 and 3.75 δ, with geminal coupling of 16 Hz, is due to $C_6H_5C\underline{H}_2$ moiety. From the above data structure **II** can be written for A26771A.

Fig. 2.

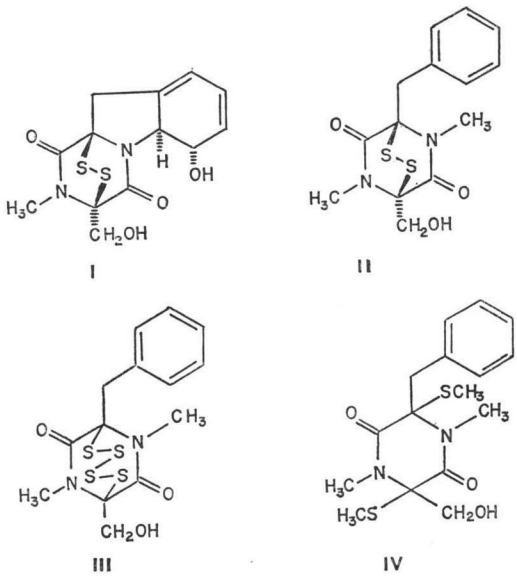


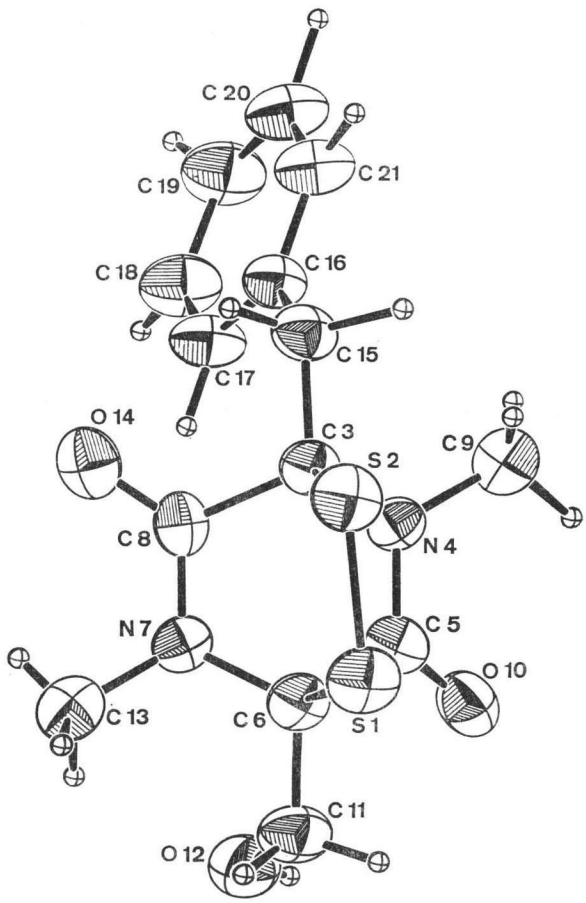
Table 4. Heavy atom coordinates (standard deviations) $\times 10^4$ for A26771A in the crystal. Atoms are numbered as in Fig. 3.

Atom	*X*	*Y*	*Z*
S(1)	−258(2)	−5038(4)	178(2)
S(2)	1689(2)	−5461(4)	896(2)
C(3)	1968(6)	−5097(10)	3067(7)
N(4)	1424(5)	−3585(8)	3359(6)
C(5)	119(7)	−3462(9)	2880(8)
C(6)	−553(6)	−5044(11)	2151(7)
N(7)	−16(6)	−6487(8)	3024(6)
C(8)	1267(7)	−6628(9)	3445(8)
C(9)	2162(8)	−2071(11)	3605(9)
O(10)	−466(5)	−2227(7)	3015(6)
C(11)	−1987(6)	−4883(13)	1821(8)
O(12)	−2382(4)	−4907(8)	3190(6)
C(13)	−802(8)	−7942(10)	2963(10)
O(14)	1853(6)	−7870(7)	4006(6)
C(15)	3414(6)	−5232(11)	3759(8)
C(16)	3932(6)	−5020(11)	5508(8)
C(17)	3236(6)	−5374(12)	6539(8)
C(18)	3798(7)	−5192(15)	8138(8)
C(19)	5020(8)	−4636(15)	8689(10)
C(20)	5745(8)	−4341(14)	7668(10)
C(21)	5184(7)	−4515(12)	6057(10)

Table 3. Crystal parameters for A26771A

Empirical formula	$C_{14}H_{16}N_2O_3S_2$
Molecular weight	324.4
a	10.911±0.003 Å
b	8.137±0.003 Å
c	8.978±0.003 Å
β	106.40 ±0.01°
Space group	$P2_1$
Molecules/cell	2
Observed density	1.39 g.cm^{-3}
Calculated density	1.41 g.cm^{-3}

Fig. 3. Conformation of A26771A in the crystal. The thermal ellipsoid are drawn at the 50 % probability level.



Antibiotic A26771A was crystallized from diethylether as colorless needles with the crystal parameters given in Table 3. The intensities of 1225 reflections were measured on a four-circle automated diffractometer using copper radiation. The sulfur positions were lacated from a sharpened PATTERSON vector map and the resultant

phase set was refined by use of the tangent formula. An electron density map based on the refined phases showed all 21 heavy atoms. The structure was partially refined by the least-squares method and all 16 hydrogen atoms were located from a difference electron density map. The final least-squares refinement with all atoms gave an R value of 0.049. The coordinates for the heavy atoms are given in Table 4.

The conformation of the molecule in the crystal is shown in Fig. 3 and the bond distances and angles are given in Table 5. Although the absolute configuration of the molecule has not been determined by X-ray diffraction, the circular dichroism studies mentioned below support the configuration shown. The chirality of each of the two asymmetric centers is *R* and the helical sense of the disulfide bond is left-handed, with a dihedral angle of 11.8°.

It should be noted that in all epidithiodiketopiperazine systems reported to date, the helicity of the disulfide bond is such that each sulfur atom is closer to the adjacent carbonyl carbon

Table 5. Bond distances and angles for A26771A

Atom			Distance (Å) or angle (°)	Atom			Distance (Å) or angle (°)
2	1	3		2	1	3	
S(1)	S(2)		2.068	C(6)	C(5)	C(11)	110.7
	C(6)		1.888		N(7)	C(11)	114.6
	S(2)	C(6)	98.0	N(7)	C(8)		1.347
S(2)	C(3)		1.909		C(13)		1.454
	S(1)	C(3)	98.4		C(6)	C(8)	116.9
C(3)	N(4)		1.422		C(6)	C(13)	119.8
	C(8)		1.549		C(8)	C(13)	119.7
	C(15)		1.528	C(8)	O(14)		1.225
	S(2)	N(4)	111.6		C(3)	N(7)	114.1
	S(2)	C(8)	98.8		C(3)	O(14)	121.4
	S(2)	C(15)	104.6		C(7)	O(14)	124.4
	N(4)	C(8)	113.4	C(11)	O(12)		1.412
	N(4)	C(15)	115.1		C(6)	O(12)	112.4
	C(8)	C(15)	111.8	C(15)	C(16)		1.521
N(4)	C(5)		1.369		C(3)	C(16)	116.8
	C(9)		1.454	C(16)	C(17)		1.384
	C(3)	C(5)	117.2		C(21)		1.378
	C(3)	C(9)	121.3		C(15)	C(17)	123.6
	C(5)	C(9)	117.9		C(15)	C(21)	116.4
C(5)	C(6)		1.534		C(17)	C(21)	119.9
	O(10)		1.215	C(17)	C(18)		1.401
	N(4)	C(6)	113.7		C(16)	C(18)	120.0
	N(4)	O(10)	123.9	C(18)	C(19)		1.362
	C(6)	O(10)	122.4		C(17)	C(19)	120.5
C(6)	N(7)		1.441	C(19)	C(20)		1.390
	C(11)		1.514		C(18)	C(20)	119.9
	S(1)	C(5)	102.1	C(20)	C(21)		1.409
	S(1)	N(7)	111.2		C(19)	C(21)	119.8
	S(1)	C(11)	104.9	C(21)	C(16)	C(20)	119.8
	C(5)	N(7)	112.3				

atom rather than the adjacent amide nitrogen atom, presumably because of electrostatic attraction. This means then, that the chirality of the asymmetric centers determines the helicity of the disulfide bond; *i.e.*, if the carbon chiralities are *R*, the disulfide bridge is left-handed and if *S*, right-handed.

Table 6. Effect of the polarity of solvent on the CD spectra of A26771A

CF_3CH_2OH	CH_3OH	CH_3CN	Dioxane
356	360	360	362
+0.40	+0.32	+0.23	+0.24
317*	313	317	316
+0.08	−0.06	−0.10	−0.12
263	263	263	265
+8.4	+8.8	+8.03	+7.1
228	233	234	237
−25.4	−25.4	−25.4	−25.5

* The ellipiticity at 317 nm is still positive ($\Delta\varepsilon$=0.08), but from the data in other solvents there should be a negative COTTON effect at about this wavelength.

By comparison of the physical-chemical data of A26771A, A26771C and A26771E, the close structural relationship of these three metabolites is apparent. Accordingly, the major difference between A26771A and A26771C is that the latter compound contains two sulfur atoms more than A26771A. Whereas, the mass spectrum A26771A affords in intense m/e=260 arising from M^+-S_2, the mass spectrum of A26771C also gives an intense m/e=260, but in the latter case due to M^+-S_4. These data establish that A26771C has the epitetrathiopiperazinedione structure **III**.

A comparison of the physical-chemical properties of A26771A and A26771E reveals that in addition to all the spectral characteristics of A26771A, the nmr spectrum of A26771E contains two three-proton singlets at 2.25 and 2.12 δ suggesting the presence of two SCH_3 groups. This conclusion is corroborated by both elemental analysis, and the composition of m/e=307.1137 as $C_{15}H_{19}N_2O_3S$ arising by the cleavage of SCH_3 gronp from A26771E. Consequently, A26771E should have structure **IV**.

The absolute stereochemistry of A26771A was derived from its CD data. The CD spectrum of A26771A shows four COTTON effects, and these are assigned[13] as follows: (1) The positive 360 nm band-disulfide $n\sigma^*$ transition, (2) The negative 313 nm band-$n_1\rightarrow\pi^*$ charge transfer band, (3) The positive 263 nm band-n_2, $n_3\rightarrow\pi^*$ charge transfer band, and (4) The negative 233 nm band-overlapping of the peptide $n\pi^*$ transitions and disulfide n_2, $n_3\rightarrow\sigma^*$ transitions. The sign of the charge transfer transition CD bands can be used to predict the absolute configuration of epidithiopiperazinedione systems[13]. The sign of the 270 nm and 310~320 nm charge transfer transition CD bands are the same in gliotoxin, acetylaranotin, acetylaporanotin and A26771A. Consequently, the absolute configuration at the epidithiopiperazinedione system in gliotoxin and A26771A are *R* and represented by structures **I** and **II**, respectively. Also, on a biogenetic basis, such an assignment is reasonable. Since A26771C and A26771E are co-produced along with A26771A in the same fermentation, probably the absolute configuration at the two asymmetric centers in all the three metabolites are identical. However, the CD spectra of these three compounds are quite different, indicating thereby that conclusions derived for the epidithiopiperazinedione system (**II**) are not valid for the epitetrathiopiperazinedione (**III**) or dimethylthiopiperazinedione (**IV**) systems.

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