Cystothiazoles A and B, New Bithiazole-type Antibiotics from the Myxobacterium *Cystobacter fuscus*

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New bithiazole-type antibiotics, cystothiazoles A ($C_{20}H_{26}N_2O_4S_2$) and B ($C_{20}H_{26}N_2O_5S_2$), have been isolated from a culture broth of the myxobacterium, *Cystobacter fuscus*. The gross structures of these compounds were elucidated by spectroscopic analysis, and their absolute stereochemistry was determined by chemical degradation of cystothiazole A. Cystothiazole A inhibits fungi and human tumor cells, whereas it is inactive against bacteria. The antifungal activity appears to result from the inhibition of submitochondrial NADH oxidation. Although these compounds are structurally related to the known antibiotic myxothiazol, cystothiazole A was more active against fungi and less cytotoxic than myxothiazol.

In recent years a number of novel secondary metabolites have been discovered from myxobacteria, which have now been recognized as a rich source of new antibiotics. One of the most significant examples are the epothilons[1], which are attracting attention of many researchers[2,3]. In the course of our search for bioactive compounds from myxobacteria by using an inhibition assay against the phytopathogenic fungus, *Phytophthora capsici*[4], we found two new antifungal substances from a myxobacterium strain AJ-13278, which was identified as *Cystobacter fuscus*. These antibiotics termed cystothiazoles A and B (Fig. 1) are structurally related to myxothiazol, an antibiotic from the myxobacterium, *Myxococcus fulvus*[5], and show a broad antifungal spectrum. This paper describes the production, isolation, physico-chemical properties, structural elucidation, absolute stereochemistry, and biological activity of cystothiazoles.

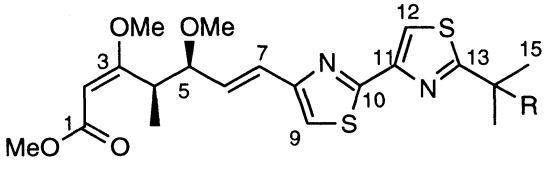
Microorganisms

Bacterial strain AJ-13278 was isolated from a soil sample collected at Kamakura, Kanagawa, Japan. According to the BERGEY'S Manual of Determinative Bacteriology, the strain was identified as *Cystobacter fuscus*, a member of fruiting gliding bacteria, myxobacteria. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, as FERM P-15997.

Fermentation

A slant culture of *C. fuscus* grown on vy/2 medium (baker's yeast 0.5%, $CaCl_2 \cdot H_2O$ 0.1%, cyanocobalamin 0.5 μg/ml, Bacto agar 1.5%, pH 7.2) was used throughout this work. A loopful of the slant culture was inoculated into a 300-ml Erlenmeyer flask containing 70 ml of a seed medium [Casitone (Difco) 1%, baker's yeast 0.5%, Malt extract (Difco) 0.2%, Yeast extract (Difco) 0.1%, $MgSO_4 \cdot H_2O$ 0.1%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.3%, HEPES 1%, Bacto agar 0.05%]. The flask was shaken on a rotary

Fig. 1. Structures of cystothiazoles A and B.



cystothiazole A, R = H
cystothiazole B, R = OH

Table 1. Physico-chemical properties of cystothiazoles A and B.

	Cystothiazole A	Cystothiazole B
Appearance	Colorless needles	White powder
mp	111~112°C	—
$[\alpha]_D^{25}$	+109° (*c* 0.24, $CHCl_3$)	+139° (*c* 0.086, $CHCl_3$)
Molecular formula	$C_{20}H_{26}N_2O_4S_2$	$C_{20}H_{26}N_2O_5S_2$
FAB-MS *m/z*	423 $(M+H)^+$, 391, 359, 279	439 $(M+H)^+$, 407, 391, 375, 295
HRFAB-MS		
Calcd for (M+H):	423.1412	439.1351
Found:	423.1426	439.1364
Elemental analysis		
Calcd:	C 56.80, H 6.20, N 6.63	—
Found:	C 56.82, H 6.29, N 6.58	—
UV λ_{max}^{MeOH} nm (ε)	223 (38,200), 242 (34,500), 310 (12,400)	222 (38,400), 243 (35,700), 311 (12,000)
IR $\nu_{max}^{CHCl_3}$ cm^{-1}	3123, 1706, 1624, 1150, 1094	3620, 3123, 1706, 1623, 1150
TLC (Rf value)		
System A[a]	0.58	0.24
System B[b]	0.21	0.41

[a] Silica gel plate, hexane-EtOAc (2:1).
[b] RP-18 plate, 80% aq. MeOH.

shaker (180 rpm) for 3 days at 28°C. Five milliliters of the seed culture thus obtained were inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a production medium. The production medium was the same as the seed medium except that 2% (w/v) adsorber resin SP207 (Mitsubishi Chemical Co.) was added to promote the productivity of the active substances. The fermentation was done on a rotary shaker (180 rpm) at 28°C for 4 days.

Isolation

The bacterial cells and the adsorber resin were collected from 2.5-liter culture broth and extracted with a mixture of acetone and methanol. The extract was partitioned between 60% aqueous methanol and dichloromethane, and the dichloromethane fraction was chromatographed on silica gel. The ethyl acetate fraction, which was active against *P. capsici* at 1 μg/disc, was subjected to silica gel medium-pressure liquid chromatography to give several active fractions. The most active fraction was purified by recrystallization to yield 31.6 mg of cystothiazole A as colorless needles. A more-polar active fraction was separated by silica gel column chromatography followed by TLC to give an active fraction, which was further purified by normal-phase HPLC to give 1.7 mg of cystothiazole B as a colorless powder.

Physico-chemical Properties

Table 1 summarizes the physico-chemical properties of cystothiazoles A and B. Both compounds were soluble in methanol, ethanol, acetone, ethyl acetate, chloroform, and benzene, slightly in hexane, and scarcely in water. Positive FAB-MS showed the pseudomolecular ion $(M+H)^+$ at 423 for cystothiazole A and 439 for cystothiazole B, and high-resolution FAB-MS revealed the molecular formulae $C_{20}H_{26}N_2O_4S_2$ and $C_{20}H_{26}N_2O_5S_2$, respectively. The molecular formula of cystothiazole A was also supported by elemental analysis. The UV spectra of cystothiazoles A (Fig. 2) and B are quite similar, suggesting that these compounds are congeners possess-

Fig. 2. UV spectrum of cystothiazole A (2.39×10^{-5} M in MeOH).

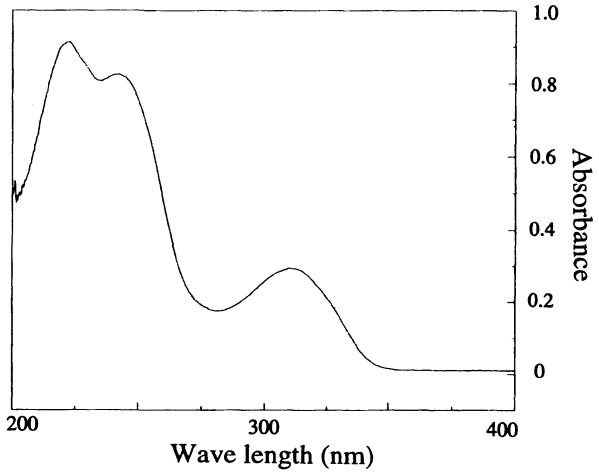


Fig. 3. IR spectrum of cystothiazole A ($CHCl_3$).

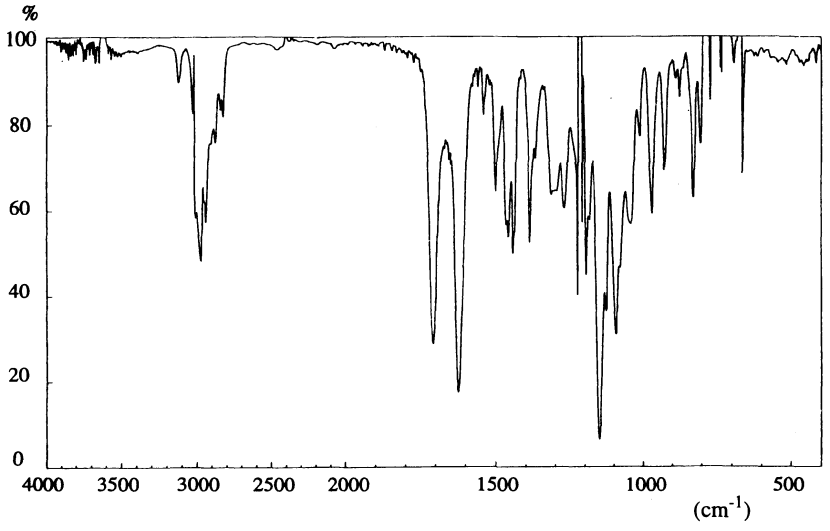


Table 2. NMR data for cystothiazoles A and B in $CDCl_3$.

Position	Cystothiazole A			Cystothiazole B
	^1H[a]	^{13}C[b]	HMBC[c]	^1H[a]
1		167.7 s	1-OMe, H-2	
1-OMe	3.66 s	50.8 q		3.67 s
2	4.96 s	91.1 d		4.97 s
3		176.7 s	H-2, 4, 3-OMe, 4-Me	
3-OMe	3.60 s	55.5 q		3.60 s
4	4.17 dq (8.0, 6.8)	39.8 d	H-2, 5, 4-Me	4.18 dq (8.0, 6.8)
4-Me	1.22 d (6.8)	14.1 q	H-4, 5	1.22 d (6.8)
5	3.81 dd (8.0, 7.6)	84.4 d	H-4, 7, 4-Me, 5-OMe	3.82 dd (8.0, 7.6)
5-OMe	3.33 s	57.0 q	H-5	3.34 s
6	6.41 dd (15.6, 7.6)	131.8 d	H-4, 7	6.42 dd (15.8, 7.6)
7	6.58 d (15.6)	125.4 d	H-5, 6, 9	6.57 d (15.8)
8		154.3 s	H-6, 7, 9	
9	7.09 s	114.9 d	H-7	7.09 s
10		162.6 s	H-9	
11		148.6 s	H-12	
12	7.87 s	115.1 d		7.90 s
13		178.6 s	H-12, 14, 15, 14-Me	
14	3.36 sept (6.8)	33.3 d	14-Me, H-15	—
14-OH	—	—		2.85 s
14-Me	1.44 d (6.8)	23.1 q	H-14, 15	1.72 s
15	1.44 d (6.8)	23.1 q	H-14, 14-Me	1.72 s

[a] Recorded at 400 MHz. Coupling constants in Hz are in parenthesis.
[b] Recorded at 100 MHz.
[c] Recorded at 400 MHz. Parameters were optimized for $J_{CH} = 10$ Hz.

ing a common chromophore. The absorption bands at 1705, 1625, and 1150 cm^{-1} in the IR spectrum of cystothiazole A (Fig. 3) are indicative of the presence of an α,β-unsaturated ester.

Structural Elucidation and Absolute Stereochemistry

The ^1H and ^{13}C NMR data for cystothiazole A were summarized in Table 2. The assignment of the direct connectivity between protons and carbons was estab-

Table 3. Antimicrobial spectrum of cystothiazole A in comparison with myxothiazol.

Microorganism	Cystothiazole A		Myxothiazol	
	Inhibition zone at 2 μg/disc (mm)	MIC (μg/ml)	Inhibition zone at 2 μg/disc (mm)	MIC (μg/ml)
Candida albicans AJ-5682	24	0.4	11	0.8
Saccharomyces cerevisiae YGSC-X-2180	30	0.1	12	0.4
Rhodotorula glutinis AJ-5012	19		13	
Torulopsis versatilis AJ-5327	26		10	
Aspergillus fumigatus AJ-117190	24	1.6	10	1.6
Botritys cinerea AJ-117140	18	1.6	16	3.2
Trichoderma reesei AJ-117127	10	6.3	11	6.3
Phytophthora capsici No. 302	16		13	
Staphylococcus aureus AJ-12510	—[a]		—	
Escherichia coli NIHJ	—		—	

[a] Inhibition was not observed.

lished by HETCOR experiments. The presence of two partial structures, an isopropyl group and $-CH(CH_3)-CH(OR)CH=CH-$, were easily determined from spin-spin coupling patterns in the ^1H NMR of cystothiazole A. The signals in the aromatic region of the ^1H and ^{13}C NMR spectra were presumed to correspond to a bithiazole substructure as in myxothiazol[5] by comparison of NMR data of both compounds. The bithiazole substructure was confirmed by ^1H-^{13}C long-range couplings determined by the heteronuclear multiple-bond correlation (HMBC) spectrum. The HMBC data are shown in Table 2. The above partial structures and the rest of the molecule [three methoxyl groups, an olefinic methine (C-2), and quaternary carbons (C-1 and C-3)] were also connected by the HMBC experiment to give the gross structure of cystothiazole A. The *E* geometry of the trisubstituted double bond at C-2 was evidenced by difference NOE data (H-2/3-OMe).

The gross structure of the minor product cystothiazole B was elucidated by comparison with the spectral data for cystothiazole A. The ^1H NMR data (Table 2) were similar to those for cystothiazole A except for the data for the isopropyl group (C-14 to C-16): the signals due to the methine proton H-14 disappeared and the methyl protons H-15 and H-16 were observed at a lower field as a singlet. These findings and the fact that the molecular formula of cystothiazole B is more than that of cystothiazole A by one oxygen suggest that cystothiazole B is 14-hydroxycystothiazole A.

The stereochemistry of cystothiazole A was determined by degradation experiments. Ozonolysis of cystothiazole A followed by an oxidative treatment with hydrogen peroxide and methylation with diazomethane gave dimethyl 2-methoxy-3-methylsuccinate. The ^1H NMR data for this diester was superimposable on the known *threo* isomer and its absolute configuration was determined to be 2*R*,3*R* by the specific rotation values ($[\alpha]_D$ $+32°$; lit.[6] $[\alpha]_D$ $+35°$), establishing the 4*R*,5*S* configuration of cystothiazole A. Both cystothiazoles A and B show similar ^1H NMR data (H-4 and H-5) and specific rotations, indicating that the absolute stereochemistry of cystothiazole B is identical with that of cystothiazole A.

Biological Properties

The minimum inhibitory amounts of cystothiazoles A and B against *Phytophthora capsici* were determined to be 0.05 and 1 μg/disc, respectively. For cystothiazole A, which was obtained in sufficient amount for the evaluation, the antimicrobial properties are investigated. The antimicrobial spectrum of cystothiazole A was summarized in Table 3 in comparison with myxothiazol[7]. Cystothiazole A was active against all fungi tested, but had no effect on bacteria. MICs were measured by serial dilution media [Potato dextrose broth (Difco)] ranging from 0.1 to 6.3 μg/ml. Although such a tendency of activity was same as that of myxothiazol, the potency of cystothiazole A was mostly higher.

Cystothiazole A was also tested for the *in vitro* cytotoxicity by using human colon carcinoma HCT-116 and human leukaemia K562 cells (Table 4). The IC_{50} values of cystothiazole A were 110~130 ng/ml, which were significantly higher than those of myxothiazol.

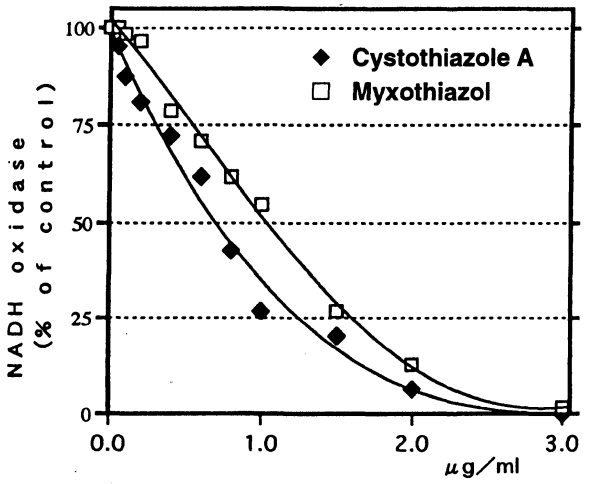
Since the mode of action of myxothiazol is known to be the inhibition of NADH oxidation of submito-

Table 4. Cytotoxic activity of cystothiazole A and myxothiazol.

Cell line	IC_{50} (ng/ml)	
	Cystothiazole A	Myxothiazol
HCT-116	130	13
K562	110	7.6

chondrial membrane fraction[8,9], the effect of cystothiazole A was tested. As shown in Fig. 4 both antibiotics showed comparable activity. Thus, the doses required for 50% inhibition of cystothiazole A and myxothiazol were 1.8 and 2.1 μM, respectively.

Fig. 4. Inhibition of NADH oxidase in a submitochondrial membrane fraction by cystothiazole A in comparison with myxothiazol.



Discussion

The cystothiazoles are bithiazole-type antibiotics, which are structurally related to myxothiazol. While cystothiazoles were isolated from *C. fuscus*, myxothiazol was from a different species of myxobacteria. Furthermore other similar antibiotics termed melithiazoles A and B were also isolated from an another myxobacterium[10]. Although these suggest the presence of a similar biosynthetic pathway in different species, myxothiazol and melithiazoles themselves were not detected in the extract of *C. fuscus*. In our assay system with the filamentous fungus *P. capsici*, cystothiazole A showed a potent inhibition, and the activity was observed up to an amount of 0.05 μg/disc. In a microscope observation, the filamentous branches decreased in both number and length. It is noteworthy that cystothiazole B is about 20-fold less active than cystothiazole A in spite of their high structural similarity. The extra hydroxyl group on the isopropyl group would serve as a detoxication factor by increasing hydrophilicity, or the isopropyl moiety may be responsible for the biological activity. The antimicrobial spectrum (Table 3) shows that the biological activity of cystothiazoles and myxothiazol is essentially same and confined within eukaryotes. The mechanism of action on fungi is supposed to be inhibition of respiration, since cystothiazole A interferes with NADH oxidation in a concentration similar to that of myxothiazol (Fig. 4). Although cystothiazole A showed potent *in vitro* cytotoxicity at IC_{50}s of 110~130 μg/ml against tumor cells tested, the IC_{50} values were significantly (10-fold) higher than that of myxothiazol. Furthermore, antimicrobial activity of cystothiazole A is a little higher than that of myxothiazol. It seems thus likely that cystothiazole A could be more promising than myxothiazol in pharmaceutical applications.

Experimental

General

Organic extracts were dried over anhydrous Na_2SO_4. Evaporation of solvents was carried out with a rotary evaporator under reduced pressure (*ca.* 3 kPa). Fuji Silysia silica gel BW-300 was employed for column chromatography. Precoated silica gel 60 F_{254} and RP-18 WF_{254} plates (E. Merck) were used for thin-layer chromatography (TLC). HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps. Melting points were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. UV spectra were recorded on a JASCO Ubest-50 UV/VIS spectrophotometer. NMR spectra were recorded on a Bruker-ARX400 (400 MHz) or a JEOL EX-270 (270 MHz). NMR chemical shifts were referenced to the solvent peak of δ_H 7.26 (residual $CHCl_3$) or δ_C 77.0 ppm for $CDCl_3$. Mass spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as a matrix.

Assay Method

Antifungal activity of fermentation broths and chromatographic fractions was evaluated by a paper disc assay using *P. capsici*. In a typical assay, an agar piece

(5 mm in diameter) containing mycelia of *P. capsici* previously grown on a synthetic agar medium (1 liter medium contains: sucrose 10 g, monosodium glutamate monohydrate 1.5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2$ 0.1 g, agar 10 g, thiamine hydrochloride 1 mg, $Na_2B_4O_7 \cdot 10H_2O$ 0.088 mg, $CuSO_4 \cdot 5H_2O$ 0.393 mg, $Fe_2(SO_4)_3 \cdot 9H_2O$ 0.91 mg, $MnCl_2 \cdot 4H_2O$ 0.072 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.05 mg, $ZnSO_4 \cdot 7H_2O$ 4.403 mg, EDTA·2Na 5 mg) was placed onto the center of a plate of the same agar medium. After incubation at 25°C with 60% humidity for 2 days, the colony was grown to 3~4 cm in diameter. Each paper disc (8 mm in diameter) containing a test sample was placed one cm away from the edge of the colony. After incubation for an additional one day under the same condition, distance between edges of the colony and the paper disc and morphological changes on growing hyphae were compared with a solvent control. Antibiotic spectra were studied by the conventional paper disc method and by MIC method determined by serial liquid media dilution. Antimicrobial tests were conducted in Müller-Hinton media (Difco) at 37°C for 24 hours for the bacteria or in Potato dextrose agar (broth) media (Difco) at 25°C for 24 hours for fungi.

Isolation

At the end of the cultivation, the bacterial cells and the adsorber resin (270 ml packed volume) were separated from 2.5 liters of the culture broth by centrifugation and extracted twice with one liter of acetone - methanol (4 : 1). The extract was concentrated, and the residue (8.2 g) was suspended in 60% aqueous methanol (100 ml) and extracted three times with dichloromethane (50 ml). After concentration, the dichloromethane extract (1.56 g) was chromatographed on silica gel (16 g) with ethyl acetate and then methanol. The ethyl acetate fraction (780 mg), which was active against *P. capsici* at 1 μg/disc, was subjected to silica gel medium-pressure liquid chromatography (Develosil LOP 60, Nomura Chemical Ltd.; 90 minutes linear gradient from hexane - ethyl acetate (9 : 1) to ethyl acetate, 5 ml/minute) to give several active fractions. The most active fraction (71.6 mg, Rt = 42~52 minutes) was purified by recrystallization from hexane - ethyl acetate (20 : 1) to yield cystothiazole A (31.6 mg) as colorless needles. A more-polar active fraction (19.3 mg, Rt = 66~86 minutes) was chromatographed on silica gel with benzene - acetone (5 : 1), and then separated by silica gel TLC with chloroform - methanol (30 : 1) to give an active fraction (3.1 mg, Rf = 0.21). The active fraction was further purified by silica gel HPLC [Develosil 60-5 (8 mm i.d. × 250 mm), hexane - dichloromethane - methanol (70 : 29 : 1), 2 ml/minute, detection at 254 nm] to give cystothiazole B (1.7 mg, Rt = 31.3 minutes) as a colorless powder.

Degradation of Cystothiazole A

A stream of 4% ozone in oxygen was passed through a solution of cystothiazole A (22.1 mg, 0.052 mmol) in dichloromethane (2 ml) at −78°C for 5 minutes. The solution was flushed with nitrogen and concentrated. The residue was dissolved in 90% formic acid (0.7 ml), and 30% hydrogen peroxide (0.3 ml) was added. After gentle heating the mixture was heated under reflux for 70 minutes. The mixture was concentrated, and the residue was dissolved in methanol (0.5 ml) and treated with ethereal diazomethane. The mixture was concentrated and the residue was chromatographed on silica gel (4 g) with hexane - ethyl acetate (9 : 1 and then 3 : 1). The fraction showing an Rf value of 0.41 on a silica gel TLC [hexane - ethyl acetate (3 : 1)] was further purified by normal-phase HPLC [Nomura Chemical Develosil 60-5 (8 × 250 mm), hexane - *t*-BuOMe (4 : 1), flow rate 2 ml/minute, UV 210 nm] to give dimethyl (2*R*,3*R*)-2-methoxy-3-methylsuccinate (Rt = 22.5 minutes) as an oil: $[\alpha]_D^{22}$ +32° (*c* 0.03, ether), ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.18 (1H, d, J = 4.8 Hz), 3.78 (3H, s), 3.72 (3H, s), 3.44 (3H, s), 2.95 (1H, dq, J = 7.2, 4.8 Hz), 1.20 (3H, d, J = 7.2 Hz).

Acknowledgments

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