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FURTHER STRUCTURE-ACTIVITY RELATIONSHIPS OF THE MYXOBACTERIAL ANTIBIOTICS CYSTOTHIAZOLES

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Abstract – Eight derivatives of the bithiazole-type myxobacterial antibiotic cystothiazole A were evaluated for antifungal activity against a phytopathogenic microorganism for a further structure-activity relationship. Two of them, an acetate and a fully conjugated non-chiral derivative, were new derivatives obtained by chemical derivatization of cystothiazole C. The results confirmed the importance of the bend conformation at C-4/C-5, the methyl ether at C-5, and the lipophilicity of the terminal alkyl group of the molecule.

INTRODUCTION

Myxobacteria are Gram-negative unicellular bacteria that globally habit soils and are characterized by their distinguishable life cycle including gliding and gathering to form multi-cellular organs, fruiting bodies.[1] During the last a few decades, the myxobacteria have been establishing themselves as proficient producers of novel secondary metabolites, in spite of their culture-resistant nature.[2-4] Hundreds of antibiotics and other secondary metabolites have been isolated from the myxobacteria, and most of them possess novel basic structures. One of the most attractive secondary metabolites is epothilones, which are promising candidates for anticancer drugs with a paclitaxel-like action mechanism.[5,6] We previously reported the isolation and structures of the antifungal and cytotoxic antibiotic cystothiazole A (**1**) and its minor congeners, cystothiazoles B−G, from the myxobacterium *Cystobacter fuscus*.[7-9] The β-methoxyacrylate (MOA) moiety is thought to bind to the target cytochrome bc_1 complex of the

mitochondrial respiratory chain system.[7] Structure-activity relationships within these natural compounds suggested the importance of the MOA pharmacophore and lipophilic alkyl substituent at the bithiazole moiety (Figure 1). In addition, the total synthesis of all the stereoisomers of **1** demonstrated that the natural 4*R*,5*S* stereochemistry was exclusive for the antifungal activity (Figure 1).[10] Further structure-activity relationships should be accumulated to verify the importance of the stereogenic centers and the lipophilic alkyl group. Here, we report the evaluation of antifungal activity of nine cystothiazole-related compounds including newly prepared derivatives, 5-acetoxy and 4,5-non-chiral derivatives, for reinforcing the structure-activity relationships.

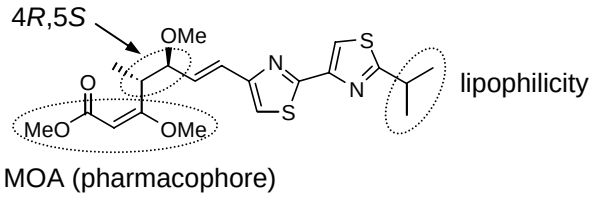
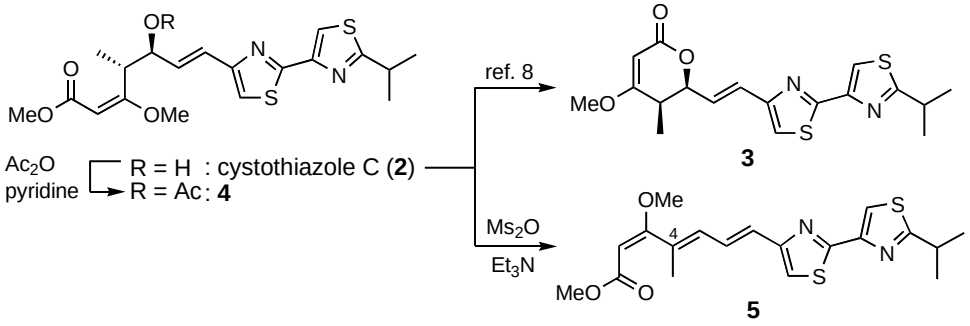


Figure 1. Hitherto known structural requirement for antifungal activity of cystothiazole A (**1**).

RESULTS AND DISCUSSION

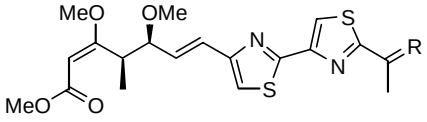
Cystothiazole C (**2**), a 5-hydroxy derivative within the cystothiazoles, would be a useful congener for chemical conversion into new derivatives. Due to the chemical lability, **2** gradually decomposed even at −30 ºC, and its antifungal activity was 100 times as low as that of cystothiazole A (**1**).[8] One of the decomposition products would be lactone (**3**), which was previously obtained from **2** under basic conditions in a moderate yield (Scheme 1).[8] Therefore, a chromatographic fraction containing **2** was used for the chemical derivatization without purification. A portion of the crude sample was first subjected to acetylation under usual conditions, and the product was chromatographed on silica gel followed by ODS to give 5-*O*-acetyl cystothiazole C (**4**). Another portion of the crude sample containing **2** was treated with methanesulfonic anhydride and triethylamine for dehydration of **2** via the mesylate. After chromatographic separation on silica gel, the dehydrated product (**5**) was obtained, instead of the corresponding mesylate, via spontaneous elimination of methanesulfonic acid during the reaction. The 4*E*



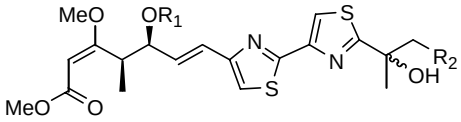
Scheme 1. Conversion of cystothiazole C (**2**) into derivatives (**3**−**5**).

geometry was determined by the NOE correlations between H-6 and 4-Me and between H-5 and 3-OMe. These three derivatives were useful for the evaluation of the importance of the substituents at the stereogenic centers.

On the other hand, some derivatives modified at the isopropyl group on the opposite molecular side were also obtained through reexamination of a myxobacterial extract or biotransformation of cystothiazole A (**1**). From mother liquids of crystallized cystothiazole A (**1**), the known compound melithiazole B (**6**)[11] was obtained after chromatographic purification. During storage in a freezer for a few years, **6** was gradually oxidized into melithiazole M (**7**).[11] The biotransformation of **1** was previously performed by the external addition of **1** into the culture of the producer myxobacterium *Cystobacter fuscus*, providing three polar derivatives (**8**–**10**).[12] These are demethylated at 5-*O*-methyl group and/or hydroxylated at the isopropyl group in **1** and seem to be useful for examining the importance of the lipophilic alkyl substituent.



melithiazole B (**6**): R = CH_2
melithiazole M (**7**): R = O



8: $R_1 = R_2 = H$
9: R_1 = Me, R_2 = OH
10: R_1 = H, R_2 = OH

The natural and unnatural derivatives described above were evaluated for the inhibition of the phytopathogenic microorganism *Phytophthora capsici* by a simple paper disk diffusion test. The minimum doses to form an inhibition zone on the agar plate are summarized in Table 1. The mother substance cystothiazole A (**1**) showed the highest activity, inhibiting *P. capsici* at 40 ng/disk or higher doses. The acetate at C-5 in **4** lowered the activity by two digits. The polarity of the terminal alkyl group significantly depressed the activity by three digits or more. A remarkable result is the complete loss of the activity of the fully conjugated non-chiral derivative (**5**) in spite of the retention of the essential β-methoxyacrylate (MOA) moiety. This result could be explained by the lack of the *cis* (or *cisoid*) framework at the C-4/C-5 position, which has been regarded as another essential factor for binding to the target.[11,13] Thus, the synthetic non-chiral compound (**11**) (= 4,5-didehydrocystothiazole A) with the *cis* framework was reported to show an antifungal activity comparable to or higher than cystothiazole A (**1**) with a *cisoid* C-4/C-5 conformation (Figure 2).[14] Similar structure-activity relationships are known for a pair of simpler structures with a β-substituted MOA, **12** and **13**,[15] and even for a set of the historically important α-substituted MOAs, oudemansin (**14**), strobilurin (**15**), and the isomer (**16**) with the *trans* framework (Figure 2).[13] Therefore, our results confirmed the importance of the *cisoid* configuration (bent shape) at C-4/C-5 even in the β-substituted MOAs.

Table 1. Minimum doses of cystothiazole derivatives for antifungal activity.

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Dose (μg/disk)	0.04	5	>100	5	>100	0.04	0.2	>25	>25	>25

In conclusion, the important factors for the antifungal activity of the cystothiazoles are: i) the cooperation

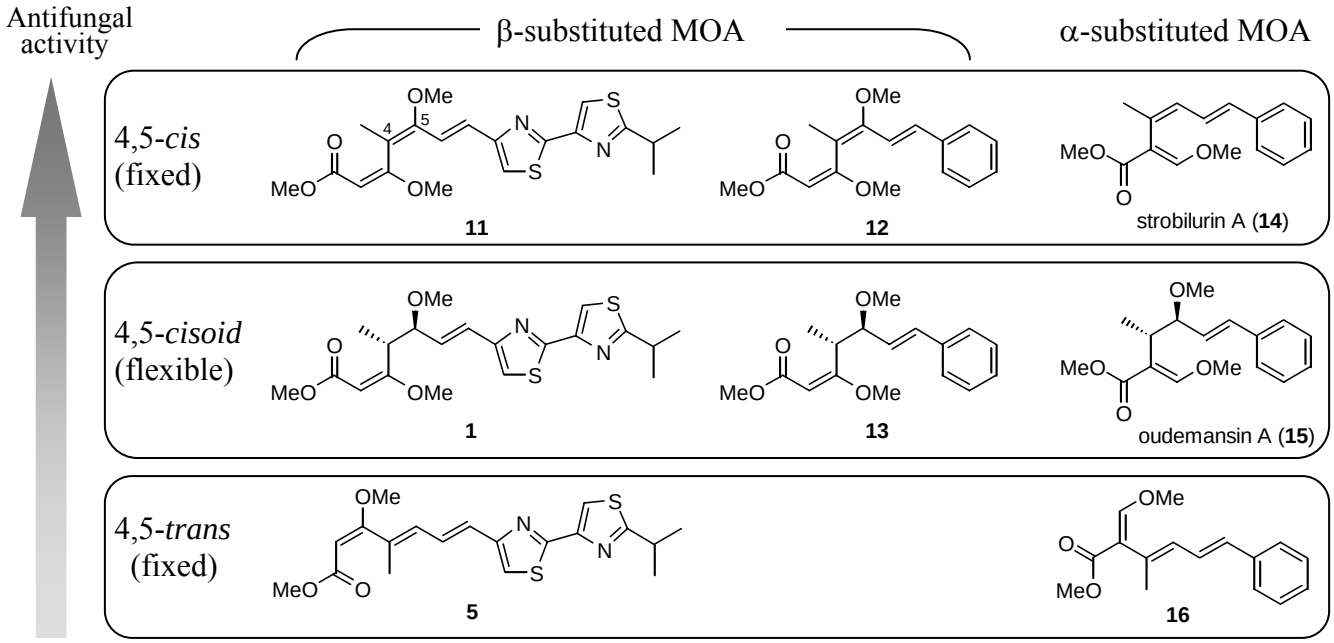


Figure 2. Importance of the C-4/C-5 conformation in some sets of β-methoxyacrylates (MOA).

of the MOA moiety and the β-substituent with the C-4/C-5 *cis* (or *cisoid*) framework to establish the ideal 90º torsion angle[11,13] between them (activity: **1** >> **3**, **5**), ii) 5-methoxy group (activity: **1** > **2**, **4**), and iii) lipophilic terminal alkyl group (activity: **1** ≈ **6** > **7** >> **8**−**10**). The stereogenic centers of cystothiazole A (**1**) is eventually not essential unless the molecule loses the C-4/C-5 *cis* framework. This rule was the important basis for the practical development of the MOA-type fungicides.[16]

EXPERIMENTAL

Silica gel 60 F_{254} (0.25 mm thickness, Merck) was used for TLC. Preparative HPLC was performed on a high-pressure gradient system equipped with PU-1586 and PU-2086 pumps and a UV-1570 detector (Jasco). Specific rotations were obtained by using a DIP-370 digital polarimeter (Jasco). IR spectra were recorded on a FT-IR-7000S spectrometer (Jasco). UV spectra were recorded on a Ubest-50 UV/VIS spectrophotometer (Jasco). MS were recorded on a Mariner Biospectrometry Workstation (Applied

Biosystems) in the positive ESI mode. A peptide mixture (angiotensin I, bradykinin and neurotensin) were used as internal standards for high-resolution (HR) MS analysis. NMR spectra were recorded on an ARX 400 (400 MHz) spectrometer (Bruker). The NMR chemical shifts (ppm) were referenced to the solvent peaks of δ_H 7.26 (residual $CHCl_3$) and δ_C 77.0 for $CDCl_3$ solutions.

Preparation of cystothiazole C-containing material.

A cystothiazole C-containing material was obtained from the large-scale fermentation broth (200 L) that was described previously.[8] The acetone extract (1-L aqueous solution after concentration) from the absorber resins was extracted with hexane-EtOAc (3:1). The cystothiazoles were previously obtained from this hexane-EtOAc layer.[8] The remained aqueous layer (1 L) was diluted with MeOH (1 L) and extracted three times with CH_2Cl_2 (0.5 L). The organic layers were combined and concentrated to give a dark brown oil (10.0 g). A half portion (5.34 g) was chromatographed on silica gel (100 g) stepwise eluted with 2% to 40% acetone in CH_2Cl_2. The fractions (1.43 g) eluted with 2% to 8% acetone in CH_2Cl_2 were combined and concentrated. The material obtained was again chromatographed on silica gel (70 g) eluted with $CHCl_3$-acetone (20:1) to give an oily material (339 mg) that contained cystothiazole C (**2**) [R_f = 0.24 on a silica gel plate developed with $CHCl_3$-acetone (15:1)].

5-*O*-acetylcystothiazole C (4).

A portion (166 mg) of the above material containing **2** was treated with Ac_2O (0.7 mL) and pyridine (1.4 mL) at room temperature for 3 h. The mixture was concentrated and the product was chromatographed on silica gel (2 g) eluted with hexane-EtOAc (4:1) to give a crude material of **4** (7.9 mg), which was further purified by HPLC [Develosil ODS HG-5 (10 x 250 mm), 75% MeOH, 2 mL/min] to give pure **4** (1.5 mg, t_R = 44.7 min): colorless oil, $[\alpha]^{27}_D$ +110° (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} 222 (ε 37000), 242 (32000), 311 (12000) nm; IR (film) ν_{max} 3107, 1741, 1713, 1624, 1235, 1146, 1033, 965, 827, 801, 760 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.84 (1H, s), 7.07 (1H, s), 6.61 (d, *J* = 15.7 Hz), 6.52 (dd, *J* = 6.8, 15.7 Hz), 5.61 (dd, *J* = 6.8, 7.2 Hz), 5.00 (1H, s), 4.35 (1H, dq, *J* = 7.2, 7.0 Hz), 3.67 (3H, s), 3.60 (3H, s), 3.36 (1H, sept, *J* = 6.9 Hz), 2.08 (3H, s), 1.43 (6H, d, *J* = 6.9 Hz), 1.18 (3H, d, *J* = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.6 (s), 175.6 (s), 170.3 (s), 167.7 (s), 162.7 (s), 154.0 (s), 148.7 (s), 129.1 (d), 125.7 (d), 116.0 (d), 114.9 (d), 91.5 (d), 75.8 (d), 55.6 (q), 50.9 (q), 38.7 (d), 33.4 (d), 23.1 (q, 2C), 21.2 (q), 13.8 (q). HR MS found *m/z* 451.1375, calcd for $C_{21}H_{27}N_2O_5S_2$ (M+H) 451.1356.

Fully conjugated non-chiral derivative 5.

A portion (66.2 mg) of the above material containing **2** was treated with methanesulfonic anhydride (0.15 g, 0.85 mmol) and triethylamine (0.12 mL, 0.85 mmol) in dichloromethane (3 mL) at 0 °C for 30 min

under argon. The reaction mixture was chromatographed on silica gel (5 g) eluted with hexane-EtOAc (4:1) to give a crude product (10.2 mg), which was then chromatographed on silica gel (1 g) eluted with hexane-Et_2O (3:1) to give a sample containing **5** (2.5 mg, R_f = 0.74 on silica gel plate with hexane-EtOAc 2:1). This was finally purified by preparative HPLC [Develosil ODS UG-5 (10 x 250 mm), 70% MeOH, 2 mL/min] to give pure **5** (0.4 mg, t_R = 48.6 min): pale yellow oil, UV (MeOH) λ_{max} 213 (ε 17000), 270 (sh., 15000), 310 (23000) nm; IR (film) ν_{max} 3100, 1717, 1602, 1436, 1382, 1265, 1235, 1211, 1192, 1146, 1129, 1108, 1042, 969, 923, 822, 794, 757 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.91 (1H, s, H-12), 7.43 (1H, dd, *J* = 11.5, 15.1 Hz, H-6), 7.12 (1H, s, H-9), 6.63 (1H, d, *J* = 15.1 Hz, H-7), 6.36 (1H, d, *J* = 11.5 Hz, H-5), 5.06 (1H, s, H-2), 3.70 (3H, s, 3-OMe), 3.66 (3H, s, 1-OMe), 3.38 (1H, septet, *J* = 6.9 Hz, H-14), 2.10 (3H, s, 4-Me), 1.45 (6H, d, *J* = 6.9 Hz, H-15, 14-Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.6, 174.2, 167.3, 162.9, 155.0, 148.7, 133.2, 131.8, 127.4, 127.0, 116.3, 115.1, 91.1, 56.1, 51.0, 33.4, 23.1 (2C), 15.9. HR MS found *m/z* 391.1173, calcd for $C_{19}H_{23}N_2O_3S_2$ (M+H) 391.1145.

Melithiazole B (6) and M (7)

A large scale purification of cystothiazole A (**1**) by a sequential recrystallization was described in our paper.[8] The two mother liquors were again recrystallized from hexane to give crystals (51 mg and 136 mg), which consisted of **1** and **6**. The combined crystals were chromatographed on silica gel (20 g) with benzene-EtOAc (50:1, 20:1, and then 10:1). Some fractions eluted with benzene-EtOAc (50:1) were combined and concentrated to give methiliazole M (**6**) (18 mg). After storage for two years, 6 partially decomposed. A portion (3 mg) was separated by preparative TLC (hexane-EtOAc 2:1) to give **6** (0.4 mg) and melithiazole M (**7**) (1.2 mg).

The full spectral data of **6** and **7** were reported in ref. 17.

Anti-*Phytophthora* assay

Detail conditions were previously reported.[7] Briefly, the phytopathogen *Phytophthora capsici* was cultured on a synthetic agar medium in a 9-cm dish at 25 °C for 2 days in the dark until the colony had grown to a size of about 3–4 cm in diameter. Each paper disk (8 mm in diameter) impregnated with a sample was placed 1 cm away from the front of the colony. After incubating for 1 day, the distance between the edge of the colony and the paper disc (control: 0 mm) was measured.

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