

Miuraenamides A and B, Novel Antimicrobial Cyclic Depsipeptides from a New Slightly Halophilic Myxobacterium: Taxonomy, Production, and Biological Properties

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Abstract A slightly halophilic myxobacterial strain, SMH-27-4, was isolated from nearshore soil and shown to belong to a new myxobacterium genus based on phylogenetic analysis. This slowly-growing myxobacterium produced the novel antibiotic depsipeptides miuraenamides A and B. Their physico-chemical properties and molecular formulas, $C_{34}H_{42}N_3O_7Br$ and $C_{34}H_{42}N_3O_7I$, were determined. Miuraenamides A exhibited potent and selective inhibition against a phytopathogenic microorganism, *Phytophthora* sp., and moderate inhibition against some fungi and yeasts, but was ineffective against bacteria. Both of the metabolites inhibited NADH oxidase at IC_{50} values of $50 \mu M$, suggesting, like β -methoxyacrylate-type antibiotics, the electron transfer system of the mitochondrial respiratory chain as the cellular target.

Keywords myxobacterium, antibiotics, depsipeptides, polyketide, electron transport inhibitor

Introduction

Myxobacteria are considered a rich source of unique antibiotics that are rarely produced by other bacteria, actinomycetes and fungi [1~4]. Although myxobacterial

strains are rather difficult to isolate and to culture, the potential usefulness of their novel secondary metabolites has been frequently mentioned [4]. Myxobacteria are not only soil bacteria, but inhabit a wide range of natural environments. For example, truly halophilic myxobacteria were isolated from the marine environment in Japan [5]. Moreover, new antifungal compounds, such as haliangicins are produced by a marine myxobacterial strain SMP-2 which belongs to the new genus *Haliangium* [6~8]. These findings suggested novel myxobacteria as promising sources of new compounds. Therefore, we have continued the effort to isolate unknown myxobacteria from the coastal

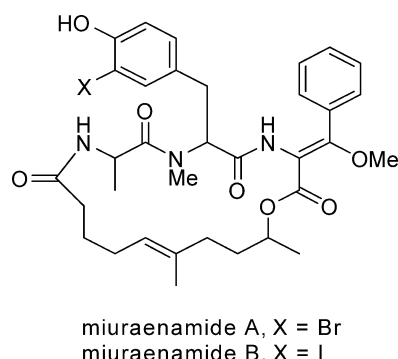


Fig. 1 Structures of miuraenamides.

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* AJ-: No. of the Ajinomoto Culture Collection.

samples. In the course of our search for myxobacterial antibiotics [9~12], miuraenamides A and B (Fig. 1) were obtained from the fermentation broth of a new slightly halophilic myxobacterium isolated from a nearshore soil in Miura Peninsula, Japan. We now describe the taxonomy and fermentation characteristics of this myxobacterium as well as the isolation, physicochemical properties and biological activity of these antibiotics.

Materials and Methods

Microorganisms

The myxobacterial strain SMH-27-4 (=AJ-110262*) was isolated from a soil sample collected in a brush vegetation near the seashore of Arai-Hama beach in the Miura Peninsula, Kanagawa, Japan. The principal method used for isolation of myxobacteria has been described previously, except that low salt concentration media (one fifth the strength of SWS solution, or 1/5SWS) were used [5]. Phylogenetic and physiological studies were conducted as described [5].

Fermentation

The myxobacterial strain was cultivated at 27°C in Vy2-1/5SWS medium. The Vy2-1/5SWS medium contained (amount/liter) Baker's yeast cake (5 g, Wako Pure Chemical, Osaka, Japan), NaCl (10 g), cyanocobalamin (0.5 mg), SWS solution [5] (200 ml) and agar (15 g), at pH 7.2 adjusted with NaOH. SWS is composed of the elements of seawater, such as magnesium, potassium, calcium, sulfate, carbonate, boric acid, strontium, iron, manganese and iodine [5]. The V-1/5SWS-Br medium was used for the production of antibiotics. The medium contained (per liter of 1/5 SWS) Baker's yeast cake (8 g, Wako Pure Chemical), Yeast Extract (0.1 g, Difco Lab., Becton Dickinson & Co., Sparks, MD, USA), NaCl (1.0 g), NaBr (7.0 g), HEPES (2.0 g) and the adsorber resin SP207 (20 g, Mitsubishi Chemical Co., Tokyo, Japan), at pH 7.3 adjusted with NaOH. Fermentation was conducted on a rotary shaker (180 rpm) at 27°C.

Chromatography and Physicochemical Properties

Flash chromatography was conducted using a low-pressure gradient system equipped with an 880-PU HPLC pump and an 880-02 gradient unit (Jasco, Tokyo, Japan). 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 determined using a DIP-370 digital polarimeter (Jasco). FT-IR spectra were recorded on a FT-IR-7000S spectrometer (Jasco).

UV spectra were recorded on an Ubest-50 UV/VIS spectrophotometer (Jasco). Mass spectra (MS) were recorded on a Mariner Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) in the positive ESI mode. A peptide mixture containing angiotensin I, bradykinin and neurotensin was used as an internal standard for high-resolution MS analysis. NMR spectra were recorded on an AMX2 600 (600 MHz) spectrometer (Bruker). The NMR chemical shifts for ^1H and ^{13}C , respectively, were referenced to the solvent peaks of δ_{H} 7.26 (residual CHCl_3) and δ_{C} 77.0 ppm for CDCl_3 solutions.

Isolation of Miuraenamides A and B

The bacterial cells and the adsorber resin were separated from 14 liters of culture broth by centrifugation followed by two extractions with acetone (1.4 liters) at room temperature for two days. The extracts were combined and concentrated to give an aqueous mixture, which was extracted three times with EtOAc (50 ml). After concentration, the residue (495 mg) was subjected to flash chromatography on silica gel [HI-FLASH SI-40W-M (12 g, 20×60 mm, Yamazen, Osaka), 5~50% (90 minutes), 50~100% (50 minutes) EtOAc in CHCl_3 , 4 ml/minute]. The fractions, eluted with 10~30% EtOAc in CHCl_3 , were combined and concentrated to produce an oil (69 mg), which was subjected to chromatography on silica gel [Wakogel C-300 (7 g, 12×130 mm, Wako Pure Chemical), hexane - EtOAc (1:1, 1:2, 1:3, and 1:4)]. The fractions eluted with hexane - EtOAc (1:3 and 1:4) were combined and evaporated. The residue (17.3 mg) was subjected to reversed-phase HPLC [Develosil ODS UG-5 (10 i.d.×250 mm, Nomura Chemical, Aichi, Japan), 70% MeOH, 8 ml/minute, detection at 260 nm] to yield miuraenamides A (10.9 mg, R_t =31.6 minutes) and B (0.5 mg, R_t =35.6 minutes).

Antimicrobial Properties

Anti-*Phytophthora* (a fungus-like phytopathogen) activity of the extracts of culture broths and chromatographic fractions was assessed according to the method of Ojika *et al.* [9]. The antimicrobial spectrum was obtained using the serial liquid media dilution method using Yeast Nitrogen Base broth (Difco) with 0.5% glucose for yeast and filamentous fungi, and Mueller-Hinton broth (Difco) for bacteria. Approximately 10^4 freshly grown cells (or spores) of the test strains were inoculated and grown at 25°C for yeast and fungi and at 37°C for bacteria. For the non spore-forming fungal species (*Phytophthora capsici* and *Trichophyton mentagrophytes*), small pieces of agar (approximately 1 mm³) containing the mycelia were used as

inocula. After incubation for 72 hours for filamentous fungi and 24 hours for yeasts and bacteria, the minimal inhibitory concentrations (MICs) were determined.

NADH Oxidase Inhibition

The inhibition rate of fungal NADH oxidase in *Candida rugosa* AJ14513 mitochondrial particles was determined using a slight modification of Thierbach *et al.* [13]. The fungus was cultured with shaking in YM medium at 25°C for 40 hours and used for further experiments. A mitochondrial fraction of the fungal cells was extracted according to the method described by Fang *et al.* [14].

Results and Discussion

Taxonomic Studies

The antibiotic depsipeptide producing strain SMH-27-4 (=AJ-110262) exhibited enlarging colony formation on agar plates and conspicuous bacteriolytic action; both features are typical characteristics of myxobacteria [4]. However, the strain did not form fruiting bodies whose shapes and structures are important taxonomic features of myxobacteria. Consequently, the strain was subjected to phylogenetic analysis based on 16S rDNA sequences of nearly 1500 bp [DDBJ (=DNA Data Bank of Japan,

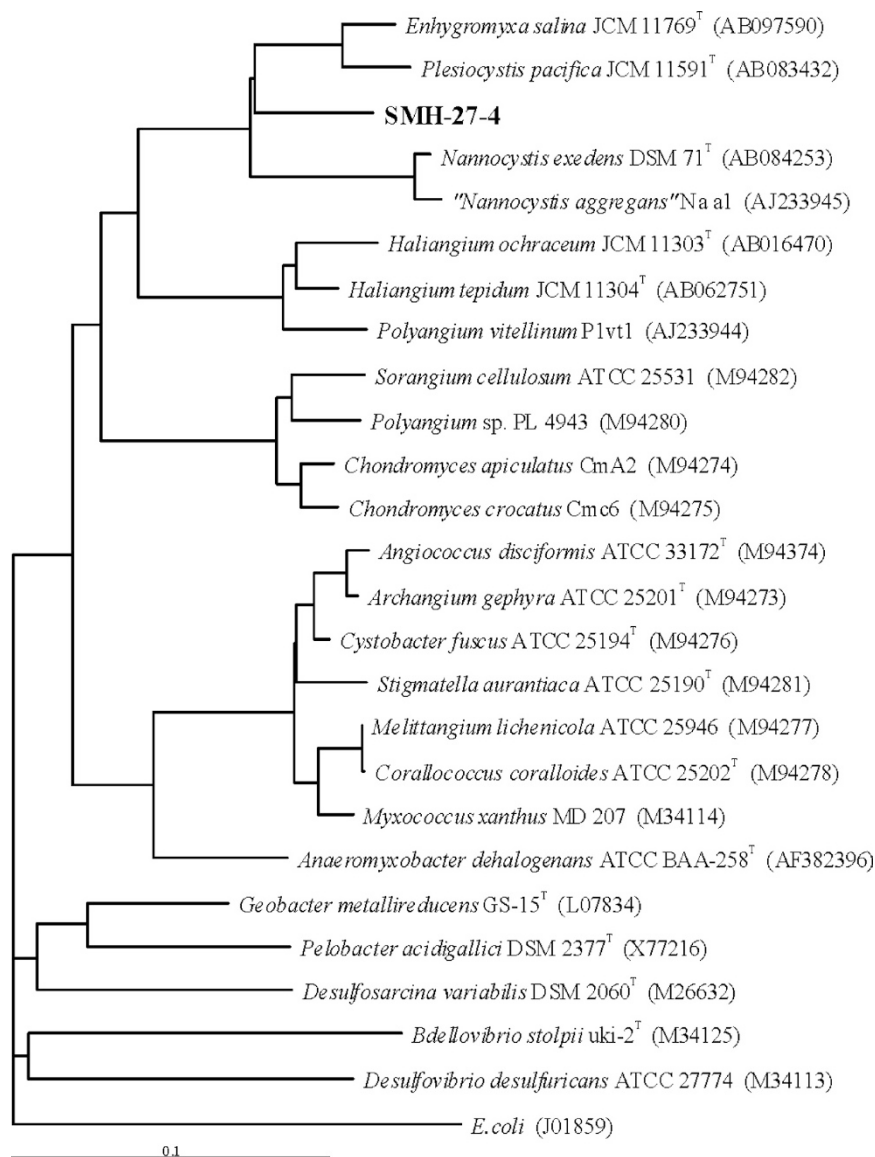


Fig. 2 Phylogenetic tree of the novel myxobacterial strain SMH-27-4 and other myxobacteria.

Bar, 0.1 changes per sequence position.

National Institute of Genetics, Mishima, Shizuoka, Japan) Accession No. for the strain: AB252740]. The strain was shown to be related to the suborder *Sorangineae* myxobacteria, and was positioned near the genus *Nannocystis* and the marine myxobacterial genera *Enhygromyxa* and *Plesiocystis* [15, 16] (Fig. 2). Homology with its closest relatives was relatively low at 93.0% with *Nannocystis exedens*, 93.2 to 93.3% with *Enhygromyxa salina*, and 91.3 to 91.5% with *Plesiocystis pacifica*. The strain showed the slight salt requirement with an optimum NaCl concentration for growth of approximately 0.5 to 1.0% (w/v), which is slightly lower than that observed for

any myxobacteria of marine origin reported to date [5, 15, 16]. Since these results strongly suggested that the strain might represent a novel taxon at the level of genus, we tentatively named it *Paraliomyxa miuraensis*. A more detailed description of the strain will be reported elsewhere.

Production and Isolation of Antibiotics

After the incubation of the myxobacterial strain on the Vy2-1/5SWS agar plates for 7 to 10 days, agar pieces, taken from the periphery of the colonies with a sterile spatula, were inoculated into 2-liter Erlenmeyer flasks containing 500 ml of the production medium. Cultivation

Table 1 Physico-chemical properties of miuraenamides

	Miuraenamide A	Miuraenamide B
Appearance	White powder	White powder
Molecular formula	C ₃₄ H ₄₂ N ₃ O ₇ Br	C ₃₄ H ₄₂ N ₃ O ₇ I
ESI-MS	684.2 and 686.2 (49, 60) [M+H] ⁺	732.2 (100) [M+H] ⁺
<i>m/z</i> (rel. int)	706.2 and 708.2 (96, 100) [M+Na] ⁺	754.2 (24) [M+Na] ⁺
HR MS found	684.2278 [M+H]	732.2139 [M+H]
calcd	684.2279 (C ₃₄ H ₄₃ N ₃ O ₇ ⁷⁹ Br)	732.2140 (C ₃₄ H ₄₃ N ₃ O ₇ I)
[α] _D ²⁵	+59 (c 0.15, MeOH)	+30 (c 0.02, MeOH)
UV λ _{max} ^{MeOH} nm (ε)	203 (49,000), 276 (11,000)	203 (53,000), 274 (11,000)
IR ν _{max} (film) cm ⁻¹	3336, 1684, 1654, 1636, 1509, 1256, 1217, 1119, 756	3336, 1683, 1636, 1507, 1261, 1217, 1119, 756
HPLC Rt ^a	16.8 minutes	19.0 minutes

^a Develosil ODS UG-5 (4.6×250 mm), 70% MeOH, 1.0 ml/minute, UV 260 nm.

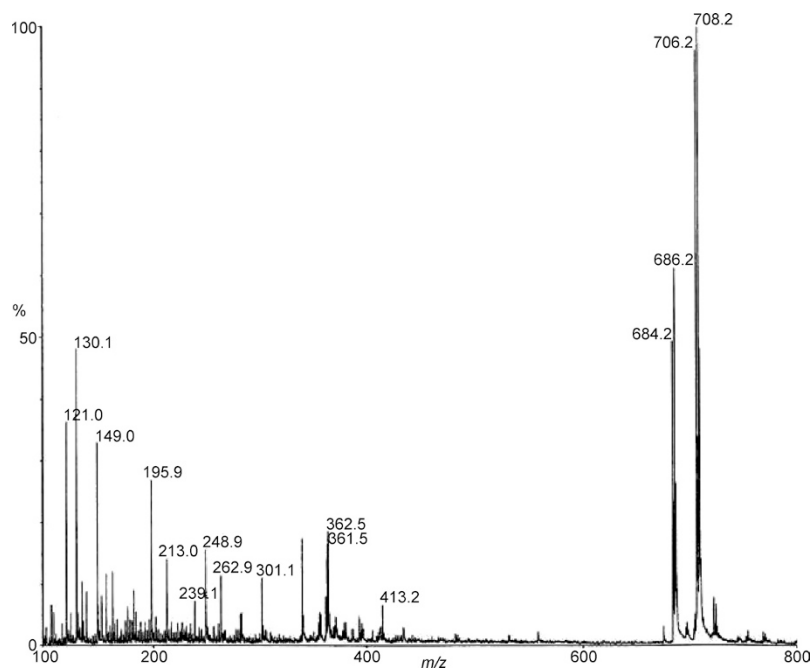


Fig. 3 ESI-TOF-MS(+) spectrum of miuraenamide A.

was performed on a rotary shaker at 27°C for 18 days. A total of 14 liters of culture broth was used for further extraction and isolation. The bacterial cells, including the adsorber resin, were extracted with acetone then separated into the EtOAc and water fractions by solvent partition. The EtOAc fraction was chromatographed twice on silica gel before being purified by reversed-phase HPLC to give miuraenamides A (10.9 mg) and B (0.5 mg).

Physicochemical Properties

Table 1 summarizes some properties of miuraenamides A and B. The electrospray-ionization time-of-flight (ESI-

TOF) MS of miuraenamide A showed protonated molecular ions at m/z 684.2 and 686.2 ($M+H$)⁺ at a ratio of approximately 1 : 1 (Fig. 3), indicating the presence of one bromine atom in the molecules. The molecular formulas were determined by high-resolution ESI-TOF MS. The IR bands of miuraenamide A at 1684 cm^{-1} (Fig. 4) and UV absorption at 276 nm (Fig. 5) suggest the presence of an unsaturated carbonyl group. The amide bands at 1654, 1636, and 1509 cm^{-1} , as well as three nitrogen atoms in the molecules, indicate the peptide nature of the molecules. The properties of miuraenamide B were relatively similar

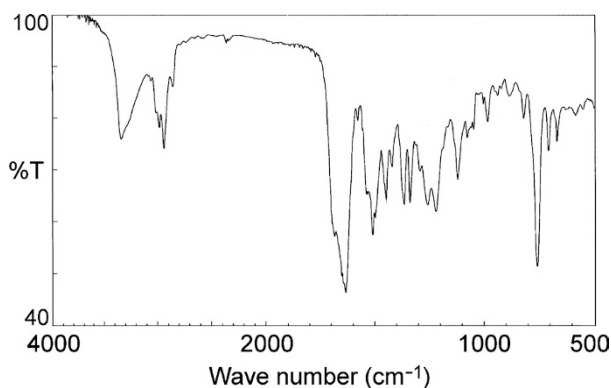


Fig. 4 IR spectrum of miuraenamide A (film).

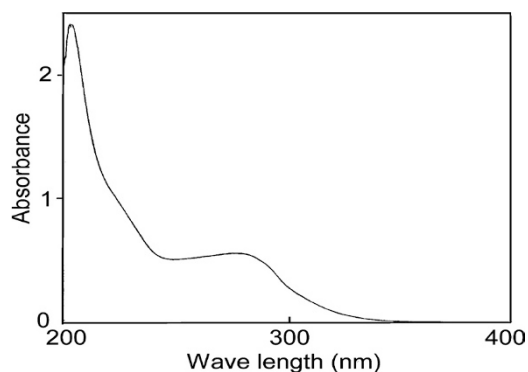


Fig. 5 UV spectrum of miuraenamide A (MeOH, $c=5.1 \times 10^{-5}$ M).

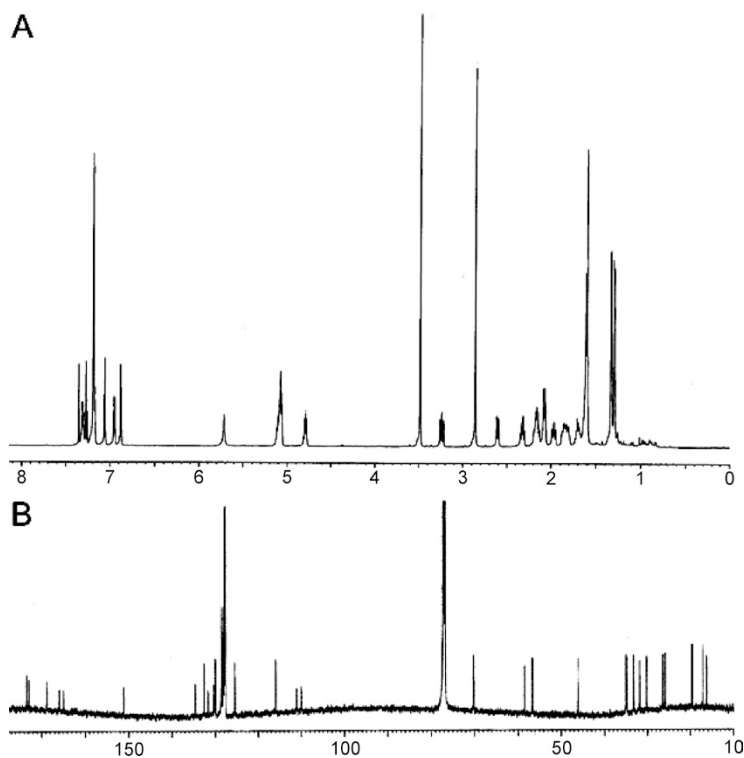


Fig. 6 ^1H (A) and ^{13}C (B) NMR spectra of miuraenamide A in CDCl_3 [600 MHz for ^1H].

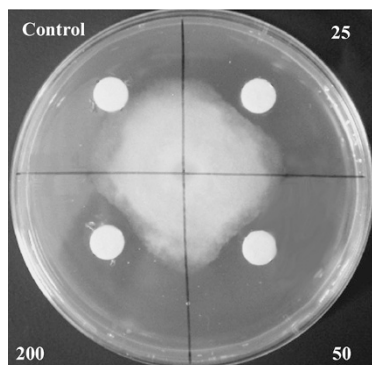


Fig. 7 Inhibition of *P. capsici* by miuraenamides A. [Doses: ng/disk].

Table 2 Antimicrobial spectrum of miuraenamides A

Tested microorganisms	MIC ($\mu\text{g/ml}$)
Fungi	
<i>Absidia spinosa</i> AJ 117196	12.5
<i>Aspergillus niger</i> AJ 117065	>50
<i>Botrytis cinerea</i> AJ 117140	>50
<i>Phytophthora capsici</i> NBRC 8386	0.4
<i>Rhizopus oryzae</i> AJ 117321	6.3
<i>Trichophyton mentagrophytes</i> AJ 11716	12.5
Yeast	
<i>Candida rugosa</i> AJ 14513	12.5
<i>Saccharomyces cerevisiae</i> AJ 14587	>50
<i>Pichia burtonii</i> AJ 14071	25
<i>Rhodotorula minuta</i> AJ 5017	12.5
Bacteria	
<i>Escherichia coli</i> NIHJ	>50
<i>Staphylococcus aureus</i> AJ 12510	>50
<i>Bacillus subtilis</i> ATCC 6051	>50

to those of miuraenamides A except for the molecular formula, in which the bromine atom was replaced by iodine (Fig. 1). The ^1H and ^{13}C NMR spectra of miuraenamides A are shown in Fig. 6. The details of the chemical attributes of the molecules such as elucidation of the structure will be published elsewhere.

Biological Activity

Miuraenamides A and B were obtained by anti-*Phytophthora* screening using a paper disk diffusion method. Both compounds inhibited the phytopathogenic oomycete, *Phytophthora capsici*, at a minimum dose of 25 ng/disk (Fig. 7). Table 2 shows the antimicrobial spectrum of miuraenamides A. We could not determine the spectrum of miuraenamides B due to the scantiness of the

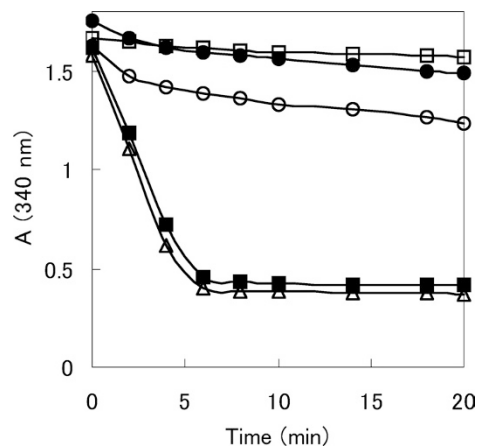


Fig. 8 Time-dependent inhibition of NADH oxidase by miuraenamides and other antibiotics.

○: miuraenamide A (100 μM); ●: miuraenamide B (100 μM); □: cystothiazole A (10 μM); ■: amphotericin B (10 μM); △: control (MeOH).

compound available. The spectrum may be very similar to that of miuraenamide A, because of the structural similarity (Fig. 1) and the same anti-*Phytophthora* activity. The compound selectively inhibited *Phytophthora* but did not inhibit bacteria. One of the interesting structural features is the β -methoxyacrylate (and enamide $\text{NH}-\text{C}=\text{C}$) moiety of the dehydrophenylalanine residue. Since the β -methoxyacrylate-type metabolites are known to be antifungal respiratory-chain inhibitors, e.g., strobilurins [17], cystothiazoles [9] and haliangicins [8], the inhibition of NADH oxidase was evaluated using *Candida*-derived mitoplast (Fig. 8) and it was found that miuraenamides inhibited NADH oxidase activity at 100 μM . Cystothiazole A, which is a typical β -methoxyacrylate compound previously isolated from a myxobacterium by us [9], was used as a positive control and completely inhibited NADH oxidase at 10 μM . On the other hand, amphotericin B, which is a potent antifungal compound of the heptaenamacrolide, serving as negative control, did not show inhibition. The IC_{50} values of 50 μM were determined in an experiment using various concentrations (data not shown). The results suggest the electron transfer system of the mitochondrial respiratory chain as cellular target, similar to β -methoxyacrylate compounds. The miuraenamides are the second reported example of α -nitrogen-linked β -methoxyacrylates produced by bacteria since the discovery of cyrmenins [18, 19].

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