Inhibitors of Microtubule Assembly Produced by the Marine Fungus Strain TUF 98F139 Collected in Palau

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Inhibitors of Microtubule Assembly Produced by the Marine Fungus Strain TUF 98F139 Collected in Palau

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Abstract: A mixture of four fatty acids, oleic acid, linoleic acid, stearic acid, and palmitic acid, was isolated as the inhibitor of microtubule assembly from mycelia of the marine fungus strain TUF 98F139, which was isolated from a marine sponge collected at Palau in 1998. The mixture, which gave a single spot on TLC, showed the inhibitory activity of 57% at 50 ppm. The bioassay with authentic specimens of fatty acids revealed that unsaturated fatty acids, oleic acid and linoleic acid, were active (52 and 58% at 50 ppm, respectively). It is the first time to report that unsaturated fatty acids inhibit microtubule assembly.

Key words: *Marine fungus, Sponge, Tubulin, Microtubule, Inhibitor, Fatty acids, Unsaturated fatty acids, Oleic acid, Linoleic acid*

Introduction

Microtubules, which are essential proteins for eukaryotes, are dynamic pipe-shape protein fibers composed of α and β-tubulin heterodimers with accompanying proteins; microtubule associated proteins and τ protein. The tubulin/microtubule system is involved in many biological functions, such as formation of the mitotic spindle, constitution of cell cytoskeleton, intracellular transport, and locomotion. Normal division of eukaryotic cells is dependent on the dynamics of microtubules during the course of mitosis and cytokinesis. Any substances able to interfere with the tubulin/ microtubule system show a potential inhibition of eukaryotic cell division. Inhibitors of cell division, which are called antimitotic agents, can be used for medicinal and agrochemical purposes as antitumor and antifungal agents[1)]. Antimitotic agents are also important biochemical tools for studying the structure and dynamics of microtubules.

The modulation of tubulin polymerization has especially been implicated as an important approach in pursuit of antitumor agents. The clinical successes of the taxanes, paclitaxel and the semisynthetic derivative docetaxel promoted this approach to search for new agents with similar mechanisms of action. These antitumor agents stabilize the microtubules and arrest dividing cells in metaphase. There is an another type of antimitotic substances such as colchicine, the vinca alkaloids, and synthetic antifungal agents. These compounds inhibit the polymerization of microtubules and also arrest dividing cells in metaphase. Antimitotic substances are, therefore, a prolific source of antitumor and antifungal agents.

We are currently involved in a research project to search for bioactive secondary metabolites from culture broths of marine fungi[2-6)]. Marine fungi have recently been recognized as a prolific source of biologically active natural products[7-10)]. During our research project, we found that the extract of mycelia of strain TUF 98F139, isolated in Palau, showed inhibitory activity to microtubule assembly (tubulin polymerization). Bioassay-guided separation of the extract of mycelia yielded a mixture of four fatty acids as responsible substance of the bioactivity. We describe here the isolation of the marine fungus strain TUF 98F139 and separation, structure assignment, and bioactivity of the inhibitors of microtubule assembly. It should be noted that this is the first time to report the inhibitory activity to microtubule polymerization by unsaturated fatty acids.

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Materials and Methods

General

^{1}H NMR spectra were measured on a JEOL GSX-400 NMR spectrometer (400 MHz) in acetone-d_6. Gas chromatograph-mass spectrometry (GC-MS) was carried out with a Hewlett Packard G1800A GCD System. Authentic samples of fatty acids (oleic acid, linoleic acid, stearic acid, and palmitic acid) were purchased from Kanto Chemical Co., Inc.

Bioassay

Preparation of microtubule proteins was performed as described previously[11]. In brief, fresh porcine brains were homogenized at 0°C in the polymerization buffer solution (100 mM 4-morphorineethansurfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 1 mM 2-mercaptoethanol, pH 6.5) and centrifuged at 50,000 *g* at 4°C. A glycerol buffer (8 M glycerol in the above buffer solution, pH 6.5) was added to the supernatant, and the mixture was incubated at 37°C for 30 min and centrifuged at 100,000 *g* to afford the precipitate (microtubule). The procedure of depolymerization and polymerization was further performed twice to purify the microtubule proteins. Concentration of proteins was quantified using the Coomassie Protein Assay Kit® (Pierce).

Microtubule assembly was observed by the turbidity at 400 nm in a glass UV cell with a Shimadzu model U-3000 spectrophotometer equipped with an electronic temperature controller. The extract of mycelia, fractions, and fatty acids were dissolved in dimethylsulfoxide (DMSO) at 5 mg/mL, and 10 or 20 μL of the solution was taken into a UV cell and diluted with the suspension of microtubule proteins in the polymerization buffer solution (1.3 mg in 1 mL) at 0°C to make 50 ppm or 100 ppm, respectively. Control experiments were done with 1% or 2% DMSO.

The turbidity was measured immediately after addition of a sample and proteins at 0°C. The mixture was then incubated for 10 min at 37°C to complete the microtubule assembly and measured the turbidity. The inhibitory activity was calculated by the following equation, in which S (sample) and C (control) show [(turbidity at 37°C) - (turbidity at 0°C)] with and without a test sample, respectively.

$$\text{Inhibitory activity (\%)} = \frac{C - S}{C} \times 100$$

Isolation of the Marine Fungus Strain TUF 98F139

An unidentified marine sponge was collected by scuba diving in the coral reef at Palau in 1998. The sponge was sealed in a sterile plastic bag in the water and stored in a cooler box with coolants. The treatment of sponge was performed in a laboratory of the training vessel Umitaka-maru. Five small pieces of the sponge, cut with a sterile pair of scissors, were taken into a sterile motor with 1 mL of sterile seawater and smashed with a pestle. The liquid (0.1 mL) was taken and spread on an agar plate (1/10 YSA: 0.02% yeast extract, 0.1% soluble starch, 1.5% agar, and 200 ppm chloramphenicol in 90% natural seawater). The plate was placed in the research room (25-26°C) of the ship and then incubated at 20°C in an incubator after brought back to the university.

The mycelia grown on the agar plate were taken and inoculated on a slant (1/10 YSA) in culture tube. The culture is maintained in Tokyo University of Fisheries as TUF strain number 98F139. Identification of the strain is not yet completed.

Separation of Inhibitors of Microtubule Assembly (Figure 1)

The fungus strain TUF 98F139 was cultured in three 500 mL flasks with each 150 mL of 1/2 PD medium (hot water extract of potato (100 g/500 mL), 10g dextrose, and 500 mL natural seawater) for three weeks at 20°C. The broth (450 mL) was filtered and mycelia was extracted with MeOH (500 mL). The MeOH extract after evaporation of solvent was chromatographed on a silica gel column with $CHCl_3$-MeOH (gradient) to give three fractions, and the bioactivity was detected in Fr. 2 (58.2 mg) eluted with 5% MeOH. Fr. 2 was dissolved in 75% MeOH in water and adsorbed on an ODS cartridge. The cartridge was washed with the same solvent mixture and eluted with MeOH. The MeOH eluate (45.7 mg) was separated by a Sephadex LH-20 column with MeOH to three fractions. Fr. 2-2 showed the bioactivity and was subjected to silica gel column chromatography with benzene-acetone (gradient) to afford three fractions. The bioactive Fr. 2-2-2 eluted with 2.5% acetone was then purified by thin layer chromatography (TLC) with benzene-acetone (11:1) to give 8.8 mg of the bioactive substance, which showed one spot on TLC.

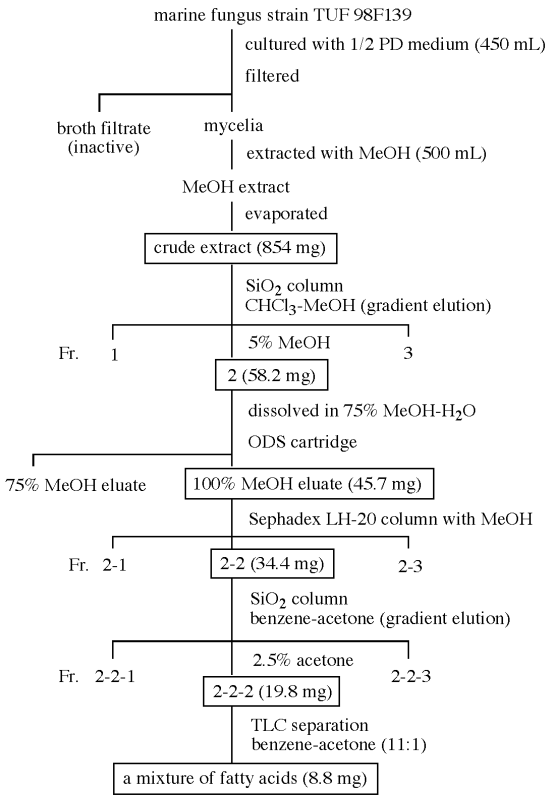

Figure 1. Separation scheme of the bioactive substance from marine fungus strain TUF 98F139.

GC-MS Analysis of Fatty Acids

Fatty acids were methylated with (Trimethylsilyl) diazomethane (Sigma-Aldrich) in hexane at room temperature for 30 min. The methyl esters were analyzed by GC-MS with a TC 70 capillary column (GL Science, 0.25 μm thick of fused silica, 0.25 mmϕ x 30 m): column temperature at 80°C for 5 min, raised to 200°C at 5°C/min and then at 200°C for 10 min; injection temperature, 200°C; detector temperature, 230°C; carrier gas, He at 1.0 mL/min. Total ions generated by an electron impact (EI) mode were monitored to obtain the chromatograms. Mass spectra of peaks appeared in the total ion chromatograms (TICs) were measured from 45 to 425 mass units. Mass spectra of the test sample were compared with those of authentic fatty acid methyl esters to identify the components (oleic acid, linoleic acid, stearic acid, and palmitic acid).

Fatty acid methyl esters in the sample were quantified by a selected ion monitor (SIM) mode (oleic acid methyl ester at *m/z* 55 and 69, linoleic acid methyl ester at *m/z* 67 and 81, stearic acid methyl ester at *m/z* 74 and 87, and palmitic acid methyl ester at *m/z* 74 and 87). The calibration curve of each fatty acid methyl ester was calculated by linear least squares regression from a calibration table created by the authentic specimen. The sample was injected and the chromatograms were integrated in terms of concentration.

Results and Discussion

Isolation of the Marine Fungus Strain TUF 98F139

The fungus was isolated from a marine sponge collected by scuba diving at Palau in 1998, during the training vessel Umitaka-maru anchored at Marakal port. The sponge resembled *Spongia officinalis*[12] and, therefore, may tentatively be identified to *Spongia* sp.

Small pieces of the sponge were smashed in a sterile motor with pestle, and the liquid obtained was spread on a 1/10 nutrient YSA plate. The strain TUF 98F139, grown on the agar plate, was isolated and stored on a slant (1/10 YSA).

Separation and Structures of Inhibitors of Microtubule Assembly

A preliminary screening bioassay of the culture broth of strain TUF 98F139 showed an inhibitory activity to microtubule assembly. The fungus was, therefore, cultured in three 500 mL flasks with each 150 mL of 1/2 PD medium for three weeks at 20°C. The broth was filtered to give mycelia and broth filtrate, and the bioactivity was detected in the MeOH extract of mycelia. Bioassay-guided separation as described in the Materials and Methods section and Figure 1 yielded a bioactive substance, which showed a single spot on TLC.

The ^{1}H NMR spectrum of the bioactive substance (Figure 2) revealed the presence of *cis*-olefin protons (δ 5.2), methylene protons between two double bonds (δ 2.65), methylene protons at the α-position of carbonyl group (δ 2.15), methylene protons next to a double bond (δ 1.9), methylene protons at the β-position of carbonyl group (δ 1.45), a number of methylene protons (δ 1.2), and methyl protons (δ 0.75). These signals were very similar to those of a polyunsaturated fatty acid. It was, however, suggested that the bioactive substance was a mixture of fatty acids since the signal due to the terminal methyl group at δ 0.75 consisted of several triplets.

The components of the mixture were identified by GC-MS analysis using the methyl ester derivatives. The EI

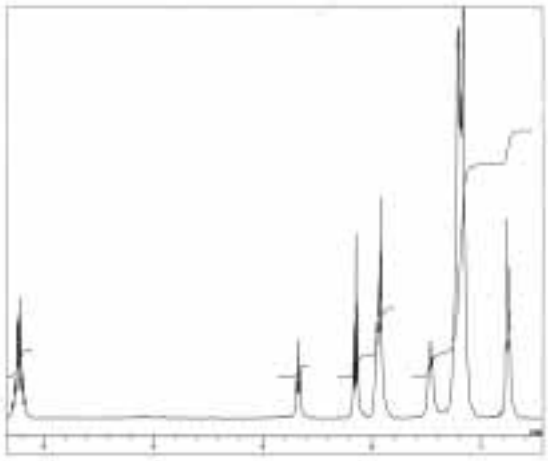

Figure 2. ^{1}H NMR spectrum (400 MHz in acetone-d_6) of the bioactive substance (mixture of fatty acids) obtained from marine fungus strain TUF 98F139.

mass spectra of four peaks appeared in the TIC were compared with the spectra of authentic fatty acid methyl esters. Oleic acid, linoleic acid, stearic acid, and palmitic acid were assigned as the components of the mixture (Table 1).

Table 1. Composition of a fatty acid mixture obtained from strain TUF 98F139

fatty acid	ratio
Oleic acid (18:1)	8
Linoleic acid (18:2)	4
Stearic acid (18:0)	3
Palmitic acid (16:0)	6

The quantitative analysis of the fatty acid derivatives was performed by the SIM mode. The calibration curves of four acid methyl esters were created by the authentic specimens. The ratio of four fatty acids contained in the mixture was calculated and listed in Table 1. A mono-unsaturated C_{18} fatty acid (oleic acid, 18:1) was the most abundant component and a saturated C_{16} acid (palmitic acid, 16:0) was the second. Contents of a C_{18} dienoic acid (linoleic acid, 18:2) and a saturated C_{18} acid (stearic acid, 18:0) were a half of oleic acid and palmitic acid, respectively.

Bioactivity

The bioactive substance (mixture of four fatty acids) showed the inhibitory activity of 57% to microtubule assembly at 50 ppm. The authentic specimens of four components detected in the mixture were tested the bioactivity. The results are listed in Table 2.

Table 2. Inhibition of microtubule assembly by fatty acids at 50 ppm

sample	molarity[*1]	activity (%)
Mixture (natural)	205 μM[*2]	57
Oleic acid (18:1)	198 μM	52
Linoleic acid (18:2)	200 μM	58
Stearic acid (18:0)	197 μM	inactive
Palmitic acid (16:0)	221 μM	inactive
Mixture (authentic)	205 μM[*2]	50

[*1] Converted 50 ppm into molar concentration.

[*2] Calculated from the average molecular weight (244) of four fatty acids in the mixture.

Unsaturated fatty acids, oleic acid and linoleic acid, showed the inhibitory activity of 52% and 58%, respectively, at 50 ppm, but two saturated fatty acids, stearic acid and palmitic acid, did not inhibit the assembly at 50 ppm or at 100 ppm (data not shown). A mixture of four authentic fatty acids of the same ratio as the natural sample showed the similar bioactivity (50%) to that of the natural mixture.

Unsaturated fatty acids are physiologically important compounds not only as the constituents of the cells and body but also as materials for biosyntheses of autacoids, such as prostaglandins and thromboxanes, and of many biologically active secondary metabolites, which have a wide range of biological and ecological functions. The growth inhibitory activities to cyanobacteria and micro- and macroalgae by unsaturated fatty acids including oleic acid and linoleic acid have been reported[13-18]. Recently, a very interesting bioactivity was reported for arachidonic acid (20:4), which stimulates the feeding for the crown-of-thorns starfish *Acanthaster planci*[19].

This is the first report on the inhibition of microtubule assembly by unsaturated fatty acids. The fungus strain TUF 98F139 may have the free unsaturated fatty acids for biological and ecological purposes.

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パラオで採集した海洋糸状菌 TUF 98F139 株が生産する微小管重合阻害活性物質

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1998年にパラオのサンゴ礁で採取した海綿から分離した海洋糸状菌TUF 98F139株の培養菌体から微小管重合阻害活性物質の単離を行い、50 ppmでコントロールに対して57%の重合阻害活性を示す脂肪酸混合物を得た。薄層クロマトグラフィーで単一スポットを与えるこの脂肪酸混合物にはパルミチン酸、ステアリン酸、オレイン酸、リノール酸が含まれていた。脂肪酸標準物質を用いて微小管重合阻害活性を調べたところ、オレイン酸とリノール酸のみが重合阻害活性を示した。その阻害活性は、50 ppmでそれぞれ52%および58%であった。飽和脂肪酸であるパルミチン酸とステアリン酸は重合阻害活性を示さなかったが、オレイン酸とリノール酸の活性を増強する効果を持つことが示唆された。

キーワード: 海洋糸状菌,海綿, チューブリン, 微小管, 阻害剤, 脂肪酸, 不飽和脂肪酸, オレイン酸, リノール酸