A growing number of biologically active metabolites have been explored from marine microorganisms during the past two decades. In recent years, marine microorganisms are paid much attention as a prodigious source for novel drug discovery. In the course of our project searching for new bioactive substances from marine organisms, we found a novel anthracycline named komodoquinone A (1) and its aglycone, komodoquinone B (2), from the solid-state fermentation (solid medium based on rice) of a marine Streptomyces sp. KS3. Komodoquinones A (1) and B (2) are characterized as a rare anthracycline having a 9-methyl substituent (e.g., nogalamycin, steflinomycin, and mutactimycin). The absolute stereostructure of komodoquinone B (2) with 7,9-syn-dihydroxyl groups has been confirmed by application of the modified Mosher’s method. The amino sugar portion in 1 was also characterized to be linked to the hydroxyl group in the D-ring of anthracyclinone. The NMR analysis of the amino sugar portion elucidated that this amino sugar is the C-4 stereo-isomer of mycaminose, which has been found as a component in macrolide antibiotics, and a novel type of amino sugar. This paper presents the elucidation of the absolute structure of the sugar portion in komodoquinones A (1), the effect of a solid-state medium on the production of 1 and its aglycone, komodoquinone B (2), and the neuritogenic activity of 1 against Neuro 2A cells.

Results and Discussion

In order to determine the absolute stereostructure of the sugar portion in 1, the following chemical conversion was executed. Compound 1 was subjected to NaIO₄ degradation followed by acid treatment (2 N aq. HCl at 50 °C) to give a C3-aldehyde, which was further treated with phenylhydrazine and acetic acid to furnish 1-(phenylhydrazo)propan-2-ol (4c). The authentic hydrazone derivatives 4a and 4b were also prepared from methyl L-rhamnoside (3) and methyl D-fucoside (5) applying the same synthetic route (Fig. 2). HPLC analysis of these hydrazone derivatives clarified that 4c was identical with 4a. Therefore, the amino sugar moiety in 1 has been defined to be L-form and the absolute stereostructure of komodoquinone A was confirmed to be 1 (Fig. 1).

Solid-state fermentation is well known to be used for production of secondary metabolites. Komodoquinones A (1) and B (2) together with staurosporine were produced by a marine Streptomyces sp. KS3 cultured in a solid-state medium based on rice. In the case that the KS3 strain was grown in a ISP1 + glucose liquid medium, no production of 1 and 2 was observed. These results led us to investigate the production of secondary metabolites. Komodoquinones A (1) and B (2) together with staurosporine were produced by a marine Streptomyces sp. KS3 cultured in a solid-state medium based on rice. In the case that the KS3 strain was grown in a ISP1 + glucose liquid medium, no production of 1 and 2 was observed. These results led us to investigate the

Fig. 1. Structure of Komodoquinones A (1) and B (2)

Fig. 2. Hydrazone Derivatives Prepared from Komodoquinone A (1), Methyl L-Rhamnoside (3) and Methyl D-Fucoside (5)
role of the solid-state fermentation on the growth of the KS3 strain and its ability to produce the secondary metabolites. We examined the effect of other cereals such as corn, soybean, and barley, on the production of 1 and 2. The solid-state medium based on corn or soybean was not able to support the growth of the KS3 strain. However, in the case of either barley or rice, the strain grew very well and produced komodoquinones A (1) and B (2). On the other hand, the cultivation of the KS3 strain in ISP1 + glucose medium-added rice produced only komodoquinone B (2). Therefore, it was presumed that a constituent contained in barley or rice was concerned with the biosynthesis of the aglycone, komodoquinone B (2), in the fermentation of the KS3 strain. However, the biosynthesis and glycosidation of the amino sugar portion in 1 must be associated with the contribution of some parameter such as osmotic pressure, which usually exert a substantial effect in solid-state fermentation. Consequently, solid-state fermentation of microorganisms is expected to be a good strategy to explore novel bioactive compounds.

Many anthracyclines have been reported from terrestrial actinomycetes, which have been documented to exhibit antimicrobial, anti-tumor and other biological activities. However, komodoquinone A (1) is the first instance of anthracycline exhibiting neuritogenic activity. Compound 1 was shown to induce a morphological change with multipolar processes emanating from the body of Neuro 2A cells at a concentration of 1 μg/ml (Fig. 3). In contrast, adriamycin (6), a representative anthracycline, showed no neuritogenic activity even at the same concentration, and komodoquinone B (2), the aglycone of 1, showed only weak activity at a concentration of 30 μg/ml. These results imply that the amino sugar moiety bound to the D-ring in 1 might play an important role for neuritogenic activity.

Next, we investigated the effect of 1 and 6 on the cell cycle of Neuro 2A cells, since neuronal differentiation was closely related to the cell cycle. The cell cycle of Neuro 2A cells treated with 1 (at 1 μg/ml) for 24 h was arrested at the G1 phase, while that treated with 6 (at 1 μg/ml) for 24 h was arrested at the G2/M phase (Fig. 4). Generally, anthracycline antibiotics, which intercalate in DNA and RNA, have been known to arrest the cell cycle at the G2/M phase. Therefore, komodoquinone A (1) may exert neuronal differential activity by a mechanism, which is different from the intercalation in DNA.

Experimental

The following instruments were used to obtain physical data: a Jasco DIP-181 digital polarimeter for specific rotations; a Hitachi 260-30 infrared spectrometer for IR spectra; a Varian Inova-600 MHz NMR spectrometer for 1H- and 13C-NMR and a Varian Inova-600 MHz NMR spectrometer for 1H- and 13C-NMR spectra. Silica gel (Fuji silysia BW-200) was used for column chromatography and TLC. Spots on TLC plates were detected by spraying vanillin/H2SO4 (vanillin 5 g, p-anisaldehyde solution (at 1.4 g) for 12 h at room temperature, and then the reaction mixture was extracted with AcOEt. The AcOEt layer was washed with brine and dried over MgSO4. Removal of solvent from the AcOEt layer under reduced pressure gave a corresponding aldehyde. A solution of the aldehyde in 2 N HCl (10 ml) was stirred for 1 h at 50 °C. After neutralization by Na2CO3, the reaction mixture was quenched with brine, then the whole was extracted with AcOEt. The AcOEt phase was dried over MgSO4 and evaporated in vacuo. The crude product was purified by reversed phase HPLC [Cosmosil 5C18-AR (10 mm i.d.×250 mm), mobile phase; MeOH–water=7:3, detection; UV (λ=268 nm), flow rate; 2.0 ml/min] to afford 1R-(phenylhydrazo)propan-2-ol 4a (11 mg). A solution of methyl β-d-fucoside (5, 80 mg) was similarly treated to obtain 13-(phenyl-
Hydrazone Derivative 4a: [ε]20 335° (e=0.2, MeOH). IR (KBr) cm⁻¹: 3345, 1653, 1585. UV λmax (MeOH) nm (ε): 268 (8600). 1H-NMR (600 MHz, CD6D6, δ): 7.2—7.0 (7H, m, –Ph, –NH, –CNH), 4.15 (1H, q, J=6.6 Hz, 2-H), 3.87 (1H, s, 2-OH), 1.11 (3H, d, J=6.6 Hz, 3-H). FAB-MS: m/z 165 (M+H). High resolution FAB-MS: m/z 165.1033 (M+H). Calcd for C10H12N2O: 165.1028.

Hydrazone Derivative 4b: [ε]20 52° (e=0.2, MeOH). IR (KBr) cm⁻¹: 3345, 1655, 1580. UV λmax (MeOH) nm (ε): 268 (8600). 1H-NMR (600 MHz, CD6D6, δ): 7.2—7.0 (7H, m, –Ph, –NH, –CNH), 4.15 (1H, q, J=6.6 Hz, 2-H), 3.85 (1H, s, 2-OH), 1.11 (3H, d, J=6.6 Hz, 3-H). FAB-MS: m/z 165 (M+H). High resolution FAB-MS: m/z 165.1033 (M+H). Calcd for C10H12N2O: 165.1028.

Chiral HPLC Analysis of Hydrazone Derivatives 4a—c: Hydrazone derivatives 4a—c were analyzed by chiral HPLC [CHIRALCEL OD-R, 4.6 mm i.d.×250 mm, mobile phase; CH3CN–H2O] and detection; UV (268 nm), flow rate; 0.5 ml/min. The retention times of 4a—c were 34.5, 30.0, and 34.5 min, respectively.

Culture of Streptomyces sp. KS3 in Other Conditions: A liter of seed medium (ISP1, 25 g of glucose, 100 mg yeast extract, 50 ml of artificial sea water) was autoclaved before use. A liter of ISP1 medium [25 g of corn, 200 mg of glucose, 100 mg yeast extract, and 50 ml of artificial sea water] in 500 ml flask was autoclaved before use. The soybean solid medium [25 g of soybean, 200 mg of glucose, 100 mg yeast extract, and 40 ml of artificial sea water] in 500 ml flask was autoclaved before use. The corn solid medium [25 g of corn, 200 mg of glucose, 100 mg yeast extract, and 40 ml of artificial sea water] in 500 ml flask was autoclaved before use. A liter of ISP1-glucose-rice medium [25 g of rice, 5 g of Bacto triptone, 3 g of yeast extract, 5 g of glucose, and artificial sea water] was autoclaved before use. The Streptomyces sp. KS3 strain was cultured in the seed medium at 30°C for 5 d. Then, the broth of the strain was inoculated into the production medium and cultured under static conditions at 30°C for 2 weeks.

Assay for Neuritogenic Activity in Neuro 2A Cells: Neuro 2A cells were grown in Dulbecco’s modified essential medium (DMEM) with 10% fetal bovine serum (FBS). The cells were kept in an incubator at 37°C with 5% CO2. The cells were plated on 24-well plates at a density of 2×10⁴ per well with 1 ml of culture medium. After 24 h cultivation, the medium was exchanged for fresh medium, and the testing sample as 10 μl of EtOH solution was added to each well. After 24 or 48 h incubation, morphological changes in the cells were observed under microscope.

Cell Cycle Analysis: The cell suspension of Neuro 2A cells (4×10⁴ cells in 1 ml of the culture medium) was plated on 24-well plate and incubated for 24 h. After medium exchange, an ethanol solution (10 μl) of the test sample was added and further incubated for 24 h. The culture medium of the cell suspension was removed by centrifugation (1000g for 3 min). The collected cells were dyed by DNA-Prep Reagents Kit for 20 min. Then, the supernatant was removed by centrifugation (500g for 5 min) and the resulting cell suspension in 500 μl of D-PBS (−) solution was filtered by 40-μm nylon mesh filter. The cell cycle analysis of the filtrate was carried out on a FACSCalibur (Becton Dickinson, λex = 493 nm, λem = 630 nm).

Acknowledgments: The authors are grateful to the Houansha Foundation, the Tokyo Biochemical research foundation, and the Uehara Memorial Foundation for financial support.

References: