VOL. 56 NO. 6, JUNE 2003 THE JOURNAL OF ANTIBIOTICS pp. 526 — 532

New Monordens Produced by Amidepsine-producing Fungus *Humicola* sp. FO-2942

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(Received for publication March 6, 2003)

Based on UV spectrum-guided purification, new monordens C, D and E, known monordens A (radicicol) and B and 5-*O*-methylsclerone were isolated from the fermentation broth of amidepsine-producing *Humicola* sp. FO-2942 by solvent extraction, silica-gel column chromatography, ODS column chromatography and HPLC. All monordens cause the cell cycle arrest at G1 and G2/M phases in Jurkat cells. But among them, monordens A and E show antifungal activity only against *Aspergillus niger*.

Based on the recent development of analytical apparatus including LC-UV, LC-MS and LC-NMR, researchers are interested in defining all the metabolites of microorganisms, leading to the current concept "metabolomix".

Humicola sp. FO-2942 was originally discovered as a producing fungus of amidepsines, inhibitors of diacylglycerol acyltransferase[1,2]. In the process of amidepsine purification, we found that the culture broth contained several metabolites showing UV spectra similar to those of amidepsines by LC-UV analyses. Unexpectedly, monorden (radicicol, monorden A in this paper)[3], monorden B (6,7,8,9-tetrahydromonorden A)[4,5], structurally related new compounds named monordens C, D and E and 5-*O*-methylsclerone[6] (Fig. 1) were isolated along with amidepsines from the culture broth. Monorden B and 5-*O*-methylsclerone were first isolated as fungal metabolites.

The physico-chemical properties and structure elucidation of new monordens are reported in the accompanying paper[7]. In this paper, fermentation, isolation and biological properties of monordens B, C, D and E, and 5-*O*-methylsclerone are described.

Materials and Methods

Materials

Humicola sp. FO-2942, previously reported as an amidepsine-producing fungus[1,2], was used for production of monordens and 5-*O*-methylsclerone. Authentic monorden A was isolated from the culture broth of a fungal strain FO-4910. A human T-cell leukemia-derived cell line, Jurkat, was kindly provided by Dr. M. SUGANUMA (CanBas Co., Ltd.). Fetal calf serum (FCS) was purchased from JRH Biosciences. Propidium iodide, Ribonuclease A and NP-40 were obtained from Sigma. AlamarBlue and RPMI 1640 medium were purchased from IWAKI. *Aspergillus niger* ATCC6275 was purchased from American Type Culture Collection (ATCC).

General Experimental Procedures

Kieselgel 60 (E. Merck) and ODS (SSC-ODS 7515-12, Senshu Sci. Co., Ltd.) were used for column chromatography. For determination of the amounts of monordens in culture broths, samples (ethyl acetate extracts) were dissolved in methanol and analyzed by LC-UV (HP1100, Hewlett Packard) under the following conditions; Symmetry C18/3.5 μm column (2.1×150 mm, Waters), a 15-minute linear gradient from 50%

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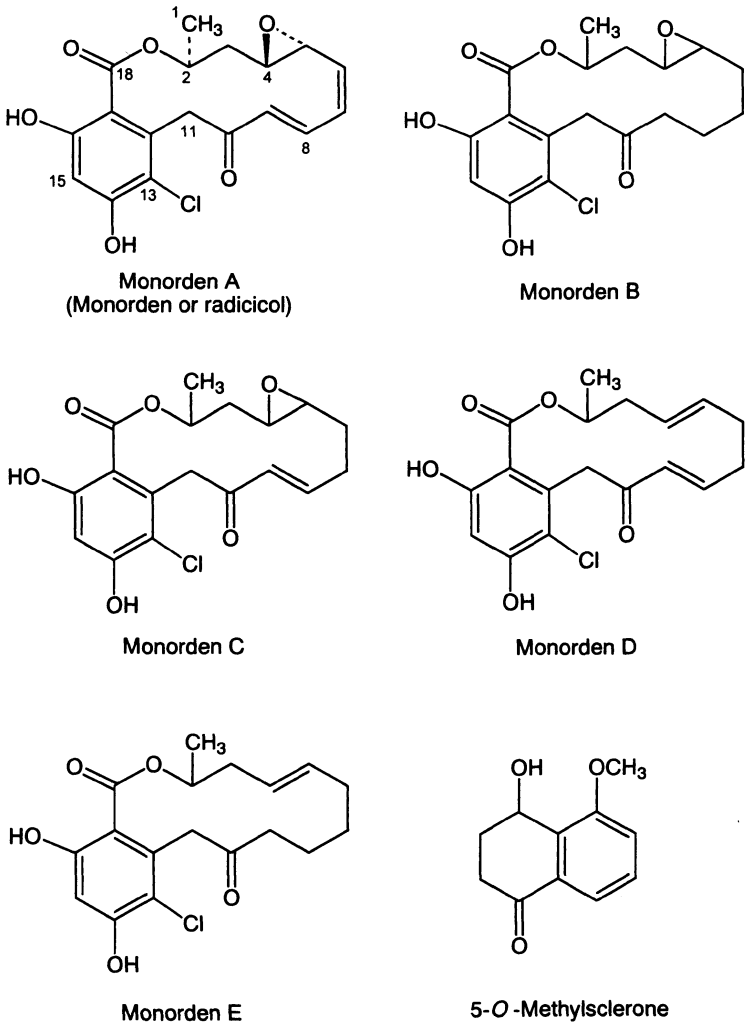
Fig. 1. The structures of monordens and 5-*O*-methylsclerone.



CH_3CN/0.05% H_3PO_4 to 60% CH_3CN/0.05% H_3PO_4, 0.2 ml/minute, and UV at 210 nm. Monordens A to E and amidepsines A to D were eluted as peaks with retention times of 2.5, 2.9, 2.7, 5.2, 7.6, 4.6, 5.9, 8.0 and 11.7 minutes, respectively (Fig. 2). 5-*O*-methylsclerone was analyzed by LC-UV as follows; Symmetry C18/3.5 μm column (2.1×150 mm, Waters), a 15-minute linear gradient from 30% CH_3CN/0.05% H_3PO_4 to 50% CH_3CN/0.05% H_3PO_4, 0.2 ml/minute, and UV at 210 nm. Under the conditions, the compound was eluted as a peak with a retention time of 3.6 minutes (data not shown).

Cell Culture and Cell Cycle Analysis

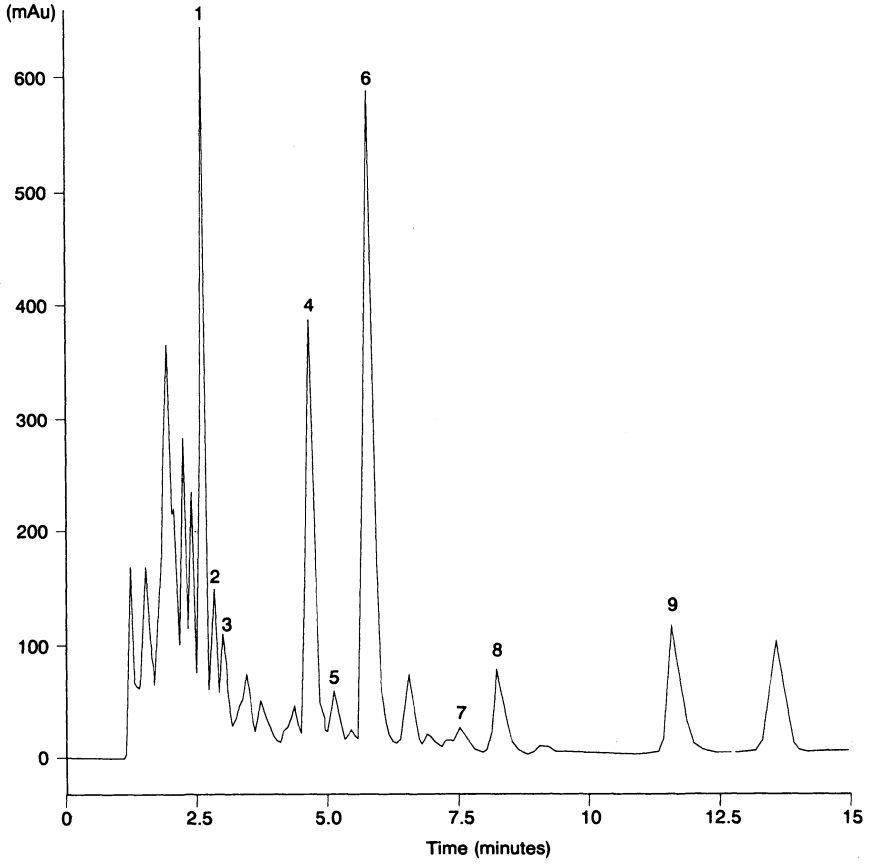
Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2.

The cell cycle status of Jurkat cells was analysed according to the method described previously[8]. In brief, Jurkat cells (1.0×10^5 cells) were treated with a compound for the indicated time at 37°C. Then, the cells were resuspended in 200 μl of 0.1% sodium citrate solution containing 50 μg/ml propidium iodide, 20 μg/ml ribonuclease A and 0.5% NP-40. After 2 hours, the cell cycle distribution was determined by the analysis of DNA content using FACSCalibur (Becton Dickinson). Cells (1.0×10^4 cells) were measured for each sample, and population of each phase (G1, S and G2/M) was calculated by the program ModiFit LT ver. 2.0 according to the manufacture's protocol.

Anti-*Aspergillus niger* Activity

The growth of *Aspergillus niger* in the presence of a

Fig. 2. HPLC chromatographic profile of ethyl acetate extracts.



Ethyl acetate extracts (20 μg in 5 μl of MeOH) were subjected to HPLC under the conditions as described in "Materials and Methods".

Peak 1, monorden A (radicicol); peak 2, monorden C; peak 3, monorden B; peak 4, amidepsine A; peak 5, monorden D; peak 6, amidepsine B; peak 7, monorden E; peak 8, amidepsine C; peak 9, amidepsine D.

sample (monordens A to E or 5-*O*-methylsclerone) was tested using AlamarBlue by the established method[9]. In brief, spores of *A. niger* ATCC6275 (2.5×10^4 cells in each well of a 96-well microplate) were plated in 175 μl of RPMI 1640 medium containing 165 mM of MOPS (pH 7.0), and then 5.0 μl of the sample in MeOH (final concentration of 0~100 μM) and 20 μl of AlamarBlue were added to each culture. Following a 24-hour incubation at 35°C, the growth of *A. niger* was measured as fluorescence intensity (Ex: 530 nm, Em: 590 nm) with CytoFluor 4000 (PerSeptive Biosystems). A sample concentration causing 50% inhibition of the growth of *A. niger* (IC_{50}) was defined as an index of anti-*A. niger* activity.

Results

Fermentation

A slant culture of the strain FO-2942 grown on YpSs agar (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4\cdot7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, pH 6.0) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 4 days. The seed culture (600 ml) was transferred into a 90-liter jar fermenter (Mitsuwa Biosystem) containing 60 liters of the production medium (sucrose 2.0%, glucose 1.0%, corn steep liquor 1.0%, meat extract 0.5%, KH_2PO_4 0.1%,

$MgSO_4 \cdot 7H_2O$ 0.05%, 360 ml of trace elements containing in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0%, $MnCl_2 \cdot 4H_2O$ 1.0%, $ZnSO_4 \cdot 7H_2O$ 1.0%, $CuSO_4 \cdot 5H_2O$ 1.0%, $CoCl_2 \cdot 2H_2O$ 1.0%, $CaCO_3$ 0.3% and agar 0.1%, pH 6.0). The fermentation was carried out at 27°C with an aeration of 5 liters/minute and an agitation of 200 rpm. On day 4, the production of monordens A to E and amidepsines A to D is 6.5, 1.6, 3.1, 1.2, 0.8, 10.9, 20.3, 3.5 and 4.9 μg/ml, respectively.

Isolation

The 4-day old culture broth (60 liters) was centrifuged to separate mycelium and supernatant. The mycelium was extracted with 36 liters of acetone. After the acetone extract was filtered and concentrated, the resulting aqueous solution was extracted with 10 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield 11.2 g of an oily material. The materials were dissolved in chloroform and applied on a silica gel column (Kieselgel 60, 600 ml) previously equilibrated with chloroform. The materials were eluted stepwise with chloroform - methanol solutions (3000 ml each, 100 : 0, 98 : 2, 95 : 5 and 0 : 100, v/v), and 150 ml of the elution was collected. All the compounds were eluted with the chloroform - methanol solution (98 : 2); amidepsines, monordens D and E were recovered from the fractions 5 to 7 (Fr. I), 5-*O*-methylsclerone from the fractions 8 and 9 (Fr. II), and monordens A to C from the fractions 10 and 11 (Fr. III). Frs. I, II and III were concentrated *in vacuo* to yield 824.6, 179.4 and 310.6 mg, respectively. Then, Fr. I was suspended in 10% aq CH_3CN, and subjected to an ODS column (80 ml). The materials were eluted stepwise with H_2O-CH_3CN solutions (400 ml each, 10, 30, 50 and 70% aq CH_3CN), and 50 ml of elution was collected. The 50% aq CH_3CN fraction was collected and concentrated, and the aquous solution was extracted with ethyl acetate to give a brownish powder (15.6 mg). The resulting materials were purified by preparative HPLC; YMC-Pack ODS-AM column (20×250 mm, YMC Co., Ltd.), 50% CH_3CN in 0.05% H_3PO_4, 6.0 ml/minute, and UV at 215 nm. Under the conditions, monordens D and E were eluted as peaks with retention times of 42.0 and 71.0 minutes, respectively. Each peak was collected and concentrated to yield pure monordens D (5.14 mg) and E (2.82 mg) as yellow oil. Frs. II and III were purified by preparative HPLC under the following conditions; PEGASIL ODS column (20×250 mm, Senshu Sci. Co., Ltd.), 40% CH_3CN in 0.05% H_3PO_4, 8.0 ml/minute, and UV at 210 nm. Under the conditions, 5-*O*-methylsclerone and monordens A to C were eluted as peaks with retention times of 10.2, 18.5, 26.1 and 23.3 minutes, respectively. Each peak was collected and concentrated to yield pure 5-*O*-methylsclerone (1.46 mg) as yellowish oil and pure monordens A (22.7 mg), B (5.10 mg) and C (20.3 mg) as yellowish powder.

Biological Properties

Effect of Monordens on the Cell Cycle

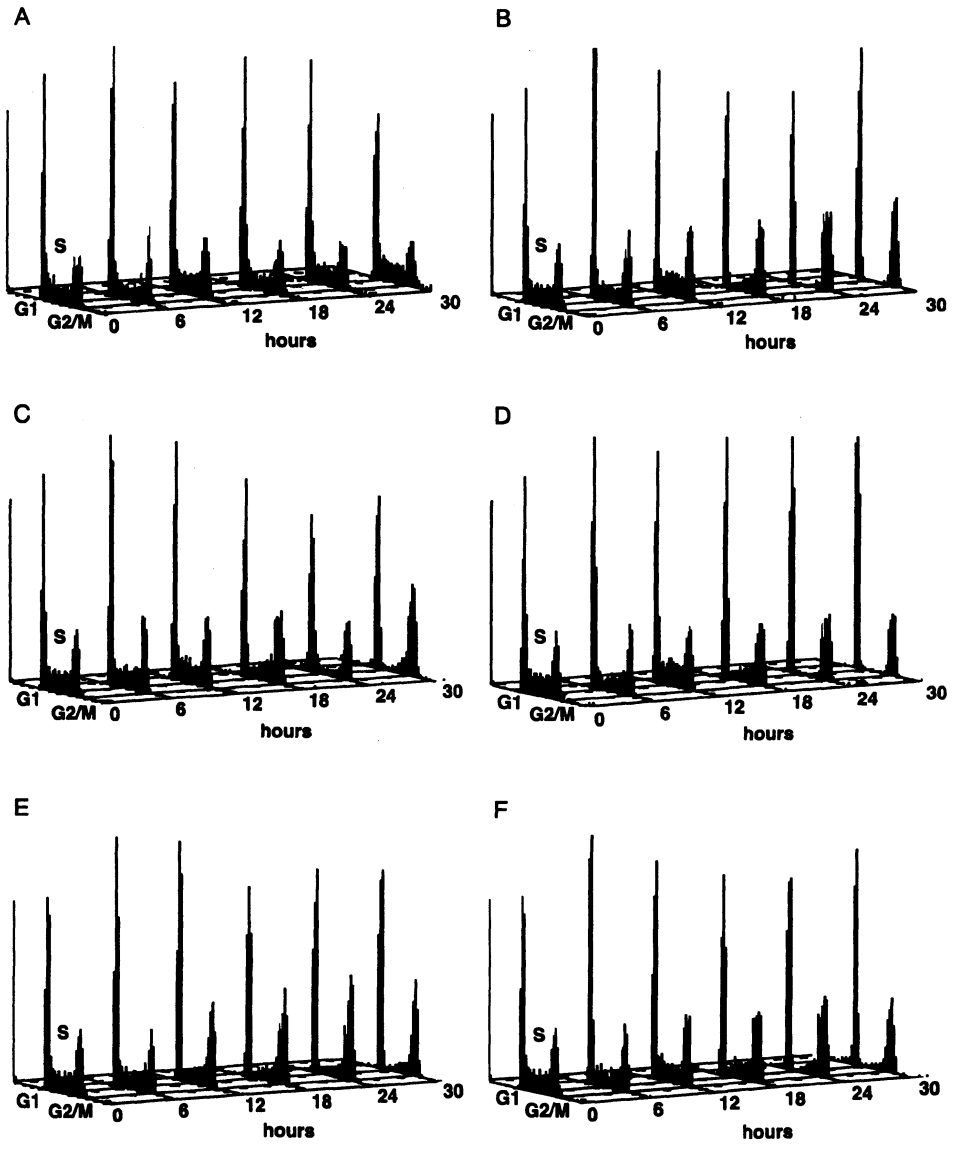
Jurkat cells were cultured in the presence of each monorden, and the cell cycle distribution was analysed every 6 hours. In the control experiments (without a sample), the distribution ratio in G1, S and G2/M phases was almost constant, that is, 49±2%, 20±2% and 23±3%, respectively, at least up to 30 hours (Fig. 3A). In the presence of monordens at the indicated concentrations (Fig. 3B~3E), the most drastic changes in the cell cycle were that Jurkat cells in the S phase decreased at 18 hours after the treatment, and almost disappeared at 24 and 30 hours. Conversely, cells in the G1 and G2/M phase increased. Although monorden A had been reported to show the activity, as reproduced in Fig 3F, monorden B to E were also found to cause the cell cycle arrest at G1 and G2/M phases.

Then, Jurkat cells were cultured with monordens at various concentrations (0~30 μM), and the cell cycle distribution was analysed at 24 hours. As the results are summarized in Table 1, all monordens showed no effect on the distribution at 3.0 μM, but decrease in the S phase cells was observed at 9.0 μM monordens A to D, and small decrease was at 9.0 μM monorden E. Such decrease was kept at 30 μM monorden B and D, and enhanced at 30 μM monorden E. Unexpectedly, the distribution in the cell cycle at a higher concentration of 30 μM monordens A and C was ostensively recovered to the control one. 5-*O*-Methylsclerone showed no effect on the cell cycle up to 30 μM (data not shown).

Anti-*Aspergillus niger* Activity

From the conventional paper disk assay, monorden A showed weak inhibition only against *A. niger* among 16 microorganisms tested[10]. Therefore, effect of all monordens was compared on the growth of *A. niger* in the liquid culture method. As shown in Fig. 4, only monordens A and E were found to inhibit the growth with IC_{50} values of 12 and 70 μM, respectively.

Fig. 3. Effect of monordens on the cell cycle in Jurkat cells.



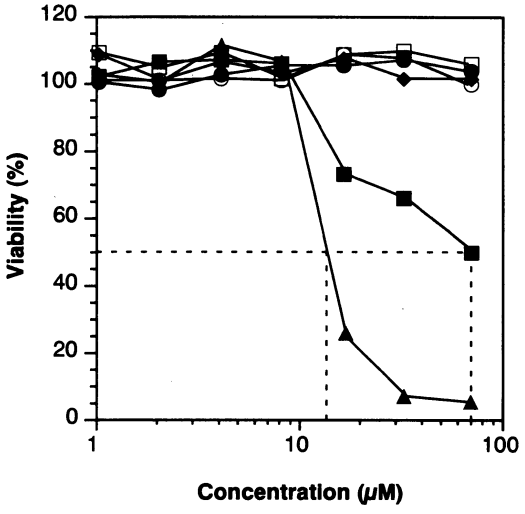
Jurkat cells were cultured in the absence (A) or presence of monorden B (30 μM, B), monorden C (9.0 μM, C), monorden D (30 μM, D), monorden E (30 μM, E) or monorden A (9.0 μM, F) and the distribution in the cell cycle was analyzed with FACS at 0, 6, 12, 18 and 24 hours after incubation.

Table 1. Effect of monordens on the cell cycle in Jurkat cells.

Concentration (μM)	Monorden A (%)			Monorden B (%)			Monorden C (%)			Monorden D (%)			Monorden E (%)		
	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
0	49.9	19.6	22.5	49.9	19.6	22.5	49.9	19.6	22.5	49.9	19.6	22.5	49.9	19.6	22.5
3.0	50.0	23.2	19.9	47.8	20.8	22.9	48.2	19.8	20.5	50.1	19.2	21.3	47.3	18.5	22.8
9.0	53.3	6.7	35.4	52.9	2.4	32.9	49.0	7.3	39.4	53.7	8.7	28.4	48.6	14.0	23.8
30	42.1	15.5	23.3	50.9	2.7	35.2	31.4	15.4	36.8	54.0	6.8	37.2	52.0	8.3	26.9

Jurkat cells were cultured at indicated concentrations of each compound for 24 hours. Cell cycle distribution was analyzed with FACS, and percentage of G1 phase, S phase and G2/M phase were caliculated by the program Modifit LT ver. 2.0.

Fig. 4. Anti-*Aspergillus niger* activity of monordens.



A. niger was grown at 35°C for 24 hours in RPMI 1640 containing 165 mM MOPS and AlamarBlue in the presence of monorden A (▲), monorden B (○), monorden C (□), monorden D (●), monorden E (■) or 5-*O*-methylsclerone (◆). The growth was measured as fluorescence intensity with a microplate reader as described in "Materials and Methods".

Discussion

Recently, avermectin-producing actinomycete was found to encode at least 30 biosynthetic genes of secondary metabolites[11], even though only several compounds including avermectins and oligomycin had been isolated from the culture broth. Therefore, metabolomix studies will be important to discover new leads for medicines and useful agents of microbial origin.

In this paper, six compounds other than amidepsines were isolated from the culture broth of amidepsine-producing *Humicola* sp. FO-2942 as possessing UV spectra similar to amidepsines. Among them, three monorden-related compounds are new, and monorden B and 5-*O*-methylsclerone are synthetically known but the first of natural origin. The six compounds did not inhibit DGAT activity, but new monordens were found to cause the cell cycle arrest at the G1 and G2/M phases.

A variety of biological activities for monorden-related compounds has been reported so far. Monorden A was first reported as an antifungal substance in 1951[3]. Then, monordens have been reported to induce cell differentiation[12], inhibit expression of cyclooxygenase gene[13], cause the cell cycle arrest at G1 and G2 phases[14] and suppress transformation of normal cells into cancer cells[15]. Several mechanisms of action have been reported; inhibition of tyrosine kinase[14], MAP kinase[14], Hsp 90 function[15,16] and ATP citrate lyase[16]. In fact, KI *et al.* demonstrated that biotinylated derivatives of monorden A (radicicol) are bound to Hsp 90 and ATP citrate lyase molecules[16]. Especially, Hsp 90 plays an important role in cellular regulatory proteins such as transcription factors and receptors, leading to a variety of biological activities due to its inhibition by monordens.

We showed that new monordens cause the cell cycle arrest at G1 and G2/M phases (Fig. 3 and Table 1) as well as monorden A[14]. From the results, monorden E showed the weakest activity among the five monordens. Monordens A and C seemed to show potent effect on the cell cycle, but at higher doses (≧15 μM) they inhibited the cell proliferation completely to freeze the cell cycle. In fact, the cell cycle distributions at 30 μM monordens A or C were almost the same as those of the control cells during the incubation periods (0~30 hours, data not shown). Comparison of the effect of synthetic derivatives of monorden on the morphological reversion activity of v-src-transformed 3Y1 cells showed that monorden A is the most potent, followed by monorden B, and that the reductive derivative of monorden B lacking the C-4 epoxide lost the activity[4]. The hierarchy of these monordens potency in the two activities (morphological reversion assay and cell cycle assay) indicates that the C-4 epoxide and the C-6 and C-8 double bonds are important for exhibiting the activities. It might be that these activities are derived from the same mechanism(s); inhibition of Hsp 90 and/or ATP citrate lyase or unknown molecule. As shown in Fig. 4, however, only monordens A and E showed the anti-*A. niger* activity. The result is not parallel to that in the cell cycle assay. It might be that monorden E has a different target in *A. niger*.

Further studies are necessary to define the correlation between the target molecules and biological activities.

Acknowledgments

We express our thanks to Ms. Y. OYAMA and Ms. N. NISHIMURA for expert technical assistance. This study was supported in part by the grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology, Japan.

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