Communications to the Editor

ISOLATION AND STRUCTURAL ELUCIDATION OF STIPIAMIDE, A NEW ANTIBIOTIC EFFECTIVE TO MULTIDRUG-RESISTANT CANCER CELLS

Sir:

Resistant of tumor cells to multiple cytotoxic drugs is a major impediment to cancer chemotherapy. Multidrug-resistance (MDR) is characterized by decreased cellular sensitivity to anticancer agents due to the increased expression of a membrane glycoprotein with a MW of approximately 170 kd, termed P-glycoprotein[1].

It is well known that colchicine-resistance cells have simultaneously acquired resistance to doxorubicin and vinblastine[2]. Thus, it could be expected that compounds active against colchicine-resistance cells will be useful for overcoming MDR to antitumor substances.

By employing colchicine-resistance KB(CH^R)[3] cells, we undertook the screening for agents which were more active to KB(CH^R) cells in the presence of colchicine†. As a result, a gliding bacterium isolated from a soil sample from Izu Peninsula, Japan was found to produce a new antibiotic named stipiamide. The producing organism was identified as *Myxococcus stipitatus* (AJ-12587) by taxonomic comparison with type strains of *Myxococcus* sp. as well as by its morphological, cultural and physiological characteristics. In this paper, we will report the fermentation, isolation and structure elucidation of stipiamide.

The organism was cultivated in 5-liter Sakaguchi flasks containing 1 liter of the Casitone liquid medium consisting of Casitone (Difco) 2% and $MgSO_4$ 0.2% (pH 7.2). The flasks were incubated at 28°C for 4 days on a reciprocal shaker at 115 rpm. In this medium, the organism grew as a homogeneous cell suspension. The cells harvested by centrifugation from the whole broth (5 liters) were stirred with 500 ml of acetone. The solvent extract was concentrated *in vacuo* to a small volume and the active material was extracted from the aqueous residue twice with each 100 ml of EtOAc. The separated organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give a dark brownish oil, which was subjected to silica gel column chromatography (Wakogel C-200, 5 × 10 cm) packed in $CHCl_3$. After developing with 800 ml of $CHCl_3$, the column was developed by a mixture of $CHCl_3$-MeOH (49 : 1). The active fractions were combined and concentrated *in vacuo* to afford a crude oil. Further purification of the oil was achieved by preparative TLC (Merck, Kieselgel 60F_{254} Art. No. 5744, 0.5 mm) developed with $CHCl_3$-MeOH (21 : 1). The active band was collected and purified once more by preparative TLC developed with hexane-EtOAc (2 : 3) to yield 106 mg of stipiamide.

Its physico-chemical properties are shown in Table 1. The molecular formula of stipiamide was determined to be $C_{32}H_{45}NO_3$ by HRFAB-MS data (*m/z* 492.3416 $(M+H)^+$, Calcd: 492.3477).

The ^{13}C and ^1H NMR spectral data of stipiamide are summarized in Table 2. The functionalities of the carbons in stipiamide were revealed by DEPT experiments to be as follows; $CH_3 \times 6$, $CH_2 \times 2$, $CH \times 16$, $CH_2O \times 1$, $CHO \times 1$, $CHN \times 1$, $C{=}O \times 1$ and $C \times 4$.

The ^{13}C resonance at δ_C 169.8 was assigned to an amide carbonyl due to an IR absorption band at 1645 cm^{-1}. The partial structures shown in Fig. 1 were determined by analysis of the DQF-COSY spectrum which showed the sequences of 1′-H to 2′-H (I), 3-H to 9-H (II), 11-H to 13-H (III), 15-H

Table 1. Physico-chemical properties of stipiamide.

Appearance	Yellowish oil
Molecular formula	$C_{32}H_{45}NO_3$
HRFAB-MS (*m/z*)	492.3416 $(M+H)^+$, Calcd: 492.3477
UV λ_{max}^{MeOH} nm (ε)	207 (9,820), 264 (6,730), 359 (31,500)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	209 (32,200), 264 (6,780), 359 (31,300)
IR $\nu_{max}^{CCl_4}$ (cm^{-1})	3375, 2950, 1645, 1505, 1455, 995, 965, 700
$[\alpha]_D^{23}$	−115.1° (*c* 1.45, MeOH)
Solubility	Soluble in CCl_4, $CHCl_3$, MeOH Insoluble in H_2O
Rf (Kieselgel 60 F_{254})	0.28 ($CHCl_3$-MeOH, 21 : 1) 0.37 (Hexane-EtOAc, 2 : 3)

† IC_{50} of colchicine to KB(CH^R) cells was 8.5 μg/ml, while that to normal KB cell was 0.08 μg/ml. KB(CH^R) cells were about 30 times as resistant to daunorubicin as KB cell.

Table 2. ^{13}C and ^{1}H NMR signals of stipiamide taken in acetone-d_6.

No.	δ_C	δ_H
1	169.8	
2	132.2	
3	133.8	7.00 (1H, d, J=11 Hz)
4	129.4	6.58 (1H, dd, J=11, 15 Hz)
5	134.2	7.08 (1H, dd, J=10, 15 Hz)
6	129.3	6.10 (1H, dd, J=10, 10 Hz)
7	133.5	6.15 (1H, dd, J=10, 10 Hz)
8	122.9	6.78 (1H, dd, J=10, 15 Hz)
9	141.8	6.39 (1H, d, J=15 Hz)
10	135.2	
11	140.0	5.64 (1H, d, J=10 Hz)
12	38.1	2.80 (1H, qdd, J=7, 7, 10 Hz)
13	82.2	3.84 (1H, dd, J=5, 7 Hz)
14	137.0	
15	133.5	5.25 (1H, d, J=10 Hz)
16	32.8	2.43 (1H, m)
17	40.8	1.50, 1.63 (2H, m)
18	34.9	2.50, 2.60 (2H, m)
19	144.0	
20	129.6	7.16 (1H, d, J=8 Hz)
21	129.4	7.23 (1H, t, J=8 Hz)
22	126.7	7.13 (1H, t, J=8 Hz)
23	129.4	7.23 (1H, t, J=8 Hz)
24	129.6	7.16 (1H, d, J=8 Hz)
2-CH_3	13.7	1.96 (3H, d, J=8 Hz)
10-CH_3	13.5	1.87 (3H, s)
12-CH_3	18.7	0.95 (3H, d, J=7 Hz)
14-CH_3	13.0	1.60 (3H, s)
16-CH_3	22.0	0.96 (3H, d, J=7 Hz)
1′	49.0	4.05 (1H, qt, J=7, 7 Hz)
2′	66.9	3.54 (2H, m)
1′-CH_3	17.8	1.17 (3H, s)

to 18-H (IV), and a phenyl ring (V).

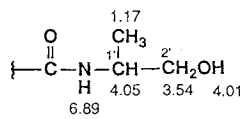
The complete connectivity of the carbon skeleton of the polyene chain and phenethyl residue could easily be established by an HMBC experiment.[4,5] Thus, the ^{13}C-^{1}H long range couplings observed with methyl groups in Fig. 2 (2-CH_3 (δ_H 1.96), 10-CH_3 (δ_H 1.87) and 14-CH_3 (δ_H 1.60)) enabled the partial structures (I) to (IV) in Fig. 1 separated by quaternary carbons to be connected to form a highly unsaturated chain. In addition, the couplings between aromatic protons (20-H, 24-H (δ_H 7.16)) and benzyl methylene (18-H (δ_H 2.50, 2.60)) proved the partial structure (IV) shown in Fig. 2. Based on these results, the total planar structure of stipiamide can be depicted as shown in Fig. 3.

Stereochemical studies were facilitated by analysis of the ^{1}H-^{1}H coupling constants (3-H to 9-H) (Fig. 1 (II)) and one dimensional NOE difference experiments. NOEs obtained by irradiating at methyl protons (2-CH_3 (δ_H 1.96), 10-CH_3 (δ_H 1.87) and 14-CH_3 (δ_H 1.60)) were as follows; (2-CH_3→4-H (δ_H 6.58), N–H (δ_H 6.89); 10-CH_3→8-H (δ_H 6.78), 12-H (δ_H 2.80), and 14-CH_3→12-H (δ_H 2.80), 16-H (δ_H 2.43)). These results established the stereochemistries of the double bonds in stipiamide as (2*E*,4*E*,6*Z*,8*E*,10*E*,14*E*). This structure was supported by the upfield ^{13}C chemical shifts of the three allylic methyl carbons (2-CH_3 (δ_C 13.7), 10-CH_3 (δ_C 13.5), 14-CH_3 (δ_C 13.0)) (γ-effect)[6].

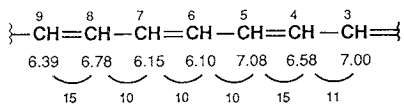
The structure of stipiamide thus established is similar to that of myxalamide B[7], an alaninol amide with a highly unsaturated branched fatty acid. The chemical shifts of C-1′ to C-2′ and C-1 to C-15 agreed very well with those of the corresponding carbons

Fig. 1. Partial structures of stipiamide as revealed by DQF-COSY.

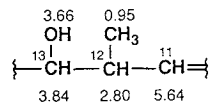
Values show ^{1}H-chemical shifts in ppm. Curves indicate coupling constants in Hz.



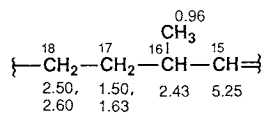
(I)



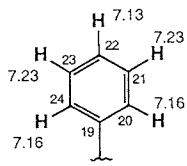
(II)



(III)



(IV)



(V)

Fig. 2. Partial structures of stipiamide as revealed by HMBC.

Values and those in parentheses show ^{13}C- and ^{1}H-chemical shifts in ppm, respectively. Arrows mean long range ^{13}C-^{1}H couplings.

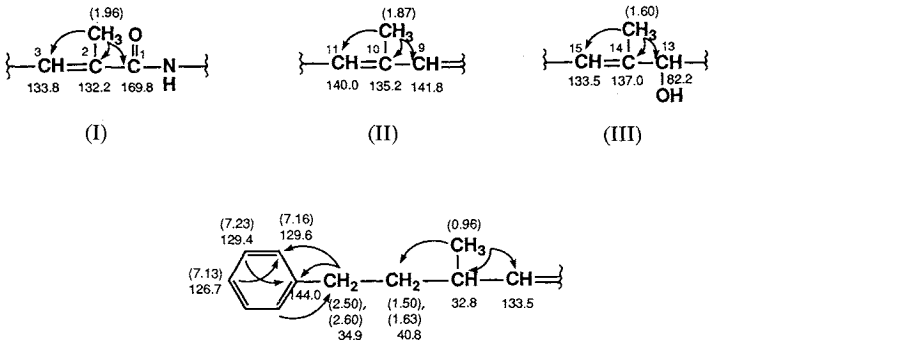
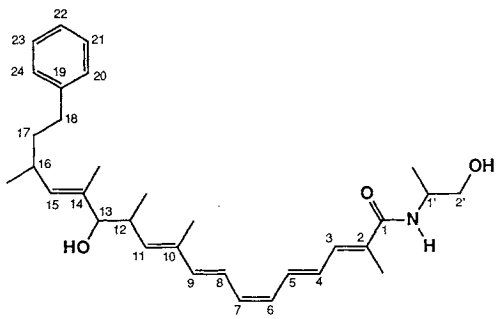


Fig. 3. Structure of stipiamide.



in myxalamide B. They differ from each other in that the terminal methyl group of the long chain in myxalamide B is replaced by a phenethyl group in stipiamide.

Stipiamide had no inhibitory effect on bacteria or yeast at a concentration of 1,000 μg/ml, while it showed weak activity against *Pyricularia oryzae* at the same concentration. Stipiamide inhibited the growth of KB cells and its colchicine-resistant mutant KB(CH^R) dose dependently in a colchicine free medium with IC_{50} being 1.51 μg/ml and 1.40 μg/ml, respectively. On the other hand, stipiamide showed stronger cytocidal activity against KB(CH^R) at IC_{50} of 0.35 μg/ml in a medium containing 1.5 μg/ml of colchicine. However, it showed slight effect to reduce the IC_{50} value against the same cell (from 1.40 to 0.8 μg/ml) in the presence of 5 μg/ml of doxorubicin. These experimental results may suggest that stipiamide possesses a strong synergistic effect to colchicine against KB(CH^R) cell but does not affect MDR. Further studies will be required to reveal the action mechanism of stipiamide.

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