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# (-)-PHENYLAHISTIN: A NEW MAMMALIAN CELL CYCLE INHIBITOR PRODUCED BY *ASPERGILLUS USTUS*

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**Abstract :** (-)-Phenylahistin is a fungal diketopiperazine metabolite consisting of L-phenylalanine and isoprenylated dehydrohistidine, and it showed an inhibitory activity on the cell cycle progression of P388 cells in the G2/M phase. © 1997 Elsevier Science Ltd.

Novel and specific cell cycle inhibitors should be useful tools for the investigation of cell cycle mechanisms and could be candidates for cancer chemotherapy[1]. During the course of screening for new cell cycle inhibitors, NSCL-96F037[2] was found in the culture broths of *Aspergillus ustus* NSC-F038 by Fukumoto *et al.*[2], but its structure and detailed biological activity were not clarified. In this study, we determined the structure of NSCL-96F037, and termed it phenylahistin. We also elucidated that phenylahistin was a mixture of enantiomers (*R*:*S* = 3:1), and only the (-)-(*S*)-enantiomer (Fig. 1) had the cytotoxic and cell cycle inhibitory activities.

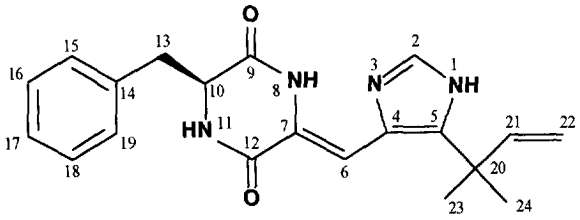


Figure 1. Structure of (-)-Phenylahistin

The production of phenylahistin by *A. ustus* NSC-F038 was closely related to the conidia formation of this fungus. Therefore, it was cultured on agar medium[3], which was suitable for the conidia formation at 28 °C for 8 days. The cultured agar media (200 plates; 4L) were then extracted with ethyl acetate. The extract was initially chromatographed on a silica gel column using an ethyl acetate-acetone stepwise gradient, followed by a second silica gel column and eluted with 2% methanol in chloroform. The fractions that exhibited cell cycle inhibitory activity were collected and evaporated, then dissolved in ethyl acetate and left two days at room temperature. The active substance was precipitated to yield 330 mg of a white amorphous powder[4].

Phenylahistin had the molecular formula, $C_{20}H_{22}N_4O_2$, which was determined by HR-FAB-MS (*m/z* found: 350.1741 ($M^+$), calcd. for $C_{20}H_{22}N_4O_2$: 350.1743). In the IR spectrum, the absorption at 3440 $cm^{-1}$

Table $^{13}C$ and $^{1}H$ NMR Assignment of Phenylahistin in $CDCl_3$

| Positions | $\delta$ C * | $\delta$ H ** |
|---|---|---|
| 1 (NH) | —— | 9.48 (1H, br s) |
| 2 | 132.56 d | 7.55 (1H, s) |
| 4 | 132.18 s | —— |
| 5 | 136.83 s | —— |
| 6 | 105.62 d | 6.88 (1H, s) |
| 7 | 123.64 s | —— |
| 8 (NH) | —— | 12.08 (1H, br s) |
| 9 | 164.73 s | —— |
| 10 | 57.14 d | 4.35 (1H, ddd, *J*=10, 4, 3# Hz) |
| 11 (NH) | —— | 5.82 (1H, br s) |
| 12 | 159.94 s | —— |
| 13 | 41.23 t | 2.95 (1H, dd, *J*=14, 10 Hz) |
| | | 3.49 (1H, dd, *J*=14, 4 Hz) |
| 14 | 135.45 s | —— |
| 15, 19 | 129.52 d | 7.25 (2H, d, *J*=7 Hz) |
| 16, 18 | 129.07 d | 7.33 (2H, t, *J*=7 Hz) |
| 17 | 127.45 d | 7.27 (1H, t, *J*=7 Hz) |
| 20 | 37.61 s | —— |
| 21 | 144.66 d | 6.02 (1H, dd, *J*=18, 11 Hz) |
| 22 | 113.29 t | 5.13 (1H, d, *J*=18 Hz) |
| | | 5.17 (1H, d, *J*=11 Hz) |
| 23, 24 | 27.97 q | 1.49 (6H, s) |

* 125 MHz, including multiplicity assignment on the basis of DEPT summary. Chemical shifts in ppm from $CDCl_3$ as an internal standard (77.00 ppm).

** 500 MHz, Chemical shifts in ppm from TMS as an internal standard (0.00 ppm).

# Coupling with 11-H was observed by decoupling experiment.

corresponds to the N-H group, and strong absorptions at 1670 and 1640 $cm^{-1}$ indicated the existence of amide groups. These findings together with the absence of the amide II band near 1550 $cm^{-1}$ in the spectrum suggested the presence of the diketopiperazine system in phenylahistin[5], which was also supported by the negative Ninhydrin reaction.

The $^{13}C$-NMR spectrum of phenylahistin (Table), which showed 17 resolved peaks with three overlapping carbon signals. The multiplicity of these peaks was determined by analysis of its DEPT spectra. The $^{1}H$-NMR spectrum displayed 22 proton signals (table) including three exchangeable protons. All bond connections between proton and carbon were interpreted by $^{1}H$ -$^{13}C$ COSY.

The $^{1}H$ NMR and $^{1}H$ -$^{1}H$ COSY spectra revealed only the presence of four partial structures (thick-linked parts in Fig. 2-**A**, **B**); a monosubstituted benzene ring, methylene protons being coupled to a methine proton, which was also coupled to an exchangeable proton, one geminal methyl group and a vinyl group. Since quaternary carbons prevented us from constructing further partial structures, we then measured the PFG-

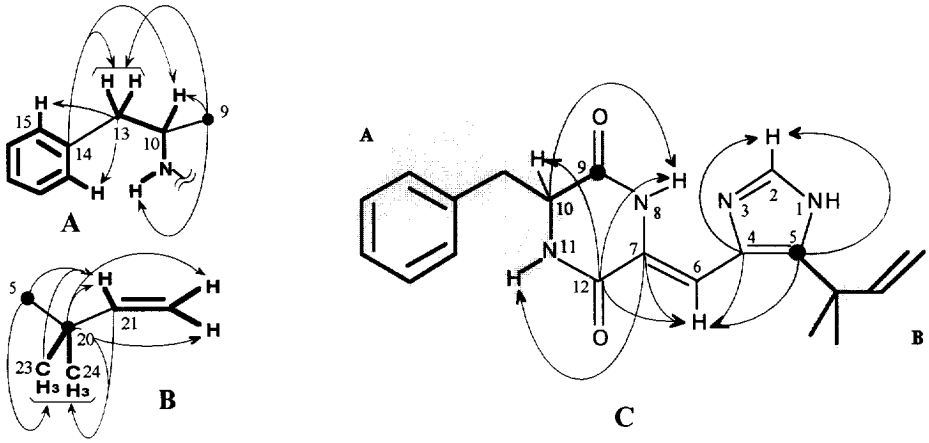


Figure 2. Partial Structurs (**A**, **B**) and Planar Structure (**C**) of Phenylahistin Determined from PFG-HMBC Spectrum

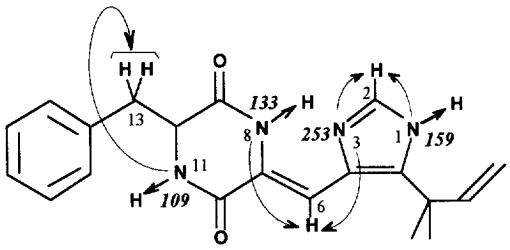

Figure 3. Results of PFG-$^{15}$N-HMBC Experiments

Italic numbers reveal $^{15}$N chemical shift obtained from the PFG-$^{15}$N-HMBC spectrum in ppm from formamide as an internal standard (112.4 ppm).

HMBC spectrum. Partial structure **A** (shown in Fig. 2) was determined on the basis of the long-range correlation signals of C-9, C-13 and C-14, which observed in the spectrum. The carbon chemical shift of C-9 ($\delta$ 164.73) indicated that it must be a carbonyl carbon. Therefore, **A** must be a phenylalanine residue. In the same way, partial structure **B** (Fig. 2) was determined from the correlation signals of C-5, C-20, C-21, C-23 and C-24, which showed an isoprenyl group binding to quaternary $sp^2$ carbon ($\delta$ 136.83). The diketopiperazine ring was also constructed from the interpretation of correlations between C-7 and 11-H, C-10 and 8-H, C-12 and 8-H and C-12 and 10-H in the PFG-HMBC spectrum (see Fig. 2-**C**). The dehydrohistidine moiety was estimated from remaining three carbons, two nitrogens and three hydrogens, and this structure was supported by correlation signals of C-4, C-5, C-7 and C-12 (see in Fig. 2-**C**). The correlation signal between C-5 and H-6 indicated that the isoprenyl group was placed in the C-5 of the imidazole ring. This structure was confirmed by analysis of the PFG-$^{15}$N-HMBC spectrum, which enabled connections between nitrogens and protons within three bonds, and the four nitrogens were assigned as shown in Fig. 3.

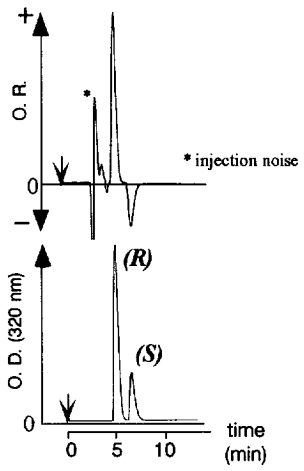


Figure 4. Resolution of Enantiomers of Phenylahistin

From these data, the planar structure of phenylahistin was established. The stereochemistry of the C-6 - C-7 double bond was suggested to be *Z* by the low field shift of 8-H (NH: $\delta$ 12.08), which was explained by hydrogen bonding between this proton and N-3 of the imidazole ring, as in the case of aurantiamine[6].

Phenylahistin has a chiral center at the C-10 position. Chiral HPLC[7] analysis of the phenylalanine [8], which was obtained from the acidic hydrolysate of the amorphous white powder of phenylahistin, indicated that the phenylahistin was a mixture of enantiomers (*R*:*S* = 3:1). In order to examine the biological activity of each enantiomer, chiral resolution was carried out by chiral HPLC[9] (Fig. 4).

The cytotoxic effects of enantiomers on P388 murine leukemia cells were examined (Fig. 5). IC50 values of (-)-phenylahistin (*S*-configuration) (92.2% e.e.) and (+)-phenylahistin (*R*-configuration) (97.8% e.e.) were 3.5 x $10^{-7}$ M and 3.8 x $10^{-5}$ M, respectively. Judging from the content of (-)-phenylahistin in (+)-phenylahistin and the mixture of enantiomers (*R*:*S* = 3:1), (+)-phenylahistin was considered to have no or a very low cytotoxicity to P388 cells.

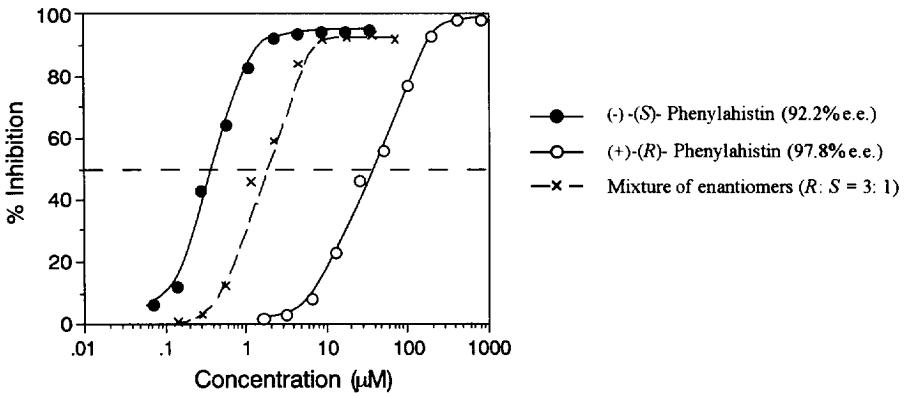


Figure 5. Cytotoxic Effects of Enantiomers of Phenylahistin on P388 Cells

P388 cells (5 x $10^3$ cells/100 μl) were seeded to 96-well plates, and cultured for 14 hours. Various concentrations of samples were added to each well. After a 40-hour incubation, cell numbers were measured by AlamarBlue® assay.

The effect of phenylahistin on the cell cycle progression of P388 cells was investigated using a flow cytometer[10]. P388 cells in the log growth phase were seeded into flasks and cultured for 14 hours, and then various concentrations of phenylahistin were added to each flask. After an 8-hour incubation, the cells were harvested and fixed with 50% MeOH at -20 °C overnight. The cells were washed with 30% MeOH and then with 10 mM PBS, and treated with 50 μg/ml propidium iodide at 4 °C for 2 hours. DNA histograms were obtained using a flow cytometer (Cyto ACE-300: JASCO).

(-)-Phenylahistin exhibited cell cycle inhibitory activity at 1 x $10^{-6}$ M (Fig. 6), but (+)- phenylahistin (97.8% e.e.) had no effect at 1 x $10^{-5}$ M (data not shown). Therefore, the active configuration of phenylahistin was determined to be *S*, and it inhibited the cell cycle progression in the G2/M phase.

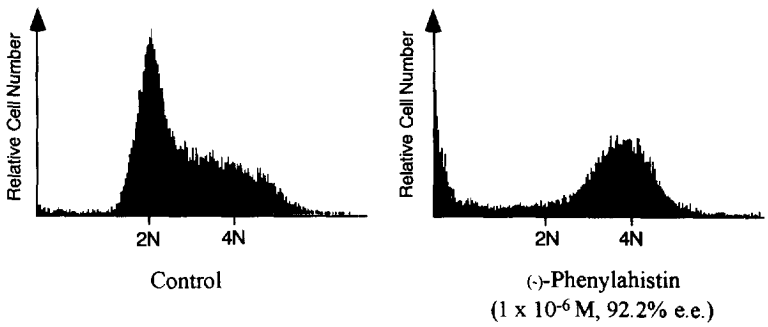


Figure 6. Effect of (-)-Phenylahistin on Cell Cycle Progression of P388 Cells

Recently, Cui *et al.* have reported that tryprostatins A and B together with their related compounds[11-12] inhibited cell cycle progression in M phase similar to (-)-phenylahistin. Tryprostatins are diketopiperazines consisting of proline and isoprenylated tryptophan residues. Because of their structural similarities, the inhibitory mechanism of (-)-phenylahistin and that of tryprostatins are probably the same. Moreover, a diketopiperazine system and an isoprenylated aromatic moiety should be important for the cell cycle inhibitory activity.

It is noteworthy that phenylahistin was produced as a scalemic mixture, and only the (-)-enantiomer showed biological activities. The stereochemical structures and activity relationships of diketopiperazines should be an important field for study in the future. And, studies on the further detailed biological activity, especially antitumor activity, and the mechanisms of the action of phenylahistin are now in progress.

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## References and Notes

1) Roberge, M.; Tudan, C.; Hung, S. M. F.; Harder, K.W.; Jirik, F. R.; Anderson, H. *Cancer Res.* **1994**, *54*, 6115.

2) Fukumoto, K.; Asari, T.; Harada, T. *Japanese Patent P409188749*, September 4, **1996** (Japanese).
*A. ustus* NSC-F038 was deposited at the National Institute of Biosciences and Human-Technology, Japan, as FERM P-15830.

3) The agar medium contained glucose (0.5%), glycerol (2%), yeast extract (0.2%), Pharmamedia® (Traders Protein) (2%), NaCl (0.25%) and agar (1.5%), adjusted to pH 6.5 before sterilization.

4) M.p. 233- 236 °C; $[\alpha]_D^{22}$ +123 ° (*c* = 0.13, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 202 (4.38), 233 sh (4.05), 320 (4.43); IR (KBr pellet) $\nu_{max}$: 3440, 3240, 1670, 1640, 1440 $cm^{-1}$; HR-FAB-MS (*m/z*) 350.1741 ($M^+$, calcd. for $C_{20}H_{22}N_4O_2$: 350.1743)

5) Steyn, P. S. *Tetrahedron* **1973**, *29*, 107.

6) Larsen, T. O.; Frisvad, J. C.; Jensen, S. R. *Phytochemistry* **1992**, *31*, 1613.

7) HPLC conditions: column; Crownpak CR(+) ϕ4.0 x 150 mm (Daicel Chemical Industries, Ltd.), mobile phase; $H_2O$ (pH 2, adjusted with perchloric acid), flow rate; 0.8 ml/min, detector; UV λ 200 nm.

8) Yamazaki, M.; Fujimoto, H.; Akiyama, T.; Sankawa, U.; Iitaka, Y. *Tetrahedron Lett.* **1975**, 27.
The phenylalanine obtained from the hydrolysate of phenylahistin was identified using an amino acid analyzer and by measurement of EI-MS [ $(m+H)^+$ : 166.2 ].

9) HPLC conditions: column; Chiracel OD ϕ4.6 x 250 mm (Daicel Chemical Industries, LTD), mobile phase; *n*-hexane/ ethanol = 75/ 25, flow rate; 1.0 ml/min, temperature; 25 °C. Absolute configuration was determined by analysis of phenylalanine obtained from (+)-phenylahistin using chiral HPLC.

10) Krishan, A. *J. Cell Biol.* **1975**, *66*, 188.

11) Cui, C.-B.; Kakeya, H.; Okada, G.; Onose, R.; Osada, H. *J. Antibiotics* **1996**, *49*, 527.

12) Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiotics* **1996**, *49*, 534.

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