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THE NICOTINIC ACID-*p*-AMINOPHENYLALANINE-HYDROXY-BENZOIC ACID TRIADS INDUCE APOPTOSIS IN HUMAN LEUKEMIA U937 CELLS

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Abstract – The nicotinic acid-*p*-aminophenylalanine-hydroxybenzoic acid triads were newly synthesized from *p*-aminophenylalanine methyl ester *via* 4 steps, and they induced apoptosis in human leukemia U937 cells. The number of hydroxyl group on benzoic acid apparently affected upon the apoptosis-inducing activity.

Apoptosis is characterized by a series of distinct morphological changes including cell shrinkage, chromatin condensation, DNA fragmentation, and cellular fragmentation into apoptosis-bodies, and it is essential for the maintenance of tissue homeostasis and the elimination of unwanted or damaged cells.¹ The compound which can specifically induces apoptosis in tumor cells is considered to be a potent candidate for anticancer drug. Numerous natural products such as apoptolidin,² stilbenoids,³ and (-)-epigallocatechin-3-gallate (EGCG)⁴ are known to induce apoptosis in cancer cells. In contrast only limited papers concerning synthetic heterocyclic compounds with the apoptosis-inducing activity have been reported.^{5,6}

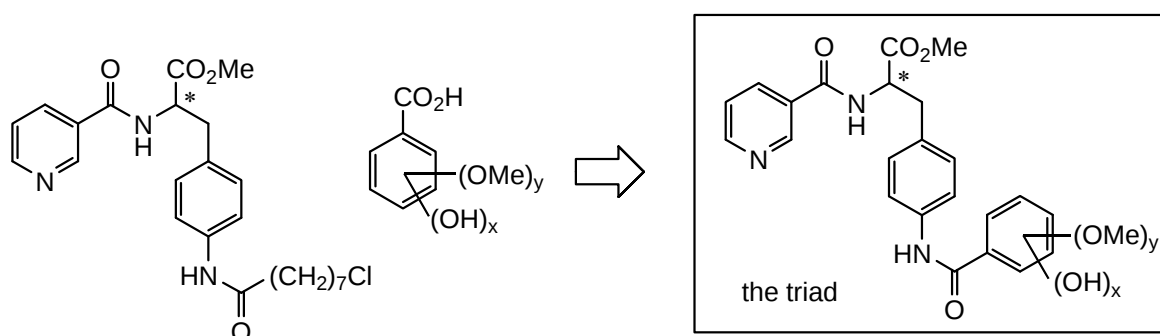
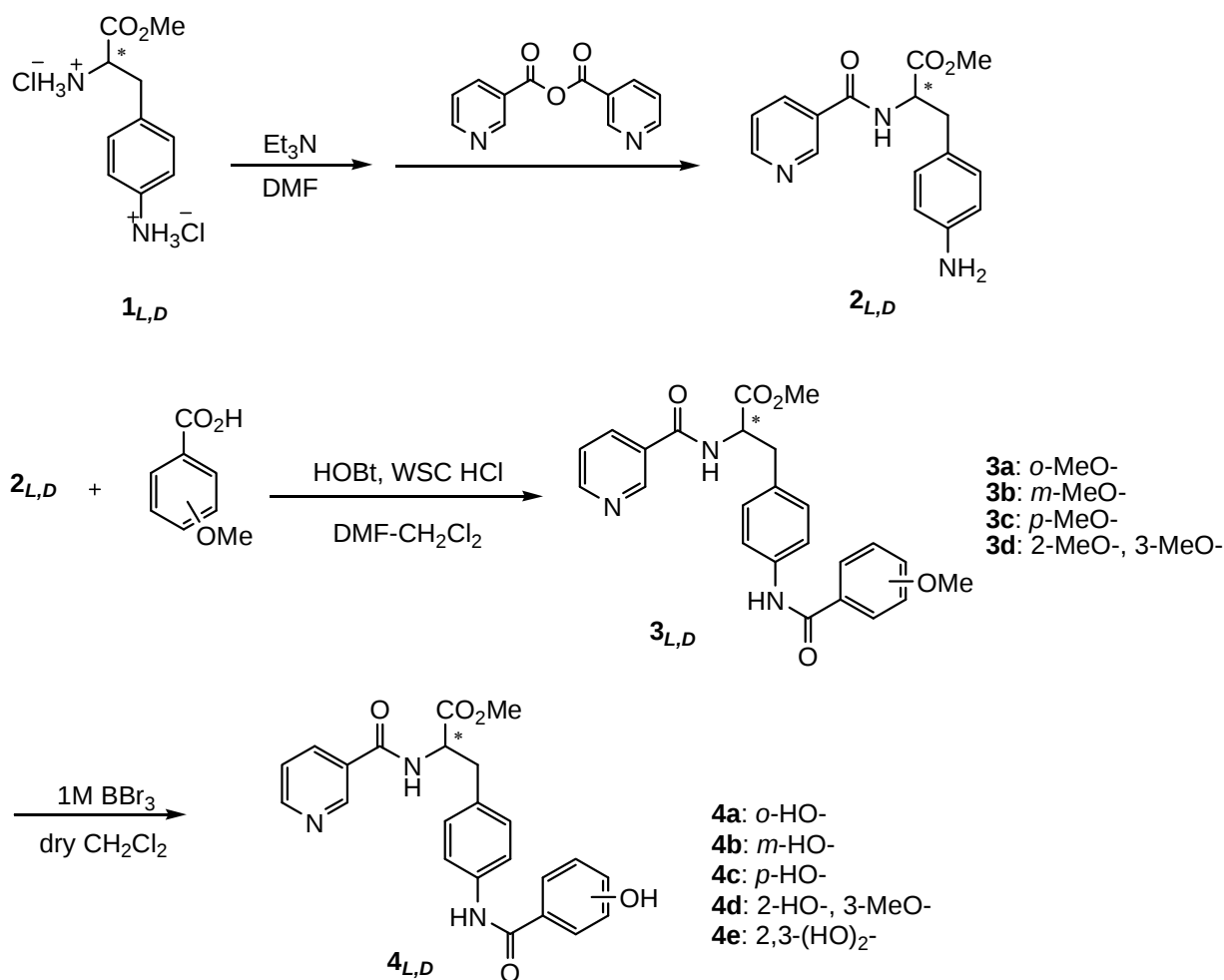


Figure 1

We have already prepared more than a thousand heterocyclic compounds containing mono- and diazines especially over two decades. Among them, 35 representative samples were selected and preliminarily tested the growth inhibitory effect on human leukemia U937. A compound which composed of nicotinic acid and *p*-aminophenylalanine as shown in Figure 1 showed the antiproliferation activity. On the other hand, phenol and polyphenol functional groups have been often seen in natural products that showed potent apoptosis activities. As intensive studies on the application of mono- and diazine-type heterocycles as chemotherapeutic agents for various diseases,⁷ we designed the triad as shown in Figure 1 and planned to evaluate its apoptosis-inducing activity with special attention to the effects of the absolute configuration and the number of the hydroxyl groups on benzoic acid on the activity.



Scheme 1

N-Nicotinyl-*p*-aminophenylalanine methyl esters (**2_L**: 43%; [α]_D²⁶ -49.0° (*c*=0.1 in MeOH), **2_D**: 67%) were synthesized from *p*-amino-L- or D-phenylalanine methyl ester dihydrochloride (**1**) and nicotinic anhydride.⁸ A typical procedure for synthesis of the triads is as follows. To a mixture of **2_D** (300 mg,

1.0 mmol), 2,3-dimethoxybenzoic acid (200 mg, 1.1 mmol), and 1-hydroxybenzotriazole (HOBt) (200 mg, 1.2 mmol) in dry DMF (7 mL) was added dropwise a solution of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (WSC·HCl) (152 mg, 1.2 mmol) in dry CH₂Cl₂ (7 mL) at 0 °C, and then the reaction mixture was stirred for 4 days at room temperature. The crude product was purified by column chromatography on silica gel with CHCl₃:acetone:EtOH (100:40:8) mixture as an eluant to give *N*-nicotinyl-*p*-(2,3-dimethoxybenzoyl)amino-*D*-phenylalanine methyl ester (**3d**; 84%) as an orange oil. A solution of 1M BBr₃ in dry CH₂Cl₂ (1.2 mL) was added dropwise to a solution of **3d** (245 mg, 0.53 mmol) in dry CH₂Cl₂ (10 mL) at -30 °C, and then the reaction mixture was stirred for 2 days at room temperature. The crude product was purified by column chromatography on silica gel with CHCl₃:MeOH (6:1) mixture to give the final product (**4de**; 14%)⁹. (Scheme 1)

At first, the growth inhibitory effect of synthesized compounds on human leukemia U937 was examined. U937 was grown in RPMI-1640 medium containing 5% penicillin-streptomycin (P/S) and 10% fetal bovine serum (FBS) under an atmosphere of 95% air and 5% CO₂ at 37 °C. The number of viable cells was determined by staining with trypan blue.¹⁰

A solution of synthesized compound in DMSO (10 mM, 40 µL) was added to the cell's suspension (0.8 × 10⁵ cells/mL, 5 mL), and the mixture was incubated for 48 h at 37 °C under an atmosphere of 95% air and 5% CO₂. Figure 2 showed the viabilities (%) of U937 cells treated by the synthesized triads together with those for DMSO and EGCG as a reference.

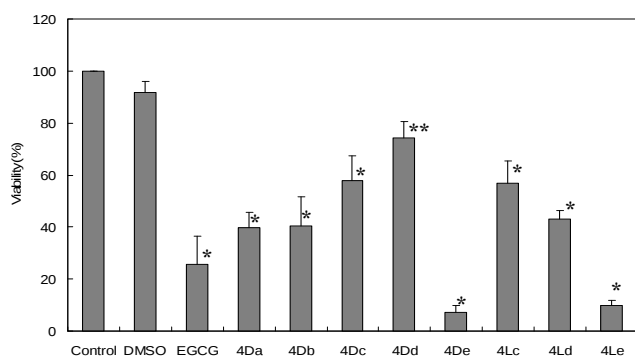


Figure 2. The viability (%) of U937 treated by nicotinic acid derivatives. Significance **P*<0.001 vs. control, ***P*<0.005 vs. control of difference between indicated data using a dunnett's test. Each value is expressed as the mean ± SD for 5 experiments.

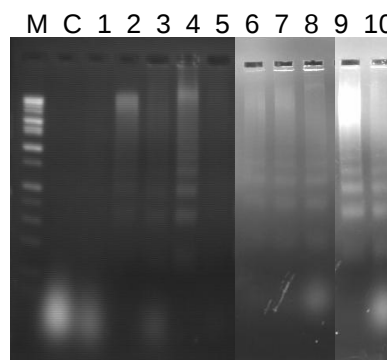


Figure 3. DNA fragmentation induced in U937 cells by synthetic triads. M: DNA marker, C: control, 1: DMSO, 2: EGCG, 3: **4Lc**, 4: **4Le**, 5: **4Ld**, 6: **4Da**, 7: **4Db**, 8: **4Dc**, 9: **4Dd**, and 10: **4De**. DNA fragmentation was analyzed by agarose gel electrophoresis and visualized with ethidium bromide staining under UV light.

These experiments revealed the following points: (1) the difference in the absolute configuration of *p*-aminophenylalanine residue hardly affected the viability, (2) 2,3-dihydroxybenzoyl derivatives **4Le**

and **4_{De}** showed higher inhibitory effect upon proliferation than natural EGCG, (3) monohydroxybenzoyl derivatives had lower activities than EGCG, and their activities were in the order of *ortho*, *meta* > *para*, and (4) the viability(%) of the dihydroxybenzoyl derivatives **4_{De}** and **4_{Le}** was significantly lower than that of monohydroxybenzoyl ones **4_{Dd}** and **4_{Ld}**, indicating that the number of the hydroxyl group on the benzene ring plays an important role in the inhibition of the proliferation of U937 cells.

Next, whether cell death was caused by apoptosis was examined. Nucleosomal DNA ladders which result from the internucleosomal degradation can be observed in agarose gel electrophoresis as a typical feature of apoptosis. To examine the induction of apoptosis in U937 cells by the nicotinic acid-*p*-aminophenylalanine-hydroxybenzoic acid triads and EGCG, the cells were treated with 80 μ M of them for 48 h. A nucleosomal DNA ladder formation was observed (Figure 3), suggesting that the apoptosis was induced by treatment with the synthesized triads.

Finally, the effect of the synthesized triads was found to be dose-dependent in the concentration range from 8 to 80 μ M. Values of IC₅₀ which is the 50% U937 growth inhibitory concentration for each compound, were estimated, and the results are summarized in Table 1, together with IC₅₀ value for EGCG as a reference. The IC₅₀ value (30 μ M) for EGCG is comparable to the values for other cancer cells.^{4a, 4c} It also can be seen that **4_{Le}** and **4_{De}** strongly inhibited proliferation of U937 cells, the activities being higher than that for natural EGCG. Further, the IC₅₀ values for **4_{Le}** and **4_{De}** on U937 cells were lower than those for synthetic α,β -unsaturated amides.^{5a} Again, the significant difference in the absolute configuration in terms of IC₅₀ value could not be observed.

Further investigation of the apoptosis-inducing activity toward other kinds of cancer and non-cancerous cells treated by the nicotinic acid-*p*-aminophenylalanine-hydroxybenzoic acid triads are currently under way. On the basis of the present results, the synthetic triad is expected to become a new candidate for the anticancer drug.

Table 1 Estimated IC₅₀ values for growth of U937 cells

Compound	IC ₅₀ value (μ M)
EGCG	30.36 $\pm$ 1.97
4_{Le}	20.69 $\pm$ 2.45*
4_{De}	15.15 $\pm$ 1.69**

Cells were treated with compounds for 48 h. Each value is expressed as the mean $\pm$ SD for 5 experiments. **P*<0.05 vs. EGCG. ***P*<0.01 vs. EGCG.

REFERENCES AND NOTES

1. a) J. F. Kerr, H. W. Andrew, and A. R. Currie, *Br. J. Cancer*, 1972, **26**, 239. b) A. H. Wyllie, J. F. Kerr, and A. R. Currie, *Int. Rev. Cytol.*, 1980, **68**, 251. c) M. J. Arends and A. H. Wyllie, *Int. Rev.*

- Exp. Pathol.*, 1991, **32**, 223. d) N. B. Blatt and G. D. Glick, *Bioorg. Med. Chem. Lett.*, 2001, **9**, 1371. e) Z. Huang, *Chem. Biol.*, 2002, **9**, 1059. f) D. D. Newmeyer and S. Ferguson-Miller, *Cell*, 2003, **112**, 481.
2. P.T. Daniel, U. Koert, and J. Schuppan, *Angew. Chem. Int. Ed.*, 2006, **45**, 872.
 3. I. Iliya, Y. Akao, K. Matsumoto, Y. Nakagawa, A. Zulfiqar, T. Ito, M. Oyama, H. Murata, T. Tanaka, Y. Nozawa, and M. Iinuma, *Biol. Pharm. Bull.*, 2006, **29**, 1490.
 4. a) Z. P. Chen, J. B. Schell, C. Ho, and K. Y. Chen, *Cancer Lett.*, 1998, **131**, 173. b) M. Roy, S. Chakrabarty, D. Sinha, R. K. Bhattacharya, and M. Siddiqi, *Mutation Res.*, 2003, **523-524**, 33. c) N. Horie, N. Hirabayashi, Y. Takahashi, Y. Miyauchi, H. Taguchi, and K. Takeishi, *Biol. Pharm. Bull.*, 2005, **24**, 574.
 5. a) V. Nesterenko, K. S. Putt, and P. J. Hergenrother, *J. Am. Chem. Soc.*, 2003, **125**, 14672. b) R. S. Dothager, K. S. Putt, B. J. Allen, B. J. Leslie, V. Nesterenko, and P. J. Hergenrother, *J. Am. Chem. Soc.*, 2005, **127**, 8686.
 6. S. Hanessian, L. Zhan, R. Bovey, O. M. Saavedra, and L. Juillerat-Jeanneret, *J. Med. Chem.*, 2003, **46**, 3600.
 7. a) J. Ohkanda and A. Katoh, *Rev. on Heteroatom Chem.*, 1998, **18**, 87. b) H. Sakurai, A. Katoh, and Y. Yoshikawa, *Bull. Chem. Soc. Jpn.*, 2006, **79**, 1645.
 8. a) A. Katoh, S. Naruse, J. Ohkanda, and H. Yamamoto, *Heterocycles*, 1997, **45**, 1441. b) R. Saito, S. Naruse, K. Takano, K. Fukuda, A. Katoh, and Y. Inoue, *Org. Lett.*, 2006, **8**, 2067.
 9. **4Le** (18%): IR (KBr): 3447 (ν N-H), 3076 (ν C-H), 2952 (ν C-H), 2861 (ν C-H), 1736 (ν C=O), 1654 (ν C=O), 1550 (δ N-H), 1429 (δ C-H), 1362, 1259 (δ O-H), 1194, 1042 (ν C-O), 833, 785, 700 cm^{-1} (γ C-H); $^1\text{H-NMR}$ (δ , CDCl_3 , 400 MHz): 3.21-3.36 (m, 2H, CH_2), 3.81 (s, 3H, CO_2Me), 5.10 (q, 1H, $J = 6.3$ Hz, CH), 6.67 (d, 1H, $J = 7.4$ Hz, NH), 6.83 (t, 1H, Ar-5-H), 7.06 (dd, 1H, $J = 1.2$ and 8.1 Hz, Ar-4-H), 7.10 (dd, 1H, $J = 1.2$ and 8.1 Hz, Ar-6-H), 7.17 (d, 2H, $J = 8.4$ Hz, Ar-2,6-H), 7.40 (t, 1H, $J = 6.4$ Hz, Py-5), 7.53 (d, 2H, $J = 8.4$ Hz, Ar-3,5), 8.02 (s, 1H, Ar-3-OH), 8.08 (dt, 1H, $J = 1.6$ and 6.4 Hz, Py-4-H), 8.75 (dd, 1H, $J = 1.6$ and 6.4 Hz, Py-6-H), 8.93 (d, 1H, $J = 1.6$ Hz, Py-2-H), and 12.26 ppm (s, 1H, Ar-2-OH); Anal. Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_3\text{O}_6$: C, 64.13; H, 5.16; N, 9.35%. Found: C, 64.03; H, 5.06; N, 9.18%; **4De** (14%): IR (KBr): 3447 (ν N-H), 3076 (ν C-H), 2952 (ν C-H), 2861 (ν C-H), 1736 (ν C=O), 1654 (ν C=O), 1550 (δ N-H), 1429 (δ C-H), 1362, 1259 (δ O-H), 1194, 1042 (ν C-O), 833, 785, 700 cm^{-1} (γ C-H); $^1\text{H-NMR}$ (δ , CDCl_3 , 400 MHz): 3.22-3.36 (m, 2H, CH_2), 3.82 (s, 3H, CO_2Me), 5.10 (q, 1H, $J = 7.0$ Hz, CH), 6.65 (d, 1H, $J = 7.0$ Hz, NH), 6.84 (t, 1H, Ar-5-H), 7.05 (dd, 1H, $J = 1.0$ and 8.1 Hz, Ar-4-H), 7.11 (dd, 1H, $J = 1.0$ and 8.1 Hz, Ar-6-H), 7.17 (d, 2H, $J = 6.9$ Hz, Ar-2,6-H), 7.40 (t, 1H, $J = 6.3$ Hz, Py-5), 7.54 (d, 2H, $J = 6.9$ Hz, Ar-3,5), 7.98 (s, 1H, Ar-3-OH), 8.07 (dt, 1H, $J = 1.9$ and 6.3 Hz, Py-4-H), 8.75 (dd, 1H, $J = 1.9$ and 6.3 Hz, Py-6-H), and

8.93 (d, 1H, $J = 1.9$ Hz, Py-2-H), 12.27 ppm (s, 1H, Ar-2-OH); Anal. Calcd for $C_{23}H_{21}N_3O_6 \cdot 0.8H_2O$: C, 61.41; H, 5.06; N, 9.34. Found: C, 61.56; H, 5.11; N, 9.32.

10. M. Ujibe, S. Kanno, Y. Osanai, K. Koiwai, T. Ohtake, K. Kimura, K. Uwai, M. Takeshita, and M. Ishikawa, *Biol. Pharm. Bull.*, 2005, **28**, 2338.