[Chem. Pharm. Bull. 24(9)2248—2250(1976)]

UDC 547.963.32.09 : 615.282.84.011.5.076.7

Synthesis and Cytotoxicity of Bredinin 5′-Monophosphate

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(Received November 29, 1975)

Bredinin 5′-monophosphate was synthesized. It lost the anticandida activity but the cytotoxicity was enhanced approximately twofold in L5178Y cells. The effect of the nucleotide on life prolongation in mice inoculated with L 1210 was much the same as that of bredinin.

Bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate), a novel imidazole nucleoside produced by the fungus *Eupenicillium brefeldianum*, has been shown to inhibit the growth of *Candida albicans* and the growth of various cells in tissue culture. It exhibits a potent immunosuppressive activity.[2]

5′-Phosphate derivatives of various nucleoside antibiotics such as cordycepin, tubercidin, sangivamycin, formycin, or pyrazomycin have been reported to have interesting biological activities.[3] These findings led us to synthesis bredinin 5′-monophosphate. In this paper we present the synthesis and the cytotoxicity of bredinin 5′-monophosphate.

Materials and Methods

Bredinin was obtained from the culture filtrate of *Eupc. brefeldianum* by a published procedure.[2a] All chemicals used were commercially available analytical reagent grades.

Escherichia coli alkaline phosphatase (510 U/mg) was a gift from Dr. Y. Horiuchi, Toyo Jozo Co., Ltd. Snake venom (5′-nucleotidase fraction from *Agkistrodon acutus*) was supplied through the courtesy of Prof. H. Sugihara, Faculty of Pharmacy, Meijo University. Bredinin was phosphorylated according to the procedure of Streeter, *et al.*[4]

A solution of phosphorus oxychloride (6.0 ml, 65.2 mmole) in trimethylphosphate (120 ml) was cooled to 0° and bredinin monohydrate (5.55 g, 20 mmole) was added with stirring. The reaction mixture was protected from moisture and was stirred at 0° for 5 hr. After reaction was complete, the solution was poured into ice water (200 ml) and was extracted twice with chloroform (300 ml) to remove trimethylphosphate. The aqueous solution was neutralized with 5 N NaOH and concentrated. The resulting salt was filtered out. The filtrate was applied on a column of Dowex 1×8 (Cl type, 200—400 mesh, 500 ml), and the column was washed with water and then eluted with 0.075 N HCl. The eluates were monitored by ultraviolet (UV) absorption at 280 nm and $FeCl_3$ reaction.

Thin-layer chromatography (TLC) was made by using cellulose plate (Merck, Art 5716) with the solvent systems of I; butanol–acetic acid–water, 3: 1: 1, and II; methanol–10% NH_4Cl–acetic acid, 8: 2: 1. Qualitative analysis for phosphate ester was done with the color test described by Hanes-Isherwood.[5]

Anticandida and cytotoxic activities were assayed by the same procedures described in previous papers.[2a,b] BDF1 mouse was used in anti-L 1210 test.

Results and Discussion

In the eluates of Dowex 1×8 chromatography, five major peaks giving UV absorption were observed and designated as BP-I, II, III, IV, and V respectively in the elution order.

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2) *a*) K. Mizuno, M. Tsujino, M. Takada, M. Hayashi, K. Atsumi, K. Asano, and T. Matsuda, *J. Antibiotics*, 27, 775 (1974); *b*) K. Sakaguchi, M. Tsujino, M. Yoshizawa, K. Mizuno, and K. Hayano, *Cancer Res.*, 35, 1463 (1975).
3) R.J. Suhadolnik, "Nucleoside Antibiotics," Wiley-Interscience, Inc., New York, 1970.
4) D.G. Streeter, J.T. Witkowski, G.P. Khare, R.W. Sidwell, R.J. Bauer, R.K. Robins, and L.N. Simon, *Proc. Natl. Acad. Sci. U.S.*, 70, 1174 (1973).
5) C.S. Hanes and F.A. Isherwood, *Nature*, 164, 1107 (1949).

Each peak was neutralized and evaporated. The powder was put through desalting by gel filtration with Sephadex G-15 and then lyophilized. Some properties of the BP samples obtained are shown in Table I.

Table I. Some Properties of Phosphorylation Products

Products	Yield (mg)	UV_{max} (nm) in 0.1N HCl	UV_{max} (nm) in 0.1N NaOH	Phosphate ester[a]	Anticandida activity I[b]	Anticandida activity II[c]
BP-I	139	229, 264	254	—	0	0
BP-II	571	235, 273	255	+	0	23.0
BP-III	600	234, 270	253	+	26.0	25.0
BP-IV	501	246, 280	278	+	0	38.5
BP-V	1900	239, 271	253	+	0	21.0
Bredinin (Control)	—	246, 280	278	—	41.5	39.0

a) The phosphate was detected by Hanes-Isherwood reagent.
b) The test solution (1000 μg/ml) was bio-assayed by the cup-diffusion procedure using *C. albicans*, and the values show inhibition-zone diameter (mm).
c) The assay was carried out after the reaction with *E. coli* alkaline phosphatase in following system; a mixture of 1.0 mg of the product, 1.0 mg of the enzyme, and 0.2 ml of 0.5M Tris-HCl buffer (pH 8.0) in a total volume of 1.0 ml was incubated at 37° for 60 min.

UV spectrum of BP-IV very much resembled that of bredinin but the other products exhibited different spectra suggesting structural change in the chromophore moiety of bredinin molecule. Anticandida activity of all phosphate derivatives except BP-III appeared after the treatment with alkaline phosphatase. Since BP-IV, among the products, could be most likely considered to be bredinin monophosphate, it was further purified by the gel filtration. The purified powder was treated with Dowex 50 W×2 (H type) column and the free nucleotide was obtained as colorless hygroscopic powder through lyophilization. The bredinin phosphate thus purified has no definite mp (darkening at 135°), and it exhibits similar UV absorption spectra to that of bredinin: λ_{max} nm ($E^{1\%}_{1cm}$), 245 (185) and 279 (400) in water, 245 (195) and 280 (384) in 0.1N HCl, 277 (450) in 0.1N NaOH. The elementary analysis afforded the empirical formula to be $C_9H_{14}O_9N_3P \cdot H_2O$ (Found: C, 30.50; H, 4.20; N, 12.03; P, 8.18. Calcd.: C, 30.27; H, 4.48; N, 11.76; P, 8.67). To determine the phosphate position in ribose moiety, Snake venom[6] was used as a specific tool. A mixture of 2 mg of the nucleotide in 1.0 ml of 0.2M glycine buffer (pH 8.0) containing 0.03M $MgSO_4$ and 0.5 ml of the enzyme solution was incubated at 37° for 60 min. The reaction mixture was directly applied on TLC. The chromatogram indicated that the nucleotide disappeared and was obviously converted to bredinin. In parallel runs, 5′-AMP was converted to adenosine but 2′(3′)-AMP and GMP were not hydrolyzed by the enzyme. Bredinin 5′-monophosphate thus obtained was

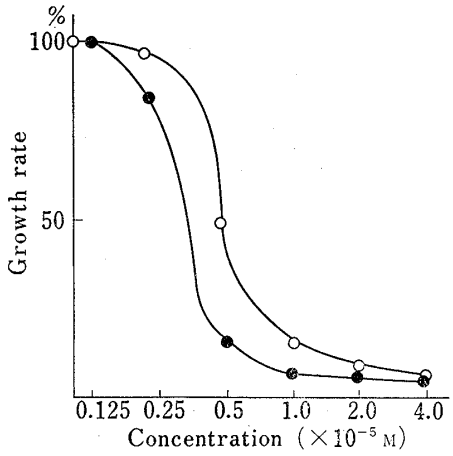

Fig. 1. Cytotoxicity of Bredinin 5′-Monophosphate and Bredinin in L5178Y Cells

Cells were incubated at 37° for 40 hr in medium (Fisher's medium supplemented 10% dialyzed bovine serum) containing various concentration of bredinin or the phosphate and then growth rate was calculated. Cell numbers were determined with a microcell counter (Toa Dempa Co., Tokyo).
—●—: bredinin 5′-monophosphate
—○—: bredinin

6) H. Sugihara, T. Nikai, M. Moriura, K. Kamiya, and T. Tanaka, *Nippon Saikingaku Zasshi*, 27, 47 (1972).

no longer active against *Candida albicans* even at the concentration of 1000 μg/ml, however, it showed about twofold stronger cytotoxicity in L5178Y cells than that of bredinin as shown in Fig. 1.

Although there is no apparent evidence, it is stated that nucleotides in general do not enter directly into cells, they can enter as nucleosides after their hydrolytic conversion by probably a phosphatase.[2b] However, the fact that bredinin 5′-monophosphate showed enhanced cytotoxicity suggested direct penetration by itself. If it were subject to hydrolysis, the cytotoxicity should be the same as that of bredinin.

Unlike the virazole 5′-monophosphate which has been reported as the active metabolite,[4] no phosphate derivatives of bredinin have been detected in L5178Y cells or a rat liver suggesting that bredinin is not phosphorylated in the cells or a rat, and it is also found that bredinin is not incorporated into nucleic acid of L5178Y cells.[7] The discrepancy between bredinin and its 5′-monophosphate in the cytotoxicity in the cells is taken into account of the presence of different cytocidal mechanisms. The loss of the anticandida activity would most likely be due to the permeability change.

On the comparison of anti-L 1210 activity between the phosphate and bredinin, they showed just the same dose response effect in molar level on life prolongation in mice. This suggested that bredinin 5′-monophosphate is converted to bredinin in the animal.

Acknowledgement The authors wish to thank Prof. H. Sugihara, Meijo University, for kind gift of Snake venom. We are also indebted to Dr. Y. Horiuchi for the supply of alkaline phosphatase and to Drs. M. Hayashi, M. Tsujino, and K. Sakaguchi, Research Laboratories of Toyo Jozo Co., Ltd. for their valuable help throughout this work.

7) K. Sakaguchi, M. Tsujino, M. Hayashi, K. Kawai, K. Mizuno, and K. Hayano, *J. Antibiotics*, "in press."

[Chem. Pharm. Bull. 24(9)2250—2253(1976)]

UDC 547.869.2.04 : 547.513.04

Facile Thermal Dimerization of a photochemically Isomerized Olefin

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(Received December 3, 1975)

In sharp contrast to 4-methyl-2-methoxycarbonylmethylene-3,4-dihydro-3-oxo-2H-1,4-benzothiazine (Z-form), the photochemically produced isomer (E-form) underwent a facile thermal dimerization to form a cyclobutane derivative. The present observation is notable for demonstrating that the apparent photodimerization of the open-chain olefin could involve primarily the thermal dimerization of its isomer formed photochemically.

A number of thermal dimerization of open-chain olefins appropriately substituted to form cyclobutanes are well-known.[2] To our best knowledge, however, the distinct difference

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2) For a review, see J.D. Roberts and C.M. Sharts, "Organic Reactions," Vol. 12, Willey, New York, N. Y., 1962, p. 1.