

A BUTYRYLCHOLINESTERASE INHIBITOR PRODUCED BY
PENICILLIUM SP. NO. C-81 AND ITS IDENTITY
WITH MYCELIANAMIDE

TORU NAGASAWA, NOBUHIRO MORI, YOSHIKI TANI and KOICHI OGATA
Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan

HIROSHI IRIE
Department of Pharmacology, Kyoto University, Kyoto, Japan

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A screening aimed at obtaining a cholinesterase inhibitor of microbial origin was carried out using *Pseudomonas* butyrylcholinesterase. A mycelium-extract of a fungus strain, belonging to the genus *Penicillium*, was found to produce such an enzyme inhibitor. The inhibitor was purified and crystallized as colorless leaflets. From physical and chemical studies, the inhibitor was identified as being identical with an antibiotic, mycelianamide, though this compound was not known to have enzyme inhibitor activity. The kinetics of the inhibition of *Pseudomonas* butyrylcholinesterase were also studied. Horse serum cholinesterase and hog liver carboxylesterase were also inhibited by the isolated *Penicillium* C-81 inhibitor, but lipase and acetylcholinesterase were not.

There are many kinds of cholinesterase (ChE) inhibitors such as organophosphates, carbamates and quaternary ammonium salts. Most of them are synthetic compounds; some of them are extracted from plants. Only few reports deal with the production of ChE inhibitors by microorganisms.^{1,2} ChE inhibitors are useful as insecticides and as chemotherapeutic agents. Thus, a systematic study to examine microorganisms as possible source for such inhibitors should be valuable.

Recently, we isolated and characterized acetyl ChE (AcChE), and butyryl ChE (BuChE) from bacterial cells.^{3,4} In some respects these enzymes resemble mammalian ChE; they are inhibited by various anticholinergic compounds. By using these easily obtainable enzymes, we began the search for ChE inhibitors of microbial origin. In a previous paper we demonstrated the presence of an AcChE inhibitor in the culture filtrate of *Aspergillus terreus*.²

In this paper, the isolation of a BuChE inhibitor from *Penicillium* sp. No. C-81, its identification as mycelianamide and the kinetic data of the enzymic inhibition are described.

Materials and Methods

Chemicals. Carboxylesterase (E.C. 3.1.1.1) from hog liver, AcChE (E.C. 3.1.1.7) from the electric eel, pseudo-ChE (E.C. 3.1.1.8) from horse serum, lipase (E.C. 3.1.1.3) from wheat germ and hog pancreas were purchased from Sigma Chemicals Co.; Silica gel, used for column chromatography was obtained from Mallinkrodt and silica gel, used for thin-layer chromatography, was Kieselgel 60 F-254, a product of Merck.

Preparation of the *Pseudomonas* enzyme. The preparation for *Pseudomonas* BuChE has been outlined in a previous report.⁴ *Pseudomonas polycolor* was cultured in a jar fermentor and a cell-free extract was prepared by disruption of the cells by means of an ultrasonic oscillator followed by centrifugation. BuChE present in the cell-free extract, was precipitated with ammonium sulfate at 0.3 to 0.8 saturation. The precipitate was dialyzed against 0.01 M potassium phosphate buffer (pH 7.4) for 20 hours. The enzyme solution, thus prepared, was stored at -20°C and was stable for several

months. The screening for ChE inhibitor was carried out with this preparation. Furthermore, the enzyme solution was applied to a CM-Sephadex column (6.0×80 cm) equilibrated with 0.05 M buffer (pH 7.4) and the enzyme was eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.3 M sodium chloride. Active fractions (134 ml) were combined and this purified enzyme solution was used in experiments dealing with the kinetics of this inhibition.

Bacterial AcChE used in our tests was prepared from the cell-extract of *P. aeruginosa* A-16 by the method described previously.³⁾

Microorganisms and screening. Microorganisms used in our screening test consisted of about 300 strains stocked in our laboratory and about 300 strains newly isolated from natural sources. Each strain was grown aerobically for 3~5 days at 28°C in the following media. Medium A used for cultivation of bacteria contained 0.5% glucose, 1.0% meat extract, 0.5% peptone, and 0.1% NaCl; medium B used for cultivation of molds contained 2.0% glucose, 2.0% malt extract, and 0.2% yeast extract, and medium C used for cultivation of actinomycetes contained 1.0% glycerol, 0.05% arginine, 0.05% K₂HPO₄ and 0.5% yeast extract. The cells and mycelia were harvested by centrifugation and filtration, respectively. Possible presence of an inhibitor was investigated both in the culture filtrate and in the mycelia. The mycelium-extract was prepared by suspending the mycelium in a 1:1 mixture of chloroform and ethanol at room temperature for 2~4 days. After the removal of the mycelium by filtration, the extract was evaporated to dryness and then dissolved in a small amount of methanol. Inhibitory activity against *Pseudomonas* BuChE was determined as follows: 0.2 ml of BuChE solution containing 1.3 units activity/ml in 0.1 M potassium phosphate buffer (pH 7.4) and 0.8 ml of the culture filtrate or 50 μ l of the mycelium-extract were mixed and incubated for 30 minutes at 30°C. The activity in 0.2 ml of the mixture was measured on a Hitachi 124 spectrometer at 412 nm with addition of 1 μ mole of 5,5'-dithio-bis-(2-nitrobenzoate) and 1.45 μ moles of butyrylthiocholine iodide and 300 μ moles of potassium phosphate buffer (pH 8.0) in a total volume of 3.35 ml.⁵⁾

Assay of enzyme activity. The activity of carboxylesterase was assayed in a reaction mixture (3.05 ml) containing *p*-nitrophenyl acetate as the substrate, 300 μ moles of potassium phosphate buffer (pH 8.0) and 4.1 units of enzyme. AcChE activity was assayed in a reaction mixture (3.02 ml) containing acetylthiocholine iodide as the substrate, 1 μ mole of 5,5'-dithio-bis-(2-nitrobenzoate), 300 μ moles of potassium phosphate buffer (pH 8.0), and 3.8 units of electric eel enzyme or 1.1 units of enzyme from *P. aeruginosa* A-16. Pseudo-ChE activity was assayed in a reaction mixture (3.10 ml) containing butyrylthiocholine iodide as the substrate, 1 μ mole of 5,5'-dithio-bis-(2-nitrobenzoate), 280 μ moles of potassium phosphate buffer (pH 8.0), and 1.23 units of enzyme. One unit of carboxylesterase, AcChE and Pseudo-ChE was defined as the amount of protein that hydrolyzed 1 μ mole of *p*-nitrophenyl acetate, acetylthiocholine and butyrylthiocholine, respectively, in the above reaction mixtures in 1 minute at 25°C. Lipase activity was assayed in a reaction mixture (4.2 ml) containing 2.8 μ moles of 2-naphtyl myristate dissolved in ethylene glycol monoethyl ether, 116 μ moles of sodium cholate, 800 μ moles of Tris-HCl buffer (pH 7.4) and an appropriate amount of lipase from wheat germ or hog pancreas. The reaction was carried out at 37.5°C for 5 hours, and the formation of 2-naphtol was measured colorimetrically.⁶⁾

Results and Discussion

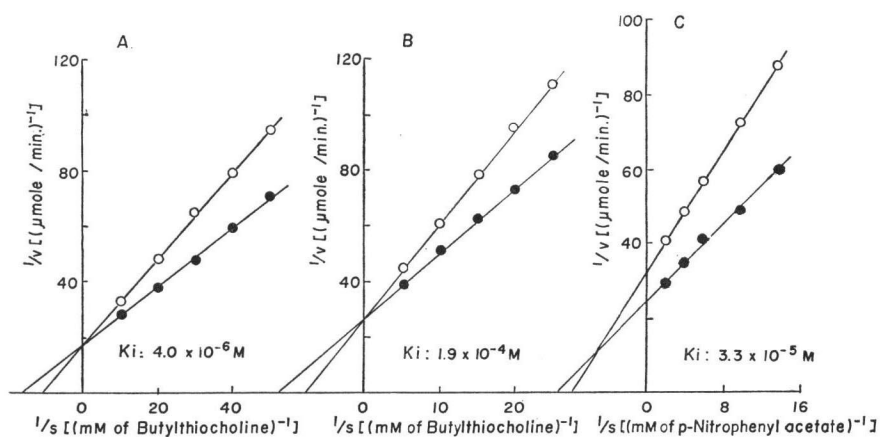
As a result of the screening, the mycelium-extract of an isolated fungus No. C-81. was found to show a strong inhibitory activity. Taxonomic study showed that the fungus belonged to the genus *Penicillium*. The following experiments were conducted with strain, *Penicillium* sp. No. C-81. Various nitrogen compounds were tested as the nitrogen source for the production of the inhibitor. Best production of the inhibitor was obtained with sodium nitrate (Table 1a). Organic nitrogen sources were not suitable. Several kinds of carbohydrates (starch, sucrose, glucose, glycerol) were effective for the production of the inhibitor (Table 1b). The fungus was cultured in shaking flasks, the progress of a typical fermentation is plotted in Fig. 1. Maximum production was reached after 8~10 days.

with a mixture of chloroform - methanol (96:4). The eluate was concentrated, and evaporated to dryness under reduced pressure. The crude powder (131 mg) thus obtained was washed with *n*-hexane, and was crystallized from acetone-water as colorless leaflets (73.9 mg).

The active compound melted at 159~160°C with decomposition. The specific rotation $[\alpha]_D^{25}$ was -133° (c 1, dioxane). Ultraviolet maxima in methanol showed λ_{\max} 325 nm (ϵ 23,500) and λ_{\max} 234 nm (ϵ 12,000). It gave a brown color reaction with ferric chloride and decolorized permanganate solution. The formula, $C_{22}H_{28}O_5N_2$, was calculated from the analytical results (calc.: C, 65.98; H, 7.05; O, 19.98, N, 7.00; found: C, 66.16; H, 6.87; N, 6.99) and was supported by the mass spectrum (m/e 400). The active compound is soluble in methanol, acetone, pyridine, chloroform and dioxane, sparingly soluble in water, and insoluble in *n*-hexane. These properties are identical with those of mycelianamide, a metabolic compound isolated in 1948 from the mycelium of strains of *Penicillium griseofulvum* DIERCKZ.⁷⁻¹¹ The identity of the inhibitor with mycelianamide (Fig. 2) was confirmed by the pmr spectra and by preparing a reduction product of the inhibitor. The pmr spectrum in $CDCl_3$ showed two methyl proton signals at δ 1.73 (6H, s) and 1.76 (3H, s), methylene proton signal at δ 4.45 (2H, d), two olefin proton signals at δ 4.95 (1H, m) and 5.45 (1H, t) and methine proton signal at δ 2.03 (4H, t) which were similar in shape to the signals and the chemical shifts of geraniol; furthermore the inhibitor showed one extra methyl proton signal at δ 1.60 (3H, d), and four aromatic protons at δ 6.79 and 7.30 as an AB-quartet ($J=8$ Hz). Mild reduction with zinc dust in acetic acid produced the compound $C_{22}H_{28}O_3N_2$ (mp. 196°C, lit. mp. 170°C⁸), lacking two oxygen atoms. It has an ultraviolet absorption very similar to that of mycelianamide itself. Only a slight alternation in the chromophore was observed on reduction (from λ_{\max} 234, 325 nm to λ_{\max} 227, 432 nm). The slight shift to shorter wavelength on reduction would be expected on removal of a hydroxyl group from nitrogen. In the ir spectrum there is a strong band at 1675 cm^{-1} which is characteristic of unstrained cyclic amides. The reduction product was hydrolyzed by acid, and alanine was detected by silica gel thin-layer chromatography developed with the solvent: $CHCl_3$ - acetone (3:1). Thus the reduced compound of the inhibitor was identical with deoxymycelianamide. From these results,

Fig. 3. Kinetics of inhibition by mycelianamide of *P. polycolor*. BuChE-butrylthiocholine system (A); Serum ChE-butrylthiocholine system (B); Carboxylesterase-*p*-nitrophenyl acetate system (C). The reaction was carried out with (○) or without (●) mycelianamide in the presence of methanol (1.6%).

10 μ g, 104 μ g and 30 μ g mycelianamide dissolved in methanol was added to the reaction system (A), (B) and (C), respectively.



the newly isolated inhibitor seemed to be mycelianamide. The identity with authentic mycelianamide which was supplied by Prof. R. BATES (University of Arizona, Tucson) was confirmed by the direct comparison of their ir spectra and by a mixed melting point determination.

The effect of mycelianamide on several esterases was studied. Mycelianamide inhibits the activity of *Pseudomonas* BuChE as shown in Fig. 3 (A). It had the highest affinity towards *Pseudomonas* BuChE among esterases investigated. Inhibition by mycelianamide was competitive with butyrylthiocholine as substrate, though mycelianamide has no structural relationship with butyrylthiocholine. The hydrolysis of butyrylcholine by horse serum cholinesterase and the hydrolysis of *p*-nitrophenyl acetate by hog liver carboxylesterase were also inhibited by mycelianamide; K_i values were 1.9×10^{-4} M and 3.3×10^{-5} M, respectively (Fig. 3 B and C). However, mycelianamide did not show an inhibitory effect on *Pseudomonas* AcChE and electric eel AcChE. The hydrolysis of 2-naphthyl myristate by lipases from wheat germ and from hog pancreas was not inhibited by mycelianamide. Also other esterases were found not to be inhibited by mycelianamide.

The distribution of mycelianamide among molds was examined in 13 genera of molds, *Aspergillus*, *Phytophthora*, *Mucor*, *Verticillium*, *Gliocladium*, *Pullularia*, *Gibberella*, *Neurospora*, *Ustilago*, *Fusarium*, *Sporotrichum*, *Isaria* and *Penicillium*. Molds were cultured in a medium containing 5% sucrose, 1% sodium nitrate and salts mixture or by the addition of 1% malt extract instead of sodium nitrate. Mycelium extracts were prepared as described in Materials and Methods. From the studies on the inhibitory activity towards the *Pseudomonas* enzyme and the Rf values on thin-layer chromatography as shown in Fig. 4, mycelianamide and mycelianamide-like compound were not detected in investigated strains of the genus *Penicillium* such as *P. chrysogenum*, *P. notatum* and *P. expansum* nor in other genera of molds; it was present only in strain C-81. *Penicillium* sp. C-81 resembles *P. griseofulvum* morphologically, but details of possible identity were not examined as yet.

As described previously,⁴⁾ the *Pseudomonas* BuChE is inhibited by organophosphorus compounds and carbamates, such as diisopropylphospho-fluoridate and eserine. Furthermore, the enzyme is inhibited by some biologically active compounds such as atropine, quinidine

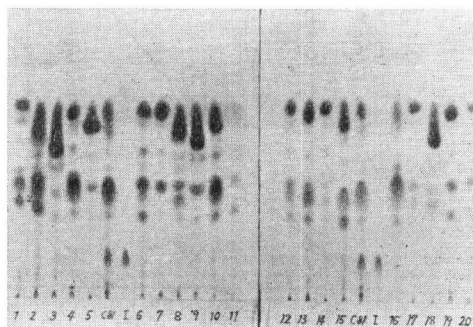
Fig. 4. Thin-layer chromatograms of mycelium-extract from molds.

Adsorbent: silica gel H.

Solvent system: benzene - methanol 9: 1.

Spray reagent: 10% phosphomolybdic acid in ethanol, followed by heating at 80°~100°C for 10 minutes.

(1)	<i>Aspergillus oryzae</i>	AKU 3301
(2)	<i>Phytophthora infestans</i>	IFO 4872
(3)	<i>Mucor lamprosporus</i>	IFO 6337
(4)	<i>Verticillium albo-atrum</i>	IFO 5922
(5)	<i>Gliocladium deliquescens</i>	IFO 6617
(6)	<i>Pullularia pullulans</i>	IFO 3562
(7)	<i>Mucor javanicus</i>	IFO 4570
(8)	<i>Gibberella fujikuroi</i>	IFO 6603
(9)	<i>Mucor racemosus</i>	IFO 4581
(10)	<i>Aspergillus niger</i>	IFO 3305
(11)	<i>Neurospora crassa</i>	IFO 6068
(12)	<i>Penicillium chrysogenum</i>	IFO 4626
(13)	<i>Penicillium notatum</i>	IFO 4640
(14)	<i>Penicillium expansum</i>	IFO 6096
(15)	<i>Aspergillus flavus</i>	IFO 5839
(16)	<i>Ustilago zeae</i>	IFO 5346
(17)	<i>Aspergillus terreus</i>	IFO 6123
(18)	<i>Fusarium anguoides</i>	IFO 4467
(19)	<i>Sporotrichum schenckii</i>	IFO 5983
(20)	<i>Isaia kogane</i>	IFO 5299
(I)	Inhibitor (mycelianamide)	
	C-81: <i>Penicillium</i> , sp. C-81.	



and quaternary ammonium salts. Above all, the high affinity for tropine derivatives (atropine, scopolamine, homatropine) is a unique feature of this enzyme. We intended to obtain a new enzyme inhibitor or active compound of microbial origin with the help of this easily obtainable enzyme. Although the isolated inhibitor was identified as mycelianamide, a compound already described as an antibiotic inhibiting the growth of Gram-positive bacteria,⁷⁾ its function was first recognized as an enzyme inhibitor in this study.

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