Isolation of Viridicatin from *Penicillium crustosum*, and Physiological Activity of Viridicatin and its 3-Carboxymethylene Derivative on Microorganisms and Plants

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An antibacterial substance isolated from the mycelial extract of a strain of *Penicillium crustosum* Thom was identified as viridicatin, 3-hydroxy-4-phenylcarbostyryl. To study the physiological and structural function of viridicatin, 3-(4-phenylcarbostyryloxy) acetic acid was synthesized from viridicatin and monochloroacetic acid, and its physiological activity on microorganisms and plants was compared with that of viridicatin. It was found that although viridicatin has a fairly strong antimicrobial activity on some gram positive bacteria and inhibits the growth of rice seedlings, the carboxymethylene derivative has a very weak activity on microorganisms but has stimulatory effect on the growth of rice seedlings.¹ The physiological significance of the substituting group in carbostyryl structure of these compounds was discussed.

Viridicatin (V-OH) had been first isolated from a strain of *Pen. viridicatum* Westling by Cunningham and Freeman,¹⁾ and successively from various strains of *Pen. cyclopium* Westling by Bracken, Pocker and Raistrick.²⁾ Lately, a strain of *Pen. puberulum* Bainier was also reported to produce V-OH, by Austin and Meyers.³⁾ The authors have found that a strain of *Pen. crustosum* Thom which has a lipase producing activity⁴⁾ produces V-OH.

V-OH possesses a carbostyryl structure (3-hydroxy-4-phenyl-carbostyryl), and partly resembles an active intermediary metabolite (3-methyleneoxindole) of auxin (indole-3-acetic acid) by microorganisms and plants.^{5~8)} How-

ever, physiological effect of V-OH on plants has never been studied, hitherto. Accordingly, it is of interest to study the structure-function relationship of V-OH with microorganisms and plants. For plant experiments, it is necessary to obtain a water soluble derivative since V-OH is hardly soluble in water. In this paper, a derivative, 3-(4-phenylcarbostyryloxy)-acetic acid (3-carboxymethyleneviridicatin, V-OCH₂COOH), which is easily soluble in water as salt was synthesized from V-OH and monochloroacetic acid, and used for the comparative physiological experiments with V-OH.

EXPERIMENTAL AND RESULTS

I) Isolation of V-OH

A strain of *Pen. crustosum* Thom was cultured on bran for nearly 20 days at 25°C.

1) K. G. Cunningham and G. G. Freeman, *Biochem. J.*, **53**, 328 (1953).

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3) D. J. Austin and M. B. Meyers, *J. Chem. Soc.*, **1964**, 1197.

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6) C. C. Still, T. T. Fukuyama and H. S. Moyed, *ibid.*, **240**, 2612 (1965).

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The bran culture was dried at 80°C and immersed in about eight volumes of 50% methanol. The supernatant was concentrated to a small volume under reduced pressure. The concentrated solution was extracted with ethyl acetate, after adjusting pH to 2.5 with hydrochloric acid solution. The extract was evaporated to a syrup *in vacuo* and dissolved in a small volume of water. The aqueous solution was mixed and shaken with ether repeatedly in a separating funnel. The separated ether layer was combined and dried over anhydrous sodium sulfate. After removing ether by evaporation, the residue was dissolved in chloroform and insoluble precipitate was removed by filtration. The chloroform solution was concentrated and subjected to a silicic acid column chromatography using chloroform as solvent.

Ferric chloride and Folin tests positive fraction was collected and, after removing chloroform by evaporation, crystalline substance remained was recrystallized from ethanol or chloroform. Colorless, shining needles were

TABLE I. ISOLATION AND PURIFICATION PROCEDURE OF V-OH

Dry powder of a bran culture of <i>Pen. crustosum</i> Thom (20 kg)	
	extracted with 50% methanol and concentrated under reduced pressure
Syrup	
	after adjusting pH to 2.5, extracted with ethyl acetate
Ethyl acetate extract	
	evaporated to a syrup <i>in vacuo</i> and extracted with ether
Ether extract	
	after removing ether by evaporation, dissolved in chloroform
Chloroform solution	
	concentrated and subjected to a silicic acid column chromatography
Ferric chloride and Folin tests positive fraction	
	concentrated
Crude crystal	
	recrystallized from ethanol or chloroform
V-OH (1 g)	

obtained in a yield of about 0.005% to the dry matter of a bran culture of the mold. The isolation and purification procedure is summarized in Table I.

II) Identification of V-OH

The crystals obtained melted at 268~269°C without decomposition and showed no optical rotation in ethanol. *Anal.* Found: C, 75.96; H, 4.75; N, 5.93. Calcd. for $C_{16}H_{11}NO_2$: C, 75.93; H, 4.67; N, 5.90%. Mass spectrum exhibited a parent peak at m/e 237 in conformity with the theoretical value. UV spectrum in ethanol showed $\lambda_{max} m\mu(\epsilon)$: 224 (37,400), 240 (18,000), 289 (7100), 310 (7600), 320 (9600), 332 (8200) which shifted to: 228 (28,400), 253 (16,400), 295 (7400), 334 (12,100), 344 (13,700) by adding 1 drop of 0.1 N sodium hydroxide solution. This indicated a highly conjugated structure and the presence of a phenolic hydroxyl group in the molecule which is also supported by a positive ferric chloride test. IR spectrum showed the presence of amide- (3340, 1660, 1560 cm^{-1}), intramolecularly bonded hydroxyl- (3200~2500 cm^{-1}) and phenyl groups (1575, 1500, 765, 720, 700 cm^{-1}), and conjugated double bond (1640 cm^{-1}). NMR spectrum showed signals at 1.42 τ (1H, singlet) due to a chelated hydroxyl proton and at 2.2~2.9 τ (10H, multiplet) due to aromatic and amide protons.

Monoacetate was prepared by treatment with pyridine-acetic anhydride (overnight at room temperature) and recrystallized from aqueous ethanol. Colorless prisms melting at 201~202°C were obtained. *Anal.* Found: C, 73.05; H, 4.84; N, 5.03. Calcd. for $C_{17}H_{13}NO_3$: C, 73.11; H, 4.69; N, 5.02%.

These data agreed with those on V-OH reported so far and this compound was identified as V-OH by the comparison of IR spectrum with that of authentic sample, as shown in Fig. 1.

III) Synthesis of V-OCH₂COOH

To 1g of V-OH dissolved in the smallest

FIG. 1. IR Spectrum of V-OH (Nujol).

(A) Isolated
(B) Authentic

amount of ethanol were added 12 ml of 33% sodium hydroxide solution and 4 g of monochloroacetic acid. After heating under a reflux in a boiling water bath for 3 hr, the mixture was cooled and diluted to about 40 ml with water. The diluted solution was acidified to pH 3.0 with hydrochloric acid and extracted with ethyl acetate. The ethyl acetate solution was washed with a small volume of water and shaken with 1N sodium bicarbonate solution. The sodium bicarbonate solution was separated and acidified with hydrochloric acid.

The precipitate formed was collected on a filter and recrystallized from ethanol. Colorless needles melting at 219~221°C were obtained. *Anal.* Found: C, 68.90; H, 4.56; N, 4.81. Calcd. for $C_{17}H_{13}NO_4$: C, 69.14; H, 4.44; N, 4.74%. IR spectrum showed the presence of a carboxyl group (3300~2200, 1750

cm^{-1}). The synthesis of $V-OCH_2COOH$ is shown in Fig. 2.

IV) Physiological activity of V-OH and $V-OCH_2COOH$

Antimicrobial activity was determined by dilution method as follows. Bacteria were inoculated in 5 ml of a peptone medium in a test tube and incubated at 37°C for 2 days. Yeast and mold were cultured on wort at 25°C for 2 days. V-OH was first dissolved in ethanol and added to the medium. The effect of ethanol was checked with control. $V-OCH_2COOH$ was dissolved in water as sodium salt and diluted by the medium.

As shown in Table II, V-OH exhibited a highly specific antibacterial activity against gram positive bacteria and completely inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus* at a concentration of 5 ppm and 100 ppm, respectively. On the other hand, $V-OCH_2COOH$ had no longer the same activity as V-OH, and, even at a very high concentration as 400 ppm, only slightly or partially inhibited the growth of these gram positive bacteria.

The effect of V-OH and $V-OCH_2COOH$ on plants was tested with rice seeds as follows. The seeds used were "Toyoato" and "Tama-

FIG. 2. Synthesis of $V-OCH_2COOH$.

TABLE II. EFFECT OF V-OH AND V-OCH₂COOH ON GROWTH OF MICROORGANISMS

Organisms	V-OH					V-OCH ₂ COOH		
	1	5	10	50	100	100	200	400
Concentration in ppm								
<i>Escherichia coli</i>	##	##	##	+	+	##	##	##
<i>Bacillus subtilis</i>	+	—	—	—	—	##	##	+
<i>Staphylococcus aureus</i>	+	+	+	+	—	##	##	##
<i>Candida utilis</i>	##	##	##	+	+	##		
<i>Saccharomyces cerevisiae</i>	##	##	##	##	+	##		
<i>Penicillium crustosum</i>	##	##	##	##	##	##		

No effect, + Slight inhibition, + Partial inhibition, — Complete inhibition

FIG. 4. Effect of V-OCH₂COOH and GA₃ on Growth of Rice Seedlings (Variety "Tamanishiki").

○—○ V-OCH₂COOH, ×—× GA₃
(Seedlings were grown for 18 days.)

nishiki". Seeds were soaked in 75% ethanol for 5 min, sterilized with mercuric chloride solution for 15 min and washed with sterilized water thoroughly. They were then, after steeping in sterilized water for 2 hr, laid on sea sand containing 20 ml of various concentration of V-OH or V-OCH₂COOH (pH adjusted to 5.0) in a Petri dish, and allowed to

FIG. 3. Effect of V-OH and V-OCH₂COOH on Growth of Rice Seedlings (Variety "Toyosato").

●—● V-OH, ○—○ V-OCH₂COOH
(Seedlings were grown for 7 days.)

germinate under fluorescent lamp with 800 lux at 30°C. After 7 days, the length of shoot and root of the seedlings was measured.

As presented in Fig. 3, V-OH at a concentration of 10~50 ppm distinctly inhibited the growth of both shoot and root, while at a limiting concentration of 3 ppm rather stimulated the shoot growth. On the other hand, V-OCH₂COOH at a concentration of 10~100

ppm remarkably stimulated the root growth and only slightly the shoot growth. However, a very high concentration of V-OCH₂COOH had an inhibitory effect on growth.

The effect of V-OCH₂COOH was then compared with gibberellin (GA₃). As presented in Fig. 4, the effect of GA₃ was specific for shoot elongation at a wide range of concentration, 1~50 ppm, while that of V-OCH₂COOH on root formation only at a comparatively high concentration, 25~50 ppm.

DISCUSSION

According to Cunningham *et al.*,¹¹ *Pen. viridicatum* lost the productivity of V-OH, later on. Several strains of *Penicillia* which have been found to produce V-OH, belong to a related species. Although the yield of V-OH from *Pen. crustosum* in this paper is tolerable as described above, it has recently decreased suddenly to a negligible amount. The biosynthetic metabolism⁹⁾ of V-OH in these strains may be very delicate and readily disordered by culture condition.

Both the antibacterial activity and inhibitory effect on plant growth of V-OH must be due to a function of the hydroxyl group involved in its structure, because V-OCH₂COOH has no longer such activity and inhibitory effect, as shown in the above experiment. Also, V-OH forms complexes with several metal ions

such as iron, copper, nickel and molybdenum, giving specific color reactions, while V-OCH₂COOH has no such reaction. Therefore, the formation of the metal complex may be involved in the action of V-OH on growth of microorganisms and plants.

On the other hand, it is of interest that V-OCH₂COOH has a weak antibacterial activity at a high concentration. In this case, the unmasking of V-OCH₂COOH, giving rise to V-OH, may take place in the cells of bacteria *per se*. This might be also the case in plants and the stimulatory effect of V-OCH₂COOH on plant growth may depend on V-OH derived from V-OCH₂COOH unmasked in the plant. However, further, investigation would be necessary to prove the presence of an activity capable of splitting V-OCH₂COOH to V-OH in microorganism and plant. There is a difference between the effect of low concentration of V-OH, and V-OCH₂COOH on plant growth; namely, the former stimulates shoot elongation but the latter root formation. There may be a physiological significance in carbostyryloxyacetic acid structure of V-OCH₂COOH. Further studies on the physiological activity of V-OCH₂COOH on plants are now in progress.

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