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AN INDUCIBLE LACTONE HYDROLASE YIELDING 2,5-DIPHENYL-3-HYDROXY-4-OXO-2-HEXENDIOIC ACID FROM PULVINIC ACID

HIROSHI IIJIMA, YUTAKA EBIZUKA, USHIO SANKAWA, ETSUO YAMAMOTO* and G. H. N. TOWERS*

Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan; *Department of Botany, University of British Columbia, Vancouver, B. C., Canada

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Key Word Index—*Letharia vulpina*; lichen; degradation; vulpinic acid; pulvinic acid; 2,5-diphenyl-3-hydroxy-4-oxo-2-hexendioic acid; lactone hydrolase.

Abstract—The metabolism of vulpinic acid by an unclassified soil micro-organism was studied. A new compound, 2,5-diphenyl-3-hydroxy-4-oxo-2-hexendioic acid (DHOHA) was isolated from the reaction mixture of a cell-free preparation and pulvinic acid. The existence of a hydrolase which catalyses the conversion of vulpinic acid to pulvinic acid was detected in cell-free preparation, and an inducible lactone hydrolase capable of converting pulvinic acid to DHOHA was purified 130-fold and characterized. This enzyme had a MW of *ca* 34 000, a K_m for pulvinic acid at pH optimum (pH 7.0) less than 10^{-6} M, pI = 5.0, and was inhibited by *p*-chloromercuriphenylsulfonate and diethylpyrocarbonate. The enzyme was highly specific for pulvinic acid. The initial degradative steps proposed for this organism are vulpinic acid → pulvinic acid → DHOHA.

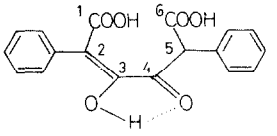
INTRODUCTION

The lichens, *Letharia vulpina* and *Cetraria pinastri*, which have been found to contain up to 10% dry wt of vulpinic acid [1, 2], the methyl ester of pulvinic acid, occur abundantly throughout southern British Columbia (Canada). These epidendric species accumulate to the extent of 1.5 tons/acre [3] and numerous fragments fall to the ground so that vulpinic acid and related antibacterial compounds [4] must present a significant biochemical entry into the soil ecosystems. Apart from usnic acid [5–7], very few studies have been made on the biodegradation of lichen compounds. A preliminary study by W. S. Chalmers [unpublished] indicated the presence, in soil samples in British Columbia, of bacterial species which were capable of the rapid degradation of vulpinic acid. This report describes both the isolation of 2,5-diphenyl-3-hydroxy-4-oxo-2-hexendioic acid as a metabolite of vulpinic acid and the purification and characterization of an enzyme which catalyses the reaction: pulvinic acid $+ H_2O \rightarrow$ DHOHA.

RESULTS AND DISCUSSION

2,5-Diphenyl-3-hydroxy-4-oxo-2-hexendioic acid (DHOHA) (**1**) was isolated from the reaction mixture of cell-free extracts of a soil bacterium incubated with pulvinic acid. The structure of DHOHA was elucidated by analyses of spectral data and by the formation of its quinoxaline (**2**). The ^{1}H NMR spectrum of the product showed, except for the aromatic proton signals, only one proton signal at δ4.60, which was unexchangeable with D_2O and assignable to H-5. The ^{13}C NMR spectrum indicated a methine carbon, two olefinic carbons and three carbonyl carbons in addition to aromatic carbons. All the signals could not be assigned, but the chemical shifts agree with the proposed structure. The IR spectrum supported the presence of carboxylic acid, and the mass spectrum gave a MW of 322. The identity of the quinoxaline (**2**) derived both from DHOHA or from dibenzylglyoxal (**3**) was confirmed on the basis of mmp's, and comparisons of IR and mass spectra. Decarboxylation of DHOHA must have occurred during the reaction with *o*-phenylenediamine, since DHOHA is a β-keto acid. Although DHOHA in solution is quite unstable, especially in acetone, the crystallized compound was stable at room temperature for a few months.

The hydrolase which catalyses the hydrolysis of the lactone moiety of pulvinic acid (**5**) was purified 130-fold with a 16% recovery by employing hydroxyapatite, Blue Sepharose CL6B, and Sephacryl S-200 CC (Table 1). Polyacrylamide gel electrophoresis of the S-200 preparation showed one major protein band, which coincided exactly with a single peak of the enzyme activity. The purified enzyme was not very stable when the protein concentration was less than 1 mg/ml. The concentrated S-200 preparation retained almost 100% of the initial activity for 2 days at 4°, but was completely denatured within 2 weeks. When the organism was grown in a medium without vulpinic acid, no hydrolase activity was detected. The repetition of preculture in the presence of vulpinic acid (**4**), as described in the Experimental, was necessary for the production of the enzyme. Calibration with MW markers on a Sephacryl S-200 column gave a MW of 34 000 for the enzyme. The apparent K_m for pulvinic acid at optimal pH (pH 7.0) was too small to measure by spectrophotometry, as it is below 10^{-6} M. A symmetric pH optimal curve was obtained in the range pH 6–8. The optimal pH in KPi buffer was 7.0–7.5. The enzyme activity was focussed at pH 5.0 by isoelectric focussing over the pH range 3.5–10. Although no cofactor requirement for NAD(P)H, NAD(P), ATP, FAD or FMN was observed, the enzyme was absorbed by Blue Sepharose, a well-known affinity dye matrix for proteins which require a dinucleotide as cofactor or which



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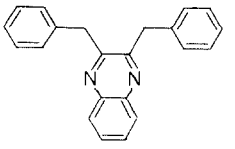

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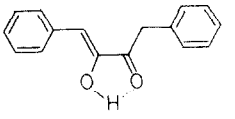

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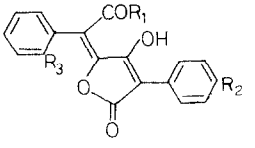
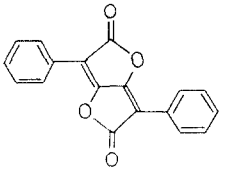


	R_1	R_2	R_3
4	OMe	H	H
5	OH	H	H
6	OMe	OMe	H
7	OMe	H	OMe
10	NHCH(CH_2Phe)COOMe	H	H
11	NH_2	H	H



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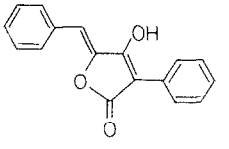

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Table 1. Purification of pulvinic acid lactone hydrolase

	vol. (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)
Crude extract	18.5	3132	66.3	47.2
Hydroxyapatite	12.9	2077	10.2	202.8
Blue Sepharose	2.5	1645	1.56	1052.8
Sephacryl S-200	0.4	499	0.032	6250.0

1 unit (U) is 1 μmol pulvinic acid degraded per min.

possess dinucleotide folding pockets. The enzyme could be eluted with pulvinic acid, but not with any of the nicotine dinucleotides, and the elution pattern (Fig. 1) indicated that the enzyme dissociated from the matrix to make an enzyme–substrate complex. Divalent ions, such as Cu^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+}, strongly inhibited the reaction (100, 100, 100 and 94% inhibition, respectively, at 1 mM, in Tris–HCl buffer, pH 7.1), whereas Mn^{2+}, Ca^{2+} and Mg^{2+} did not. Addition of EDTA accelerated the reaction. The enzyme resembles the lactone hydrolases of bacterial origin which attack the lactone moiety of compounds such as actinomycin, or triacetic acid lactone, in its pH optimum (pH 7–8), and inhibition by Cu^{2+} and Zn^{2+} [8, 9]. The results of the experiments with some protein alkylating and acylating reagents are shown in Table 2. Incubation with diethylpyrocarbonate or with *p*-chloromercuriphenylsulfonate, a specific inhibitor for sulfydryl residues [10] was strongly inhibitory. On the other hand, diisopropylfluorophosphate, tosylphenylalanine chloromethylketone and phenylmethylsulfonyl fluoride, which are known, respectively, as an alkylating reagent for serine residues, an alkylating reagent for the histidine residue of chymotrypsin and an acylating reagent for serine residues [11], did not inactivate the enzyme. The fact that protonated (i.e. ionized) imidazole could not be acylated by diethylpyrocarbonate [12] is in

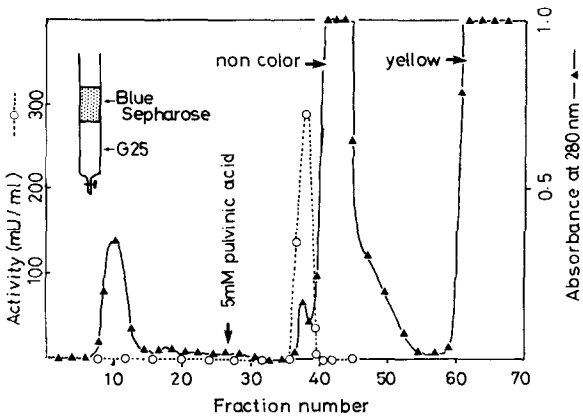

Fig. 1. Blue Sepharose CL6B CC. The non-color peak which came just after the enzyme activity was that of DHOHA formed during the run.

Table 2. Effect of enzyme inhibitors (a) and effect of pH on the inhibition by DEP (b)

(a) Inhibitor (1 mM)	Relative activity (%)
No addition	100
DEP	4
PCMPS	4
DFP	96
TPCK	96
PMSF	96

(b)	Activity (ΔA/min)		Relative
pH	Control	DEP (0.1 mM)	activity (%)
5.5	1.62×10^{-2}	1.22×10^{-2}	76
8.5	1.60×10^{-2}	0	0

The final concentration of DEP was 0.1 mM for the experiment b. Relative activity was expressed against the enzyme treated with no protein modifying reagent. DEP, diethylpyrocarbonate; PCMPS, *p*-chloromercuriphenylsulfonic acid monosodium salt; TPCK, tosylphenylalanine chloromethylketone; PMSF, phenylmethylsulfonylflouride.

good accord with the effects of pH on the inactivation of the enzyme (Table 2b), suggesting the existence of a histidine residue, whose pK_a is *ca* 7.

Pulvinic acid (**5**) was the only compound which served as substrate for the purified lactone hydrolase, whereas the crude enzyme decomposed vulpinic acid and pinastric acid (**6**) as well as pulvinic acid. The relative activity of the crude enzyme against pulvinic acid was 65% for vulpinic acid and 5% for pinastric acid. This result suggests that a methyl ester hydrolase is present in the crude extract. Leprapinic acid (**7**) was not degraded even by the crude enzyme. Pulvinic dilactone (**8**), a compound which has two lactone rings, and pulvinone (**9**) were not degraded either by the crude enzyme or by the S-200 preparation, indicating that the carboxylic acid moiety in pulvinic acid is important. Rhizocarpic acid (**10**) and pulvinamide (**11**) were not hydrolysed even by the crude enzyme, suggesting that amide hydrolase activity was not induced when the organism was grown in the presence of vulpinic acid.

The initial steps in the metabolism of vulpinic acid by the soil micro-organism are vulpinic acid → pulvinic acid → DHOHA. In the biosynthesis of vulpinic acid, oxidative ring cleavage of polyporic acid may be considered to be a key step in the pathway [13]. The hypothetical cleaved compound would be identical to DHOHA. It is interesting that in the biodegradative pathway of vulpinic acid, the same intermediate is involved.

EXPERIMENTAL

Organism and culture conditions. The micro-organism capable of metabolizing vulpinic acid was isolated by the enrichment culture technique [14]. Soil samples (0.5 g) collected in Lytton, B.C., where *Letharia vulpina* is abundant, were suspended in 100 ml medium A in 500-ml flasks. (Medium A: NaCl 1 g, yeast extract 2 g, glucose 3 g, powdered vulpinic acid 0.5 g, H_2O 1 l.) The culture flasks were shaken for 2 days at 25°. The culture which lost its yellow color most rapidly was selected and *ca* 100 μl of the broth was withdrawn and transferred onto agar plates ($NaCl$ 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, $(NH_4)_2HPO_4$ 1 g, KH_2PO_4 0.5 g, glucose 3 g, yeast extract 0.5 g, powdered vulpinic acid 0.5 g, agar 15 g, H_2O 1 l.) A colony which formed a decolorized zone around itself was selected, and after several transfers onto agar plates, the bacterial species was isolated and used in further work. The bacterium grew very well at 37° on normal nutrient agar plates, but not on the vulpinic acid–agar plates at the same temp. In the presence of vulpinic acid the optimal growth temp. was found to be 25° and, thus, cultivation in the presence of vulpinic acid was carried out at this temp. One or two colonies from stock culture plates (vulpinic acid–agar) were inoculated to 20 ml medium B in 100-ml flasks. (Medium B: NaCl 1g, $MgSO_4 \cdot 7H_2O$ 1 g, $(NH_4)_2HPO_4$ 1 g, KH_2PO_4 0.5 g, yeast extract 2 g, glucose 3 g, powdered vulpinic acid 0.25 g, H_2O 1 l.) The culture flask was shaken for 48 hr at 25°. 2.5 ml of this culture was then transferred into 50 ml medium B in a 300-ml flask and shaken for 24 hr. 2.5 ml of this culture was again transferred into 50 ml medium B in a 300-ml flask and shaken for 24 hr. This seed culture (10 ml) was used as the inoculum for 500-ml flasks containing 200 ml medium B. These flasks were incubated at 25° on a rotary shaker (*ca* 10 cm stroke, 160 rpm) for 16–17 hr, which is the end of the log phase and which contained *ca* 10^{11} cells/ml. The micro-organism is maintained in the micro-organism culture collection of Tokyo University.

Identification of 2,5-diphenyl-3-hydroxy-4-oxo-hexendioic acid. A mixture of 2.5 units of crude enzyme and 200 mg pulvinic acid was incubated in 600 ml KPi buffer (100 mM, pH 7.0) at 30°, until the yellow color of the reaction mixture had faded (*ca* 5 hr). The mixture was acidified to pH 3.0 with 1 N HCl, and extracted with EtOAc (500 ml, × 3). The organic layer was dried on Na_2SO_4. Removal of the solvent under red. pres. gave an oily residue (260 mg), which was dissolved in 200 μl MeOH and 2 ml $CHCl_3$. The soln was chromatographed on 20 g of an oxalic acid impregnated Si gel column first with $CHCl_3$, then $CHCl_3$–MeOH (100:1). The fractions containing the reaction product were combined (89 mg) and recrystallized from pure $CHCl_3$ to yield 37.4 mg of colorless crystals subliming at 160–180°. R_f 0.2 on oxalic acid impregnated Si gel TLC with solvent system $CHCl_3$–MeOH (20:1); UV λ_{max}^{EtOH} nm (log ε): 270 (4.04), (KPi buffer, pH 7.0) 282 (4.09), 263 (4.14), (pH 3.5) 274 (4.01); IR ν_{max}^{KBr} cm^{-1}: 3200, 1750, 1710, 1430; MS: *m/z* 322 $[M]^+$, 308, 282, 264, 238, 147, 119, 9, 65; ^1H NMR (d_8-dioxane–TMS, 100 MHz): δ 4.60(1H, *s*, H-5), 7.1–7.8(10H, *m*, ar.-H), 6.73(< 1H, *s*, OH), 12.12(< 1H, *s*, OH); ^{13}C NMR (d_8-dioxane–TMS, 25 MHz): δ 59.63(*d*, C-5), 100.53 (*s*, C-2 or C-3), 103.19 (*s*, C-2 or C-3), 127.54, 128.60, 129.30, 130.27, 130.97 (ar.

C), 169.00 (*s*), 170.38 (*s*), 178.58 (*s*, C-1, C-6 or C-4). (Found: C, 64.47; H, 4.99. $C_{18}H_{14}O_6$ requires: C, 65.85; H, 4.91%.)

Quinoxalines of the enzyme reaction product and of dibenzylglyoxal. To a mixture of 35 mg DHOHA and 200 μl EtOH was added a soln of 16 mg *o*-phenylenediamine in 400 μl EtOH. The mixture was heated at 50° for 40 min. *Ca* 10 ml H_2O was added and the mixture was centrifuged at 3000 *g* for 15 min. The ppt obtained was recrystallized from EtOH (pale brown plates 10 mg, mp 119°). Similarly 10 mg of chemically synthesized dibenzylglyoxal (**3**) and *o*-phenylenediamine were reacted as described above, and 3.1 mg of crystals were recovered (mp 119°, lit. 119° [15]). MS: *m/z* 310 $[M]^+$, 295, 231, 219, 165, 149; IR ν_{max}^{KBr} cm^{-1}: 1600, 1485, 760, 710.

Dibenzylglyoxal. Dibenzylglyoxal was synthesized by standard procedures [15, 16], mp 88–88.5° (colorless needles from C_6H_6). IR ν_{max}^{KBr} cm^{-1}: 1640, 1620; MS: *m/z* 238 $[M]^+$, 147, 119, 91, 65; ^{1}H NMR (CD_3OD–TMS, 100 MHz): δ4.15 (2H, *s*, $CH_2C=O$), 6.69(1H, *s*, HO–C=C<u>H</u>), 7.3–7.8 (10H, *m*, ar.H); ^{13}C NMR(CD_3OD–TMS, 25 MHz): δ 43.1(*t*, <u>C</u>H_2C=O), 115.4 (*d*, HO–C=<u>C</u>H), 127–146 (ar. C), 148.3 (*s*, –<u>C</u>OH–CH), 196.4(*s*, C=O).

Enzyme assay. The standard assay mixture consisted of 300 nmol pulvinic acid, enzyme (5–100 μl) and an appropriate vol. of 100 mM KPi buffer (pH 7.0) to give a total vol. of 3 ml. Decrease in *A* at 358 nm was observed at 30° ($\varepsilon_{358\,nm}^{pH\,7.0} = 9.12 \times 10^3$). 1 unit of enzyme activity was equal to 1 μmol pulvinic acid degraded per min. Under these conditions, the proportionality of enzyme concn to reaction velocity, and the linearity between reaction rate and incubation time were confirmed.

Enzyme extraction and purification. All operations described below were carried out at 2–5°. The 17 hr culture (1 l.) was centrifuged at 3000 *g* for 20 min. The supernatant was decanted and the cells were washed with 30 ml KPi buffer A (100 mM, pH 7.0) and centrifuged again. The washed cells were homogenized with the aid of alumina in a mortar, extracted with 30 ml buffer A and followed by centrifugation at 10000 *g* for 15 min. The supernatant was stored and the ppt was homogenized and extracted again. To the combined supernatant, 2% (w/v) protamine sulfate was added dropwise to give a final concn of 0.1%. The soln was gently stirred for 30 min and centrifuged at 20000 *g* for 30 min. The supernatant (crude enzyme preparation) was applied to a hydroxyapatite column (1.3 × 18 cm) equilibrated with 20 mM KPi buffer (pH 7.0). The column was washed with the starting buffer, and the absorbed protein was eluted with a linear gradient prepared with each 100 ml 20 mM KPi buffer (pH 7.0) and 200 mM KPi buffer (pH 7.0) (35 ml/hr, 3 ml/fr.). The active fractions were combined and concd, by ultrafiltration. (Amicon PM-10), to 12.9 ml. The hydroxyapatite preparation was charged on the top of a Blue Sepharose column (1.7 × 7 cm). The column was washed with buffer A and active enzyme was eluted by a pulse of 8 ml 5 mM pulvinic acid soln. The lower-half of the column was packed with a 26 ml bed vol. of Sephadex G-25(M) for the purpose of removing excess pulvinic acid and the enzyme reaction product from active fractions. The active fractions were combined and concd by ultrafiltration. The Blue Sepharose preparation was chromatographed on a Sephacryl S-200 column (2.5 × 34 cm) equilibrated with 20 mM KPi buffer (pH 7.0) (24 ml/hr, 2 ml/fr.). The active fractions were combined and concd to 400 μl in a collodion bag (Sartorius SM) as soon as chromatography was completed. Protein concn was determined by the modified method of Lowry *et al.* with BSA as standard [17]. The MW was determined using the Sephacryl S-200 column calibrated with MW markers; BSA (67000), ovalbumin (43000), chymotrypsinogen (25000), ribonuclease A (13700). S-200 preparations (5 μl) and pulvinic acid (100 μM) were assayed in KPi buffers (900 μl) of various pHs for the determination of the pH optimum. The enzyme preparations were analysed electrophoretically using the modified method of Davis [18]. After the electrophoresis, gels were sliced and protein was extracted from the homogenized slices with buffer A for 2 hr at 4°. Enzyme activity was assayed using standard assay conditions.

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