Phytochemistry, 1972, Vol. 11, pp. 3215 to 3218. Pergamon Press. Printed in England.

BIOSYNTHESIS OF CITREOVIRIDIN

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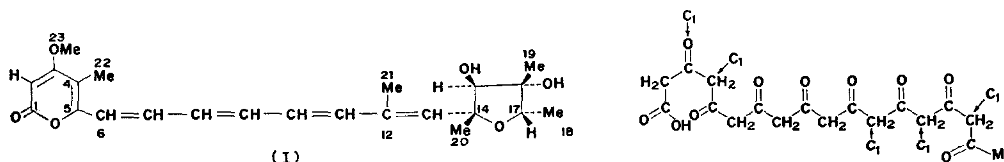
(*Revised Received* 6 *May* 1972. *Accepted* 1 *June* 1972)

Key Word Index—*Penicillium pulvillorum*; Aspergillaceae; Fungi; citreoviridin biosynthesis; polyene neurotoxin.

Abstract—The biosynthesis of citreoviridin (I) by *Penicillium pulvillorum* Turfitt was investigated. Evidence is presented that citreoviridin was derived from nine molecules of acetic acid and five molecules of methionine.

INTRODUCTION

CITREOVIRIDIN (I) is a polyene neurotoxin elaborated by strains of *Penicillium citreoviride* Biourge,[1] *P. ochrosalmoneum*,[2] *P. pulvillorum* Turfitt[3] and *P. fellutanum* Biourge.[4] *P. pulvillorum* CSIR 1406 was selected for this biosynthetic study by virtue of its ability to produce I in high yield on both solid[3] and stationary liquid medium (Ushinsky).



It can be postulated that citreoviridin is derived from one acetate unit, eight malonate units and methionine. A polyketide chain which arises as a result of the head-to-tail condensation of the acetate and malonate units is C-alkylated in four positions by methyl groups of methionine. The methoxyl group at the C-3 position also arises from this amino acid, as shown above.

RESULTS AND DISCUSSION

Citreoviridin was produced by *P. pulvillorum* in media to which methyl-^{14}C-methionine and [2-^{14}C-]acetate were added in separate experiments about nine days after inoculation.

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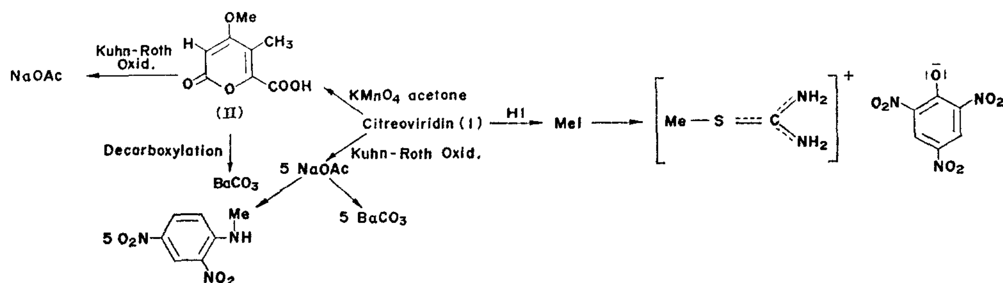
The citreoviridin was purified by chromatography and recrystallized to constant activity. The following incorporations were obtained: 70 and 5% from labelled methionine and labelled acetic acid, respectively. The citreoviridin was chemically degraded as depicted in Scheme 1.



SCHEME 1. DEGRADATION SCHEME OF CITREOVIRIDIN.

The methyl-^{14}C-methionine labelled I was treated with HI in refluxing acetic anhydride and the MeI formed converted to the methylthioureapicrate. The activity of the methoxyl group represented 20·4% of the total activity of citreoviridin, indicating that four of the five C-methyl groups were derived from the C_1-pool; in the postulated biosynthesis, C-18 would be derived from acetate.

Kuhn–Roth oxidation of methyl-^{14}C-methionine labelled I yielded acetic acid. The corresponding *p*-bromophenacylacetate had a specific molar activity of 80·8% of that of the starting material. Schmidt decarboxylation of the acetic acid showed that all its activity was contained in the methyl group, which was determined as 2,4-dinitro-*N*-methylaniline.

A further degradation step was the oxidation of methyl-^{14}C-methionine labelled I with $KMnO_4$ in acetone. The corresponding coumalic-acid-(6) (II) showed specific molar activity of 39·7% of that of the starting material, in agreement with our previous findings, as the methyl group attached to the pyrone moiety should also be derived from the C_1-pool.

In the second approach, cultures of *P. pulvillorum* were incubated with [2-^{14}C-]acetate. Kuhn–Roth oxidation of the labelled I yielded *p*-bromophenacylacetate which had a specific molar activity of 51·8% of that of the starting material (it should be 5/9 = 55·5%, assuming a total of nine labels). Schmidt decarboxylation of the acetic acid showed that the methyl group contained 1/9 of the activity of that of the starting material and the carboxyl groups 31·4%, counting efficiency of $BaCO_3$ uncorrected.

The oxidation of [2-^{14}C-]acetate labelled I yielded the acid II which contains 3/9 of the activity of I. To establish the labelling of the C-6, the acid was submitted to a Schmidt decarboxylation. However, the activity of the $BaCO_3$ obtained was much lower than calculated. This $BaCO_3$ represented by weight a yield substantially more than the theoretical. Additional decarboxylation therefore occurred during the reaction. A Kuhn–Roth degradation was carried out on II. The acetic acid (as *p*-bromophenacylacetate) had a specific molar activity of 1/3 of that of II, or 1/9 of that of citreoviridin.

From the labelling experiments with methionine it was evident that the C-22 is derived from methionine: that determines the C-4 as being derived from the methyl group of the acetate. As citreoviridin is formed from one acetate unit and eight malonate units, the C-18

must be the beginning of an unbranched polyketide chain which contained four *C*-methyl groups and one *O*-methyl group, derived from the C_1-pool.

TABLE 1. THE INCORPORATION OF PRECURSORS INTO CITREOVIRIDIN

Precursor	Activity of precursor added (μCi)	Vol. of culture fluid (ml)	Yield of citreoviridin (mg)	Total activity of citreoviridin (μCi)	Incorporation (%)
[^{14}C-CH_3]-L-Methionine	50	200	38	35	70
[2-^{14}C-]Acetate	250	600	80	12·5	5

EXPERIMENTAL

Radioactivity was assayed on a Packard Tri-Carb Liquid Scintillation Spectrometer Model 574; organic compounds were analysed in 0·01% POPOP and 0·4% PPO Toluene : Triton X100 (2:1) scintillation solution. ^{14}C–$BaCO_3$ was assayed by suspension scintillation counting[5] in the same scintillation mixture which contained *ca.* 4% (w/v) of Cab-O-sil as gelling agent. Precoated Merck Silica Gel F_{254} chromatoplates were used for TLC. All labelled compounds were recrystallized to constant activity. M.ps were determined on a Kofler Block and are uncorrected.

Preparation of labelled citreoviridin. P. pulvillorum strain CSIR 1406 was grown in 100 ml aliquots of Ushinsky's medium[2] in 500 ml cotton-plugged Erlenmeyer flasks in stationary culture at 25°. When the production of citreoviridin started to rise rapidly (usually 9 or 10 days after inoculation), the precursor substrates were added to the culture. In the case of labelling experiments in which methionine and acetic acid were added to the cultures, citreoviridin was harvested after an additional incubation period of 10 and 3 days, respectively. The filtrate was extracted with $CHCl_3$. The mycelium was extracted with $CHCl_3$–MeOH (1:1) and combined with the previous $CHCl_3$-extract. The crude extract was partitioned between 90% MeOH–hexane and the concentrated MeOH-layer was separated on formamide-impregnated cellulose column. The column was eluted with a mixture of hexane and benzene (see Table 1). In each case the labelled material was diluted with inactive citreoviridin to obtain sufficient material for chemical degradation work. Specific activity of citreoviridin: methyl-^{14}C-methionine 5·15 × 10^6 dpm/mmol: [2-^{14}C-]acetate 2·46 × 10^6 dpm/mmol.

Demethylation of citreoviridin labelled with methionine. I (100 mg) was treated with HI (5 ml) in refluxing Ac_2O[6] (2 ml) for 2·5 hr and the MeI trapped in EtOH (2·5 ml) at −70°. This was converted to the methylthioureapicrate[7] (m.p. 223° from EtOH) which had SA 1·05 × 10^6 dpm/mmol, corresponding to 20·4% of the activity of compound I.

Oxidation with $KMnO_4$ *in acetone of* I *labelled with methionine.* I (201 mg, 0·5 mmol) in acetone (7 ml) was treated with powdered $KMnO_4$ (*ca.* 700 mg) over a period of about 3 hr at 5–15° until the solution remained red. H_2O (7 ml) was added and SO_2 was passed through the solution until it became clear. The solution was acidified (3 ml conc. HCl) and extracted with Et_2O (3 × 150 ml). The combined Et_2O extracts were extracted with 10% $NaHCO_3$-solution. The aqueous layer was acidified with conc. HCl and extracted again with Et_2O. Removal of the Et_2O gave a crude residue (50 mg) which was washed with $CHCl_3$ to remove impurities. Recrystallization from H_2O gave the acid II (10 mg). M.p. 220–222°, lit.[8] m.p. 216°. R_f = 0·27 on SiO_2 TLC, solvent benzene–MeOH–HOAc, 20:3:2. λ_{max}^{MeOH} 292 nm (ϵ 5880), M^+ 184. Activity 2·04 × 10^6 dpm/mmol, corresponding to 39·7% of the total activity of I.

Kuhn–Roth degradation of I *labelled with methionine.* I (50 mg) in 4 N chromic acid–conc. H_2SO_4 (4:1) (4 ml) was refluxed for 2 hr. The HOAc (29 mg) was collected and a part converted to the *p*-bromophenacylacetate[9] which had SA 4·17 × 10^6 dpm/mmol, corresponding to 80·8% of the activity of compound I. A second portion as dry NaOCOMe (15 mg) was submitted to a Schmidt decarboxylation[10] at 45° in conc. H_2SO_4 (0·9 ml) with NaN_3 (60 mg). The CO_2 formed trapped as $BaCO_3$ showed no activity. Excess alkali

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was added to the acid solution and the $MeNH_2$ was steam-distilled and trapped in 0·5% 2,4-dinitrofluorobenzene in EtOH. 2,4-Dinitro-*N*-methylaniline was crystallized overnight. M.p. 177° (from EtOH). SA $4{\cdot}15 \times 10^6$ dpm/mmol, corresponding to 80·5% of the activity of the starting material.

Kuhn–Roth degradation of I *labelled with* [2-^{14}C-]*acetate.* I (50 mg) was submitted to a Kuhn–Roth oxidation under the same conditions as before and yielded HOAc (27 mg). The *p*-bromophenacylacetate had SA $1{\cdot}275 \times 10^6$ dpm/mmol, corresponding to 51·8% of the activity of I. The 2,4-dinitro-*N*-methylaniline had SA $2{\cdot}72 \times 10^5$, corresponding to 11·05% of the total activity of I. The $BaCO_3$ had SA $7{\cdot}7 \times 10^5$ dpm/mmol uncorrected, corresponding to 31·4% of the total activity of I.

Oxidation with $KMnO_4$ *of* I *labelled with* [2-^{14}C-]*acetate.* I (201 mg) was oxidized and worked up as described above. The acid II had SA $7{\cdot}6 \times 10^5$ dpm/mmol, corresponding to 31% of the activity of I. Subsequent Kuhn–Roth oxidation of the acid II (10 mg) gave acetic acid (3·2 mg) which was converted to the *p*-bromophenacylacetate which had SA $2{\cdot}61 \times 10^5$, corresponding to 10·6% of the activity of I.

Decarboxylation of II *formed by oxidation of* I *labelled with* [2-^{14}C-]*acetate.* II was inert towards standard Schmidt decarboxylation (H_2SO_4–NaN_3). Treatment of II (10 mg) with $SOCl_2$ (3 ml) gave the acid chloride; this was converted to the acid azide shown by the characteristic band $\nu_{max}^{CHCl_3}$ 2150 cm^{-1} (–CON_3). Heating the azide with conc. H_2SO_4 (0·4 ml) in trichloro-ethylene (2·5 ml) yielded CO_2 trapped as $BaCO_3$ (32 mg) which had SA $8{\cdot}2 \times 10^4$ dpm/mmol, corresponding to 3·3% of the activity of I.