

THE STRUCTURE AND BIOGENESIS OF DESOXYVERRUCARIN E, A METABOLITE OF *EUPENICILLIUM HIRAYAMAE*

R. R. ARNDT, C. W. HOLZAPFEL

Department of Chemistry Rand Afrikaans University, Johannesburg, South Africa

N. P. FERREIRA and J. J. MARSH

Microbiology Research Group, Council for Scientific and Industrial Research,
Pretoria, South Africa

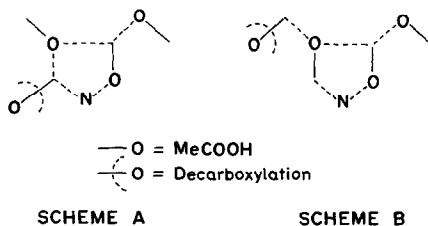
(Revised Received 5 February 1974)

Key Word Index—*Eupenicillium hirayamae*; structure; biogenesis; pyrrole; desoxyverrucarin E.

Abstract—Desoxyverrucarin E is the main nitrogenous metabolite of *Eupenicillium hirayamae*. On the basis of chemical and spectroscopic data the compound was identified as 3-acetyl-4-methylpyrrole. Radiochemical data showed that the compound is biogenetically derived from acetate.

INTRODUCTION

IN THE course of an investigation on the secondary nitrogenous products of the genus *Eupenicillium* a simple pyrrole compound was isolated from cultures of *E. hirayamae* Scott and Stolk. This product was identified as 3-acetyl-4-methylpyrrole and named desoxyverrucarin E (1). A closely related compound, verrucarin E (2), has previously been isolated from a culture of *Myrothecium verrucarium*.¹⁻³



RESULTS AND DISCUSSION

Desoxyverrucarin E synthesis was examined in batch cultures of *E. hirayamae* employing synthetic media. In this investigation, optimum yields of the metabolite were obtained in media which contained two carbon and energy sources. The two carbon sources were utilized in a typical diauxic growth cycle and the metabolite appeared in the culture filtrate after both substrates were exhausted.

¹ HARRI, E., LOEFLERR, W., SIGG, H. P., STÄHELIN, H., STOLL, CH., TAMM, CH and WIESINGER, D. (1962) *Helv. Chim. Acta* **45**, 839.

² FETZ, E. and TAMM, CH. (1966) *Helv. Chim. Acta* **49**, 349.

³ PFÄFFLI, P. and TAMM, CH. (1969) *Helv. Chim. Acta* **52**, 1911.

The compounds listed in Table 1 were utilized as carbon and energy source in the first phase of the growth cycle and the di- or tricarboxylic acids listed in Table 2 satisfied both the carbon and energy requirements of the fungus in the second phase of the growth cycle. Glucose and L(+)-tartaric acid were employed as carbon and energy sources in all the experiments described in this paper.

TABLE 1. THE EFFECT OF VARIOUS CARBON AND ENERGY SOURCES ON THE PRODUCTION OF DESOXYVERRUCARIN E IN THE BASAL MEDIUM PLUS 0.33% AMMONIUM TARTRATE

Compound added	Dry wt (g/200 ml)	Product yield (mg/l)
Glucose	1.40	32.4
Fructose	1.25	18.8
Sucrose	1.40	28.5
Maltose	1.40	27.6
Sodium acetate + glucose	1.35	18.6
Starch	0.70	12.3
Glycerol	1.16	15.6

The concentration of the carbon and energy sources added to the culture medium was equivalent to 1.25% glucose. Incubation period 6 days.

TABLE 2. THE EFFECT OF VARIOUS ORGANIC ACIDS ON THE PRODUCTION OF DESOXYVERRUCARIN E IN THE BASAL MEDIUM PLUS 1.25% GLUCOSE

Organic acid	Dry wt (g/200 ml)	Desoxyverrucarin E concentration (mg/l)
L(+)-Tartaric acid	1.20	28.5
Citric acid	1.26	25.5
Succinic acid	1.29	23.25
Malic acid	1.30	13.5
Lactic acid	1.39	7.5
Acetic acid	1.21	—

The concentration of organic acid added to the culture medium was equivalent to 0.33% tartaric acid. Incubation period 6 days.

The results, summarized in Table 3, show that the concentration of glucose has a marked effect on the utilization of tartrate. The metabolism of this acid in the second phase of the growth cycle is inhibited in cultures in which the glucose concentration was varied above or below the optimum concentration level. This inhibition of the second phase of the growth cycle has an adverse effect on the production of the metabolite.

The effect of different isomers of tartaric acid on the production of desoxyverrucarin E is shown in Table 4. D(–)-tartaric acid was not utilized by the organism and media containing this isomer did not support the production of the metabolite. The addition of D(–)-tartaric acid to a medium containing L(+)-tartaric acid or mesotartaric acid did not, however, affect the utilization of the latter isomers or the production of desoxyverrucarin E. These results suggest that the occurrence of a diauxic growth cycle is essential for the production of desoxyverrucarin E.

TABLE 3. THE EFFECT OF THE GLUCOSE CONCENTRATION ON THE PRODUCTION OF DESOXYVERRUCARIN E IN A MEDIUM CONTAINING 0.33% L(+)TARTARIC ACID

Glucose concentration (g/200 ml)	Dry wt (g/200 ml)	Residual tartaric acid (mg/ml)	Desoxyverrucarin E concentration (mg/l.)
0.25	0.71	3.0	3.25
0.5	1.23	1.09	5.0
1.25	1.60	0.0	16.3
2.0	2.05	1.2	6.0
4.0	2.42	2.9	0.0
6.0	2.74	3.1	0.0

Incubation period 6 days.

TABLE 4. THE EFFECT OF TARTARIC ACID ISOMERS ON GROWTH AND DESOXYVERRUCARIN E PRODUCTION IN A MEDIUM CONTAINING 1.25% GLUCOSE

Tartrate isomer	Dry wt (g/200 ml)	Residual tartaric acid (mg/ml)	Desoxyverrucarin E concentration (mg/l.)
L(+) -Tartrate	1.15	0.0	24.5
Meso-tartrate	1.21	—	23.2
D(−) -Tartrate	0.86	3.1	—
L(+) -Tartrate + D(−) -tartrate	1.10	1.45	22.6
Meso-tartrate + D(−) -tartrate	1.15	1.54	24.4
	0.96	—	—

Tartaric acid isomers were added to the culture medium in concentration of 0.33%. Incubation period 6 days.

Purification of the metabolite was carried out by chromatography on a formamide impregnated cellulose column followed by chromatography on silica gel and finally crystallization. Accurate mass determination of the metabolite indicated a molecular formula of C_7H_9NO . It gave a red colour with Ehrlich's reagent suggesting that it is a substituted pyrrole. The UV spectral max 247 and 277 nm (*sh*), ϵ 11 070 and 2204 respectively, is typical of a β -acetylpyrrole.⁴ The IR spectrum showed *inter alia* absorption bands at 3480 cm^{-1} (vinylogous amide NH) and 1660 cm^{-1} (vinylogous amide CO).

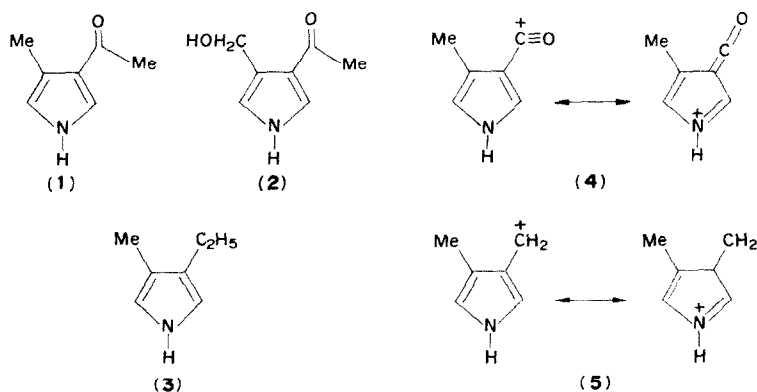
The PMR spectrum showed two singlets each representing three protons at δ 2.26 and δ 2.38. These signals are ascribed to an acetyl methyl and a vinyl methyl group. The remaining two signals each representing one vinylic proton appeared at δ 6.42 and δ 7.25. Both these peaks showed some fine splitting.

The MS was most informative with regard to the placing of the acetyl group on the pyrrole skeleton. The base peak of the spectrum appeared at M-15 (*m/e* 108) which indicated the loss of a methyl group to give a stabilized acylium ion **4**. Only a small peak at M-43 (*m/e* 80) is present due to the loss of the acetyl group. This fragmentation pattern leads to the conclusion that the acetyl group is situated in the β -position because α -acetylated

⁴ EISNER, U. and GORE, P. H. (1958) *J. Chem. Soc.* 922.

pyrroles are known to give strong M-43 peaks while β -acetylated do not lose the acetyl group readily on electron bombardment.⁵

On reduction with LiAlH_4 a new compound with molecular formula $\text{C}_7\text{H}_{11}\text{N}$ ($M^+ 109$) was obtained. The IR spectrum of this reduction product did not show any carbonyl absorption. The PMR spectrum showed a typical A_2X_3 pattern of signals: a triplet at δ 1.18 (J 7 Hz) and a quartet at δ 2.45 (J 7 Hz). This pattern is ascribed to an ethyl group linked to a carbon-carbon double bond. A three proton singlet at δ 2.04 is in accordance with reported resonance frequency of a methyl on the C-3 of the pyrrole ring.⁶ In the vinylic proton region a signal representing two protons is present at δ 6.46. A very small splitting ($J=0$, 5 Hz) that disappears on treatment with D_2O is observable in this signal. In the mass spectrum the base peak appears at $M-15$ (m/e 94) which is the expected major fragment **5** in the mass spectra of C-ethylpyrroles.⁵ It is known that 3-acetylpyrroles being vinylogous amides are reduced to 3-ethylpyrroles with LiAlH_4 hydride.⁷



The above data indicate that the metabolite isolated is 3-acetyl-4-methylpyrrole (**1**), the desoxy derivative of verrucarín E^{2,3} (**2**) and therefore it follows that the reduction product is the 3-ethyl-4-methylpyrrole (**3**). Final proof of structure **1** was obtained by direct comparison with an authentic sample of the synthetic 3-acetyl-4-methylpyrrole.*

In view of the similarity in structure of desoxyverrucarin E (**1**) to verrucarín E (**2**)^{2,3} it appeared likely that these compounds have the same biogenetic origin. Pfäffli and Tamm⁸ showed that verrucarín E (**2**) is branch-chain acetate derived, either according to scheme A or B. In order to establish whether desoxyverrucarin E is derived in a similar manner, acetate-[1-¹⁴C] was administered to a culture of *E. hirayamae*. The radioactive label was incorporated into desoxyverrucarin E to the extent of 2%. Acetate-[2-¹⁴C] was incorporated to the same extent. A Kuhn-Roth oxidation of the [1-¹⁴C] labelled product yielded two equivalents of acetic acid, a portion of which was converted into its *p*-bromophenacyl ester and recrystallized to constant activity. It was found that the specific activity of the acetic acid was approximately a quarter of that of the original compound. Degradation

* Authentic sample was generously supplied by Prof. Ch. Tamm, Institut für Organische Chemie, Basel.

⁵ BUDZIKIEWICZ, H., DIERASSI, C., JACKSON, A. H., KENNER, G. W., NEWMAN, D. J. and WILSON, J. M. (1964) *J. Chem. Soc.* 1949.

⁶ HINMAN, R. L. and THEODOROPULOS, S. (1963) *J. Org. Chem.* **28**, 3052.

⁷ TREIBS, A. and SCHERRER, H. (1952) *Liebigs Ann. Chem.* **577**, 139.

⁸ PFÄFFLI, P. and TAMM, CH. (1969) *Helv. Chim. Acta*, **52**, 1921.

of the labelled acetic acid by the Schmidt procedure showed that the compound contained all its activity in the carboxyl moiety. These results indicate that desoxyverrucarin E is constructed from 4 acetate units according to either scheme A or B.

According to Scott⁹ the conidial state of *E. hirayamae* bears a striking morphological resemblance to *Penicillium thomii* and *P. sclerotiorum*. Moreover, the major component of the colouring matter in *E. hirayamae* was reported to be a laevorotary isomer of sclerotiorin, the red pigment which is commonly produced in *P. sclerotiorum*. This pigment is synthesized according to the polyketide scheme.¹⁰ In the genus *Eupenicillium*, *E. hirayamae* is placed near *E. parvum*. This latter fungus in turn closely resembles *E. javanicum* and *E. lapidosum*.

Representative strains of all the aforementioned fungal species were, therefore, screened for their ability to synthesize pyrrole metabolites. The synthetic culture medium formulated for the study of desoxyverrucarin E synthesis as well as Czapek–Dox solution and a Czapek–Dox solution supplemented with 1% corn steep liquor were employed as culture media in this survey. It was found that none of the morphologically related species produced pyrrole type compounds in detectable amounts.

EXPERIMENTAL

M.p.s were determined on a Kofler apparatus. UV spectra were recorded in 96% EtOH, IR spectra in CHCl_3 . PMR spectra were taken on a 60 MHz instrument with TMS as internal standard in CDCl_3 soln. Radioactivity was assayed on a Packard Tri-Carb liquid scintillation spectrometer Model 574. Organic compounds were analysed in toluene as scintillation solvent containing PPO and DM-POPOP as scintillation solute. ^{14}C - BaCO_3 was assayed by suspension scintillation counting¹¹ in the same scintillator mixture which contained ca 4% (w/v) of Cab-o-sil as gelling agent. All samples were counted for a minimum of 10^4 counts.

Organism. *Eupenicillium hirayamae* Scott and Stolk CSIR 1112 was used throughout this investigation. The strain was maintained on oatmeal agar in the ascospore stage. Ascospores were obtained after a 4–6 week incubation period.

Inoculum and cultural conditions. Ascospores were obtained by scraping the surface growth of a 6-week-old culture. The ascospores were activated by heating at 63° for 5 min and used to inoculate 200 ml of the culture medium in a 500 ml flask. The mycelium was harvested by centrifugation after a 48 hr incubation period on a rotary shaker at 180 rev/min and 28° , and washed twice with sterile distilled H_2O by centrifugation. The washed mycelium was resuspended in 100 ml sterile distilled H_2O and homogenized aseptically for 10 sec. Five ml of the resultant homogenate was used to inoculate 200 ml of the culture medium in a 500 ml flask. All cultures were incubated for 6 days under similar conditions to those used for the preparation of the inoculum.

Culture medium. The basal culture medium had the following composition: KH_2PO_4 5.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.5 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 24.2 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 21.99 mg; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 5.95 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 3.93 mg; $(\text{NH}_4)_6\text{Mo}_7 \cdot 4\text{H}_2\text{O}$ 2.52 mg; distilled H_2O 1 l. Nitrogen was supplied in the form of the ammonium salts of organic acids in a final concentration of 0.4 g N/l. The final pH of the medium was adjusted to pH 6.0 with KOH. Carbon and energy sources are described in the appropriate context.

Conc of desoxyverrucarin E. The conc desoxyverrucarin E was calculated using the molar extinction coefficient $\epsilon 11070$ at 247 nm.

TLC. Extracts containing desoxyverrucarin E were spotted on Merck silica G plates and developed in CHCl_3 –MeOH (9:1). The chromatoplates were sprayed with Ehrlich's indole reagent. Desoxyverrucarin E appeared as a clear red spot.

Analytical methods. Tartrate was determined as described by La Rivière.¹²

Isolation and purification of desoxyverrucarin E. The culture filtrate was acidified with 2 N H_2SO_4 to pH 2 and extracted with hexane to remove fats, waxes and other neutral material. The aq. soln was basified with NH_4OH to pH 9 and extracted with methylene chloride. The crude basic material was then chromatographed on cellulose powder impregnated with formamide. The metabolite was eluted with 10% CHCl_3 in C_6H_6 . The partially purified metabolite was chromatographed on silica eluted with CH_2Cl_2 , crystallized from a mixture of C_6H_6 and

⁹ SCOTT, DE B. (1968) Ph.D. Thesis. University of Cape Town.

¹⁰ UDAGAWA, S. (1963) *Chem. Pharm. Bull.* **11**, 366.

¹¹ CHILEY, H. J. (1962) *Analyst* **87**, 170.

¹² LA RIVIÈRE, J. W. M. (1958) Thesis. Technical University Delft.

hexane as colourless crystals m.p. 115°. The crystalline metabolite sublimed at 40/0.2 mm Hg. Found M^+ 123.0684. Calculated for C_7H_9NO , M^+ 123.0684.

3-Ethyl-4-methylpyrrole. 3-Acetyl-4-methylpyrrole (200 mg) was dissolved in absolute ether (40 ml) and $LiAlH_4$ added (200 mg). The mixture was heated under reflux for 5 hr, when a few drops of saturated aqueous Na_2SO_4 soln were added. The mixture was again heated under reflux for 0.5 hr. The reaction mixture was filtered and the ether evaporated. The residue was a colourless oil (140 mg). According to TLC this product was homogenous and pure, M^+ 109 $C_7H_{11}N$ calc. MW 109.

Preparation of labelled desoxyverrucarin E. Sodium acetate- $[1-^{14}C]$ (25 μ Ci) was added to 4-day-old cultures and the metabolite extracted 24 hr after the addition of the labelled substrate. Inactive desoxyverrucarin E (100 mg) was added to the crude extract. This material was chromatographed on a column packed with formamide impregnated cellulose (10 g). The crystalline desoxyverrucarin E was sublimed (1×10^{-3} mm Hg, 60°) to constant activity to yield colourless crystals (118 mg) m.p. 115°, with specific activity (SA) 1.132×10^6 counts/min $mmol^{-1}$ (incorporation 1.98%). Incubation of the fungus with 25 μ Ci of acetate- $[2-^{14}C]$ yielded labelled desoxyverrucarin E with SA 0.966×10^6 counts/min $mmol^{-1}$ (incorporation 1.7%).

Kuhn-Roth degradation¹³ of desoxyverrucarin E labelled with acetate- $[1-^{14}C]$. Labelled desoxyverrucarin E (60 mg 0.488 mmol); in 4 N chromic acid-conc H_2SO_4 (4:1), v/v (4.5 ml) was heated under reflux for 2 hr. The acetic acid was collected (1.95 mmol) and part converted into the *p*-bromophenacylacetate¹⁴ which, after recrystallization to constant activity had SA 3.022×10^5 cpm $mmol^{-1}$ corresponding to 26.1% of the sp act. of desoxyverrucarin E. A second portion as the dry $Na OCOCH_3$ (10.0 mg) was submitted to the Schmidt decarboxylation¹⁵ at 40°C in conc H_2SO_4 (0.6 ml) with $Na N_3$ (40 mg). The CO_2 formed, trapped as $BaCO_3$, had SA 2.915×10^5 cpm $mmol^{-1}$ corresponding to 95% of the activity of the acetic acid. The methylamine from the reaction was trapped as the picrate and contained no radioactivity.

¹³ WIESENBERGER, E. (1948) *Mikrochim. Acta* **33**, 51.

¹⁴ VOGEL, A. I. (1961) *Practical Organic Chemistry*, p. 362. Longmans, London.

¹⁵ PHARES, E. F. (1951) *Arch. Biochem. Biophys.* **33**, 173.