Phytochemistry, 1977, Vol. 16, pp. 899–901. Pergamon Press. Printed in England.

SYNTHESIS OF TRYPTOPHOL AND *O*-ACETYLTRYPTOPHOL FROM TRYPTOPHAN BY *CERATOCYSTIS FAGACEARUM*

PATRICK FENN*, RICHARD D. DURBIN and JAMES E. KUNTZ

Plant Disease Resistance Research Unit, ARS, USDA, Department of Plant Pathology, University of Wisconsin, Madison, WI 53706 U.S.A.

(*Received* 3 *November* 1976)

Key Word Index—*Ceratocystis fagacearum*; fungus; oak wilt; biosynthesis; indoles; tryptophan; tryptophol; *O*-acetyltryptophol.

Abstract—Tryptophol (TOL) and *O*-acetyltryptophol (*O*AcTOL) were identified as tryptophan metabolites of *C. fagacearum*; *O*AcTOL was the only metabolic product of TOL detected. Yields of both indoles were increased by buffering the cultures during growth with $CaCO_3$. This is the first report of *O*AcTOL synthesis by a fungus.

INTRODUCTION

Tryptophol (indole-3-enthanol) (TOL) occurs as a natural product in plants [1–3], and has been frequently reported as a tryptophan (TPP) and tryptamine (TNH_2) metabolite in plant tissues and microorganisms where it accumulates as a byproduct of IAA synthesis [1–6]. Except for the oxidation of exogenous TOL to IAA by plant tissues and *Agrobacterium tumefaciens* [2–5] and the conversion of TOL to TOL-β-D-glucopyranoside in pea (*Pisum sativum*) [5], no other pathways of metabolism of TOL have been reported.

Investigations of the TPP metabolism of a strain of *Bacillus cereus* revealed two compounds tentatively identified as acetylated derivatives of TOL, but neither compound was fully characterized nor were details of their syntheses reported [6]. However, a significant result of this study was the finding that the conversion of TPP to TNH_2, TOL and other indoles was sensitive to culture pH [6]. The influence of pH and other culture conditions on the syntheses of simple indoles by microorganisms has rarely been reported and is inadequately understood [7].

The present paper reports the identification of *O*-acetyltryptophol (*O*AcTOL) and TOL as TPP metabolites of *C. fagacearum*, and the conversion of TPP and TOL to *O*AcTOL by this fungus. Also reported is the effect of culture pH on the synthesis of these indoles.

RESULTS

Two compounds (R_fs 0.42 and 0.55 on Si gel G, $CHCl_3$–HOAc, 19:1), which gave characteristic violet reactions for 3-substituted indoles with acid *p*-dimethylaminobenzaldehyde (DMAB), were detected in the neutral and basic EtOAc-soluble extract from filtrates of TPP-supplemented cultures. A purified sample of the compound with R_f 0.42 and an authentic sample of TOL showed identical mobility on Si gel G; $CHCl_3$–HOAc, (19:1), R_f 0.42; $CHCl_3$–MeOH–HOAc, (15:4:1), R_f 0.86; *iso*PrOH–NH_4OH–H_2O, (10:1:1), R_f 0.90; and gave the same violet color with DMAB and a brown color with $FeCl_3$ in $HClO_4$ [8]. MS of the unknown and of TOL were identical and were in agreement with published spectra [1, 9].

The less polar compound (R_f 0.55) was not identical on TLC to authentic samples of the following common neutral and basic indoles: indole, indole-3-acetonitrile, indole-3-acetamide, indole-3-aldehyde, indole-3-acetaldehyde, ethyl indole-3-acetate, tryptamine and a prepared sample of *N*-acetyltryptamine. It was extracted from culture filtrates at pH 8.5 equally with either EtOAc, Et_2O or CH_2Cl_2, which indicated that the compound was not an artifact of EtOAc extraction at alkaline pH [10]. TLC of a basic hydrolysate (10% NaOH at 24° for 3 hr) of the compound revealed TOL as the only DMAB-reactive product. MS of the unknown showed M^+ at 203, base ion at *m/e* 143 (M^+-HOAc), and a major ion at *m/e* 130 which is characteristic of indoles of the indole-3-CH_2R type [9]. These data suggested that this compound was the *O*-acetyl ester of TOL. Subsequent comparison of the unknown with a synthetic sample of *O*AcTOL showed that they had identical MS and the same chromatographic mobility in the following solvent systems on Si gel G; MeCOEt–*n*-hexane (1:1), R_f 0.78; $CHCl_3$–HOAc (19:1), R_f 0.53; $CHCl_3$, R_f 0.35; *iso*PrOH–NH_4OH–H_2O (10:1:1), R_f 0.85; CH_2Cl_2, R_f 0.47; Et_2O–*n*-hexane (1:1), R_f 0.44.

An *O*AcTOL conc of 0.19 μM was found in filtrates from 12-day-old cultures (late growth phase) on BM plus 1 mM L-TPP. Small amounts of TOL and *O*AcTOL were detected by TLC in the EtOAc-soluble compounds from MeOH extracts of mycelium. Neither TOL nor *O*AcTOL were detected in extracts of culture filtrates or mycelium from cultures not supplemented with TPP.

When TOL-[2-^{14}C) was fed to mycelium, 7.3% of the ^{14}C initially added was recovered in *O*AcTOL present in the culture filtrate after 4.5 days incubation (Table 1). Between 1.5 and 4.5 days, a 3.4% (84.4–81.0) decrease in the initial ^{14}C in TOL was more than accounted for by a 5.1% (2.2–7.3) increase in the initial ^{14}C in *O*AcTOL. During this conversion, the total

* Present address, Department of Plant Pathology and Agricultural Entomology, University of Sydney, Sydney, New South Wales 2006, Australia.

Table 1. Conversion of tryptophol-[2-^{14}C] to *O*-acetyl tryptophol-[^{14}C] by *C. fagacearum*

Incubation period* (days)	% initial ^{14}C in TOL	% initial ^{14}C in *O*AcTOL	% initial ^{14}C in TOL + *O*AcTOL	% initial ^{14}C unaccounted for in TOL and *O*AcTOL	Culture pH
0	100	0	100	0	6.4
1.5	84.4	2.2	86.6	13.4	6.0
2.5	83.7	3.7	87.4	12.6	5.7
3.5	82.9	6.5	89.4	10.6	5.5
4.5	81.0	7.3	88.3	11.7	5.5

* Mycelium from a 3-day-old culture on BM plus 0.5 mM L-TPP was washed with sterile dist H_2O and transferred to 100 ml of fresh BM containing 0.5 mM L-TPP and 0.1 mM TOL-[2-^{14}C] (sp. act. 25 μCi/mmol). At the times indicated, 10 ml aliquots of the culture medium were removed and the ^{14}C in TOL and *O*AcTOL determined. Initial ^{14}C = 86 500 cpm as TOL-[2-^{14}C] per 10 ml culture medium.

^{14}C recovered in TOL and *O*AcTOL remained constant at 87–89% of the initial ^{14}C added as TOL-[2-^{14}C]. TLC and radioscans of the EtOAc-soluble neutral and basic fraction revealed TOL and *O*AcTOL as the only radiolabeled compounds. No radioactivity was detected in the EtOAc-soluble acid (pH 3) fraction. These results indicate that the only detectable pathway of TOL metabolism in *C. fagacearum* involves its conversion to *O*AcTOL, since metabolism by other routes would have resulted in a detectable increase in the ^{14}C unaccounted for in TOL and *O*AcTOL during incubation. The 10–13% of the initial ^{14}C unaccounted for probably represented the intramycelial pool of labeled TOL and *O*AcTOL maintained during the experiment.

When DL-TPP-[2-^{14}C] (sp. act. 0.081 mCi/mmol) was added to cultures, the TOL and *O*AcTOL isolated after 12 days both had a sp. act. of 0.068 mCi/mmol. This supports the conclusion that TOL is the immediate precursor of *O*AcTOL and suggests that TOL is an intermediate in the synthesis of *O*AcTOL from TPP.

Accumulation of TOL and *O*AcTOL in the culture filtrates during fungal growth showed that *O*AcTOL production lagged behind that of TOL, as would be expected if TOL were the precursor of *O*AcTOL. The increases in TOL and *O*AcTOL in culture filtrates did not coincide with the period (3–15 days) of maximum growth (dry wt increase) of the fungus (Fig. 1a). It was found that the decline in culture pH during growth (Fig. 1b) had a limiting effect on the metabolism of TPP to TOL and *O*AcTOL. TOL accumulated rapidly between 3 and 5 days on basal medium plus 0.5 mM DL-TPP-[2-^{14}C], but showed little change thereafter (Fig. 1c). *O*AcTOL levels were very low but showed a slight increase during the rapid growth phase. Addition of $CaCO_3$ as a buffer resulted in large increases; 3 times for TOL and 7 times for *O*AcTOL, as compared to unbuffered cultures (Fig. 1c and d). The inability to completely remove the finely powdered $CaCO_3$ from the mycelium in the buffered cultures prevented accurate dry wt determinations. However, comparable amounts of growth were observed in the buffered and unbuffered cultures, and any small differences could not have accounted for the large differences in the amounts of TOL and *O*AcTOL produced. Also, *O*AcTOL was shown to be stable in aq. soln at pH 3–7 (28°, 18 hr); therefore nonenzymic degradation could not account for the smaller amounts of *O*AcTOL found in unbuffered cultures. The similarities in the amounts of TOL and *O*AcTOL found in the buffered and unbuffered cultures from 0–5 days, followed by the great increases in the buffered treatment after day 5 indicated that the effect of culture acidity on TOL and *O*AcTOL production commenced when the pH dropped below *ca* 5.8.

DISCUSSION

*O*AcTOL was identified as a TPP metabolite of *C. fagacearum*. This is the first report of the synthesis of this indole by a fungus. Indeed, except for the possible occurrence of *O*AcTOL as one of the TOL derivatives of *B. cereus* [6], OAcTOL has not been reported from any other natural source.

*O*AcTOL was the only product of the metabolism of TOL by this fungus. This fact and the identical sp. act. of TOL-[^{14}C] and *O*AcTOL-[^{14}C] produced from

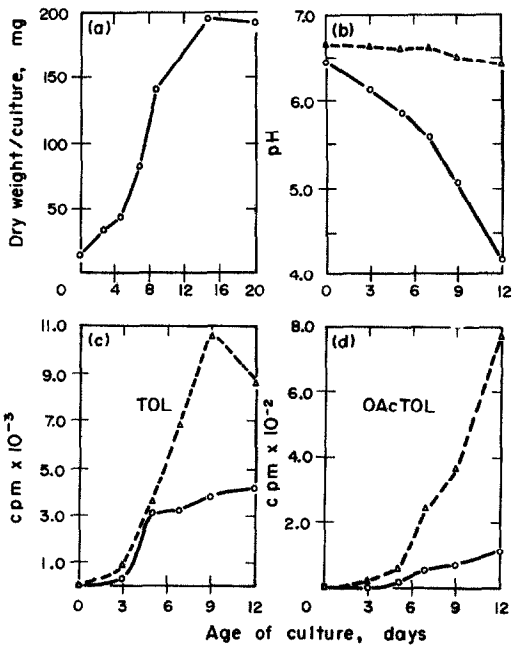

Fig. 1. Growth of *C. fagacearum* and production of TOL-[^{14}C] and *O*AcTOL-[^{14}C] from TPP-[^{14}C] in unbuffered (O———O) and $CaCO_3$ buffered (Δ------Δ) cultures. (a) growth rate, (b) pH of culture, (c) TOL-[^{14}C] in culture filtrates, (d) *O*AcTOL-[^{14}C] in culture filtrates. Cultures (200 ml) contained 0.5 mM L-TPP plus 12 μCi DL-TPP-methylene-[^{14}C] (sp. act. 57 mCi/mmol). Buffered cultures contained in addition, 1 g $CaCO_3$. Data points for growth rate represent average dry wt of 5 cultures. Data points for radioactivity are cpm in TOL or *O*AcTOL from aliquots (20 ml) of culture filtrate.

TPP-[^{14}C] suggest that TOL is an intermediate in the synthesis of *O*AcTOL from TPP. However, the sp. act. data do not rule out the possibility of other pathways of conversion of TPP to *O*AcTOL not involving TOL as an intermediate. Also, because of the long feeding period (12 days) necessary to obtain sufficient *O*AcTOL for accurate measurement and the possible randomization of the ^{14}C, localization of the ^{14}C in TOL and *O*AcTOL would be necessary to support the intermediate role of TOL in *O*AcTOL synthesis.

The effect of culture acidity in suppressing conversion of TPP to TOL and *O*AcTOL was shown by the several-fold increases in the production of both indoles in buffered cultures. Data on pH changes in the culture medium during the conversion of TOL to *O*AcTOL (Table 1) showed that this conversion was not limited at pHs <5.8. The amount of ^{14}C incorporated into *O*AcTOL, 3.6%, (3.7 to 7.3%) between 2.5 and 4.5 days when the pH changed from 5.7 to 5.5, was equal to that incorporated between 0 and 2.5 days when the pH dropped from 6.4 to 5.7. This evidence suggests then, that the accumulations of TOL and *O*AcTOL in unbuffered cultures did not parallel fungus growth because $[H_3O^+]$ became limiting to the conversion of TPP to TOL below a pH of *ca* 5.8.

A similar and unexplained effect of culture pH on the metabolism of TPP to simple indoles was reported for a strain of *B. cereus* [6]. TNH_2, TOL, and several acetylated indoles were not detected in cultures when the pH ranged from 5 to 6.5, but only in old stationary cultures when the pH was >6.5. Adjustment to pH 8 of cultures at maximum cell density resulted in production of these indoles from TPP within 5 hr.

How culture pH could affect the metabolism of TPP to TOL and other indoles is unknown. Altered growth and instability of the products were not responsible for the pH effect observed in the present study. Cell-free extracts from *B. cereus* were not active in converting TPP to TOL and lacked any acetylation activity, so that the pH effect on the enzymes could not be tested [6]. TPP uptake by fungi has been shown to decline with decreased culture pH [11]. However, *C. fagacearum* continued to convert TPP to IAA in growing cultures when the pH decreased from 6.4 to 4.5. This suggests that TPP uptake was not a limiting factor in TOL synthesis (Fenn and Durbin, unpublished). A possible explanation for these pH effects is the presence of extracellular enzymes capable of converting TPP to simple indoles whose catalytic activity and/or stability is affected by changes in culture pH. Similar enzymes which convert TPP to IAA were demonstrated recently in the cell-free filtrate of soybean suspension cultures [12].

EXPERIMENTAL

The isolate of *C. fagacearum* (Bretz) Hunt and the maintenance of stock cultures have been described [13]. The fungus remained pathogenic to oak throughout these studies. The basal medium (BM) contained; 3 g L-asparagine . H_2O, 1 g K_2HPO_4, 1 g KH_2PO_4, 0.5 g $MgSO_4 . 7H_2O$, 0.01 g $FeCl_3 . 6H_2O$, 0.304 mg $MnSO_4 . H_2O$, 0.822 mg $ZnSO_4 . 7H_2O$, 0.375 mg $CuCl_2 . 2H_2O$, 0.25 mg $Na_2MoO_4 . 2H_2O$, 0.028 mg H_3BO_3, and 25 g D-glucose per l. and was autoclaved at 121° and 1.3 kg/cm^2 for 15 min. DL- and L-tryptophan were sterilized by filtration and TOL was dissolved in a min. vol. of EtOH before addition to the cool, sterile BM. Cultures (80 ml/500 ml flask) were inoculated with 7 mm diam disks cut from 12- to 15-day-old cultures on BM plus 1.0 mM L-TPP and 2% agar. Cultures were incubated without shaking in the dark at 28°. Culture filtrates were adjusted to pH 8.5 with M Na_2CO_3 and extracted $\times 3$ with EtOAc or in some cases with Et_2O or CH_2Cl_2. Solvent phases were dried (Na_2SO_4) and the solvent removed under red. press. at 35°. Residues were fractionated by TLC on Si gel G or H. Compounds were detected under UV with parallel markers of TOL and *O*AcTOL and/or with acid DMAB [8]. TOL and *O*AcTOL were eluted from Si gel with MeOH (85–90% recovery) and determined colorimetrically [14]. $A_{580\,nm}$ was read 80 min after combining the sample and reagents. Concn was proportional to A_{589} (0–2) for both indoles. For radio-TLC, the Si gel was scraped into scintillation vials containing 10 ml of cocktail (4 g PPO, 0.2 g DiMePOPOP, 60 g naphthalene, 100 ml MeOH, 20 ml ethylene glycol, and *p*-dioxane to one l.) and after 24 hr the samples were counted for 10 min. Samples for MS were purified by TLC (Si gel H) from the EtOAc-sol fraction from 15 l. of culture filtrate. Plates were developed with $CHCl_3$–HOAc (19:1) and TOL and *O*AcTOL were eluted with MeOH; TOL was purified by TLC with $CHCl_3$–EtOH (9:1) and eluted with EtOAc. *O*AcTOL was purified by TLC with $CHCl_3$ and eluted with EtOAc. *O*AcTOL was prepared from TOL, Ac_2O and Py (100°, 2 hr). The cooled mixture was diluted with H_2O, neutralised and extracted with EtOAc. *O*AcTOL was purified by successive TLC (Si gel H) and elution steps with $CHCl_3$–HOAc (19:1) and CH_2Cl_2 solvent systems. The product, a light yellow oil, was homogeneous on TLC (3 systems) and gave a MS (probe) 70 eV *m/e* (rel. int.): 203 M^+ (10), 144 (M^+-$C_2H_3O_2$; 22); 143 (M^+-HOAc; 100), 130 (M^+-$C_3H_5O_2$; 81), 103 (130-HCN; 12). 77 (103-C_2H_2; 30), 51 (77-C_2H_2; 12) as predicted for *O*AcTOL [5, 9, 15]. TOL-[2-^{14}C] was prepared by $LiAlH_4$ reduction of IAA-[2-^{14}C] in dry Et_2O (24°, 30 hr). After addition of H_2O, the Et_2O phase was evaporated and the residue chromatographed on Si gel G with $CHCl_3$-HOAc (19:1). The TOL-[^{14}C] was eluted and chromatographed on Si gel H with the same solvent system. The product was homogeneous on TLC (2 systems) and $\geq 98\%$ radiochemically pure. *N*-acetyltryptamine was prepared from tryptamine HCl according to ref. [16]. Radiochemicals were purchased from Amersham–Searle and were used without purification.

REFERENCES

1. Rayle, D. L. and Purves, W. K. (1967) *Plant Physiol.* **42**, 520.
2. Rajagopal, R. (1967) *Physiol. Plantarum* **20**, 655.
3. Rayle, D. L. and Purves, W. K. (1967) *Plant Physiol.* **42**, 1091.
4. Kaper, J. M. and Veldstra, H. (1958) *Biochim. Biophys. Acta* **30**, 401.
5. Magnus, V., Iskrić, S. and Kveder, S. (1973) *Planta* **110**, 57.
6. Perley, J. E. and Stowe, B. B. (1966) *Biochem J.* **100**, 169.
7. Gruen, H. E. (1959) *Ann. Rev. Plant Physiol.* **10**, 405.
8. Sequeira, L. and Kelman, A. (1962) *Phytopathology* **52**, 439.
9. Jameison, W. D. and Hitzinger, O. (1970) *Phytochemistry* **9**, 2029.
10. Hutinger, O. (1969) *Anal. Biochem.* **30**, 153.
11. Wiley, W. R. and Matchett, W. H. (1966) *J. Bacteriology* **92**, 1698.
12. Black, R. C. and Hamilton, R. H. (1976) *Plant Physiol.* **57**, 437.
13. Fenn, P., Durbin, R. D. and Kuntz, J. E. (1975) *Phytopathology* **65**, 1381.
14. Anthony, A. and Street, H. E. (1970) *New Phytol.* **69**, 47.
15. Powers, J. C. (1968) *J. Org. Chem.* **33**, 2044.
16. Späth, E. and Lederer, E. (1930) *Ber. Dtsch. Chem. Ges.* **63**, 120.