PROC. N.S. INST. SCI. (1995)
Volume 40, Part 2, pp. 61-65.

THE BIODEGRADATION OF L-TYROSINE BY *Trichoderma hamatum* TO TRICHOVIRIDIN AND RELATED COMPOUNDS#§

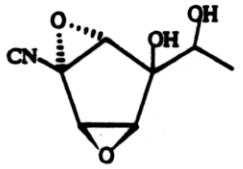
A.J. McALEES† and A. TAYLOR*
National Research Council Canada, Institute for Marine Biosciences
1411 Oxford Street, Halifax B3H 3Z1

Trichoviridin (**1**) is shown to be a degradation product of L-tyrosine by cultures of *Trichoderma hamatum*. The specific radioactivity of trichoviridin thus formed was lower than that of 3-isocyanocyclopent-2-enylidene propionic acid (**2**) a congenic catabolite which contains an additional labelled carbon atom from U-^{14}C-L-tyrosine. Evidence is presented that two other neutral isocyanides produced by the fungus are also degradation products of tyrosine.

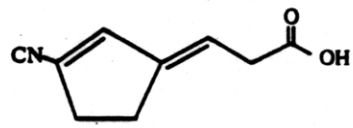
On a démontre que le trichoviridine est un produit de dégradation de L-tyrosine dans les cultures de *Trichoderma hamatum*. La radioactivité spécifique du trochoviridine obtenu ainsi est plus basse que celle de l'acide propionique de 3-isocyanocyclopent-2-enylidine, un métabolite congénère qui contient un atome de carbone étiqueté supplémentaire obtenu de la U-^{14}C-L-tyrosine. On présente l'évidence que deux autres isocyanures neutres produits par ce mycète sont aussi produits de dégradation de la tyrosine.

The biosynthesis of the acidic isocyanide (**2**) has been shown to involve the degradation of the amino acid L-tyrosine by cyclization of the side chain and oxidative fission, with loss of one carbon atom, of the phenolic ring (Baldwin, et al., 1985).

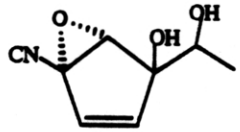
The antibiotic **2** is one of about 15 congeners (Boyd, et al., 1991), many of which are neutral C_8 entities (Fujiwara, et al., 1982). The most abundant of these bears the trivial name trichoviridin (**1**); its isolation and structure were reported by Nobuhara et al., (1976). The similarities in the structures of metabolites **1** and **2**, which both contain a five-carbon ring bearing an isocyano substituent and a side chain in a 1,3 relationship suggest that trichoviridin may be the result of further biological degradation of L-tyrosine.



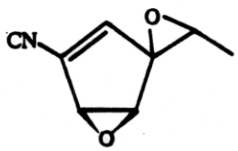
1



2



3



4

§ NRCC No. 38098

Dedicated to the memory of Walter J. Chute

* Author to whom correspondance should be addressed: present address 14 Canterbury Street, Dartmouth, Nova Scotia, B2Y 1S7.

† Present address: Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, N1G 2W1

U-^{14}C-L-Tyrosine (203 μCi) was added to a *Trichoderma hamatum* fermentation on a salts-glucose defined medium (Brewer et al., 1982) 24 h after inoculation and the culture was harvested 48 h later. The acidic isocyanide (**2**, 20 mg L^{-1}, ε_{270} 12000 dm^3 mol^{-1} cm^{-1}) was isolated in the usual way (Brewer et al., 1982) and its specific radioactivity was 2.76 mCi $mmol^{-1}$, representing a recovery of ^{14}C of 2.04%.

The ethyl acetate raffinate from which the acidic catabolites had been removed was radioactive (4.19 μCi = 2.06% of the radioactivity added to the fermentation). Chromatography gave three radioactive fractions which collectively accounted for 90% of the radioactivity of the ethyl acetate solution. Analytical thin layer chromatography showed that the fraction of greatest R_f co-chromatographed with trichoviridin. Colorless crystals (28.1 mg, m. p. 89-92°, 1.9 μCi $mmol^{-1}$) were obtained by slow evaporation of this fraction; they were collected and recrystallized from diethyl ether-hexane giving trichoviridin (**1**, 21 mg, m. p. 92° 1.82 μCi $mmol^{-1}$). This material eluted as a single Gaussian shaped peak on reversed phase partition chromatography of its Rh{η^5-$C_5Me_5(SCN)_2$} complex (Brewer et al., 1993), and its specific radioactivity was unchanged upon further recrystallization.

This trichoviridin was further characterized by reaction with veratric acid and dicyclohexylcarbodiimide in the presence of a trace of 4-dimethylpyridine (McAlees et al., 1990). The crystalline ester obtained had a single sharp absorption band at 3560 cm^{-1}; by contrast trichoviridin has absorption bands at 3580 and 3400 cm^{-1}. In the ^{1}H n.m.r. spectrum of trichoviridin the hydrogen on the carbon atom bearing the secondary hydroxyl group resonated at δ_H 3.76, but in the ester this quartet was shifted downfield to δ_H 5.11; the chemical shifts of all other hydrogens remaining approximately the same. It follows as would be expected from the kinetic data reported by Neises and Steglich (1978) that only the secondary hydroxyl group in trichoviridin had been esterified; another example of the remarkable selectivity of this reagent (Grove et al., 1988). This veratryl ester was recrystallized to constant specific radioactivity (1.82 μCi $mmol^{-1}$) and it can therefore be concluded that trichoviridin is a degradation product of L-tyrosine.

Among other C_8 isocyanides produced by *T. hamatum* growing on defined salts and glucose media are 1-(2,3-epoxy-1-hydroxy-3-isocyanocyclopent-4-enyl)ethanol (**3**) and isonitrin A (**4**, Fujiwara et al., 1982). The former (**3**) was characterized as its η^5-ethyltetramethylcyclopentadienyl)-di(thiocyanato)-rhodium complex (Boyd et al., 1991) but the catabolite was not purified to constant specific radioactivity. The sample used for preparing the complex was radioactive ($2.4x10^4$ d min^{-1} mg^{-1}). Fractions containing 'isonitrin A' (**4**) were also radioactive ($2.7x10^4$ d min^{-1} mg^{-1}) but we remain unable to thoroughly characterize this material (Boyd et al., 1991). If the radioactivities found for **3** and **4** are associated with these compounds rather than with accompanying minor impurities, it would appear that they are also formed by degradation of L-tyrosine.

Experimental

Details of instruments used for spectroscopic measurements and of the purification of reagents are given by McAlees et al. (1990). All extractions were done using a vibromixer in an efficient ventilated space. Effluent air from the fermentor was vented through an incinerator and a stack.

Fermentation, harvesting and extraction of cultures of Trichoderma hamatum - The organism was maintained and inocula grown as previously described (Brewer et al.,

1982). The production medium (10 L) containing glucose (400 g), potassium nitrate (20 g) and salts (Brewer et al., 1982) was transferred to a glass fermentor (capacity 15 L) which was stirred at 240 r min^{-1} and sparged with air (4 L min^{-1}) at 25°. The fermentor was inoculated with a culture (500 mL) 48 h old and the pH of the fermentation adjusted to 6.0. The fermentation conditions (volume, pH, air flow, temperature and viscosity) were controlled by the system described by Brewer and his coworkers (1987). After 24 h growth the culture was treated with a solution (2.5 mL) of U-^{14}C-L-tyrosine (203 μCi, 88 mg) in hydrochloric acid (*N*). Forty eight hours after addition of the tyrosine the pH of the culture was adjusted to 8.5 and the fermentation broth separated by filtration. The filtrate was cooled to 4° and then acidified to pH 4.5, when it was extracted (3 x 3L) with ethyl acetate precooled to -15° The ethyl acetate emulsion was broken by filtration through a pad of celite 535 and was then extracted with phosphate buffer (*M*/15, pH 8.5, 3 x 2L). 3-(3-isoCyanocyclopent-2-enylidene-)propionic acid (**2**) was recovered from the buffer solution as described (Brewer et al., 1982). The ethyl acetate raffinate was concentrated to 3 L, the concentrate kept for 24 h at -15°, when the ice was separated and the filtrate evaporated at <5° to 200 mL.

Chromatography of the neutral ethyl acetate soluble isocyanides - Half of the ethyl acetate solution described in the preceding paragraph (100 mL) was evaporated, the residue dissolved in diethyl ether (50 mL) and petroleum ether (b. p. 30-60°, 8 mL) added. The slightly cloudy solution was applied to the top of a silica gel (80 g, Merck 'for thin layer chromatography') column (4.5 x 12 cm) packed at 4° overnight with the solvent diethyl ether-petroleum ether-acetic acid (300:99:1). The loaded column was developed and eluted with the latter solvent (500 mL), then with diethyl ether-petroleum ether-acetic acid (400:99:1, 250 mL), then diethyl ether-acetic acid (499:1, 250 mL) and finally ethyl acetate (250 mL). The following fractions were collected after application of the sample: 1-6 (each 100 mL), 7-18 (each 50 mL) and 19 (250 mL). Analytical thin layer chromatography (tlc) showed that fractions 4 and 5 contained trichoviridin (3.8 x 10^6 d min^{-1}) and 6,7 and 8 mostly 1-(2,3-epoxy-1-hydroxy-3-isocyanocyclopent-4-enyl)ethanol (**3**, 1.6 x 10^6 d min^{-1}). A third radioactive component (?**4**, 1.2 x 10^6 d min^{-1}) was eluted in fractions 9-11. The second half of the ethyl acetate extract was processed in the same way. The crystalline material from fractions 4 of both chromatograms were combined and dissolved in ether (4 mL), the solution cooled to 0° and hexane (3 mL) layered onto the surface. After 24 h at 4° clumps of colorless crystals (50 mg, 1.9 μCi $mmol^{-1}$) separated. They were collected and recrystallized from ether-hexane (1:2) giving trichoviridin (45 mg, m. p. 92°, $[\alpha]_D^{24}$ -41° (c, 0.6, $CHCl_3$) 1.82 μCi $mmol^{-1}$). Fractions 6-11 inclusive (from two chromatograms) were combined and applied as described above to a silica gel (150 g) column prepared with diethyl ether-petroleum ether (1:1). The column (diameter 5.08 cm) was eluted with diethyl ether-petroleum ether mixtures as follows: 1:1, 200 mL; 3:2, 250 mL; 3:1, 250 mL, 4:1, 500 mL and then ether (1 L). The following fractions were collected: 1, (500 mL); 2-6 (each 100 mL); 7-18 (each 50 mL) and 19 (~500 mL). The crystalline material in fraction 15 (16.2 mg, (2.4 x 10^4 d min^{-1} mg^{-1})), was treated in dichloromethane with bis(η^5-ethyltetramethylcyclopentadienyl)di(dithiocyanato)di(μ-thiocyanato)di-rhodium(III) (McAlees et al., 1990, 25 mg), the solution kept 3 h at 4°, evaporated and the complex thus formed purified by chromatography on preparative thin layer plates (silica gel, 0.1 x 20 x 40 cm). The complex separated from methyl *t*-butylether as glistening yellow plates (35 mg, m. p. 126°) whose infrared spectrum was identical to that of the complex of 1-(2,3-epoxy-1-hydroxy-3-isocyanocyclopent-4-enyl)ethanol (**3**, Boyd et al., 1991). Tlc (silica gel, solvent diethyl ether) of fraction 19 indicated that it contained a single isocyanide that was radioactive (2.7 x 10^4 d min^{-1} mg^{-1}). It was

converted into its $Rh(\eta^5\text{-}C_5EtMe_4)(SCN)_2$ complex m. p. 133° as described (Boyd et al., 1991).

Veratryl trichoviridinate - Trichoviridin (**1**, 152 mg) was dissolved in dichloromethane (3 mL) and veratric acid (149 mg) added. A solution (6 mL) of dicyclohexylcarbodiimide (170 mg) and 4-dimethylaminopyridine (20 mg) in dichloromethane was added to the stirred and cooled (-15°) reaction mixture. After 24 h at -15° a solution (4 mL) of dicyclohexylcarbodiimide (85 mg) was added and the reaction mixture stirred for a further 48 h when the urea (142 mg, 77%) was collected, washed with ice-cold dichloromethane (3 x 2 mL) and the colorless filtrate concentrated to 5 mL. This solution was applied to a silica gel column (4.5 x 15 cm) which was made up and eluted with diethyl ether. Fractions (~ 15 mL) were collected immediately after application of the sample to the column. Fractions 13-16 inclusive contained a single component with greater R_F (ether-petroleum ether, 9:1, silica gel, 0.56) than trichoviridin (0.15). The fractions were combined, evaporated and the colorless crystalline residue recrystallised from carbon tetrachloride giving needles (110 mg, decomp. >50°, m/z 347; υ_{max} (CCl_4) 3560, 2134, 1725, 1720 cm^{-1}; δ_H (CCl_4-$CHCl_3$, 9:1) 7.59, (*J* 8.5 Hz, *J* 1.8 Hz), 7.42 (*J* 1.8 Hz), 6.77(*J* 8.5 Hz), 5.11 (*J* 6.43 Hz), 4.03, 3.86 (6H), 3.44 (m), 3.32 (m), 2.51, 1.44 (3H, *J* 6.43 Hz), 1.82 μCi $mmol^{-1}$). The 4-methoxybenzoyl ester (136 mg, 80%) was obtained in the same way from 4-methoxybenzoic acid (80 mg) and trichoviridin (97.2 mg) as a colorless oil (R_F 0.52; trichoviridin 0.21, ether-petroleum ether 1:1; υ_{max} (CCl_4) 3580, 2131, 1725, 1340, 1165 cm^{-1}; δ_H (CCl_4-$CHCl_3$ 9:1) 7.97 (2H, *J* 9.5 Hz, *J* 2.6 Hz), 6.87 (2H), 5.16 (*J* 6.5 Hz), 4.07 (*J* 2.5 Hz), 3.86 (3H), 3.47, 3.37 (*J* 2.5 Hz), 2.52, 1.48 (3H, *J* 6.5 Hz).

References

Baldwin, J.E., Bansal, H.S., Chondrogianni, J., Field, L.D., Taha, A.A., Thaller, V., Brewer, D. and **Taylor, A.** 1985 Biosynthesis of 3-(3'-isocyanocyclopent-2-enylidene)propionic acid by *Trichoderma hamatum* (Bon.) Bain. Aggr. *Tetrahedron*, 41: 1931-1938.

Boyd, R.K., McAlees, A.J., Taylor, A. and **Walter, J.A.** 1991 Isolation of new isocyanide metabolites of *Trichoderma hamatum* as their (η^5-pentamethylcyclpentadienyl)- or (η^5-ethyltetramethyl-cyclopentadienyl)-di-μ-thiocyanato-rhodium complexes. *J. Chem. Soc. Perkin Trans.* I. 1461-1465.

Brewer, D., Feicht, A., Taylor, A., Keeping, J.W., Taha, A.A. and **Thaller, V.** 1982 Ovine ill-thrift in Nova Scotia. 9. Production of experimental quantities of isocyanide metabolites of *Trichoderma hamatum*. *Can. J. Microbiol.*, 28: 1252-1260.

Brewer, D., Greenwell, M. and **Taylor, A.** 1993 Studies of *Trichoderma* isolates from *Mytilus edulis* collected on the shores of Cape Breton and Prince Edward Islands. *Proc. Nova Scotian Inst. Sci.*, 40: 29-40.

Brewer, D., Mason, F.G. and **Taylor, A.** 1987 The production of alamethicins by *Trichoderma* species. *Can. J. Microbiol.*, 33: 619-625.

Fujiwara, A., Okuda, T., Masuda, S., Shiomi, Y., Migamoto, C., Sekine, Y., Tazoe, M. and **Fujiwara, M.** 1982 Fermentation, isolation and characterization of isonitrile antibiotics. *Agric. Biol. Chem.*, 46: 1803-1809.

Grove, J.F., McAlees, A.J. and **Taylor, A.** 1988 Preparation of ten gram quantities of 15-O-acetyl-4-deoxynivalenol. *J. Org. Chem.*, 53: 3860.

McAlees, A.J., Schwartzentruber, K. and **Taylor, A.** 1990 Esterification of isocyanide carboxylic acids and hydrolysis of their esters. *J. Chem. Soc. Perkin Trans.* I 2115-2118.

Neises, B. and **Steglich, W.** 1978 Simple method for the esterification of carboxylic acids *Angew. Chemie, Int. English Ed.*, 17: 522-523.

Nobuhara, M., Tazima, H., Shudo, K., Itai, A., Okamoto, T. and **Iitaka, Y.** 1976 A fungal metabolite, novel isocyano epoxide. *Chem. Pharm. Bull.*, 24: 832-834.

(Received 23 February 1995)