Pergamon

0031-9422(95)00256-1

Phytochemistry, Vol. 40, No. 1, pp. 41-43, 1995

0031-9422/95 $9.50 + 0.00

4-NITROTRYPTOPHANS ASSOCIATED WITH THE *IN VITRO* PRODUCTION OF THAXTOMIN A BY *STREPTOMYCES SCABIES*

RUSSELL R. KING and C. HAROLD LAWRENCE

Agriculture and Agri-Food Canada, Research Branch, Fredericton Research Centre, Fredericton, New Brunswick, Canada E3B 4Z7

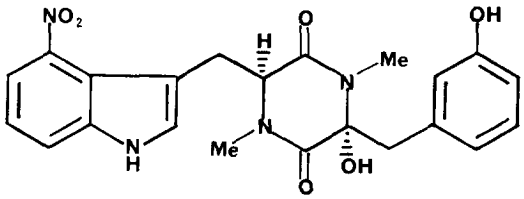
(*Received in revised form* 15 *February* 1995)

Key Word Index—4-Nitrotryptophans; phytotoxins; thaxtomin A; *Streptomyces scabies*.

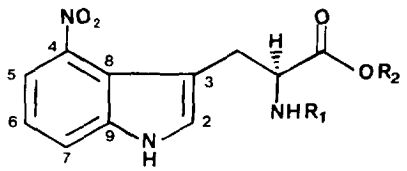
Abstract—Non-phytotoxic exudates associated with the *in vitro* production of thaxtomin A by *Streptomyces scabies* yielded two 4-nitrotryptophan derivatives, *N*-acetyl-4-nitrotryptophan and *N*-methyl-4-nitrotryptophan.

INTRODUCTION

Our investigations of phytotoxins generated by *Streptomyces scabies* (Thaxt.) Lambert and Loria [1] (the causal organism of potato common scab) have resulted in the isolation and characterization of a series of unique 4-nitroindol-3-yl containing 2,5-dioxopiperazines typified by the predominant representative thaxtomin A [2, 3]. A subsequent detailed examination of the non-phytotoxic exudates associated with phytotoxin production in an oatmeal broth [4] yielded two new metabolites which also exhibited UV, ^{1}H NMR and mass spectral data characteristic of 4-nitroindol-3-yl containing moieties.



Thaxtomin A



1 R_1 = Ac R_2 = H

2 R_1 = Me R_2 = H

3 R_1 = Ac R_2 = Me

4 R_1 = Me R_2 = Me

RESULTS AND DISCUSSION

Fractionation by silica gel TLC of the water-soluble residues from oatmeal broth cultures of pathogenic *Streptomyces scabies* isolates [5] consistently yielded two non-phytotoxic, highly polar, yellow coloured compounds that exhibited UV spectral data characteristic of 4-nitroindole containing moieties [6]. These compounds were rechromatographed on silica gel TLC and purified further by reversed-phase TLC.

Characterization of the two metabolites proved relatively straightforward. Although lacking significant $[M]^+$ ions, their mass spectral data strongly indicated that both were nitroindol-3-yl containing moieties, i.e. base fragment ions at *m/z* 175 ($C_9H_7N_2O_2$). The ^{1}H NMR and COSY spectral data of the compounds again reflected the similarities of their structures, i.e. aromatic protons compatible with a 4-nitroindole nucleus [7] and a geminal proton pair further coupled to an adjacent methine were present in both (Table 1). Two major differences were also readily explicit in the ^{1}H NMR spectral data of these compounds. First the apparent presence of an *N*-acetyl substituent (δ 1.83) in one compound contrasted with the evidence for an *N*-methyl group (δ 2.33) in the other. Next, the methine proton of the *N*-methyl containing compound was shifted significantly upfield, i.e. δ 3.26 vs 4.60 for the *N*-acetyl analog.

Resolution of the identity of the *N*-acetyl containing compound as *N*-acetyl-4-nitrotryptophan (**1**) was confirmed on the basis of comparing its chromatographic and spectral data with that of an available synthetic

Table 1 ^{1}H NMR spectral assignments for compounds **1**–**4**

H	1	2	3	4
2	7.42 *s*	7.41 *s*	7.36 *s*	7.36 *s*
5	7.73 *dd* (7.9,1.0)	7.75 *dd* (7.9, 1.0)	7.84 *dd* (7.9, 1.0)	7.84 *dd* (7.9, 1.0)
6	7.17 *dd* (8.0, 1.0)	7.15 *t* (8.0)	7.21 *t*(8.0)	7.22 *t* (8.0)
7	7.68 *dd* (8.1, 1.0)	7.65 *dd* (8.1, 1.0)	7.71 *dd* (8.1, 1.0)	7.72 *dd* (8.1, 1.0)
10	3.18 *dd* (14.4, 6.0)	2.99 *dd* (13.2, 6.2)	3.24 *ddd* (14.5, 6.2, 0.8)	3.15 *ddd* (13.1, 6.1, 0.8)
	3.52 *dd* (14.4, 8.4)	3.23 *dd* (13.0, 5.6)	3.46 *ddd* (14.5, 8.4, 0.8)	3.40 *ddd* (13.1, 6.1, 0.8)
11	4.60 *dd* (6.0, 8.4)	3.26 *dd* (6.2, 5.8)	4.58 *dd* (6.2, 8.4)	3.42 *t* (6.1)
CH_3C	1.83 *s*		1.87 *s*	
CH_3N		2.33		2.33
CH_3O			3.61 *s*	3.49 *s*

Coupling constants (J in Hz) in parentheses.

sample [8] and by conversion to the related methyl ester (**3**)

Unequivocal evidence for characterization of the *N*-methyl containing compound as *N*-methyl-4-nitrotryptophan (**2**) was also obtained by comparison of its methyl ester analog (**4**) with that of a synthetic sample. In this instance, the synthetically equivalent sample was prepared by selective nitration with nitric acid–acetic acid [6] of N-methyl tryptophan methyl ester [9].

^{13}C NMR spectra compatible with the assigned structures were also recorded for the methyl ester derivatives **3** and **4** (Table 2). The ^{13}C chemical shift assignments for the proton substituted carbons were made in a straightforward manner from HETCOR spectra. Assignment of the remaining fully substituted carbons were made by reference to previous NMR studies on indole derivatives [10] and empirical chemical shift calculations.

The respective roles of **1** and **2** as likely intermediate in the production of the thaxtomins have not yet been elaborated. It is of interest to note, however, that condensation of **2** with phenylalanine would produce 12-*N*-methylcyclo-(L-nitrotryptophyl-L-phenylalanyl) a member of the thaxtomins that is the logical precursor for the synthesis of all other thaxtomins presently identified in association with *Streptomyces scabies* [3].

Table 2. ^{13}C NMR spectral assignments for compounds **3** and **4**

C	3	4
2	130.7 *d*	130.9
3	110.7 *s*	110.4
4	143.9 *s*	144.0
5	118.5 *d*	118.4
6	121.2 *d*	121.2
7	119.0 *d*	119.1
8	120.7 *s*	120.0
9	141.1 *s*	141.2
10	30.7 *t*	31.8
11	55.4 *d*	65.9
12	174.0 *s*	175.5
CH_3N		34.4 *q*
CH_3O	52.6 *q*	52.1
$CH_3\overset{O}{\overset{\|}{C}}$	22.3 *q* 173.2 *s*	

EXPERIMENTAL

General. Mp: uncorr. IR spectra were determined on a Perkin-Elmer 467 grating infrared spectrophotometer. UV spectra were recorded in absolute EtOH using a Beckman DU-7 spectrophotometer. MS (EI and FAB) were obtained on a Finnigan MAT 312 mass spectrometer. NMR spectra were recorded for solutions in MeOH-d_4 on a Varian Unity 400 operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts were measured downfield from the signal of internal tetramethylsilane. TLC was performed on 0.25 mm Merck silica gel $60F_{254}$ plates and 0.2 mm Whatman $KC_{18}F$ plates.

Metabolite production and isolation procedures. Pathogenic isolates of *Streptomyces scabies* [5] were maintained and subcultured on a solid modified glucose medium as detailed previously [5]. Oatmeal broth medium was prepared by boiling 40 g of oatmeal per 800 ml H_2O for 5 min. in a microwave oven. The broth was cooled to approximately 50°C and filtered through a fine mesh cheesecloth. The filtrate was adjusted to one liter with distilled water and 2.0 mg of $ZnSO_4 \cdot 7\ H_2O$ added. After adjusting to pH 6.8 with 0.1 M NaOH, 100 ml portions of the medium were dispensed into 500 ml flasks and sterilized at 15.0 lb for 20 min. The oatmeal medium was inoculated with 5 ml of a 3-day-old shake culture of the test organism, and the cultures then incubated at 26°C on a rotary shaker. At maximum phytotoxin production (5–6 days), the cell cultures were extracted twice with equivalent quantities of EtOAc to remove the known phytotoxins (i.e. thaxtomin A, etc.) and the remnant mixture diluted with a 3-fold excess of MeOH. Suspended particulates were removed by filtration through Whatman # 1 filter paper and the MeOH plus H_2O removed *in vacuo* at 30°C. The residue was then triturated with MeOH (3 × 10 ml). The MeOH was removed *in vacuo*

and the residual material that dissolved in MeOH (2 ml) fractionated by TLC on silica gel with EtOAc–H_2O–HOAc–HCO_2H (10:2.5:1:1) to yield *ca* 0.2 mg each of crude *N*-acetyl-4-nitrotryptophan (**1**) (R_f 0.85) and *N*-methyl-4-nitrotryptophan (**2**) (R_f 0.35). Both compounds proved non-phytotoxic when assayed for scab-inducing activity on potato mini-tubers [5]. The compounds were rechromatographed on silica gel and purified further by reversed-phase TLC with Me_2CO–H_2O (3:2) to yield pure *N*-acetyl-4-nitrotryptophan (**1**) (R_f 0.93) as a yellow solid with UV λ_{max}^{EtOH} 345 (ε 3410) 385 (ε 4020) nm, MS (FAB) *m/z* 291 [M^+, $C_{13}H_{13}N_3O_5$], 175 (base peak) and pure *N*-methyl-4-nitrotryptophan (**2**) (R_f 0.84) as a yellow solid with UV λ_{max}^{EtOH} nm: 345 (ε 3390) 384 (ε 4010), MS (FAB) *m/z* 263 [M^+, $C_{12}H_{13}N_3O_4$], 175 (base peak). ^{1}H NMR spectral data for both compounds is outlined in the text.

N-acetyl-4-nitrotryptophan methyl ester (**3**). *N*-Acetyl-4-nitrotryptophan (1) (12 mg) in dry MeOH (5 ml) was treated at room temp. with BF_3–MeOH (0.2 ml) overnight. The solvents were removed *in vacuo* and the organic residues taken up in EtOAc (20 ml) and washed successively with satd $NaHCO_3$ solution (10 ml) and H_2O (10 ml). The EtOAc layer was dried over Na_2SO_4, concd *in vacuo* and the residue crystallized from Me_2O to yield *N*-acetyl-4-nitrotryptophan methyl ester (3) as yellow prisms with mp 205–206 °: IR ν^{nujol} cm^{-1} 1730, 1650; UV λ_{max}^{EtOH} nm: 344 (ε 3480) and 385 (ε 4070). MS (FAB) *m/z* 305 [M]$^+$, ^{1}H and ^{13}C NMR spectral data are outlined in the text.

N-methyl-4-nitrotryptophan methyl ester (**4**). *N*-Methyl-4-nitrotryptophan (**2**) (9 mg) in a solution of HCl saturated MeOH (20 ml) was refluxed for 30 min. The MeOH solution was removed *in vacuo* and the organic residue taken up in EtOAC (25 ml) and washed successively with satd $NaHCO_3$ soln (10 ml) and H_2O (10 ml). The EtOAc layer was dried over Na_2SO_4 concd *in vacuo* and the residue purified by TLC on silica gel with MeOH–$CHCl_3$ (1:9) to yield *N*-methyl-4-nitrotryptophan methyl ester (4) as a yellow solid with R_f 0.36 UV λ_{max}^{EtOH} nm: 346 (ε 3360) 384 (ε 3980), MS (FAB) *m/z* 277 [M]$^+$. ^{1}H and ^{13}C NMR spectral data are outlined in the text.

A synthetic sample of the title compound was prepared by treating *N*-methyltryptophan methyl ester (40 mg) with HNO_3–HOAc 1:10 (5 ml) at room temp. until all the *N*-methyltryptophan methyl ester was transformed (*ca* 45 min.). The mixture was then taken up in EtOAc and neutralized with aqueous $NaHCO_3$ soln. The EtOAc layer was dried over Na_2SO_4 and the EtOAc removed *in vacuo*. Pure *N*-methyl-4-nitrotryptophan methyl ester (**4**) was obtained by fractionation of the nitration mixture by TLC on silica gel with MeOH–$CHCl_3$ (1:9).

Acknowledgements—We thank Peter Penner (Department of Chemistry, University of New Brunswick, Fredericton) for recording the NMR spectra and Pierre Lapointe (Plant Research Centre, Ottawa, Ontario) for mass spectral determinations.

REFERENCES

1. Lambert, D. H. and Loria, R. (1989) *Int. J. Syst. Bacteriol.* **39**, 387.
2. King, R. R., Lawrence, C. H., Clark, M. C. and Calhoun, L. A. (1989) *J. Chem. Soc., Chem. Commun* **13**, 849.
3. King, R. R., Lawrence, C. H. and Calhoun, L. A. (1992) *J. Agric. Food Chem.* **40**, 834.
4. Babcock, M. J., Eckwall, E. C. and Schottel, J. L. (1993) *J. Gen. Microbiol.* **139**, 1579.
5. King, R. R., Lawrence, C. H. and Clark, M. C. (1991) *J. Am. Potato Assoc.* **68**, 675.
6. Hester, J. B. (1964) *J. Org. Chem.* **29**, 1158.
7. Somei, M., Inoue, S., Tokutake, S., Yamata, F. and Kaneko, C. (1981) *Chem. Pharm. Bull.* **29**, 726.
8. Endo, Y., Shudo, K., Itai, A., Hasegawa, M. and Sakai, S. (1986) *Tetrahedron* **42**, 5905.
9. Hoshino, T. (1935) *Ann.* **520**, 31.
10. Morales-Rios, M. S. Espineira, J. and Joseph-Nathan, P. (1987) *Magn. Reson. Chem.* **25**, 377.