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UNUSUAL PRODUCTION OF 5-NITROANTHRANILIC ACID BY *STREPTOMYCES SCABIES*

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Key Word Index—nitroanthranilic acids; phytotoxins; thaxtomins; *Streptomyces scabies*.

Abstract—Examination of non-phytotoxic extracts associated with the *in vitro* production of thaxtomin A by various *Streptomyces scabies* isolates consistently yielded a nitroaromatic compound that was characterized by spectral and synthetic means as 5-nitroanthranilic acid (2-amino-5-nitrobenzoic acid). Investigations examining its possible role in the biosynthesis of thaxtomin A were inconclusive. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Investigations of phytotoxins generated by *Streptomyces scabies* (Thaxt.) Lambert and Loria [1] (the main 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 [2–4]. Structural requirement determinations for the observed phytotoxicity of these compounds demonstrated a necessity for the presence of 4-nitrotryptophan and phenylalanine groups linked in an L,L-configured cyclodipeptide [3].

Since the production of organic compounds containing nitro groups is a relatively unusual occurrence in nature, only a limited number of investigations detailing the biosynthetic origins of the nitro group have been explored. In all but one of these examples it has been demonstrated that the nitro group is derived from the oxidation of an amino group rather than by direct nitration [5]. A potential nitration stage in production of the thaxtomins by *S. scabies* has previously been identified, i.e. nitration of the tryptophan indole moiety appears to precede formation of the diketopiperazine ring [6].

In the process of examining thaxtomin-producing *Streptomyces* isolates from various regions of North America [7, 8] we observed that several of them consistently generated *in vitro*, measurable quantities of an hitherto unreported metabolite. The spectral characteristics of this metabolite were consistent with those of a nitroanthranilic acid but the substitution pattern did not conform with that expected for a 4-nitroindol-3-yl group precursor. This paper details isolation and characterization of the nitroanthranilic acid, the scope of its generation by various *S. scabies* isolates and investigations examining its possible significance in the biosynthesis of thaxtomin phytotoxins.

RESULTS AND DISCUSSION

Fractionation by silica gel TLC of the ethyl acetate extract residues from oatmeal broth cultures of *S. scabies* isolates consistently yielded minor quantities (usually $< 5\%$ of the total phytotoxins produced) of a relatively polar, light-yellow coloured non-phytotoxic compound. It was then further purified by repeating the silica gel fractionation step.

The molecular formula of the compound was tentatively deduced as $C_7H_6N_2O_4$ from EI-MS (M^+, m/z 182) and DEPT-edited ^{13}C NMR experiments [$7sp^2$ (4s and 3d) hybridized carbon signals observed]. Detailed examination of the ^1H-NMR spectrum revealed the typical pattern for a 1,2,4-trisubstituted aromatic ring, i.e. the three signals of an AMX spin system ($J_{ortho} = 8.0$ Hz, $J_{meta} = 2.7$ Hz and $J_{para} = 0$, Hz). This data taken in conjunction with the observation of a significant fragmentation ion at m/z 164 ($M^+ - H_2O$) in the EI-MS suggested that the compound was 5-nitro anthranilic acid (1). Unequivocal evidence for this structure was obtained by comparison (MS, NMR, UV, TLC and HPLC parameters) with a synthetic sample of 5-nitroanthranilic acid (Aldrich Chemical Co.).

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A rationale for the production of 5-nitroanthranilic acid by *S. scabies* is not readily apparent. To our knowledge this is the first report of its isolation from a natural source. It is difficult to represent the compound as a potential 4-nitrotrytophan precursor in that a unique 1,2-migration of the nitro group would eventually be required. Its addition to the culture medium of thaxtomin A producing isolates did not lead to increased yields or more rapid production of the phytotoxin. Nor were the 5-nitroanthranilic acid supplements noticeably metabolized. On expanding the scope of our investigations, however, it became obvious that 5-nitroanthranilic acid was being generated to some degree by a significant number of *S. scabies* isolates, several from very geographically diverse areas of the world (i.e. Australia, Germany and Japan). This additional evidence led us to theorize that 5-nitroanthranilic acid might represent a by-product from generation of the more biosynthetically appropriate 6-nitroanthranilic acid (2). Thereafter, we proceeded to examine our *S. scabies* culture extracts via TLC and HPLC for the presence, however minimal, of 6-nitroanthranilic acid. When these efforts proved negative, we investigated the effects of adding 6-nitroanthranilic acid to the culture medium. These additions, however, did not lead to any change in phytotoxin or 5-nitroanthranilic acid production and the 6-nitroanthranilic acid supplements were not noticeably metabolized. In many of the foregoing analyses traces of anthranilic acid were also detected. However, supplementary additions of this potential precursor failed to elicit any change in phytotoxin or nitroanthranilic acid production.

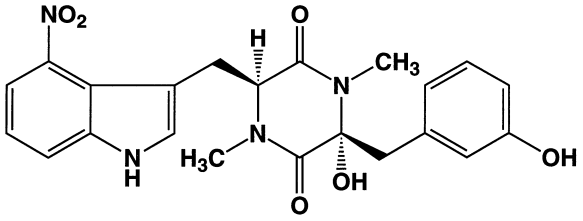
In summation, the co-generation of 5-nitroanthranilic acid has not been linked to any definitive role in the biosynthesis of thaxtomin A by *S. scabies*. The findings, however, have interesting implications for further research into the vital nitration step, i.e. it may well take place at a much earlier stage in the phytotoxin biosynthetic sequence than had previously been envisaged (6).

EXPERIMENTAL

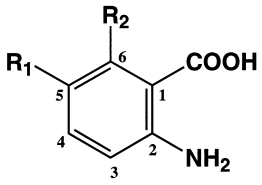
General

MS (EI) were obtained on a Finnigan MAT 312 mass spectrometer. NMR spectra were recorded for solutions in MeOH-d_4 on a Varian Unity 400 spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts were referenced to the solvent resonances (δ_H, 3.30 ppm and δ_c, 49.00 ppm). TLC was performed on Merck silica gel $60F_{254}$ plates.

HPLC studies utilized a Shimadzu LC-10AS pump and a LiChrosorb RP_{18} column 10 μm × 25 cm eluted with 0.5% formic acid–methanol 8:3 (v/v) at a flow



Thaxtomin A



1. $R_1 = NO_2$ $R_2 = H$

2. $R_1 = H$ $R_2 = NO_2$

rate of 1.0 ml/min and monitored with a Shimadzu diode array detector.

Metabolite production and isolation procedures

S. scabies isolates were maintained and subcultured on solid modified glucose medium as detailed previously [3]. Oatmeal broth medium was prepared by boiling 40 g of oatmeal/800 ml of water 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 1 l with distilled water and 2.0 mg of $ZnSO_4 \cdot 7H_2O$ was added. After the pH was adjusted to 6.8 with 0.1 N 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 then inoculated with 5 ml of a 3-day old shake culture of the test organism. The cultures were then incubated at 29°C on a rotary shaker. At maximum phytotoxin production (4–5 days), the cell cultures were transferred to a separatory funnel and extracted twice with 150 ml portions of ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate and the ethyl acetate was removed *in vacuo* at 25°C. In the feeding experiment, 5 ml aqueous solutions of anthranilic acid, 5-nitroanthranilic acid or 6-nitroanthranilic acid at concentrations of 0.5 and 1.0 mg/ml, respectively, were sterilized by passage through a 22 μm millipore sterile filter and added to the culture medium.

The residue was taken up in acetone and fractionated on 250 μm silica gel 60 plates with chloroform/methanol (9:1). A light yellow coloured non-phytotoxic (assayed for scab inducing activity on potato mini-tubers [7]) band (R_f, 0.12) was accumulated (yields ranged from *ca.* 25 to 100 μg per flask measured by HPLC). The accumulated fractions were further purified on silica gel plates with ethyl acetate/methanol (4:1) to yield 5-nitroanthranilic acid (1) (R_f, 0.44) as a light yellow solid with MS (EI) *m/z* 182 [M^+ $C_7H_6N_2O_4$], ^{1}H NMR δ 6.65 (d, 1H, J=8.0 Hz, H-3) 7.94 (dd, 1H, J=8.0, 2.7 Hz, H-4), 8.77 (d, 1H, J=2.7 Hz, H-6). ^{13}C NMR δ 117.13 (d, C-3), 111.46 (s, C-1) 129.60 (d, C-4), 130.28 (d, C-6), 137.33 (s, C-5) 157.72 (s, C-2), 170.09 (s, CO_2H).

6-*Nitroanthranilic acid* (2)

3-Nitrophthalamide was converted into a mixture of 3-nitroanthranilic acid and 6-nitroanthranilic acid as previously described [9]. After purification on 500 μm silica gel 60 plates with ethyl acetate/methanol (4:1) 6-nitroanthranilic acid (2) (R_f, 0.25) was isolated as a yellow solid with MS (EI) *m/z* 182 [M^+ $C_7H_6N_2O_4$]. ^{1}H NMR δ 6.92 (dd, 1H, J=8.0, 2.8 Hz, H-3) 7.03 (dd, 1H, J=8.0, 2.8 H, H-5) 7.10 (t, 1H, J=8.0 Hz, H-4). ^{13}C NMR δ 112.46 (d, C-5), 120.77 (d, C-3) 121.60 (s, C-1), 129.17 (d, C-4) 148.03 (s, C-6)†, 150.43 (s, C-2)† 172.92 (s, CO_2H).

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† Assignments may be interchanged.

REFERENCES

1. Lambert, D. H. and Loria, R., *Int. J. Syst. Bacteriol.*, 1989, **39**, 387.
2. King, R. R., Lawrence, C. H., Clark, M. C. and Calhoun, L. A., *J. Chem. Soc. Chem. Commun.*, 1989, **13**, 849.
3. King, R. R., Lawrence, C. H. and Calhoun, L. A., *J. Agric. Food Chem.*, 1992, **40**, 834.
4. King, R. R. and Lawrence, C. H., *J. Agric. Food Chem.*, 1996, **44**, 1108.
5. Carter, G. T., Nietsche, J. A., Goodman, J. J., Torrey, M. J., Dunne, T. S., Siegel, M. M. and Borders, D. B. (1989) *J. Chem. Soc. Chem. Commun.*, 1271.
6. King, R. R. and Lawrence, C. H., *Phytochemistry*, 1995, **40**, 41.
7. King, R. R., Lawrence, C. H. and Clark, M. C., *J. Am. Potato Assoc.*, 1991, **68**, 675.
8. King, R. R., Lawrence, C. H. and Clark, M. C., *Am. Potato J.*, 1992, **69**, 591 (Abstr.).
9. Nagai, U., *Chem. Pharm. Bull.*, 1975, **23**, 1841.