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Infectopyrone, a potential mycotoxin from *Alternaria infectoria*

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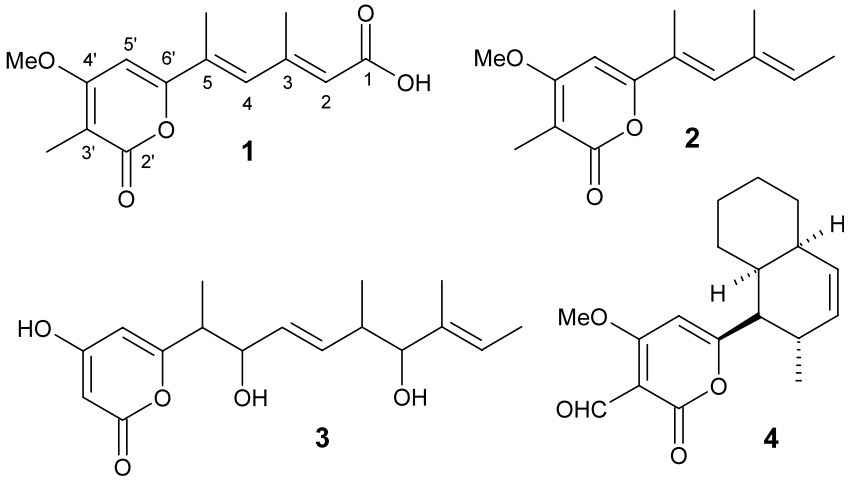
Abstract—A new metabolite, infectopyrone (**1**), has been isolated from the filamentous fungus *Alternaria infectoria*. The structure of **1** was elucidated by analysis of 2D NMR spectroscopic data. Compound **1** is an α-pyrone resembling known toxins, and is a useful phenotaxonomic marker for the *A. infectoria* species-group. Infectopyrone (**1**) was also produced by species within *Stemphyllium* and *Ulocladium*, and found in mouldy food. © 2003 Elsevier Science Ltd. All rights reserved.

Infections of cereals and feedstuffs with filamentous fungi such as *Alternaria* are of growing concern since they can produce a broad range of secondary metabolites including potential mycotoxins.[1] In temperate regions such as Denmark, members of the *A. infectoria* species-group are by far the most common species infecting barley.[2] No metabolites have yet been structurally assigned from species within this group, however, the presence of six unknown metabolites with characteristic UV spectra has previously been reported from isolates of *A. infectoria*.[2–4] In the present study we report the isolation and structural assignment of one of these metabolites as a new compound named infectopyrone (**1**). Some initial results on the occurrence of **1** in fungi and in the food chain are also given.

An *A. infectoria* isolate (IBT 9373)[5] was cultured as three point mass inoculations for 14 days on 100 Petri dishes (ca. 2.5 L) of DRYES agar medium.[6] The contents of the dishes were extracted with EtOAc and **1** was purified by various chromatographic steps.[7]

The NMR spectra of **1** (DMSO-d_6) revealed the presence of 15 non-exchangeable protons and 14 carbons (Table 1), consistent with the molecular formula ($C_{14}H_{16}O_5$), established by accurate mass measurement of the protonated molecular ion.[8] This formula indicated a compound with seven double bond equivalents, in accordance with a longest wavelength UV absorbance at 343 nm[8] indicating that at least six conjugated double bonds were likely to be present in **1**.



Keywords: *Alternaria infectoria*; *Stemphyllium*; *Ulocladium*; α-pyrone; chemotaxonomy.
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Table 1. NMR data for infectopyrone **1**[a]

Position	^{13}C	^{1}H[b]	HMBC[c]	NOESY
1	167.3	–	2, 3-Me (w)*	–
2	121.5	5.90 (1H, m, 1.5)	3-Me, 4	4, 5-Me
3	150.5	–	3-Me	–
4	133.3	6.96 (1H, br s)	2, 3-Me, 5-Me	2, 5′, 3-Me
5	129.6	–	2 (w)*, 4, 5-Me, 5′	–
2′	163.3	–	3′-Me	–
3′	101.4	–	3′-Me, 5′	–
4′	165.9	–	3′-Me, 4′-OMe, 5′	–
5′	95.5	6.79 (1H, m, 1.5)	3′-Me (w)*, 4′-OMe (w)*	4, 4′-OMe, 5-Me
6′	158.5	–	4, 5-Me, 5′	–
3-Me	18.8	2.36 (3H, m, 1.5)	2, 4, 5-Me (w)*	4, 5-Me
5-Me	14.1	2.19 (3H, m, 1.5)	4	2, 5′, 3-Me
3′-Me	8.8	1.93 (3H, s)	5′ (w)*	4′-OMe (w)
4′-OMe	57.0	4.08 (3H, s)	NO	5′ (w), 3′-Me

[a] In DMSO-d_6 at 298 K; ^{1}H at 500 MHz, ^{13}C at 125 MHz in ppm.
[b] Multiplicity: m = multiplet, br = broad, s = singlet; coupling in Hz.
[c] ^{13}C to H; w = weak; * = 4 or 5-bond correlation; NO = none observed.

A carboxylic acid group was indicated by a broad OH stretching band in the IR spectrum at 3600–2600 cm^{-1} and a strong C=O stretching band at 1686 cm^{-1} within the expected range for an α,β-unsaturated carboxylic acid.[8] The NMR spectra of **1** (Table 1) showed three methyl groups, one methoxy group, and three sp^2 hybridized CH groups. The other seven carbons were quaternary and sp^2 hybridized. Four of these had chemical shift values above 155 ppm indicating the attachment of oxygen. Since only long range couplings were present in the proton spectra (^{1}H and COSY) of **1**, no partial structures could be established before the HMBC experiment was interpreted. These data (Table 1) made it possible to connect ten carbons in a straight sequence as well as placing the three methyl groups and the methoxy group (Fig. 1). The lactone linkage required by the molecular formula was shown to be between C-2′ and C-6′ by the very similar ^{1}H and ^{13}C shift values of **1** compared to the α-pyrone **2**.[9] Placing a carboxylic acid group at C-1 completed the proposed structure of **1** and explained the chemical shift differences between **1** and **2**. The observed NOE correlations are in good accordance with the stereochemistry of the proposed structure **1** (Fig. 1), and showed the stereochemistry about both C-2–C-3 and C-4–C-5 to be *E*. Since H-5′ correlated with both H-4 and Me-5, and H-2 with both H-4 and Me-5 no specific conformation of **1** about C-5–C-6′ or C-3–C-4 is preferred in DMSO. We propose the name infectopyrone for this new compound **1**.

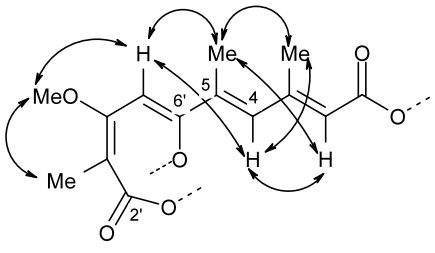

Figure 1. Partial structure of infectopyrone (**1**) from HMBC data, showing important NOE correlations.

A few other α-pyrones have been reported from *Alternaria* species: ACRL toxin II (**3**) and related compounds from *A. citri*,[10] and solanopyrone A (**4**) from *A. solani*.[11] At present **1** is the only known metabolite from the *A. infectoria* species group. Screening HPLC chromatograms of 153 *Alternaria* isolates prepared by Andersen et al.[4] showed that 33 isolates were able to produce **1** (referred to as 'unknown 2' by Andersen and Thrane[2]), and all 33 isolates belonged to the *A. infectoria* species group. Neither *A. alternata* nor isolates from the *A. arborescens* and the *A. tenuissima* species groups were shown to produce compound **1**. The *Alternaria* species producing **1** were *A. arbusti*, *A. conjuncta*, *A. infectoria*, *A. metachromatica*, *A. origonensis* and *A. triticimaculans*. In the study by Andersen and Thrane[2] it was shown that *Stemphylium sarciniforme* was also able to produce 'unknown 2' **1**. Screening of HPLC chromatograms of *Stemphylium* and *Ulocladium* isolates showed that *S. sarciniforme*, *S. vesicarium* and *Ulocladium consortiale* could also produce **1**.

Infectopyrone (**1**) was also detected in two incidences of mouldy tomatoes, where the fungus responsible for the decay was identified as *Stemphylium* sp. Furthermore, infectopyrone (**1**) was produced by isolates in the *A. infectoria* species group when grown on apple juice agar, which suggest that this compound may also be found in apple juice made from mouldy apples. Infectopyrone was not found to be cytotoxic,[12] but in view of the toxicities reported for various α-pyrones,[10,11] infectopyrone (**1**) is a potential mycotoxin whose biological activities should be further investigated.

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7. Isolation of **1**: agar plates were extracted with EtOAc (2 L) to give a crude extract (4.1 g), which was subjected to vacuum liquid chromatography on silica gel (heptane, heptane–EtOAc, EtOAc–EtOH, EtOH as eluents) to give 6 fractions. The most polar fraction (0.6 g) was chromatographed on a Sephadex LH20 (25×800 mm) column and collected into 50 fractions using MeOH–$(CH_3)_2CO$–H_2O (3:1:1) as mobile phase. Fractions 16 and 17 were combined to give 50 mg of enriched **1**. This fraction was subjected to HPLC on a Waters Prep Nova-Pak C18 cartridge (100×25 mm i.d., 6 μm) using H_2O–CH_3CN–CHOOH (50:50:0.1) as mobile phase at 20 mL/min flow rate, to give **1** (28 mg).
8. Data for **1**: pale yellow amorphous solid; UV (MeOH) λ_{max} nm (log ε) 216 (3.79), 262 (3.56), 343 (3.58); IR (KBr) 3418, 2921, 2585, 1686 (br), 1624, 1384, 1253, 1166, 1012 cm^{-1}; NMR, Table 1; ES-HRMS ($M+H^+$) m/z 265.1083 (+ 0.7 mmu calcd for $C_{14}H_{17}O_5$); Analytical HPLC conditions: Gradient: A: water+50 μl trifluoroacetic acid; B: acetonitrile+50 μl trifluoroacetic acid. Time: 0.0 min, A: 90%; Time: 30.0, A: 50%; Time: 40.0, A: 0%; Time: 45.0, A: 0%; Time: 53.0, A: 90%. Column: BDS C_{18} 2×125 mm (id) at 40°C; Flow: 0.3 ml/min. Rentention index (RI) = 882, measured according to: Frisvad, J. C.; Thrane, U. *J. Chromatogr*. **1987**, *404*, 195–214.
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