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Novel Chemotaxonomic Markers of the *Alternaria infectoria* Species-Group

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Two new structurally related metabolites, novae-zelandin A (**1**) and novae-zelandin B (**2**), as well as the novel metabolite 4*Z*-infectopyrone (**3**) were purified from extracts of filamentous fungi belonging to the *Alternaria infectoria* species-group. The structures were elucidated by a combination of 1D and 2D NMR spectroscopic data and MS data. **1**−**3** are important chemotaxonomic markers of the *A. infectoria* species-group and exhibit structures similar to those of known biologically active compounds, suggesting that they could be potential phytotoxins.

KEYWORDS: ***Alternaria*; chemotaxonomy; α-pyrone; infectopyrone; novae-zelandin; phytotoxin**

INTRODUCTION

Contamination of food products and feedstuffs with species of *Alternaria* is of growing concern because of their deterioration of the product and their production of mycotoxins (*1*). Among the field fungi found in cereals, *Alternaria* is the dominant genus, and within this habitat, taxa of the *A. infectoria* species-group predominate by far (*2*). Information available on the *A. infectoria* species-group is limited as the taxa it comprises have often been misidentified as other small-spored *Alternaria* species (e.g. *Alternaria alternata*, *Alternaria tenuissima*, or *Alternaria arborescence*), due to the use of insufficient methods for identification (*3*, *4*). Members of the *A. infectoria* species-group are morphologically distinguishable from other small-spored species of *Alternaria* by their production of long secondary conidiophores (*5*) and formation of white or gray colonies on dichloran rose bengal yeast extract sucrose media (DRYES) (*4*). Furthermore, this species-group is the only one in the genus of *Alternaria* where the teleomorph, *Lewia*, has been identified (*6*). To date, the *A. infectoria* species-group comprises the known species *Alternaria arbusti* (*4*), *Alternaria conjuncta* (*4*), *A. infectoria* (*6*), *Alternaria oregonensis* (*6*), *Alternaria tritici-maculans* (*6*), *Alternaria metachromatica* (*6*), *Alternaria viburni* (*7*), *Alternaria intercepta* (*7*), and *Alternaria novae-zelandiae* (*7*), as well as an unknown number of distinct taxa yet to be described (*4*).

Members of the *A. infectoria* species-group produce a range of unique secondary metabolites that are useful for metabolic profiling and chemotaxonomy of *Alternaria* (*4*, *8*), but until now only one of these metabolites, infectopyrone (**4** in **Figure 1**), has been identified (*9*). Production of infectopyrone (**4**) has also been reported in other fungal genera and is known to be produced by *Stemphylium sarciniforme* (*9*), *Stemphylium versicarium* (*9*), *Stemphylium eturmiunum* (*10*), and *Ulocladium consortiale* (*9*). Furthermore, infectopyrone (**4**) has been found in mouldy tomatoes infected with *S. eturmiunum* (*10*). Infectopyrone (**4**), which was detected in more than 94% of all isolates belonging to the *A. infectoria* species-group (*4*), is often

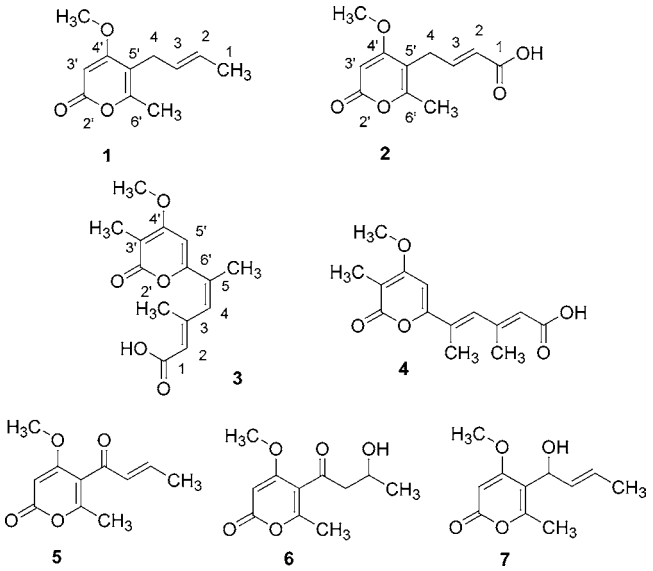

Figure 1. Structures of novae-zelandin A (**1**), novae-zelandin B (**2**), 4*Z*-infectopyrone (**3**), infectopyrone (**4**), and pyrenocines A (**5**), B (**6**), and C (**7**).

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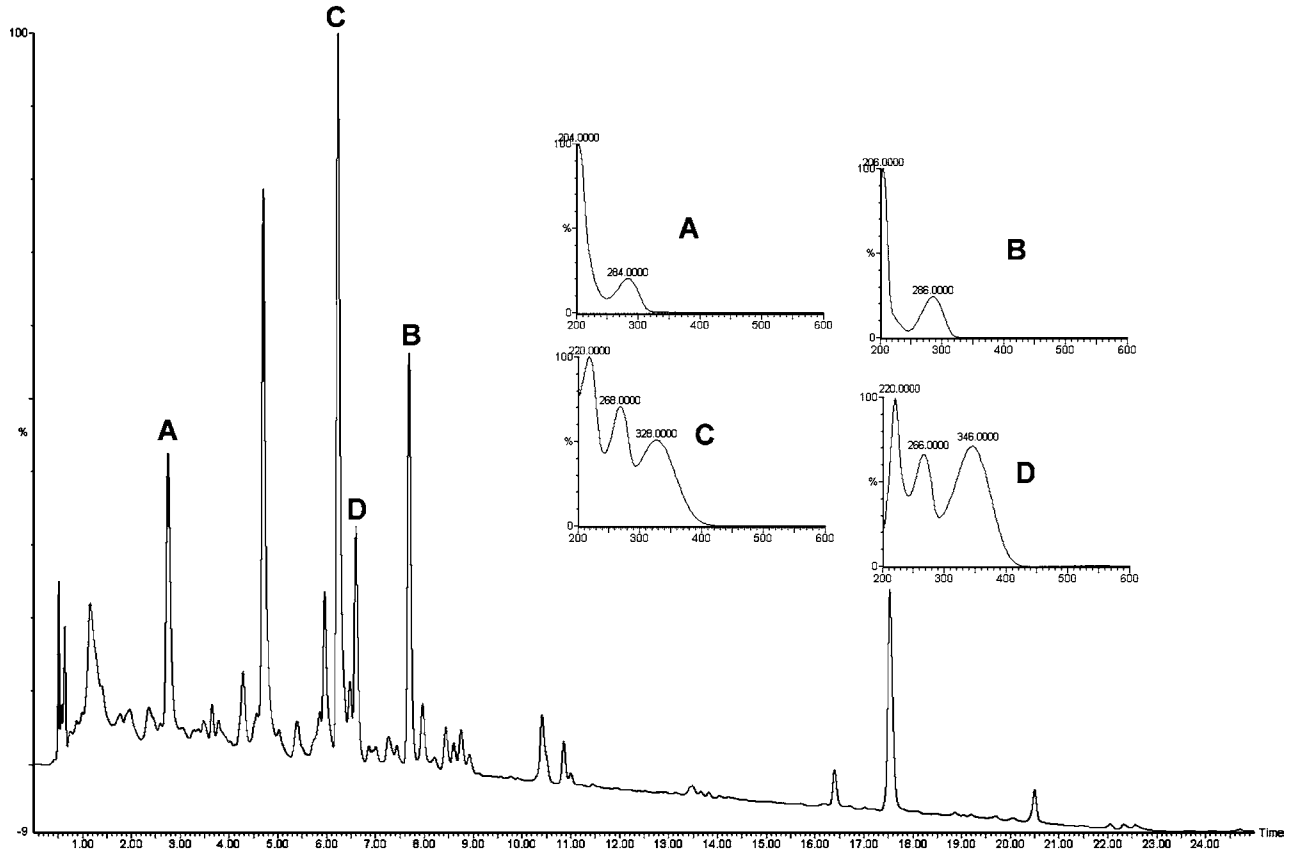


Figure 2. Chromatogram of the crude extract of BA1293 along with the UV spectra of (**A**) novae-zelandin A (**1**), (**B**) novae-zelandin B (**2**), (**C**) 4*Z*-infectopyrone (**3**), and (**D**) infectopyrone (**4**).

Table 1. Experimental NMR Data for 2 in Acetone-d_6 and for 1 in Chloroform-*d*

	novae-zelandin B (**2**)			novae-zelandin A (**1**)		
position	^{13}C	^{1}H	HMBC	^{13}C	^{1}H	HMBC
1	167.8	–	–	17.8	1.63 (3H, d, 5)	C2, C3, C4 (w), C5 (w)
2	123.2	5.89 (1H, dt, 2, 16)	C1, C4	127.0	5.38 (1H, m)	C1, C2, C3, C4, C5 (w)
3	146.8	6.99 (1H, dt, 6, 16)	C1, C4, C5	126.1	5.38 (1H, m)	C1, C2, C3, C4, C5 (w)
4	27.8	3.42 (2H, dd, 1.5, 6)	C1 (w), C2, C3, C4, C5, C6	26.8	2.99 (2H, d, 4)	C2, C3, C4, C5, C6
2	164.2	–	–	164.7	–	–
3	89.0	5.59 (1H, s)	C2, C4, C5	87.8	5.44 (1H, s)	C2, C4, C5
4	171.4	–	–	170.6	–	–
5	108.7	–	–	109.9	–	–
6	161.0	–	–	158.7	–	–
4-OMe	57.6	4.00 (3H, s)	C3, C4	56.1	3.80 (3H, s)	C3 (w), C4
6-Me	17.9	2.32 (3H, s)	C4 (w), C5, C6	17.1	2.19 (3H, s)	C4 (w), C5, C6

accompanied by other distinct secondary metabolites. One such metabolite is "unknown X" in the study of Andersen et al. (*4*), which co-occurred with infectopyrone in 80% of the *A. infectoria* species-group isolates studied (*4*). In this study, the isolation and structural elucidation of unknown X and two other important chemotaxonomic markers are undertaken.

MATERIALS AND METHODS

Chromatographic Separation. An isolate of the *A. infectoria* species-group, BA1293, from the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark, was three-point inoculated onto 200 plates of DRYES (*11*) and incubated for 14 days at 25 °C in darkness. The plates were extracted overnight with EtOAc:1% formic acid (10 mL per plate), the extract was then filtered, and the solvent was removed under vacuum to give a dry red solid (2384 mg). A portion of the extract (850 mg) was fractionated in a 25 mm i.d. glass column packed with 25 g of C_{18} obtained from Sigma-Aldrich and preconditioned with H_2O and eluted with a 5% stepwise gradient from 100% H_2O to 100% MeCN (50 mL fractions each), followed by 50% MeCN in $CHCl_3$ (50 mL) and then 100% $CHCl_3$ (100 mL). One hundred fourteen fractions (10 mL each) were obtained and on the basis of thin-layer chromatography (TLC) combined into 14 fractions. Merck TLC aluminum roll, 500 × 20 cm, silica gel 60 F_{254} was used for TLC, and 100% EtOAc was the mobile phase. All TLC plates were inspected by ultraviolet (UV) light and subsequently visualized by dipping in vanillin−sulfuric acid and heating (*12*). Fractions 7 and 3 contained novae-zelandin A (**1**) (23.8 mg) and novae-zelandin B (**2**) (5 mg), respectively (**Figure 1**). The high performance liquid chromatography (HPLC) trace of the crude extract of BA1293 along with the UV spectra of the described compounds (**1**−**4**) is given in **Figure 2**.

Novae-zelandin A (1): brownish oily solid; UV (EtOH) λ_{max} nm (ϵ) 209 (38 700) 283 (17 200); Infrared spectroscopy (IR) (film on NaCl) 1705, 1562, 1455, 1407, 1249 cm^{-1}; high-resolution electrospray ionization mass spectrometry (HRESIMS) $[M + H]^+$ *m/z* 195.1024 (195.0977 calculated for $C_{11}H_{15}O_3$); ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR, see **Table 1**. HPLC conditions: solvent A, water with 50 ppm trifluoroacetic acid (TFA); solvent B, MeCN with 50 ppm

TFA; gradient, 0.0 min A 85%, 40.0 min A 15%, 43.0 min A 15%, 50.0 min A 85%; column, 100 mm × 2 mm i.d., 3 μm, C_{18} Luna II (Phenomenex, UK) at 40 °C; flow, 0.4 mL/min; retention index (RI) = 897 (*13*).

Novae-zelandin B (2): pale yellow oil; UV (EtOH) λ_{max} nm (ϵ) 205 (8800) 283 (1740); IR (film on NaCl) 3276, 2926, 1692 (br), 1562, 1454, 1408, 1250 cm^{-1}; HRESIMS $[M + H]^+$ *m/z* 225.1022 (225.0718 calculated for $C_{11}H_{13}O_5$); ^{1}H NMR and ^{13}C NMR, see **Table 1**. HPLC conditions were the same as for **1**; RI = 688 (*13*).

An extract of a second member of the *A. infectoria* species-group, BA1286, from the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark, was prepared in the same way as with BA1293 to give an oily red solid (1956 mg). A portion of the extract (470 mg) was fractionated as with the BA1293 extract. One hundred fifteen fractions (10 mL each) were obtained and on the basis of TLC combined into 15 fractions. Fractions 7 (2.6 mg) and 8 (9.2 mg) both contained a 50:50 mixture of 4*Z*-infectopyrone (**3**) and infectopyrone (**4**).

4*Z*-Infectopyrone (3): UV λ_{max} nm 218, 268, 328; HRESIMS $[M - H]^-$ *m/z* 263.0723 (263.0723 calculated for $C_{14}H_{15}O_5$); ^{1}H NMR 500 MHz (acetone-d_6) δ 6.64 (H5′, 1H, m), 6.50 (H4, 1H, s), 5.87 (H2, 1H, m), 4.05 (H4′-OMe, 3H, s), 2.27 (H3-Me, 3H, m), 2.20 (H5-Me, 3H, J = 1.5 Hz, d), 1.93 (H3′-Me, 3H, s); ^{13}C NMR 125 MHz (acetone-d_6) δ 168.2 (C1), 166.7 (C4′), 164.9 (C2′), 160.6 (C6′), 154.2 (C3), 136.8 (C4), 133.7 (C5), 121.1 (C2), 103.2 (C3′), 98.2 (C5′), 57.7 (C4′-OMe), 23.3 (C5-Me), 19.1 (C3-Me), 9.3 (C3′-Me); NOESY H2 (H4), H4 (H2, H5-Me), H5′ (H5-Me, H3′-Me, H4′-OMe), H5-Me (H4, H5′), H3′-Me (H5′, H4′-OMe), H4′-OMe (H5′, H3′-Me).

Infectopyrone (4) (*9*): ^{1}H NMR 500 MHz (DMSO-d_6) δ 6.96 (H4, 1H, s), 6.79 (H5′, 1H, m), 5.90 (H2, 1H, m), 4.08 (H4′-OMe, 3H, s), 2.36 (H3-Me, 3H, m), 2.19 (H5-Me, 3H, m), 1.93 (H3′-Me, 3H, s); ^{13}C NMR 125 MHz (DMSO-d_6) δ 167.3 (C1), 165.9 (C4′), 163.3 (C2′), 158.5 (C6′), 150.5 (C3), 133.3 (C4), 129.6 (C5), 121.5 (C2), 101.4 (C3′), 95.5 (C5′), 57.0 (C4′-OMe), 18.8 (C3-Me), 14.1 (C5-Me), 8.8 (C3′-Me); NOESY H2 (H4, H5-Me), H4 (H2, H5′, H3-Me), H5′ (H4, H5-Me, H4′-OMe), H3-Me (H4, H5-Me), H5-Me (H2, H5′, H3-Me), H3′-Me (H4′-OMe), H4′-OMe (H5′, H3′-Me).

NMR spectra were recorded at 25 °C on a Varian Unity Inova instrument operating at 499.74 MHz for ^{1}H and 125.69 for ^{13}C. Spectra were referenced to acetone for **2** and **3** (δ_H 2.15, δ_C 30.5) and $CHCl_3$ for **1** (δ_H 7.25, δ_C 77.0). All coupling constants are given in hertz.

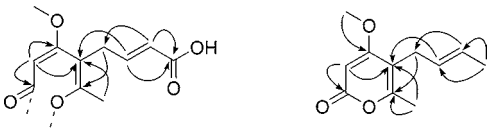
RESULTS AND DISCUSSION

Novae-zelandin B (**2**) was isolated first; therefore, the structural elucidation was performed on this metabolite, rather than the lower mass novae-zelandin A (**1**).

HRESIMS of **2** gave an $[M + H]^+$ ion corresponding to 225.1022 Da and, combined with NMR spectroscopic data, indicated a molecular formula of $C_{11}H_{12}O_5$ (six double bond equivalents). The IR spectrum indicated the presence of a carboxylic acid group with a broad hydroxyl stretching band between 3600 and 2500 cm^{-1} and a strong broadened carbonyl stretching band at 1692 cm^{-1}.

The structure of **2** was established by 1D and 2D NMR analysis. The 1D NMR spectra showed one methyl group (δ_H 2.32, δ_C 17.9), one methoxy group (δ_H 4.00, δ_C 57.6), a methylene group (δ_H 3.42, δ_C 27.8), and three sp^2 hybridized methine groups (δ_H 5.59, δ_C 89.0; δ_H 5.89, δ_C 123.2; δ_H 6.99, δ_C 146.8). Four of the remaining five sp^2 hybridized quaternary carbons (δ_C 108.7, δ_C 161.0, δ_C 164.2, δ_C 167.8, δ_C 171.4) had shifts of more than 160 ppm, indicating that they were oxygenated aromatic or vinylic carbon atoms (**Table 1**).

The observed heteronuclear multiple bond correlations (HMBC) enabled the structure of **2** to be determined (**Figure 3**). Protons H3 and H4 both correlated to C5′; with H4 also correlating to C4′ and C6′. Correlations were seen from the C6′-Me protons to C5′ and C6′, with a weak four bond correlation to C4′ also observed (ω-coupling) (*14*), establishing the attachment of the methyl group to C6′. The H3′ singlet correlated to C4′ and C5′, thereby linking C3′ to C4′. The H3′ singlet also correlated to the low-field carbon signal (δ_C 164.2) C2′, connecting this to C3′. The H2 vinyl proton (δ_H 5.89) correlated to C4 and C1 (**Figure 3**). A correlation from the methoxy group (δ_H 4.00, δ_C 57.6) to C4′ was also observed. To fulfill the requirement for six double bond equivalents, C2′ and C6′ were connected via the available oxygen on C6′ to make a lactone bridge and thereby giving novae-zelandin B (**2**). All of the assignments are in good agreement with those reported for similar α-pyrone derivatives (*9*, *15*−*18*). The *E*-configuration of the double bond was determined from the large coupling constant observed between H2 and H3 (16 Hz) in the ^{1}H NMR spectrum. Comparison of the low-field shift of the carbon signal for C1 and those for C2 and C3 with those observed for C1, C2, and C3 in **3** (*9*) suggested that C1 was the carboxylic acid group previously indicated in the IR spectrum.



2 1

Figure 3. Selected HMBC correlations for novae-zelandin B (**2**) and novae-zelandin A (**1**).

Novae-zelandin A (**1**) possessed a similar UV spectrum to that of **2**, with HRESIMS and NMR data indicating a molecular formula of $C_{11}H_{14}O_3$ (five double bond equivalents). The decrease of 30 mass units when compared to **2**, the similar UV spectra, and similar NMR data to that acquired for **2** (**Table 1**) indicated that **1** had a methyl group in place of the carboxylic acid moiety observed in **2**. An HMBC experiment confirmed the structure of **1** with correlations from the vinylic methyl group (δ_H 1.63, δ_C 17.8) to the two vinylic carbons (δ_C 126.1, δ_C 127.0) (**Figure 3**). The indicated *E*-configuration of the double bond is based on the fact that this is the lowest energy form and therefore the most likely. In this case no coupling constants could be measured due to overlap of the vinylic signals in a variety of solvents. For a cyclohexanone derivative with a similar 2-butenyl side chain to that of **1**, the shift for the terminal methyl group has been reported as δ_C 17.8 for the *E*-configuration, and for the Z-configuration, the much lower shift, δ_C 12.81, due to the γ-effect (*19*) adding evidence for the suggested *E*-configuration of **1**.

In a second extract (BA1286), three metabolites with identical masses and similar UV spectra to the previously identified infectopyrone (**4**) (*9*) were detected, but only one (**3**) was partially purified due to stability problems. The structural elucidation of **3** was performed on a 50:50 mixture with **4**, as a high degree of isomerization was observed due to the fact that infectopyrone is the lowest energy state of the *E*/*Z*-isomers. The differences between 4*Z*-infectopyrone (**3**) and **4** were as follows: The H4 proton and the C5 methyl signals in **4** had shifted from δ_H 6.96 and δ_C 14.1 to δ_H 6.50 and δ_C 23.3 in **3**, suggesting a change in the configuration of the C4−C5 double bond from *E* to *Z*. This has been observed for the two *E*/*Z*-isomers, placidene A and isoplacidene, for which the shift of the methyl group changed from δ_C 16.0 in placidene A (*E*) to δ_C 21.8 in isoplacidene (*Z*) (*20*). The observed nuclear Overhauser enhancement spectroscopy (NOESY) correlations between H4 (δ_H 6.50) and the 5-Me protons (δ_H 2.20) for **3** confirmed the change in configuration of the double bond.

α-Pyrone derivatives with structures similar to those of **1** and **2** have been isolated from a number of other fungal species,

many of these exhibiting biological activity, mostly as phytotoxins (*15*, *16*, *18*, *21*, *22*). The phytotoxic compounds pyrenocines A (**5**), B (**6**), and C (**7**), produced by *Penicillium citrionigrum* (=*P. citreo-viride*) (*17*) and *Pyrenochaeta terrestris* (*18*, *23*, *24*), are structurally similar to metabolites **1** and **2** with differences observed in the C_4-side chain (**Figure 1**). Compounds **5** and **6** have also been found in a novel large-spored *Alternaria* species (erroneously recorded as *Alternaria helianthi* (*1*)) isolated from the leaves of *Helianthus tuberosus* (*25*). Compound **5** has been found to be the major toxin of the pyrenocines in all performed assays, and this is thought to be because of the α,β-unsaturated carbonyl of the side chain, which is lacking in **6** and **7** (*26*, *27*). Tests have shown that compounds **1** and **2** do not possess any antitumor or antiviral activity. However, **1** and **2** were mildly active against *Bacillus subtilis* at 60 μg/disk, giving inhibition zones of 3 and 1 mm, respectively. Due to the close structural similarity between the metabolites **1** and **2** and the pyrenocines it is believed that they are potential phytotoxins.

The results from this study show that *Alternaria cetera*, *A. infectoria*, *A. intercepta*, *A. novae-zelandiae*, *A. triticimaculans*, and *A. viburni* are able to produce both **1** and **2**. Furthermore, a screening of 140 cultures belonging to unknown taxa in the *A. infectoria* species-group isolated from cereals, grasses, carrots, and caprifoliaceous shrubs in Denmark, The Netherlands, and New Zealand showed that 88 cultures were able to produce both **1** and **2**. The production of **1** and **2** were not detected in *A. arbusti*, *A. conjuncta*, *A. metachromatica*, or *A. oregonensis* in the *A. infectoria* species-group or any other culture belonging to other small-spored *Alternaria*, such as *A. alternata* or the *A. arborescence* and *A. tenuissima* species-groups. Neither was the production of **1** and **2** detected in any *Stemphylium* or *Ulocladium* species. Our results also show that *A. arbusti*, *A. conjuncta*, *A. infectoria*, *A. intercepta*, *A. metachromatica*, *A. novae-zelandiae*, *A. oregonensis*, *A. triticimaculans*, and *A. viburni* were able to produce **3** and **4** and that **3** and **4** co-occur in more than 90% of the cultures belonging to the *A. infectoria* species-group, but they are never detected in other small-spored *Alternaria*. On the other hand, the production of **3** is not detected in any *Stemphylium* or *Ulocladium* species.

The findings in this study show that a combination of the metabolites **1**−**4** can be used as chemotaxonomic marker for the *A. infectoria* species-group and thereby exclude *Stemphylium*, *Ulocladium*, and other small-spored *Alternaria*, which produce alternariols and tenuazonic acid, during identification procedures of food-borne fungi. Furthermore, if **4** is detected in cereal-based foods or feedstuff, where *A. infectoria* species-group predominates, one should always look for **3** as well, since these two metabolites appear to co-occur in more than 90% of cultures in the *A. infectoria* species-group.

ABBREVIATIONS USED

DRYES, dichloran rose bengal yeast extract sucrose agar; RI, retention index; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; HRESIMS, high-resolution electrospray ionization mass spectrometry; UV, ultraviolet; IR, infrared spectroscopy; TFA, trifluoracetic acid; HPLC, high performance liquid chromatography; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy; w, weak (in NMR); s, singlet (in NMR); d, doublet (in NMR); dd, double of doublets (in NMR); m, multiplet (in NMR).

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