Isolation and Structure Elucidation of the New Fungal Metabolite (−)-Xylariamide A

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Received January 24, 2005

Chemical investigations of the terrestrial microfungus *Xylaria* sp. have afforded the new natural product (−)-xylariamide A (**1**). The gross structure of **1** was determined by interpretation of 1D and 2D NMR, UV, IR, and MS data. Confirmation of the structure and the absolute stereochemistry of **1** were determined by the total synthesis of (+)-xylariamide A (**2**). Synthetic **2** was produced by *N*,*O*-bis(trimethylsilyl)-acetamide-induced coupling of 3-chloro-L-tyrosine (**3**) with (*E*)-but-2-enedioic acid 2,5-dioxo-pyrrolidin-1-yl ester methyl ester (**4**). Optical rotation comparison of **1** with **2** indicated that the natural product (**1**) contained 3-chloro-D-tyrosine. Both enantiomers of xylariamide A were tested in a brine shrimp lethality assay, and only the natural product (**1**) showed toxicity.

Several thousand organohalogen compounds have been identified as biosynthetic products from living organisms.[1] While the oceans are the single largest source of naturally occurring organohalogens, many terrestrial plants, bacteria, insects, and lichens have also been identified as producers of halogenated natural products.[2] Both marine- and terrestrial-derived fungi produce chlorinated metabolites; examples include pestalone,[3] trichodermamide B,[4] helicusins A−D,[5] and lachnumon.[6] Chlorinated fungal metabolites containing the 3-chloro-4-hydroxyl phenyl subunit are rare. To date only four such compounds have been isolated;[7] these include coniothyriomycin,[8] 3-chloro-4-hydroxybenzeneethanol,[9] 3-chloro-4-hydroxyphenylacetic acid,[10] and 3-chloro-4-hydroxyphenylacetamide.[11] We have recently embarked on a research program looking for new structural and bioactive metabolites from microfungi isolated from Australian endemic plants.[11,12] Examination of a local rainforest tree, *Glochidion ferdinandi* (family Euphorbiaceae), afforded several microfungal strains, one of which was identified as *Xylaria* sp. (family Xylariaceace). Chemical research on this particular strain has already yielded two new xanthones[12] and the novel metabolite 3-chloro-4-hydroxyphenylacetamide.[11] Ongoing investigations of the EtOAc extract resulting from the fungal isolate being cultured on solid media have resulted in the isolation of a new chlorinated natural product. Herein we report the isolation, structure elucidation, and absolute stereochemical determination of the novel metabolite that we have named (−)-xylariamide A (**1**).

The fungus *Xylaria* sp. (FRR 5657) was grown on damp white rice under static conditions, then extracted with EtOAc. This extract was initially separated by C18 flash column chromatography using H_2O and increasing amounts of CH_3OH. An early eluting fraction was subjected to C18 preparative HPLC (CH_3OH/H_2O) followed by phenyl preparative HPLC (CH_3OH/aq TFA) to yield pure (−)-xylariamide A (**1**, 0.9 mg).

Compound **1** was isolated as an optically active pale yellow gum and was assigned the molecular formula $C_{14}H_{14}NO_6Cl$ on the basis of (−)-HRESIMS and ^1H and ^{13}C NMR spectral data. The (−)-LRESIMS isotopic pattern of **1** confirmed the presence of one chlorine atom.[13] The IR spectrum displayed two strong and broad absorptions at 1716 and 1667 cm^{-1}, suggesting that **1** contained two or more carbonyl groups. The ^1H NMR spectrum of **1** contained two exchangeable signals [δ 9.95, 8.80], five sp^2 methine signals [δ 7.17, 7.05, 6.97, 6.84, 6.54], three midfield multiplets [δ 2.77, 3.00, 4.46], and a methoxyl singlet [δ 3.71]. The ^{13}C NMR spectrum of **1** displayed 14 signals, of which 11 resonated between δ 119 and 173. The HSQC spectrum enabled all the proton signals to be assigned to their directly attached carbons. The three aromatic methine signals at δ 7.17 (d, J = 1.5 Hz, 1H), 6.84 (d, J = 8.5 Hz, 1H), and 6.97 (d, J = 8.5, 1.5 Hz, 1H) were assigned to a 1,3,4-trisubstituted benzene ring. This system was oxygenated at C-4 of the aromatic ring on the basis of strong $^3J_{CH}$ HMBC correlations from both H-2 (δ 7.17) and H-6 (δ 6.97) to a carbon at δ 151.6. H-2 and H-6 also showed strong HMBC correlations to a carbon at δ 35.5, to which the diastereotopic protons δ 2.77 (H-7a) and 3.00 (H-7b) where attached. ROESY correlation between H-7a and H-7b with both methine protons H-2 and H-6 confirmed that C-7 (δ 35.5) was a benzylic carbon. The gCOSY spectrum and ^1H−^1H coupling constants revealed that the diastereotopic protons H-7a [δ 2.77 (dd, J = 14.5, 10.0 Hz, 1H)] and H-7b [δ 3.00 (dd, J = 14.5, 5.0 Hz, 1H)] were positioned next to the aliphatic methine proton H-8 [δ 4.46 (ddd, J = 10.0, 8.0, 5.0 Hz, 1H)], which also showed a strong COSY correlation to the exchangeable proton at 8-NH [δ 8.80 (d, J = 8.0 Hz, 1H)]. These characteristic data suggested that (−)-xylariamide A contained a derivative of the aromatic α-amino acid tyrosine. Strong HMBC correlations from H-7a, H-7b, and H-8 to a carboxylic acid carbonyl at δ 172.3 provided further proof for this assignment. The olefinic protons at δ 6.54 (dd, J = 15.5 Hz, 1H) and 7.05 (d, J = 15.5 Hz, 1H) were assigned to an isolated *E*-double bond on the basis of the multiplicity and magnitude of the coupling constants.[13] Both these protons showed HMBC correlations to the carbonyl carbons at δ 162.7 and 165.4. The latter carbon was assigned to a methyl ester moiety on the basis of the only observed HMBC correlation from the methoxyl singlet at δ 3.71 (3H) to C-13. Linkage of this methyl ester terminated *E*-double bond to the amino acid portion of the molecule was achieved via HMBC correlations from both the olefinic proton (H-11) and amide proton (8-NH) to the amide carbonyl at δ 162.7 (C-10). A strong ROESY correlation between H-11 and 8-NH further supported this linkage. Although no 2D NMR correlations were

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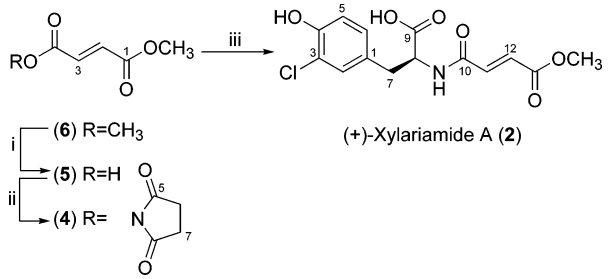
10.1021/np050025h CCC: $30.25
Published on Web 04/14/2005

Scheme 1. Total Synthesis of (+)-Xylariamide A (**2**)[a]



[a] (i) LiOH, acetone, rt, 1 h. (ii) *N*-Hydroxysuccinimide, EDCl, CH_3CN, rt, 24 h. (iii) 3-Chloro-L-tyrosine (**3**), BSA, DMF, 55 °C, 24 h.

observed for the broad exchangeable signal at δ 9.95, this was assigned to a hydroxyl group attached to C-4 of the aromatic ring on the basis of the ^{1}H chemical shift of this hydroxyl moiety and the ^{13}C chemical shift of this carbon (δ 151.6). By default, the chlorine atom present in **1** was attached to C-3 of the trisubstituted benzenoid system. Hence a 3-chloro-4-hydroxyphenyl system was established. The NMR data for this system showed only minor discrepancies (^{13}C, <1.5 ppm; ^{1}H, <0.09 ppm) with the aromatic portions of the previously isolated fungal metabolites, 3-chloro-4-hydroxyphenylacetic acid and 3-chloro-4-hydroxyphenylacetamide.[11] Although the carboxylic acid proton (9-OH) was not observed in the ^{1}H NMR spectrum of **1**, the (−)-LRESIMS data provided evidence of a carboxylic acid group due to the fragment ions corresponding to $[M - 45]^-$.[13] Hence the gross structure for **1** was assigned to xylariamide A.

To determine the absolute stereochemistry of **1** and obtain more material for future biological testing, we synthesized xylariamide A using previously published peptide chemistry.[14] Regardless of which enantiomer of xylariamide A we synthesized, the comparison of optical rotation sign would enable us to unequivocally assign the absolute stereochemistry of the natural product. The synthetic chemistry involved the use of the silylating agent *N*,*O*-bis(trimethylsilyl)acetamide (BSA), which has been shown to induce amide bond formation between an unprotected amino acid and an *N*-succinimide-activated ester.[14] For the synthesis of xylariamide A we used the commercial reagent 3-chloro-L-tyrosine (**3**), (*E*)-but-2-enedioic acid 2,5-dioxo-pyrrolidin-1-yl ester methyl ester (**4**), and BSA in DMF at 55 °C for 24 h (Scheme 1). Reaction workup, which included C18 column chromatography and Sephadex LH-20 gel permeation separation, afforded pure xylariamide A (**2**, 47 mg, 48% yield). The NMR, MS, UV, and IR data for the synthetic compound were essentially identical to the natural product (**1**). However the optical rotation of synthetic xylariamide A (**2**) ($[\alpha]^{23}_D$ +17° (CH_3OH)) had the opposite sign of the naturally occurring material (**1**) ($[\alpha]^{24}_D$ −22° (CH_3OH)). Hence the synthetic material (+)-xylariamide A (**2**) was the enantiomer of the natural product, and thus 3-chloro-D-tyrosine was determined to be a constituent of (−)-xylariamide A (**1**).

Both synthetics **4**[15] and **5**[16,17] have been previously synthesized; however both were only partially characterized by spectroscopic methods. 1D and 2D NMR experiments were performed on both compounds in DMSO-d_6, and full NMR assignments were made. Initially, the assignment of the ^{13}C chemical shifts for C-2 and C-3 of **4** and **5** was not possible since both olefinic protons H-2 and H-3 in **4** and **5** had identical or similar ^{1}H chemical shifts and they both showed strong HMBC correlations to the carbonyl carbons C-1 and C-4. The full carbon NMR assignments for **4** and **5** were made possible due to gHMBC

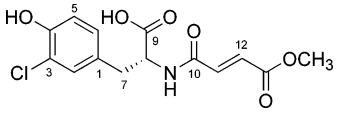


Figure 1. Structure of (−)-xylariamide A (**1**).

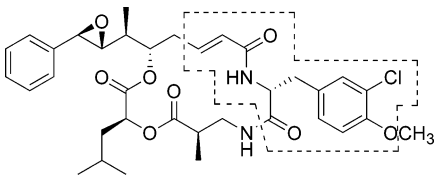


Figure 2. Structure of cryptophycin A (**7**). Shared structural motif of **7** with **1** is outlined (- - -).

experiments performed on a 600 MHz Varian spectrometer equipped with a triple resonance cold probe. These data showed a $^4J_{CH}$ correlation from 1-OCH_3 to C-2 in both compounds **4** and **5**.

Interestingly, the natural product (−)-xylariamide A (**1**) shares a structural motif with the cyanobacteria-derived depsipeptide and potent antitumor agent cryptophycin A (**7**)[18] (Figure 2). Although compound **1** shared structural homology with only a small portion of cryptophycin A (**7**), this observation prompted us to test the natural product (**1**) and synthetic enantiomer (**2**) for toxicity.

Both (−)-xylariamide A (**1**) and (+)-xylariamide A (**2**) were screened for toxicity in a brine shrimp (*Artemia salina*) lethality assay.[19] The synthetic enantiomer (**2**) showed no toxicity when screened at 20 or 200 μg/mL, while the natural product (**1**) showed 0% and 71% lethality at 20 and 200 μg/mL, respectively. Unfortunately, lack of material of **1** prevented us from calculating a LD_{50} value in the brine shrimp lethality assay or testing (−)-xylariamide A in a panel of human carcinoma cell lines. The synthesis of (−)-xylariamide A (**1**) is currently underway; more material will allow further biological evaluations of this new chlorinated fungal natural product to be performed.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 30 °C on either a Varian 500 or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ 2.49 and 39.51, respectively. LRESIMS were recorded on a Fisons mass spectrometer. HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier transform mass spectrometer. FTIR and UV spectra were recorded on a Perkin-Elmer 1725X spectrophotometer and a GBC UV/vis 916 spectrophotometer, respectively. Optical rotations were recorded on a Jasco P-1020 polarimeter. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Sephadex LH-20 packed into an open glass column (45 mm × 450 mm) was used for gel permeation chromatography. A Waters AP-2 MPLC column (20 mm × 90 mm) packed with Alltech Davisil silica 30−40 μm 60 Å was used for synthetic reaction purification. A Phenomenex Strata Si-1 or C18-E SPE cartridge (1 g, 50 μm, 70 Å) was used for synthetic reaction cleanup. A Waters 600 pump equipped with a Waters 996 PDA detector and a Rheodyne injector were used for HPLC. Thermo Hypersil C18 and phenyl BDS 5 μm 143 Å preparative columns (21.2 mm × 150 mm) were used for HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade, and the H_2O used was Millipore Milli-Q PF filtered. All synthetic reagents were purchased from Sigma-Aldrich. All fungal culture media were purchased from Difco.

Fungal Material. The collection and identification of *Xylaria* sp. (FRR 5657) are detailed elsewhere.[12]

Fermentation, Extraction, and Isolation. The fungal isolate was initially grown in three culture tubes each containing malt extract broth (10 mL) at 30 °C for 5 days. These cultures were transferred to three conical flasks (500 mL) each containing sterilized damp white rice (50 g of rice plus 100 mL of H_2O), and the fermentation was allowed to proceed under static conditions at 25 °C for 28 days. EtOAc extraction of the cultures followed by removal of the solvent in vacuo yielded a dark green gum (1.57 g). This extract was preabsorbed to C18 silica, then loaded onto a C18 flash column, and a 20% stepwise gradient was performed from 100% H_2O to 100% CH_3OH. The 20% CH_3OH/80% H_2O elution was further purified by C18 preparative HPLC using a linear gradient from 100% H_2O to 50% CH_3OH/50% H_2O in 50 min at a flow rate of 8 mL/min. Fraction 14 (11.6 mg, t_R = 38−41 min) was subjected to phenyl preparative HPLC using a linear gradient from 100% aqueous TFA (1.0%) to 100% CH_3OH in 20 min at a flow rate of 6 mL/min and yielded pure (−)-xylariamide A (**1**, 0.9 mg, t_R = 17.2 min).

(−)-Xylariamide A (1): stable yellow gum; $[\alpha]^{24}_D$ −22° (*c* 0.060, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 202 (4.05), 217 (sh) (3.74), 278 (3.12) nm; IR ν_{max} (NaCl) 3600−3100, 1716, 1667, 1544, 1511, 1438, 1293, 1197, 1024, 975, 826 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 2.77 (1H, dd, J = 14.5, 10.0 Hz, H-7a), 3.00 (1H, dd, J = 14.5, 5.0 Hz, H-7b), 3.71 (3H, s, 13-OCH_3), 4.46 (1H, ddd, J = 10.0, 8.0, 5.0 Hz, H-8), 6.54 (1H, d, J = 15.5 Hz, H-12), 6.84 (1H, d, J = 8.5 Hz, H-5), 6.97 (1H, dd, J = 8.5, 1.5 Hz, H-6), 7.05 (1H, d, J = 15.5 Hz, H-11), 7.17 (1H, d, J = 1.5 Hz, H-2), 8.80 (1H, d, J = 8.0 Hz, 8-NH), 9.95 (1H, brs, 4-OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 35.5 (C-7), 52.0 (13-OCH_3), 53.8 (C-8), 116.4 (C-5), 119.2 (C-3), 128.6 (C-12), 128.6 (C-6), 128.9 (C-1), 130.2 (C-2), 136.9 (C-11), 151.6 (C-4), 162.7 (C-10), 165.4 (C-13), 172.3 (C-9); (−)-LRESIMS *m/z* (rel int) 168 (15), 167 (5), 282 (10), 284 (3), 326 (100), 328 (33); (−)-HRESIMS *m/z* 326.04257 ($C_{14}H_{13}NO_6{}^{35}Cl$ $[M - H]^-$ requires 326.04369).

Synthesis of (*E*)-But-2-enedioic Acid Monomethyl Ester (5). The commercial reagent (*E*)-but-2-enedioic acid dimethyl ester (**6**, 1.44 g, 10 mmol) was dissolved in acetone (70 mL) at room temperature, and 1 N LiOH (10 mL, 10 mmol) was slowly added over 15 min to the stirred solution. The reaction was stirred for 1 h, diluted with 2 N HCl (200 mL), saturated with NaCl, and then extracted with EtOAc (3 × 200 mL). The EtOAc layer was slowly evaporated, and the resulting precipitate was filtered and dried to yield pure (*E*)-but-2-enedioic acid monomethyl ester (**5**, 1.13 mg, 87% yield) as a white amorphous solid: mp 142−143 °C (lit. mp 141−141.5 °C);[16,17] UV (CH_3OH) λ_{max} (log ϵ) 209 (4.15) nm; IR ν_{max} (NaCl) 1803, 1775, 1751, 1656, 1441, 1425, 1362, 1314, 1286, 1204, 1101, 1049, 977, 962, 886, 809, 759, 736, 651 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 3.72 (3H s, 1-OCH_3), 6.69 (2H, s, H-2, H-3), 13.18 (1H, brs, 4-OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 52.3 (1-OCH_3), 132.3 (C-2), 134.7 (C-3), 165.1 (C-1), 165.7 (C-4); (−)-LRESIMS *m/z* (rel int) 129 (100); (−)-HRESIMS *m/z* 129.01930 ($C_5H_5O_4$ $[M - H]^-$ requires 129.01933).

Synthesis of (*E*)-But-2-enedioic Acid 2,5-Dioxo-pyrrolidin-1-yl Ester Methyl Ester (4). (*E*)-But-2-enedioic acid monomethyl ester (**5**, 260 mg, 2 mmol), EDCI (768 mg, 4 mmol), and *N*-hydroxysuccinimide (690 mg, 6 mmol) were dissolved in dry CH_3CN (5 mL), and the reaction was stirred at room temperature for 24 h. The reaction mixture was preabsorbed to silica, then loaded onto a glass column and flushed with 100% EtOAc (50 mL). The EtOAc wash was evaporated to dryness, redissolved in 100% DCM, and injected onto a MPLC silica-packed column using isocratic conditions of 40% EtOAc/60% hexanes at a flow rate of 6 mL/min for 30 min. This yielded pure (*E*)-but-2-enedioic acid 2,5-dioxo-pyrrolidin-1-yl ester methyl ester (**4**, 180 mg, 40% yield, t_R = 12.0 min) as a white amorphous solid: mp 93−95 °C (lit. mp 93.5−94.5 °C);[15] UV (CH_3OH) λ_{max} (log ϵ) 213 (3.47), 263 (sh) (2.53) nm; IR ν_{max} (NaCl) 1722, 1682, 1633, 1440, 1413, 1317, 1286, 1266, 1177, 997, 918, 778, 653, 562 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 2.85 (4H, s, H-6, H-7), 3.78 (3H s, 1-OCH_3), 7.07 (1H, d, J = 16.0 Hz, H-3), 7.11 (1H, d, J = 16.0 Hz, H-2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 25.5 (2C, C-6, C-7), 52.6 (1-OCH_3), 126.9 (C-3), 137.9 (C-2), 163.9 (C-1), 160.6 (C-4), 169.8 (2C, C-5, C-8); (+)-LRESIMS *m/z* (rel int) 228 (20), 250 (100); (+)-HRESIMS *m/z* 250.03208 ($C_9H_9NO_6Na$ $[M + Na]^+$ requires 250.03221).

Synthesis of (+)-Xylariamide A (2). *N*,*O*-Bis(trimethylsilyl)acetamide (439 μL, 1.8 mmol) was added to (*E*)-but-2-enedioic acid 2,5-dioxo-pyrrolidin-1-yl ester methyl ester (**4**, 68 mg, 0.3 mmol) and 3-chloro-L-tyrosine (**3**, 195 mg, 0.9 mmol) in dry DMF (3 mL), and the reaction was heated at 55 °C for 24 h. Upon cooling, the DMF was removed in vacuo and the residue was preabsorbed to C18. The C18 material was added to a C18 SPE cartridge, and 20% stepwise elutions were run from 100% aqueous TFA (0.1%) to 100% CH_3OH (10 mL washes). Fractions 3 and 4 were combined, then subjected to Sephadex LH-20 chromatography using 100% CH_3OH as the eluent at a flow rate of 4.5 mL/min. All resulting fractions were analyzed by TLC and identical fractions combined to yield pure (+)-xylariamide A (**2**, 47 mg, 48% yield) as a stable yellow gum: $[\alpha]^{23}_D$ +17° (*c* 0.313, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 207 (4.18), 218 (sh) (4.08), 278 (3.51) nm; IR ν_{max} (NaCl) 3400−3100, 1716, 1667, 1541, 1512, 1437, 1293, 1227, 1197, 1167, 1056, 1020, 977, 818 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 2.77 (1H, dd, J = 14.0, 9.5 Hz, H-7a), 3.00 (1H, dd, J = 14.0, 4.5 Hz, H-7b), 3.71 (3H, s, 13-OCH_3), 4.44 (1H, ddd, J = 9.5, 8.5, 4.5 Hz, H-8), 6.54 (1H, d, J = 16.0 Hz, H-12), 6.85 (1H, d, J = 8.5 Hz, H-5), 6.97 (1H, d, J = 8.5 Hz, H-6), 7.06 (1H, d, J = 16.0 Hz, H-11), 7.16 (1H, s, H-2), 8.77 (1H, d, J = 8.5 Hz, 8-NH), 10.08 (1H, brs, 4-OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 35.6 (C-7), 52.0 (13-OCH_3), 54.1 (C-8), 116.4 (C-5), 119.1 (C-3), 128.5 (C-12), 128.6 (C-6), 129.1 (C-1), 130.2 (C-2), 137.1 (C-11), 151.6 (C-4), 162.6 (C-10), 165.4 (C-13), 172.4 (C-9); (−)-LRESIMS *m/z* (rel int) 168 (20), 167 (7), 282 (10), 284 (3), 326 (100), 328 (33); (−)-HRESIMS *m/z* 326.04419 ($C_{14}H_{13}NO_6{}^{35}Cl$ $[M - H]^-$ requires 326.04369).

Brine Shrimp Lethality Assay. Brine shrimp eggs were obtained from Bio-Marine Aquafauna and were hatched in artificial seawater prepared from Instant Ocean (Aquarium Systems). The hatching, harvesting, and dispensing of nauplii were performed in a manner similar to that described by Solis et al.[19] Compounds **1** and **2** were added to live nauplii (10−20 per vial, V_T = 500 μL) in glass vials (2 mL), and following 24 h of drug treatment the brine shrimp lethality was measured by counting the number of dead (nonmotile) nauplii per vial. Compounds **1** and **2** were tested in triplicate at 20 and 200 μg/mL and were solubilized in 100% DMSO with a final DMSO concentration of 2% in each vial. The brine shrimp responded typically when treated with the laboratory standards, lissoclinotoxins E and F.[20] For example, the LD_{50} of lissoclinotoxin F toward the brine shrimp was ~5 μg/mL.

Acknowledgment. The author thanks the Eskitis Institute and Griffith University (NRG) for research funding, and Drs. J. I. Pitt, A. Hocking, and N. Tran-Dinh from Food Science Australia for fungal identification. Dr. J. Mitchell from Griffith University is acknowledged for obtaining the HRESIMS data.

Supporting Information Available: ^1H and ^{13}C NMR spectra and LRESIMS data for compounds **1**, **2**, **4**, and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP050025H