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NAPHTHOFURANONE PHYTOALEXINS FROM THE GREY MANGROVE, *AVICENNIA MARINA*

DAVID C. SUTTON*, FRANK T. GILLAN† and MICHAEL SUSIC

Australian Institute of Marine Science, P.M.B. No. 3, Townsville M.C., Queensland 4810, Australia

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Key Word Index—*Avicennia marina*; Verbenaceae; *Phytophthora* sp.; mangrove; phytoalexins; quinones; naphthofuranones.

Abstract—Infection of wound tissue of *Avicennia marina* seedlings by a fungus belonging to the genus *Phytophthora* induced the production of three chemically-related phytoalexins. After isolation by extraction, partition and HPLC separation, one was identified as naphtho[1,2-b]furan-4,5-dione, and the other two tentatively as 3-hydroxy-naphtho[1,2-b]furan-4,5-dione and 2-[2′-(2′-hydroxy)propyl]-naphtho[1,2-b]furan-4,5-dione.

INTRODUCTION

Phytoalexins, plant antimicrobial compounds formed after infection, have been reported from a number of plant families since the first convincing report of their detection in bean [1] and the first characterization of a phytoalexin, pisatin, from pea [2]. Since these initial reports, a diverse range of chemical compounds have been identified as phytoalexins, including flavonoids, terpenes, polyacetylenes, polyketides, alcohols and stilbene-derived compounds [3]. Available evidence indicates that particular plant families do not produce this diversity of structural types but synthesize only specific classes of phytoalexins.

Major questions remain concerning the role of phytoalexins in host defence mechanisms. Contributing to this are the relatively few plant species belonging to only a limited number of plant families that have yet been examined for the occurrence of phytoalexin formation. In general, the studies undertaken have been restricted to economically important herbaceous annual species [4], and reports of phytoalexin formation by perennial species are rare. However, the ability of these types of plants to produce phytoalexins has been indicated by the recent demonstration of their formation in the European lime [5]. Studies of this nature are necessary if the ubiquity of phytoalexin formation in the plant kingdom, and hence the role of this phenomenon as part of general plant defense mechanisms, is to be ascertained.

This paper reports the detection and identification of three naphthofuranone phytoalexins from the perennial mangrove species *Avicennia marina* L., in response to infection by *Phytophthora* sp.

RESULTS AND DISCUSSION

Phytophthora sp. (Australian Collection of Marine Microorganisms, FN 005) isolated from *A. marina* leaf litter was tested for its ability to induce phytoalexin formation following infection of wound sites on *A. marina* seedlings. Inoculation of these sites resulted in the formation of brown restricted necrotic lesions within 7–10 days. Aqueous or ethanolic extracts of macerated lesion tissue inhibited the growth of *Phytophthora* sp. in *in vitro* bioassays, whilst extracts of macerated uninoculated wound tissue did not.

Partition of the aqueous extract with ethyl acetate resulted in recovery of the inhibitory activity in the organic phase. Since aqueous extracts of *A. marina* tissue contain large amounts of sucrose [6], oxalate, citrate, malate [7], and inorganic ions [7], which would not partition into ethyl acetate, this partition step is a simple method for initial sample clean up. Further fractionation of the extract was achieved by reversed-phase HPLC using methanol–water mixtures as eluents, and UV (254 nm) detection. Many UV-absorbing components were present. Three major components (**1–3**, Fig. 1) present in the extract of the inoculated tissue were found to be inhibitory, and these components accounted for nearly all of the activity of the extract. The same components were present in both aqueous and ethanolic extracts of lesion tissue. This indicates that they are not artifacts of the extraction procedure (cf. ref. [8]). Insufficient material was available from a single HPLC separation to permit characterization of the compounds. Indeed, the pooling of isolated compounds **1–3** from a large number of HPLC separations was necessary to obtain sufficient of each component (100–400 μg) for further characterization.

Identification of active compounds 1–3

Packed column GC/MS analyses were performed on the three compounds. No peak was evident in the analysis of **1**, indicating the presence of polar functional groups

*Present address: Sir George Fisher Centre, James Cook University of North Queensland, Townsville, Australia.

†Author to whom correspondence should be addressed.

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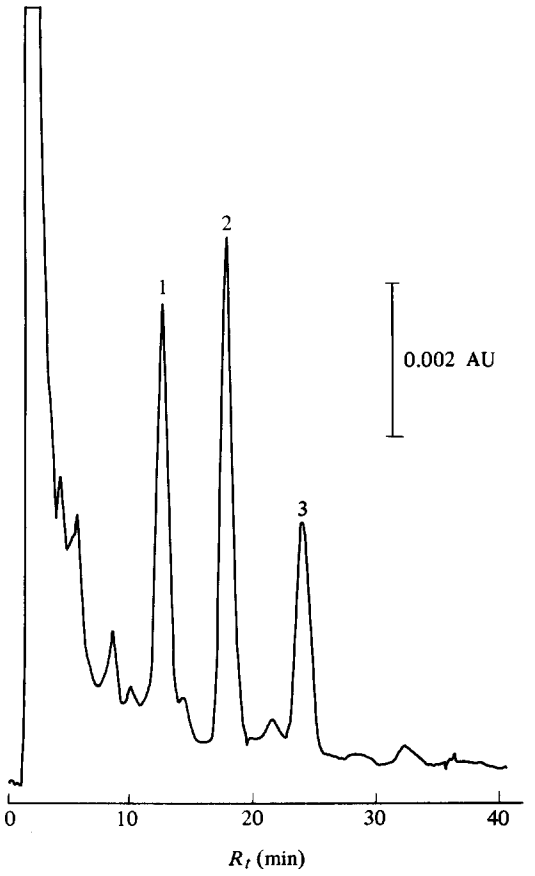

Fig. 1. Isocratic HPLC separation of phytoalexins present in aqueous extracts from inoculated *A. marina* wound tissue. Compounds 1–3 were found to be biologically active. Eluent 35% methanol; detection: UV 254 nm.

(which would lead to poor chromatographic performance on the dimethylsilicone column used for this analysis) and/or thermal instability. Compounds **2** and **3** both yielded single GC peaks with distinctive mass spectra.

Computer search of the National Bureau of Standards (NBS) mass spectral library yielded a 96% fit between **2** and naphtho[1,2-b]-furan-4,5-dione. This identification was confirmed by comparison of the reported UV spectrum for naphtho[1,2-b]furan-4,5-dione [9], the reported ^{1}H NMR chemical shifts for the furan protons [9, 10] and the reported mass spectrum [9, 11] with those of **2**, and by computer simulation of the proton NMR of vicinal disubstituted benzenes, which confirmed this substitution pattern in the aromatic nucleus. Compound **2** is thus identified as naphtho[1,2-b]furan-4,5-dione.

The EI mass spectrum of **3** exhibited a molecular ion at *m/z* 256 which was confirmed by methane CI-MS (base peak $[M+H]^+$ at *m/z* 257). The base peak (*m/z* 241) in the EI-MS of **3** corresponded to loss of a methyl substituent. Computer search of the NBS library resulted in no fits over 85% and thus no tentative identification was made. The presence of a low intensity ion at *m/z* 199 in the EI-MS cf. $[M]^+$ (2) *m/z* 198 and the close similarity of the UV spectra of **2** and **3** indicated that **2** and **3** were probably structurally related. The low field region of the ^{1}H NMR of **3** was identical to that of compound **2** except for the absence of the furan α-proton resonance and the presence of a singlet from the furan β-proton. It was concluded that **3** was a 2-substituted naphtho[1,2-b]furan-4,5-dione. The molecular ion mass difference (**3** – **2**) of 58 corresponded to $C_2H_2O_2$ or C_3H_6O. The proposed substituent was thus either $C_2H_3O_2$ or C_3H_7O. The high-field region of the proton NMR exhibited an intense (6H) singlet at δ 1.66 corresponding to two equivalent methyl groups, and $[M+H-18]^+$ was a dominant mass peak in the CI-MS (94%) indicative of a hydroxyl substituent. We thus concluded that the substituent was $Me_2C(OH)$–. Two low intensity fragment ions in the EI-MS at $[M-17]^+$ (–hydroxyl) and $[M-18]^+$ ($-H_2O$) provided further confirmation of this structure. The EI-MS base peak at $[M-15]^+$ is readily explicable as due to the allylic cleavage of a C–C bond resulting in a resonance-stabilized carbonium ion. Compound **3** is thus tentatively identified as 2-[2'-(2'-hydroxy)propyl]-naphtho[1,2-b]furan-4,5-dione.

Identification of **1** posed more difficulties. Its UV spectrum was similar to those of **2** and **3**, but the absorption bands were shifted to much longer wavelengths (20 nm shift). The ^{1}H NMR spectrum revealed two complex multiplets centred at δ 7.05 and 8.03 accounting for 2H each and a broad singlet at δ 7.49 (1H). The line broadening was quite marked. By analogy with **2** and **3**, the NMR data was consistent with a vicinal disubstituted benzene ring and an α-furan proton. That is, **1** can be tentatively assigned as a 3-substituted naphtho[1,2-b]furan-4,5-dione.

In our initial HPLC studies, the peak for compound **1** was observed to split into two overlapping peaks. It was found that the formation of the doublet was dependent as the absence of traces of acid, and that careful adjustment of the pH resulted in reversible splitting/coalescence of the peaks of compound **1**. The behaviour is explicable if **1** exists as two tautomers with similar equilibrium concentrations, and if the rate of equilibration is slow in the absence of acid. More rapid interconversion (due to acid) would readily explain the NMR band broadening. A 3-hydroxy substituent would be expected to exist in both keto and enol forms with the latter stabilized by intramolecular hydrogen bonding.

On the basis of this proposed structure, **1** was acetylated with acetic anhydride–pyridine and the product purified by HPLC and subjected to probe MS. HPLC analysis of the crude acetylation mixture revealed only one product. This compound (**4**) eluted just after **2**. Probe MS (EI, 70 eV) revealed a molecular ion at *m/z* 256 (I = 14) and a base peak at *m/z* 214. No $[M-15]^+$ fragment ion was observed. The base peak can be explained by loss of ketene via a six-membered transition state to produce the conjugated trione **5** (Fig. 2). Ions corresponding to the further loss of CO (*m/z* 186) and 2 × CO (*m/z* 158) were evident (I = 27 and 12 respectively) in the spectrum of **4** as is expected for a substituted ortho-quinone [11]. In addition, a strong *m/z* 69 fragment ion (I = 62) supported the proposed presence of a 3-oxygen substituent (Bowie *et al.* [12] have suggested that naphthoquinones containing the O–C–C–C–O unit yield a characteristic *m/z* 69 fragment in their mass spectra). These data support the assignment of **1** as 3-hydroxynaphtho[1,2-b]furan-4,5-dione. The differences between the UV and NMR spectra of **1** compared with **2** and **3**, are readily explicable on the basis of the proposed keto–enol tautomerism.

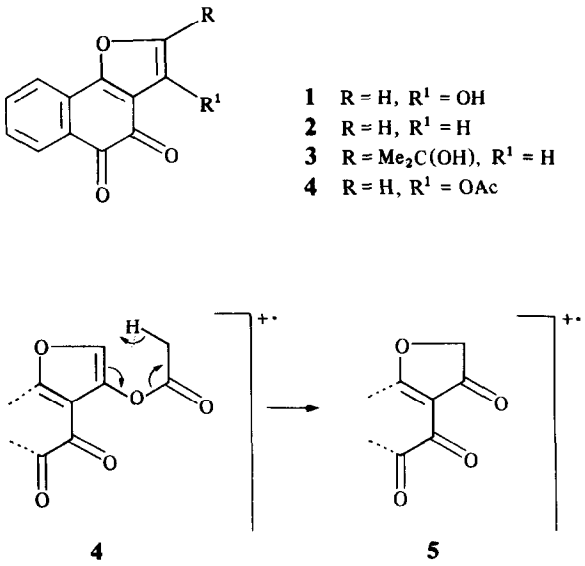

Fig. 2. Chemical structures of the compounds reported in the text, and proposed fragmentation pathway (−ketene) for compound 4.

EXPERIMENTAL

Fungal and plant materials. A species of fungus (A.C.M.M. FN 005, Centre for Tropical Marine Studies, J.C.U.) belonging to the genus *Phytophthora* was isolated from *Avicennia marina* leaf litter and maintained on potato dextrose agar at 24°. Plugs of mycelium (0.5 cm dia.) cut from the margin of 14-day-old cultures served as inoculum. Seedlings of *Avicennia marina* were grown from field collected propagules, transplanted into vermiculite in plastic trays in a shadehouse at 50% ambient sunlight and at ambient temp. (20–28°). Two to three month old seedlings were inoculated at wound sites made at 1 cm intervals along the hypocotyl and epicotyl lengths, and incubated at 24° in humid chambers. After 7–10 days restricted necrotic lesions had formed at inoculated sites. After 10 days the inoculum plugs were removed and the inoculated tissues excised and extracted. Equivalent wounded but uninoculated tissues served as control.

Tissue extractions. For aq. extractions, excised plant tissues were extracted twice by maceration in H_2O and the filtrate partitioned against EtOAc (×3). The macerate was extracted with MeOH, the extract evaporated to dryness, and the residue partitioned between H_2O and EtOAc. All EtOAc extracts were combined, evaporated, dissolved in MeOH and subjected to HPLC. For ethanolic extractions, excised lesion tissue was soaked in EtOH for 48 hr. The EtOH was taken to dryness. The residue was dissolved in MeOH and subjected to HPLC.

Bioassay. Individual extract components, separated by HPLC, were tested for the effect on the growth of the *Phytophthora* isolate in a bioassay previously established for *Sclerotinia sclerotiorum* [13].

HPLC. Instrumentation consisted of a Spectra-Physics solvent delivery system and computing integrator with a Waters injector and absorbance detector (model 440) operating at 254 nm. A Brownlee Labs column (10 cm × 4.6 mm i.d.) packed with Spherisorb RP-8 (5 μ) was used. The mobile phase solvent (aq. 35% MeOH) was delivery isocratically at 0.8 ml/min. Samples were injected as solns in aq. 50% MeOH.

GS/MS. Analyses were performed on a Finnigan 4510 MS at low resolution (1000), 2 s/scan, 40–500 amu. Spectra were run at 70 eV. Separations were performed on a 2 m × 3 mm packed 5% OV-101 column, temp. programmed from 180 to 260° at 8°/min. Carrier gas was He at 30 ml/min.

NMR. Spectra were accumulated at 200 MHz using a Varian XL-200 spectrometer. CD_3OD was used as solvent.

Naphtho[1,2-b]furan-4,5-dione (2). Compound 2 was identified by MS library search (NBS) which yielded a 96% match to NBS spectrum no. 12615 (CA no. 32358-83-1). UV λ_{max}^{EtOH} nm: 243, 275; ^{1}H NMR (CD_3OD): δ7.08 (1H, *d*, *J* = 1.6 Hz, furan β-H), 7.85 (2H, *m*), 8.06 (1H, *d*, *J* = 1.6 Hz, furan α-H) and 8.22 (2H, *m*); MS *m/z* (rel. int.): 199 (12), 198 (100), 171 (6), 170 (45), 142 (25), 115 (7), 114 (67), 113 (33), 88 (17), 87 (14), 76 (25), 75 (12), 74 (13), 71 (17), 63 (15), 62 (11), 57 (12), 51 (6) and 50 (31); CI(CH_4)-MS *m/z*: 199 (100).

2-[2′-(2′-*Hydroxy*)*propyl*]-*naphtho*[1,2-*b*]*furan*-4,5-*dione* (3). Compound 3 was isolated by HPLC (Fig. 1) and characterized on the basis of its MS, NMR and UV spectra. UV λ_{max}^{EtOH} nm: 248, 274; ^{1}H NMR (CD_3OD): δ1.66 (6H, *s*), 6.90 (1H, *s*, furan β-H), 7.86 (2H, *m*), and 8.21 (2H, *m*); MS *m/z* (rel. int.): 256 (6), 242 (16), 241 (100), 239 (3), 238 (3), 214 (11), 213 (8), 199 (10), 115 (6), 114 (6), 113 (5), 76 (13), 63 (7) and 50 (10); CI(CH_4)-MS *m/z*: 257 (100), 239 (94).

3-*Acetyl-naphtho*[1,2-*b*]*furan*-4,5-*dione* (4). Compound 1 was isolated by HPLC (Fig. 1) and acetylated with Ac_2O–pyridine. The product was subjected to HPLC and the single derivative isolated. Several μg were available for MS characterization. MS *m/z* (rel. int.): 256 (14), 215 (14), 214 (100), 187 (4), 186 (27), 167 (10), 158 (12), 149 (48) and 69 (62).

3-*Hydroxynaphtho*[1,2-*b*]*furan*-4,5-*dione* (1). This compound was isolated by HPLC and subjected to GC/MS (unsuccessfully), NMR and UV analysis. UV λ_{max}^{EtOH} nm: 264, 301. ^{1}H NMR (CD_3OD): δ7.05 (2H, *m*), 7.49 (1H, *s*, *br*) and 8.03 (2H, *m*).

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