Notes

Cryphonectric Acid and Other Minor Metabolites from a Hypovirulent Strain of *Cryphonectria parasitica*[1]

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Investigations carried out on secondary metabolites produced in culture by a hypovirulent strain of *Cryphonectria parasitica* allowed the isolation of several compounds which were characterized by NMR analysis and derivatization reactions. The most abundant metabolite was a new compound, called cryphonectric acid (**1**). Other metabolites were diaporthin, the only known phytotoxic compound isolated from both virulent and hypovirulent strains of *C. parasitica*, (+)-orthosporin, and L-*p*-hydroxyphenyllactic acid (HOPLA). Root growth activity of the purified compounds was evaluated both on tomato seedlings and maize subapical segments.

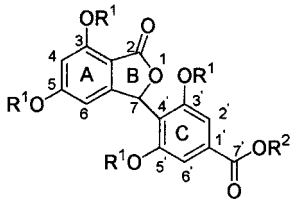
Cryphonectria (formerly *Endothia*) *parasitica* (Murr.) Barr is the causal agent of chestnut blight, responsible for the chestnut decline in many areas of Europe and North America, where very susceptible western *Castanea* species (*C. sativa* Mill. and *C. dentata* Borkh.) are present.[2] The healing and nonlethal cankers discovered on *C. sativa* in Europe led to the isolation of *C. parasitica* strains characterized by a reduced virulence when inoculated on susceptible chestnuts.[3,4] The aim of the present work is either to isolate and characterize secondary metabolites produced by a hypovirulent isolate showing a broad range of vegetative compatibility or to assess their biological activity on selected test plants.

The EtOAc crude extracts obtained from cultures of a hypovirulent strain of *C. parasitica* designated E13, grown on MPGA, were separated using a series of silica gel chromatography columns and stepwise elution with organic solvents. Four of the several compounds obtained were completely characterized: metabolite **1**, a new aromatic acid, along with diaporthin, previously isolated from cultures of virulent strains of *C. parasitica*,[5] a trace of (+)-orthosporin,[6] and L-*p*-hydroxyphenyllactic acid (+)-HOPLA, also produced by *Candida* spp. and various bacterial species.[7]

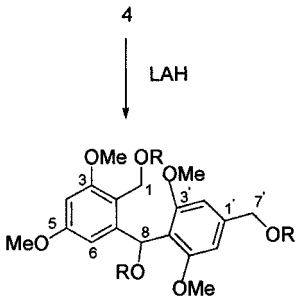
Cryphonectric acid (**1**) is the metabolite most abundantly produced by E13, representing more than 20% in weight of the crude extract; **1** is a highly polar solid, slightly soluble in organic solvents. Cryphonectric acid **1**, isolated as a pale yellow powder, mp >300 °C (dec), $[\alpha]_D$ +9.2° (*c* 0.1, MeOH), analyzed for $C_{15}H_{10}O_8$, and the formula was supported by EIMS (M^+, 318). Its IR spectrum (KBr) showed bands at 3430 cm^{-1} (OH) and at 1750 cm^{-1}, attributable to an ester-like function; the absorption at 1720 cm^{-1} indicated a further carbonyl function; the UV spectrum (EtOH) showed absorptions at 206, 256, and 293 nm (ϵ 26350, 9600, and 3500).

Acetylation of **1** gave the tetraacetate derivative **2**, which showed a molecular peak at *m*/*z* 486 (EIMS). The ^{1}H NMR spectrum revealed a broad signal at δ 9.60 (1′-CO_2H) (two *meta*-coupled aromatic protons H-4 and -6, J = 2.0 Hz), of a broad singlet (H-7), which was correlated with an sp^3 oxygen-bearing carbon; also present were two more signals attributable to acetate groups (3′- and 5′-OAc) and to two aromatic protons (H-2′ and -6′), which sharpened by heating at ∼50 °C, indicating restricted rotation of the phenyl group about the C(7)−C(4′) bond. The ^{13}C NMR spectrum gave four acetate signals and two carbonyl signals (C-2 and -7′) and confirmed that one of the two tetrasubstituted aromatic rings has a symmetry axis passing through the C(1′), C(4′) carbons, since C-2′ and -6′ and C-3′ and -5′ presented double intensity.

The relative disposition of substituents around the aromatic rings was deduced along the following lines. The



1 $R^1 = R^2 = H$
2 $R^1 = Ac$; $R^2 = H$
3 $R^1 = Ac$; $R^2 = Me$
4 $R^1 = R^2 = Me$
5 $R^1 = H$; $R^2 = Me$



6 R = H
7 R = Ac

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carboxylic moiety was placed at C-1′, since irradiation of H-2′ and -6′ caused the removal of three-bond couplings from C-7′; C-7 was linked to both the aromatic rings since H-7 presented long-range couplings with C-2a, -6, -6a, -3′, -4′, and -5′, while one of the two *meta* protons was placed at C-6 since it presented mutual NOEs with H-7 (3%). On the basis of these results, the four acetoxy functions were located at C-3, -5, -3′, and -5′ and the C-2 lactone carbon was joined to C-2a to complete the molecular formula of **2**. Compound **2** was reacted subsequently with a solution of diazomethane, giving the ester **3**. The structure **1** proposed for cryphonectric acid was confirmed by reaction of the fully methylated compound **4** with LAH, to obtain the trihydroxy derivative **6** via the opening of the lactone moiety and the reduction of the two carbonyl ester functions. Long-range coupling of 4.0 Hz between C-6 and H-8 and not between C-6 and H_2-1 in the corresponding triacetate derivative **7**, attributable to a three-bond interaction between the two atoms, placed C-8 at C-7.[8] Finally, the mutual NOEs observed between H-8 and H_2-1 (Experimental Section) indicated that C-1 and C-8 are *ortho*-positioned.

As far as the stereochemistry of C-7 is concerned, the CD values of **1** were very low (Experimental Section) and the [α] value changed with time from +9.2° (0 time) to +27.7° (1 day). Moreover in compound **6**, CD and [α] values were null; in our opinion the C-7 center must be racemic and the optical activity must be attributed to atropoisomerism around the chirality axis C7−C4′ due to the steric hindrance of the two substituents in the *ortho* position of ring C. This hypothesis is in agreement with the broadening of the NMR signals of **2**, which disappears by heating at ∼50 °C. Cryphonectric acid (**1**) is unusual, but norwedelic acid, a benzofuranic acid isomer of **1**, occurs in *Wedelia calendulaceae*.[9]

Metabolites **1**, diaporthin, and (±)-HOPLA, tested using a tomato seedling bioassay up to 2 mM, did not induce any necrotic symptoms on leaves. However, formation of tomato seedlings was totally inhibited by 100 μM **1** and 200 μM diaporthin, while a 75 and 50% inhibitory effect was observed by using 100 and 10 μM diaporthin, respectively. No root inhibition was detected in the presence of 10 μM **1** and 2 and 0.2 mM (±)-HOPLA. In a second bioassay on elongation of subapical segments of maize roots, 2 and 0.2 mM (±)-HOPLA determined respectively a 16 and 35% increase in the length of the treated samples, comparing with the control. The results are in agreement with the interference with cell differentiation shown by the culture filtrates of E13 *C. parasitica* strain on chestnut calli of *C. sativa* and *C. mollissima*.[10] Further work is required in order to characterize the physiological effects of the compounds produced by E13 on the cultured cells of both susceptible and resistant *Castanea* species.

Investigations on the potential angiogenesis inhibition by **1**,[11] carried out at two different concentrations, 50 and 12.5 μM, showed neither inhibition of the migration of endothelial cells nor cytotoxicity or morphological differentiation of bovine endothelial cells into capillary-like structures, suggesting that **1** does not represent a potential angiogenic inhibitor.

Experimental Section

General Experimental Procedure. Flash CC was performed with Merck silica gel (0.04−0.06 mm) and TLC with Merck HF_{254} and RP-18 F_{254}. IR values were determined on Perkin-Elmer 177 spectrophotometer, MS on a Finnigan-MAT-TSQ70 spectrometer, and optical rotations on a JASCO-500 DIP-181 polarimeter. NMR spectra were recorded on a Bruker AC 250L spectrometer operating at 250.1 MHz for ^{1}H NMR and 62.9 MHz for ^{13}C NMR; chemical shifts are in ppm (δ) from TMS as internal standard. HPLC analyses were performed on a LiChrograph L-6000A (Merck-Hitachi) equipped with a L-4000 detector (λ = 225 nm) and D-2500 integrator, using a Merck RP-18, 0.45 × 25 cm column with CH_3CN-H_2O (1−1.5, v/v) as eluent at a nominal flow rate of 0.5 cm^3 min^{-1}.

Isolation of 1, Diaporthin, (+)-Orthosporin, and (+)-HOPLA. A hypovirulent strain of *C. parasitica*, E13, from the Mycological Collection of the Plant Pathology Institute, University of Milan, was grown on MPGA (malt extract, peptone, glucose, agar, 20−2−20−15 g L^{-1}, pH 6.8) medium in 30 Roux flasks at 24 °C in the dark for 14 days**.** The mycelia were extracted twice with ethyl acetate containing 1% MeOH, and the extracts (0.8 g) were chromatographed on a silica gel column with stepwise elution using CH_2Cl_2−MeOH to obtain diaporthin (30 mg), (+)-orthosporin (2 mg), (+)-HOPLA (15 mg), and cryphonectric acid (**1**) (220 mg). The known compounds were further purified by PLC in hexanes−EtOAc (1:1) or CH_2Cl_2−MeOH (15:1), while **1** was isolated using an RP-18 silica gel HPLC column, using acetone−H_2O (1:1) as the eluent.

Diaporthin and (+)-Orthosporin. Both compounds were identified by comparison of mp, $[\alpha]_D$, MS, and ^{1}H NMR with literature data.[6]

L-p-Hydroxyphenyllactic acid: mp 165−168 °C, $[\alpha]_D$ +18° (*c* 0.5, MeOH); 5 mg was treated with an ethereal solution of CH_2N_2 to obtain the methylester, EIMS, *m/z* 196[M]$^+$(10%), 178[M − 18]$^+$(15), and 107(100); the compound was identified by TLC, MS, and ^{1}H NMR to a methyl ester of a commercial sample of (±)-HOPLA.

Cryphonectric acid (1): EIMS *m/z* 318[M]$^+$(20%), 300[M − 18]$^+$(28), and 107(100); CD (*c* 0.1 mg/mL, MeOH) 208 and 223 nm (Δε −2.2 and −1.0); ^{1}H NMR (acetone-d_6) δ 9.10 (5H, br signal, 4 × OH and 1′-CO_2H), 7.14 (2H, s, H-2′ and −6′), 6.95 (1H, br s, H-7), 6.38 and 6.28 (2H, br d, *J* = 2.0 Hz, H-4 and -6). *anal*. C 56.45%, H 3.10%, calcd for $C_{15}H_{10}O_8$, C 56.61%, H 3.17%.

Compound 2. Compound **1** (30 mg) was dissolved in dry pyridine (0.5 mL) containing Ac_2O (0.8 mL), and the solution was kept at 0 °C for 12 h. The mixture was then poured into ice−water and extracted with CH_2Cl_2. From PLC in hexanes−EtOAc (1:1) of the residue, tetraacetate **2** (25 mg) was isolated as a white solid: mp 160−165 °C, $[\alpha]_D$ +48° (*c* 0.2, MeOH); EIMS *m/z* 486 [M]$^+$(4%), 444(50), 402(50), 384(40), 360(58), 318(65), and 300(40); ^{1}H NMR ($CDCl_3$) δ 9.60 (1H, br signal, 7′-OH), 7.73 (2H, br signal, H-2′ and -6′), 7.03 (1H, br d, *J* = 2.0 Hz, H-4), 6.99 (1H, dd, *J* = 2.0 and 0.9 Hz, H-6), 6.52 (1H, br s, H-7), 2.44 and 2.27 (6H, s, 3-and 5-OAc), 2.15 (6H, br signal, 3′- and 5′-OAc); ^{13}C NMR ($CDCl_3$) δ 168.7 (s, 3′-and 5′-O*CO*Me), 168.6 (s, C-7′), 168.5 and 168.2 (s, 3-and 5-O-*CO*Me), 166.6 (s, C-2), 156.6 (s, C-5), 150.8 (s, C-6a), 150.1 (s, C-3′ and -5′), 149.0 (s, C-3), 132.4 (s, C-1′), 124.8 (s, C-4′), 122.6 (d, C-2′ and C-6′), 117.4 (d, C-4), 115.6 (s, C-2a), 113.8 (d, C-6), 73.5 (d, C-7), 21.0 and 20.6 (q , 3- and 5-OCO*Me*), 20.4 (q, 3′- and 5′-OCO*Me*). *anal*. C 56.53%, H 3.78%, calcd for $C_{23}H_{18}O_{12}$, C 56.79%, H 3.73%.

Compound 3. Compound **2** (10 mg) dissolved in dry CH_2Cl_2 (5 mL) was treated with an ethereal solution of CH_2N_2 for 10 min, and evaporation of the solution gave **3**: EIMS, *m/z* 501[MH]$^+$, MALDI, *m/z* 593.5[M + K]$^+$, and 523.3[M + Na]$^+$; ^{1}H NMR ($CDCl_3$) δ 7.71 (2H, s, H-2′ and -6′), 7.03 and 6.99 (2H, br d, *J* = 2.0 Hz, H-4 and -6), 6.49 (1H, br s, H-7), 3.92 (3H, s, 7′-OMe), 2.43 and 2.26 (6H, s, 3-and 5-OAc), 2.17 (6H, br signal, 3′- and 5′-OAc); HREIMS *m/z* 500.0939 (calcd for $C_{24}H_{20}O_{12}$, 500.0948).

Compound 4. A solution of compound **1** (100 mg), in CH_2Cl_2−MeOH (9:1, v/v), was reacted repeatedly with a solution of CH_2N_2 in dry CH_2Cl_2, until only one spot appeared on TLC plates; PLC of the residue on CH_2Cl_2−MeOH (15:1) gave compound **4** as white solid: mp 170−175 °C; $[\alpha]_D$ −7.6° (*c* 0.2, MeOH); IR (KBr) ν_{max} 1753, 1726 and 1614 cm^{-1}; UV(EtOH) λ_{max} 212, 255, 290, and 310sh nm (ε 40850, 25500, 7900, and 4250); EIMS, *m/z* 388 [M]$^+$(50%), 357(100), 343(35), 193(50),

and 165(100); ^{1}H NMR ($CDCl_3$) δ 7.22 (2H, br signal, H-2′ and -6′), 6.89 (1H, dd, J = 1.0 and 0.5 Hz, H-7), 6.40 (1H, dd, J = 2.0 and 0.5 Hz, H-4), 6.19 (1H, dd, J = 2.0 and 1.0 Hz, H-6), 3.98 (3H, s, 3-OMe), 3.93 (3H, s, 7′-OMe), 3.80 (3H, s, 5-OMe), 3.75 (6H, br signal, 3′- and 5′-OMe); NOE experiments ($CDCl_3$), {H-4} enhanced 3-OMe (3%) and 5-OMe (1%), {H-6} enhanced H-7 (6%) and 5-OMe (2.5%), {H-7} enhanced H-6 (4%), {H-2′ and -6′} enhanced 3′- and 5′-OMe (4%), {3-OMe} enhanced H-4 (19%), {5-OMe} enhanced H-4 (5%) and H-6 (17%); ^{13}C NMR ($CDCl_3$) δ 169.2 (s, C-2), 166.5 (s, C-5), 166.4 (s, C-7′), 159.1 (s, C-3), 158.9 (s, C-3′ and -5′), 155.2 (s, C-6a), 132.3 (s, C-1′), 116.8 (s, C-4′), 107.7 (s, C-2a), 105.6 (d, C-2′ and -6′), 98.5 and 97.1 (d, C-4 and -6), 73.2 (d, C-7), 56.3 (q, 3′- and 5′-OMe), 56.0 and 55.8 (q, 3- and 5-OMe), 52.4 (q, 7′-OMe). *anal*. C 62.10%, H 5.10%, calcd for $C_{20}H_{20}O_8$ C 61.85%, H 5.19%.

Compound 5. A solution of **1** (15 mg), in CH_2Cl_2−MeOH, was treated with CH_2N_2 for 5 min. From PLC of the residue **5** was isolated as an oil: ^{1}H NMR (acetone-d_6) δ 9.28 and 8.41 (2H, br s, 3- and 5-OH), 9.05 (2H, br s, 3′- and 5′-OH), 7.08 (2H, s, H-2′ and -6′), 6.92 (1H, dd, J = 1.0 and 0.5 Hz, H-7), 6.36 (1H, dd, J = 1.9 and 0.5 Hz, H-4), 6.25 (1 H, dd, J = 1.9 and 1.0 Hz, H-6), 3.83 (3H, s, 7′-OMe); HREIMS m/z 332.0531 (calcd for $C_{16}H_{12}O_8$, 332.0528).

Compound 6. LAH (40 mg) was added to a solution of **4** (50 mg) in dry THF and the mixture stirred for 1 h at 40 °C; the reaction was quenched with EtOAc, then acidified with diluted HCl and extracted with EtOAc; PLC of the residue gave 35 mg of compound **6**: white crystals, mp 130−135 °C; UV (EtOH)λ_{max} 209 and 283 nm (ϵ 48 700 and 4500); CIMS (isobutane) m/z, 347[MH − 18]$^+$(70%), 346(50), 329(100), and 179(60); ^{1}H NMR (acetone-d_6) δ 6.79 (2H, s, H-2′ and -6′), 6.50 (1H, br d, J = 10.9 Hz, H-8), 6.49 and 6.25 (2H, br d, J = 2.4 Hz, H-4 and -6), 4.92 (1H, d, J = 10.9 Hz, 8-OH), 4.82 (1H, dd, J = 11.7 and 8.7 Hz, H-1a), 4.66 (2H, d, J = 5.7 Hz, H_2-7′), 4.65 (1H, dd, J = 11.7 and 3.8 Hz, H-1b), 4.19 (1H, t, J = 5.7 Hz, 7′-OH), 3.84 and 3.66 (6H, s, 3- and 5-OMe), 3.82 (6H, s, 3′- and 5′-OMe), 3.46 (1H, dd, J = 8.7 and 3.8 Hz, 1-OH); HREIMS m/z 364.1523 (calcd for $C_{19}H_{24}O_7$, 364.1515).

Compound 7. Compound **6** was acetylated to obtain **7** as an oil: CIMS (isobutane) m/z 491[MH]$^+$, 431[MH − 60]$^+$; ^{1}H NMR (acetone-d_6) δ 7.58 (1H, dd, J = 0.8 and 0.5 Hz, H-8), 6.86 (1H, dd, J = 2.5 and 0.8 Hz, H-6), 6.67 (2H, br s, H-2′ and -6′), 6.49 (1H, dd, J = 2.5 and 0.5 Hz, H-4), 5.06 (2H, br s, H_2-7′), 4.99 and 4.93 (2H, d, J = 11.6 Hz, H_2-1), 3.84 (3H, s, 5-OMe), 3.79 (3H, s, 3-OMe), 3.73 (6H, s, 3′- and 5′-OMe), 2.07, 2.05, and 1.71 (9H, s, 1-, 8-, and 7′-OAc); selected NOE experiments (acetone-d_6), {H_2-1} enhanced H-8 (20%), {H-4} enhanced 3-OMe (3%) and 5-OMe (1.5%), {H-6} enhanced H-8 (2%) and 5-OMe (2%), {H-8} enhanced H_2-1 (4%) and H-6 (2%), {H_2-7′} enhanced H-2′ and −6′ (6.5%), {3-OMe} enhanced H_2-1 (0.5%) and H-4 (19%), {5-OMe} enhanced H-4 (8.5%) and H-6 (12.5%), {3′-and 5′-OMe} enhanced H-2′ and -6′ (13%); ^{13}C NMR (acetone-d_6) δ 171.8, 171.5, and 170.6 (s, 1-, 8-, and 7′-O*C*OMe), 162.0 and 161.0 (s, C-3 and -5), 160.3 (s, C-3′ and -5′), 144.9 and 140.6 (s, C-6a and -1′), 116.7 and 114.4 (s, C-2 and -4′), 106.3 (d, C-6), 105.7 (d, C-2′ and -6′), 98.0 (d, C-4), 67.6 (d, C-8), 67.0 (t, C-7′), 58.7 (t, C-1), 56.9 (q, 3′- and 5′-OMe), 56.8 and 56.2 (q, 3- and 5-OMe), 21.6, 21.4, and 21.3 (q, 1-, 8-, and 7′-OCO*Me*). *anal*. C 60.95%, H 6.20%, calcd for $C_{25}H_{30}O_{10}$, C 61.21%, H 6.16%.

Tomato Seedling Bioassay. Seedlings of tomato cv. Super Marmande, grown until the fourth fully expanded leaf, were cut and transferred to 50 mL of distilled H_2O in glass vials placed in a growth chamber at 24 °C, 70% relative humidity, and 14/10 photoperiod (light/dark). After 24 h conditioning, H_2O was replaced with test solutions. Compound **1**, diaporthin, and (±)-HOPLA (Sigma) were tested for phytotoxic effects and root growth inhibition at 24, 48, and 72 h, at 2, 0.2, 0.1, and 0.01 mM. All the metabolite solutions were filter sterilized through 0.2 μm filters. Each assay was performed twice on 10 replicates of tomato seedlings.

Subapical Maize Root Segment Bioassay. Maize seedlings (Dekalb cv. DK 300), washed in tap H_2O for 2 h, were incubated in 2 L beakers containing 0.5 M $CaSO_4$ for 24 h. Root apical tips were then eliminated, and the subapical root segments (0.6 cm) were conditioned in 0.5 M $CaSO_4$ for 2 h. Afterward, the segments were measured on millimeter paper and transferred to buffer solution (0.5 mM $CaSO_4$, 0.5 mM KCl, 0.1 mM Mes-Na, pH 6), and buffer solution was added with 0.2 and 2 mM (±)-HOPLA. At the end of incubation period carried out for 4 h on an orbital shaker (80 rpm) at 26 °C, the segments were remeasured in order to assess their elongation. The mean values were evaluated on three replicate trials carried out on 40 segments.

References and Notes

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