Metabolites of *Colletotrichum gloeosporioides,* an Endophytic Fungus in *Artemisia mongolica*

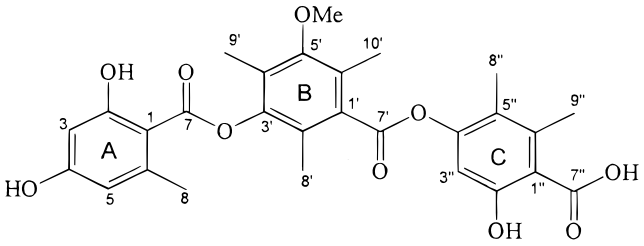
W. X. Zou,[†] J. C. Meng,[†,‡] H. Lu,[†] G. X. Chen,[§] G. X. Shi,[§] T. Y. Zhang,[⊥] and R. X. Tan*,[†]

Institute of Functional Biomolecule, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, People's Republic of China, Department of Plant Protection, Shandong Agricultural University, Taian 271018, People's Republic of China, National Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, People's Republic of China, and Jiangsu Key Laboratory of Bioresource Engineering, Nanjing Normal University, Nanjing 210095, People's Republic of China

Received April 21, 2000

A new antimicrobial metabolite, named colletotric acid (**1**), was isolated from a liquid culture of *Colletotrichum gloeosporioides*, an endophytic fungus colonized inside the stem of *Artemisia mongolica*. The structure was determined using spectroscopic methods (EIMS and FABMS,^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HMBC, and HMQC). Compound **1** inhibited the growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea* with minimal inhibitory concentrations (MICs) of 25, 50, and 50 μg/mL, respectively, and the crop pathogenic fungus *Helminthosporium sativum* (MIC: 50 μg/mL).

The genus *Artemisia* L. (Asteraceae) with some 185 species growing in China is well known for its resistance to herbivores and to most of the bacterial and fungal pathogens that may live on other neighboring plants with greater ease. This observation prompted us to launch a program aimed at the characterization of novel bioactive metabolites from cultures of endophytes colonized inside *Artemisia* species. Some bioactive metabolites have been characterized from a *Colletotrichum* sp., an endophytic fungus inside the stem of *Artemisia annua*.[1] We now present the characterization and antimicrobial action of a new secondary metabolite from *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., an endophyte in stems of *Artemisia mongolica* (Fisch. ex Bess.) Nakai. We have observed *A. mongolica* to be strongly resistant to insects and pathogens.



1

The FABMS of **1** exhibited a quasi-molecular ion at *m*/*z* 525 [M + H] $^+$, which, together with HRFABMS and ^{1}H and ^{13}C NMR data, determined its molecular formula as $C_{28}H_{28}O_{10}$. The ^{13}C NMR spectrum of **1** gave three carboxyl, six aromatic methyl, and 18 aromatic carbon resonance lines, suggesting that it was a tridepside.[2] This assumption was confirmed by extensive 2D NMR analyses (^{1}H−^{1}H COSY, HMBC, and HMQC), which led to the assignments of most of the proton and carbon signals. A pair of singlets at δ 6.33 and 6.44, broadened due to mutual coupling, was ascribed to H-3 and H-5 in ring A, respectively, while another aromatic proton singlet at δ 6.70 was attributed to H-3″ in ring C. A three-proton singlet at δ 3.87 indicated the presence of a methoxyl group which was found to be anchored on C-5′ by its long-range correlation with this carbon in the HMBC spectrum. The EIMS of **1** exhibited two significant fragments at *m*/*z* 151 and 193, corresponding respectively to rings A and B, which were produced through the sequential ester cleavages.[3] The formulated connectivity of the three rings was also intensified by its FAB mass spectrum, in which typical fragments at *m*/*z* 343 and 375 were afforded by the successive elimination of rings C and A from the parent ion.[3] For simplicity, we have named the compound colletotric acid.

The known secondary metabolites were identified on the basis of spectroscopic methods (IR, MS, ^{1}H and ^{13}C NMR) as uracil, lumichrome,[4] and 3β,5α,6β-trihydroxyergosta-7,22-diene.[1] The spectra of these products are obtainable on request from the corresponding author.

Antimicrobial bioassay revealed that compound **1** was inhibitory to the bacteria *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea* with MICs of 25, 50, and 50 μg/mL, respectively (the MICs of ampicillin against these microorganisms: 0.05, 0.5, and 0.01 μg/mL), and to the fungus *Helminthsporium sativum* with an MIC of 50 μg/mL (the MIC of triadimefon used as a positive control: 20 μg/mL).

There are more than 2000 taxa on at least 470 different host genera reported within the group *C. gloeosporioides*.[5] Most of them are host-specific pathogens causing anthracnose of host plants, whereas some are of value for microbial control of weeds.[6−8] To our knowledge, this is the first report of *C. gloeosporioides* as an endophyte strain in the family Asteraceae, although it has been reported as an endophyte of other plants.[9−11] However, further investigation is desired to ascertain the correlation between the natural adaptability of *A. mongolica* and the presence of *C. gloeosporioides* as an endophyte strain.

Experimental Section

General Experimental Procedures. Melting points were determined with a YANACO-MT-S2 apparatus and were not corrected. Infrared spectra were obtained on a Perkin-Elmer 577 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker AM 400 FT-NMR spectrometer at 400 and 125 MHz, respectively. Chemical shifts were expressed in δ values relative to an internal TMS and/or solvent

* To whom correspondence should be addressed. Tel./Fax: 86 25 3593201. E-mail: rxtan@netra.nju.edu.cn.
† Nanjing University.
‡ Lanzhou University.
§ Nanjing Normal University.
⊥ Shandong Agricultural University.

10.1021/np000204t CCC: $19.00 © 2000 American Chemical Society and American Society of Pharmacognosy
Published on Web 09/27/2000

resonance lines. EI (70 eV) and FAB (positive mode) mass spectra were obtained on a VG-ZAB-HS mass spectrometer. All chemicals used in the study were of analytical grade.

Fungal Isolation, Identification, and Preservation. *A. mongolica* was collected in May and July 1997 at several hillsides of different altitudes of the Zijin Mountain in the suburb of Nanjing, China. A voucher specimen, authenticated by Prof. L. X. Zhang, was preserved under the number ZLX 970715 in the Herbarium of Nanjing University. Thus, apparently healthy plants were uprooted and subjected immediately to the isolation of endophytes. Five stem rods (approximately 10 cm in length) were cut from each plant and washed carefully in running water in the laboratory. The subsequent surface-sterilization and isolation procedure was identical to that adopted previously.[1] A total of 35 isolates were isolated from 100 stem segments.

For inducing sporulation, each of the fungal isolates was separately inoculated on PDA, corn meal agar (CMA), oatmeal agar (OA), water agar with wheat stem (WSA), and potato carrot agar (PCA) in Petri dishes, respectively. Specifically, the inoculated plates were incubated for up to 5 weeks at 26 °C with 12 h co-illumination 45 cm over the Petri dishes utilizing near-ultraviolet (40 W) and cool white fluorescent (40 W × 2) lights followed by 12 h standing in darkness. All experiments and observations were repeated at least twice.

C. gloeosporioides has been deposited in the China Center for Type Culture Collection (CCTCC) under the number AF99012. Other living isolates were preserved in our laboratory on PDA slants and in 2 mL vials with sterile distilled water at 4 °C, and the dried culture mats of these isolates were deposited in the Herbarium of Nanjing University.

The identification of *C. gloeosporioides* was mainly based on the cultural morphology, which was very comparable with the earlier description.[5,12,13] Colonies of *C. gloeosporioides* (strain G501) on PDA grew rapidly, attaining 82−85 mm in diameter in 10 days, with a regular and even margin, white aerial mycelium, velvety initially and then greenish gray from the center and collapsing after 3 days, with pale orange conidial masses forming, reverse white and greenish gray in the center. Colonies on PCA were water soaked-like, reaching 80 mm in 10 days, with hyaline to slightly white immersed hyphae and without or with sparse diffuse gray aerial mycelium; pale orange conidial masses formed inside and on the surface of substrate where hyphae disappeared and the substrate became extremely transparent. Sclerotia were absent. Setae, occasionally present on PDA, are produced singly, dark brown at the base, lighter brown toward the top, 1−3 septate and tapering gradually into the pointed tip (3.4−5.1 × 81−94.5 μm). Conidia are straight, cylindrical, rounded at the apexes, 3.8−4.7 (−5.6) × 9.4−13.5 μm in size. Appressoria on PCA were absent. The teleomorph was not found in culture. These morphological characteristics led to identification of G501 as *C. gloeosporioides*.[5,12−14] Other strains identified as *C. gloeosporioides* were G801, H402-1, H404, and VII401.

Fermentation. The fresh mycelium grown on PDA at 28 °C for 5 days was inoculated into 500 mL Erlenmeyer flasks containing 100 mL of potato sucrose broth (PSB). After 2 days of incubation at 28 °C on rotary shakers at 150 rpm, 50 mL of culture liquid was transferred as seed into each 1000 mL Erlenmeyer flask containing 500 mL of PSB. Cultivation was kept at 28 °C and 150 rpm for 10 days on rotary shakers. The procedure was repeated until sufficient biomass was accumulated.

Extraction and Isolation. The culture liquid (ca. 100 L) was extracted exhaustively with ethyl acetate. Evaporation of solvent in vacuo gave a brown oily residue (43 g), which was then refluxed with 300 mL of MeOH. After cooling, the solution was kept at 4 °C for 10 h and −20 °C for 24 h to remove waxy material. Filtration of the cooled solution gave a brown precipitate (ca. 20 g) and a filtrate that afforded a residue (23 g). The residue was separated by column chromatography (CC) over silica gel (200−300 mesh, 600 g) eluting successively with $CHCl_3$ (2.0 L), a $CHCl_3$/MeOH gradient (100:1→1:1, 11.2 L), and MeOH (2.0 L). Based on the TLC monitoring, the CC fractions (300 mL each) were combined into six parts (F-1: 3.2 g, F-2: 0.9 g, F-3: 1.7 g, F-4: 1.6 g, F-5: 1.2 g, and F-6: 6.4 g). F-1 and F-2 were complex mixtures containing mainly lipids with no antimicrobial activity. F-3 was subjected to CC (90 g) using $CHCl_3$/MeOH (20:1, 0.8 L) and yielded 3β,5α,6β-trihydroxyergosta-7,22-diene[1] (12 mg). CC of F-4 over silica gel (100 g) with a $CHCl_3$/MeOH gradient (50:1→1:1, 1.2 L) afforded two parts (F-4-1 and F-4-2). F-4-1 was subjected to further CC fractionation over silica gel with a $CHCl_3$/MeOH (20:1, 0.4 L) mixture, yielding lumichrome[4] (10 mg). Further CC separation of F-4-2 over silica gel with $CHCl_3$/MeOH (10:1, 0.4 L), followed by gel filtration over Sephadex LH-20, gave compound **1** (26 mg). Repetitious gel filtration of F-5 over Sephadex LH-20 with $CHCl_3$/MeOH (1:1) yielded uracil (134 mg) as a white solid. F-6 was found to contain mainly polar pigment and saccharides as indicated by the spraying agents. The known compounds were identified by comparison with reference compounds and literature data.

Colletotric acid (1): white solid; mp 85−86 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 6.33 (1H, br s, H-3), 6.44 (1H, br s, H-5), 2.67 (3H, s, H-8), 2.14 (3H, s, H-8′), 2.17 (3H, s, H-9′), 2.40 (3H, s, H-10′), 6.70 (1H, s, H-3″), 2.19 (3H, s, H-8″), 2.55 (3H, s, H-9″), 3.87 (3H, s, −OMe); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.9 (s, C-7″ or C-7′), 170.2 (s, C-7′ or C-7″), 167.1 (s, C-7), 166.8 (s, C-2), 164.3 (s, C-4), 160.2 (s, C-2″), 154.0 (s, C-5′), 153.5 (s, C-4″), 150.4 (s, C-3′), 144.9 (s, C-6), 141.8 (s, C-6″), 133.6 (s, C-1′), 127.7 (s, C-6′), 126.7 (s, C-2′), 122.9 (s, C-4′), 121.8 (s, C-5″), 115.2 (s, C-1″), 113.3 (d, C-5), 108.7 (d, C-3″), 104.1 (s, C-1), 101.8 (d, C-3), 62.7 (q, −OMe), 24.8 (q, C-8), 18.7 (q, C-9″), 16.9 (q, C-10′), 13.1 (q, C-8′), 12.6 (q, C-8″), 10.3 (q, C-9′); EIMS *m*/*z* 193 (63.1), 151 (9.1), 124 (66.6), 45 (100); FABMS *m*/*z* 525 $[M + H]^+$ (22), 375 (55), 343 (63); HRFABMS *m*/*z* 525.1765 $[M + H]^+$ (calcd for $C_{28}H_{28}O_{10}$, 525.1761).

Antimicrobial Activity. MICs of the pure substances were determined by paper-disk assay on LB (5.0 g L^{-1} yeast extract, 10.0 g L^{-1} peptone, 5.0 g L^{-1} NaCl, and 20.0 g L^{-1} agar, pH 7.0) and/or PDA plates seeded with 10^6 cells (and/or spores) mL^{-1} suspension of tested bacteria and fungi, followed by incubation at 37 °C for bacteria (48 h) and 28 °C for fungi (96 h), respectively. All isolates were dissolved in EtOH and applied to disks at different concentrations. The test microorganisms were *Bacillus subtilis* (Ehrenberg) Cohn, *Staphylococcus aureus* Rosenbach, *Sarcina lutea* Schroeter, *Pseudomonas* sp., *Candida albicans* (Robin) Berkhout, *Aspergillus niger* van Tieghem, *Cunninghamella elegans* Lendner, *Helminthosporium sativum* Pamm. King & Bakke, and *Trichophyton rubrum* Castellan. Only **1** showed antimicrobial activity.

Acknowledgment. This work was co-financed by grants for R.X.T. from NNSF (Nos. 39725033 and 39970083).

References and Notes

(1) Lu, H.; Zou, W. X.; Meng, J. C.; Hu, J.; Tan R. X. *Plant Sci.* **2000**, *151*, 67−73.
(2) Narui, T.; Takatsuki, S.; Sawada, K.; Okuyama, T.; Culberson, C. F.; Culberson, W. L.; Shibata, S. *Phytochemistry* **1996**, *42*, 839−842.
(3) Huneck, S.; Porzel, A.; Schmidt, J.; Feige, G. B.; Posner, B. *Phytochemistry* **1993**, *32*, 475−477.
(4) Brown, P.; Hornbeck, C. L.; Cronin, J. R. *Org. Mass Spectrom.* **1972**, *6*, 1383−1399.
(5) Sutton, B. C. *The coleomycetes, fungi imperfecti with pycnidia, acervuli and stromata*; Commonwealth Mycological Institute: Kew, U.K., 1980; pp 523−537.
(6) Zhang, T. Y. *Acta Mycol. Sinica* **1985**, *4*, 234−239.
(7) Hildebrand, P. D.; Jensen, K. I. N. *Can. J. Plant Pathol.* **1991**, *13*, 60−70.
(8) Killgore, E. M.; Sugiyama, L. S.; Barreto, R. W.; Gardner, D. E. *Plant Dis.* **1999**, *83*, 964−970.
(9) Smith, C. S.; Chand, T.; Harris, R. F.; Andrews, J. H. *Appl. Environ. Microbiol.* **1989**, *55*, 2326−2332.
(10) Okane, I.; Nakagiri, A.; Ito, T. *Inst. Ferment. Res. Commun. (Osaka)* **1997**, 45−51.
(11) Okane, I.; Nakagiri, A.; Ito, T. *Can. J. Bot.* **1998**, *76*, 657−663.
(12) Davis, R. D.; Boland, R. M.; Howitt, C. J. *Mycol. Res.* **1992**, *96*, 128−134.
(13) Arx, J. A. von. *Phytopath. Z.* **1957**, *29*, 413−468.
(14) Gunnell, P. S.; Gubler, W. D. *Mycologia* **1992**, *84*, 157−165.

NP000204T