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Colletofragarones A1 and A2, Novel Germination Self-Inhibitors from the Fungus *Colletotrichum fragariae*

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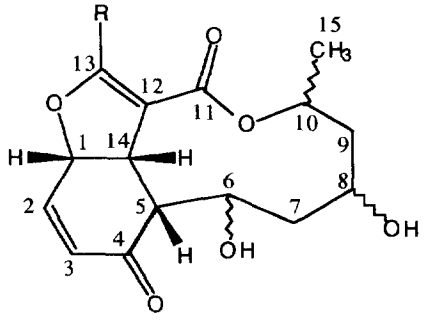
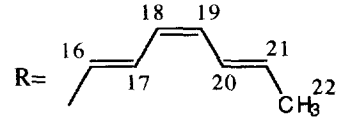
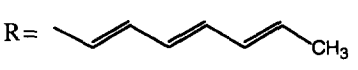
Abstract: The structures of colletofragarones A1 and A2, germination self-inhibitors from *Colletotrichum fragariae*, have been elucidated by spectroscopic analysis. Copyright © 1996 Elsevier Science Ltd

The conidial germination of *Colletotrichum fragariae* is dependent on the population, germination being inhibited at higher concentrations of conidia in water. Repeated washing of the conidia cancels out its inhibitory activity. This phenomenon is considered to be due to a chemical substance(s), called self-inhibitor(s), secreted from the spores. We have detected five active principles from the acetone extract of the potato-sucrose-agar (PSA) plate culture of the fungus, and three compounds were identified as (*E*)- and (*Z*)-(3-indolyl)propionic acid and (2*R*)-(3-indolyl)propionic acid[1]. Other active principles were considered to be a pair of isomers by a preliminary study[2], and were named colletofragarones A and B. We report here the isolation and structure elucidation of the germination self-inhibitors named colletofragarones A1(**1**) and A2(**2**).





Colletofragarone A1 (**1**)

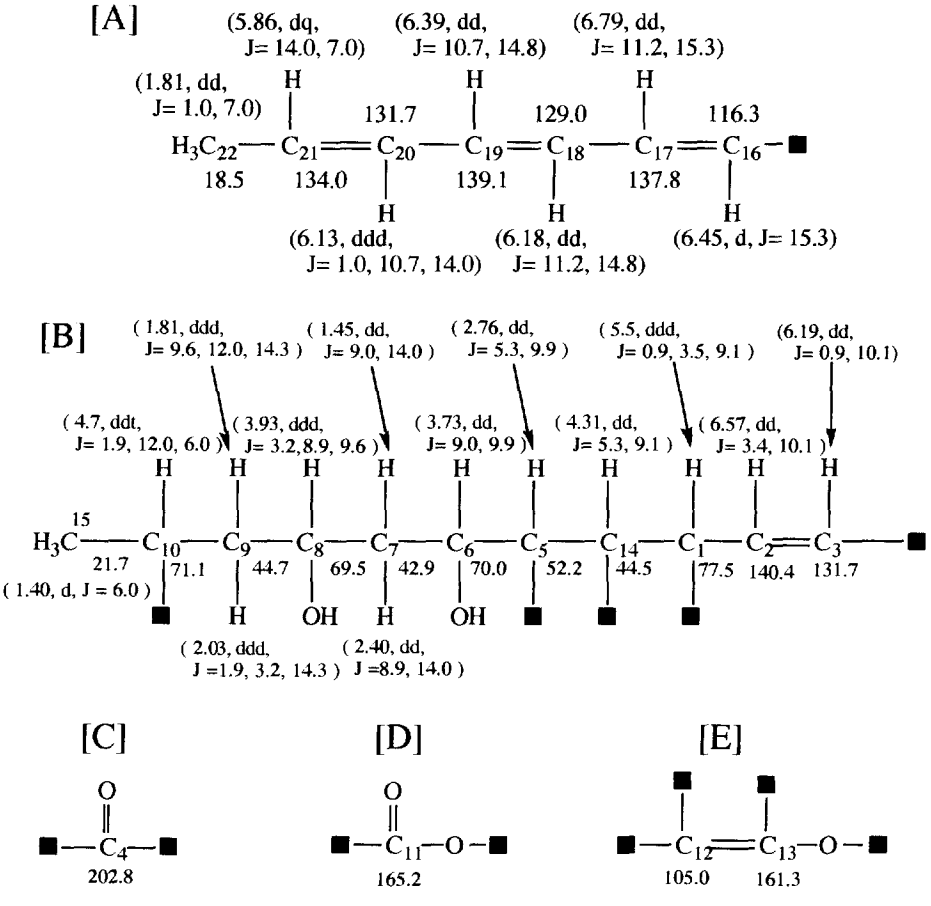


Colletofragarone A2(**2**)

Active principles were extracted with acetone from the PSA-plate cultures (300 plates, 90 mm in diameter) of *C. fragariae*. The extract was evaporated under reduced pressure to obtain an aqueous solution, which was then extracted with EtOAc. The EtOAc extract was concentrated and chromatographed on a silica gel column, using *n*-hexane-$CHCl_3$-MeOH as the solvent. The active fractions of $CHCl_3$ / MeOH(97/3 and 95/5) and 100% MeOH were combined and purified by normal phase HPLC (Cosmosil 10SL; Hexane/2-propanol (85/15-70/30) and subsequently by reversed phase HPLC (Cosmosil 5C18AR; MeOH / H_2O, 70/30)

to give **1** and **2**[3]. LC-MS analyses revealed M+H$^+$ ions at *m/z* 387 for both **1** and **2**, suggesting that they were isomers of each other.

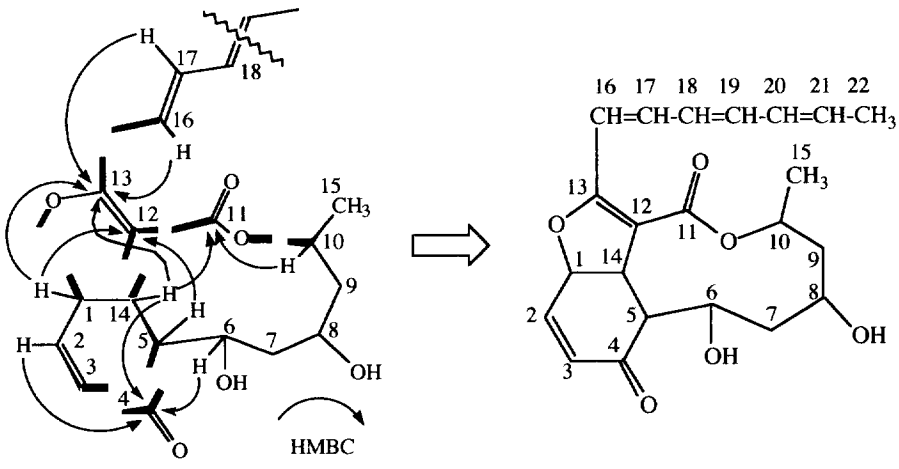
The molecular formula of **2**[4] was determined to be $C_{22}H_{26}O_6$ on the basis of the observed molecular ion at *m/z* 386.1741(calcd. 386.1728) by EIHRMS. The ^{13}C-NMR spectrum of **2** together with DEPT and/or ^{13}C-^{1}H COSY experiments indicated the presence of 4 x C; 14 x CH; 2 x CH_2; 2 x CH_3. Using DMSO-d_6 as solvent the ^{1}H-NMR spectrum of **2** showed the presence of two hydroxyl groups (δ_H 4.33 and 4.61). Five partial structures from [A] to [E] were deduced on the basis of the ^{1}H-^{1}H, ^{13}C-^{1}H COSY experiments.



[δ in $CDCl_3$ / CD_3OD= 9/1, ^{13}C-NMR(75MHz), ^{1}H-NMR(300MHz)]

C-6 and C-8 were substituted with OH because H-6 and H-8 shifted from δ 3.73 to 5.31 and δ 3.93 to 5.18 after acetylation. C-1(δ_C 77.5) and C-10(δ_C 71.1) were assigned to O-bearing carbons on the basis of their ^{13}C-NMR chemical shifts data. HMBC experiments (delay time 60 msec) revealed the formation of cyclohexenone from the correlations between signals C-4 (δ_C 202.8) and H-2, H-6, H-14. Correlations were also observed between C-12 (δ_C 105.0) and H-1, H-5; between C-13 (δ_C 161.3) and H-1, H-14, H-16, H-17, respectively. The conectivities of the partial structures and C-11 were clarified by extending delay time to 120

msec on HMBC. Long-range couplings were observed between C-11 (δ_C 105.0) and H-10, H-14, which revealed the presence of a 10-membered ring lactone. Thus, the plane structure of **2** was constructed reasonably.



The relative stereochemistry of **2** was determined based on ^{1}H-^{1}H coupling constants and NOESY experiments. The *trans*, *trans*, *trans*-conjugated triene system of the side chain was deduced from the coupling constants of $J_{16\text{-}17}$=15.3 Hz, $J_{18\text{-}19}$=14.8 Hz and $J_{20\text{-}21}$=14.0 Hz. In the NOESY, the presence of cross peaks between H-1, H-14 and H-5 suggests that these protons are situated in a cis relation. Determination of the absolute configuration of **2** is now in progress.

The structure of colletofragarone A1(**1**) was determined from data of a mixture sample of **1** and **2** without purification, because **1** is unstable and easily changes to **2**. The ^{1}H and ^{13}C-NMR spectra[5] indicated that **1** is a geometrical isomer of **2** in terms of the conjugate triene system. The observed coupling constants of olefinic protons in **1** were as follows : H16-H17, trans(J= 15.3 Hz); H18-H19, cis(J= 11.4 Hz); H20-H21, trans(J= 14.0 Hz).

The culture of *C. fragariae* in the presence of [1,2-^{13}C]-acetate yielded ^{13}C-labeled **2**[6]. ^{13}C-NMR analysis indicated that ^{13}C atoms were incorporated to all carbons in **2**, giving triplet-like signals, and therefore colletofragarone was found to be of acetate origin.

The mixture of **1** and **2** reduced germination of *C. fragariae* conidia by approximately 50% at a concentration of 20 μg/ml[2]. These compounds were also detected in the conidia suspension under crowded conditions[2], and thus, we concluded that colletofragarones A1 and A2 act as germination self-inhibitors.

Acknowledgments

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References and Notes

1. T. Tsurushima, T. Ueno, H. Fukami, H. Irie and M. Inoue, *Molecular Plant-Microbe Interactions.* **1995**, 8, 652-657.
2. M. Inoue, N. Mori, H. Yamanaka, T. Tsurushima, H. Miyagawa, and T. Ueno, *J. Chem. Ecol.*, submitted.
3. Yield of **1** and **2** mixture was about 25 mg in this experiment. Compound **1** easily changed to **2** after HPLC separation.
4. Physico-chemical properties of colletofragarone A2(**2**) were : $[\alpha]_D^{25}$ +476.1°($CHCl_3$+MeOH, c 0.83), UV ($CHCl_3$+MeOH) λ_{max} nm (log ε) 247 (3.80), 336 (4.47) and IR ($CHCl_3$)ν_{max}(cm^{-1}) 3430, 1704, 1681, 1618.
5. ^{1}H-NMR (300 MHz, $CDCl_3/CD_3OD$=9/1) δ 1.40 (3H, d, J=6.0 Hz, H-15), 1.45 (1H, J=9.1, 14.0, H-7α), 1.85 (3H, dd, J=1.0, 7.0 Hz, H-22), 1.81 (1H, ddd, J=9.6, 12.0, 14.2 Hz, H-9β), 2.03 (1H, ddd, J=1.9, 3.2, 14.2 Hz, H-9α), 2.40 (1H, dd, J=9.0, 14.0 Hz, H-7β), 2.76 (1H, dd, J=5.3, 9.9 Hz, H-5), 3.73 (1H, dd, J=9.0, 9.9 Hz, H-6), 3.93 (1H, ddd, J=3.2, 8.9, 9.6 Hz, H-8), 4.32 (1H, dd, 1H, J=5.3, 9.1 Hz, H-14), 4.70 (1H, ddt, 1.9, 12.0, 6.0 Hz, H-10), 5.54 (1H, ddd, J=0.9, 3.4, 9.1 Hz, H-1), 5.89 (1H, dq, J=14.0, 7.0 Hz, H-21), 5.95 (1H, dd, J=11.4, 11.9 Hz, H-18), 6.18 (1H, dd, J=10.0, 1.4 Hz, H-19), 6.21 (1H, dd, 0.9, 10.1 Hz, H-3), 6.46 (1H, d, J=15.3 Hz, H-16), 6.59 (1H, ddd, J=1.0, 10.0, 14.0 Hz, H-20), 6.62 (1H, dd, J=3.4, 10.1 Hz, H-2), 7.23 (1H, dd, J=11.9, 15.3 Hz, H-17).

   ^{13}C-NMR (75 MHz, $CDCl_3/CD_3OD$=9/1) δ_C 202.7 (C-4), 165.2 (C-11), 161.1 (C-13), 140.4 (C-2), 139.1 (C-19), 137.8 (C-17), 133.9 (C-21), 131.7 (C-3), 131.7 (C-20), 129.0 (C-18), 116.2 (C-16), 105.0 (C-12), 77.5 (C-1), 71.2 (C-10), 70.0 (C-6), 69.5 (C-8), 52.1 (C-5), 44.6 (C-9), 44.5 (C-14), 42.9 (C-7), 21.7 (C-15), 18.5 (C-22).
6. The labeled compound was obtained by adding [1,2-^{13}C]-acetate to the cultures of *C. fragariae*. *C. fragariae* was cultured on PSA agar plates fortified with [1,2-^{13}C]-acetate at a rate of 1 g/l. After a 2 week culture, the isotope enriched **2** was isolated from acetone extraction and purified by SiO_2 column chromatography and preparative HPLC, as described in the text.

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