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Malettinin A: a new antifungal tropolone from an unidentified fungal colonist of *Hypoxylon* stromata (NRRL 29110)

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Abstract—Malettinin A (**1**) has been isolated from cultures of an unidentified fungus encountered as a colonist of *Hypoxylon* stromata. The structure of **1** was proposed by analysis of NMR and MS data, and confirmed by X-ray diffraction analysis of a methanol adduct. Malettinin A shows significant activity in assays against *Aspergillus flavus*.

Our ongoing studies of mycoparasitic and fungicolous fungi have led to the discovery of a variety of new natural products.[1–4] The occurrence of antifungal metabolites is proving to be common among these fungi, as might be predicted based on their tendency to cause damage to host species.[5] Our current approach involves studies of fungi that attack and colonize long-lived, nutrient-rich fungal bodies, such as stromata.[6] During the course of this project, isolates obtained from an *Hypoxylon* sp. stromatal specimen collected in New Mexico included a non-sporulating *Mycelia sterilia* culture (MYC-155 = NRRL 29110).[7] The organic extract from solid-substrate fermentation cultures of this isolate displayed potent antifungal activity. Chemical investigation of this extract led to the isolation of a novel antifungal polyketide-derived tropolone that we named malettinin A (**1**).

Fractionation of the crude EtOAc extract of solid-substrate fermentation cultures[8] of NRRL 29110 by Sephadex LH-20 column chromatography afforded malettinin A (**1**)[9] as the major component. The molecular formula of **1** was established as $C_{16}H_{16}O_5$ (nine unsaturations) on the basis of FABMS and HREIMS data. ^1H and ^{13}C NMR data (Table 1) revealed the presence of three methyl groups, one methylene unit, one sp^3 methine unit, one doubly oxygenated quaternary sp^3 carbon, two carbonyls, and eight olefinic or aromatic sp^2 carbons, three of which appeared to be oxygenated. Addition of one exchangeable proton would account for the molecular formula of the compound. Accordingly, a tricyclic structure was required for **1** to fulfil the unsaturation requirement.

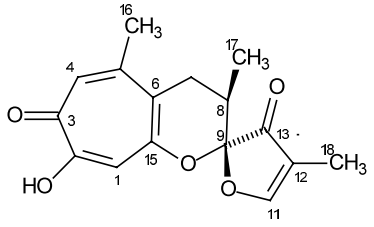

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Table 1. NMR data for malettinin A (**1**)

Pos. #	δ_H (mult., J in Hz)[a]	δ_C[b]	HMBC (H→C#)
1	6.96 (s)	112.2	2, 3, 6, 15
2	–	163.2	–
3	–	173.0	–
4	7.20 (s)	126.5	2, 3, 6, 16
5	–	149.8	–
6	–	120.3	–
7_{eq}	2.75 (dd, 17, 5.7)	30.3	5, 6, 8, 9, 15, 17
7_{ax}	2.52 (dd, 17, 13)		5, 6, 8, 17
8	2.36 (m)	29.6	7, 9, 13, 17
9	–	101.1	
11	8.02 (q, 1.1)	173.4	9, 12, 13, 18
12	–	114.8	
13	–	198.8	
15	–	157.7	
16	2.39 (s)	26.8	4, 5, 6
17	0.95 (d, 6.5)	13.9	7, 8, 9
18	1.77 (d, 1.1)	5.3	11, 12, 13

[a] Recorded using $CDCl_3$ at 600 MHz.
[b] Recorded using acetone-d_6 at 600 MHz.

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Preliminary analysis of the NMR data initially led to speculation that **1** contained an oxygen-substituted benzoic acid moiety with a carboxy carbon at δ_C 173.0. However, some of the chemical shift values and HMBC correlations (Table 1) were inconsistent with those expected for such a unit. Detailed analysis of the data and consideration of other possibilities led to incorporation of the δ_C 173.0 carbon into the ring, resulting in recognition of a tropolone unit. This assignment was supported by HMBC correlations of H-1 to C-2, C-3, C-6, and C-15, and of H-4 to C-2, C-3, and C-6. Additional correlations from H_3-16 to the tropolone ring carbons C4, C-5, and C-6 enabled location of this methyl group at C-5. Assignment of a tropolone unit was further supported by comparison of the NMR data with those observed for known tropolones with a similar substitution pattern.[10–14]

The isolated spin system $^{7}CH_2$-^{8}CH-$^{17}CH_3$ was assigned on the basis of ^{1}H–^{1}H decoupling experiments. Attachment of this unit to the tropolone ring at C-6 was evident from HMBC correlations of H_2-7 to carbons C-5, C-6, and C-15. The doubly oxygenated quaternary carbon C-9 (δ_C 101.1) was connected to C-8 based on HMBC correlations of H_3-17, H_2-7, and H-8 to C-9. The presence of an α,β-unsaturated ketone subunit (C-11/C-12/C-13) was suggested by the ^{13}C NMR signals at δ_C 173.4, 114.8, and 198.8. This unit was confirmed by HMBC correlations of H-11 (δ_H 8.02) and H_3-18 (δ_H 1.77) to C-13 (δ_C 198.8). H_3-18 showed HMBC correlations to C-11 and C-12, as well as allylic coupling (1.1 Hz) to H-11. The downfield shifts of the signals for both H-11 and C-11 (δ_H 8.02; δ_C 173.4) indicated that C-11 bears an oxygen atom. An HMBC correlation of H-11 with C-9 led to attachment of C-11 to C-9 through an oxygen atom. Connection of C-9 to ketone carbon C-13 to complete the furanone ring was established by an HMBC correlation of H-8 with C-13. The chemical shifts of the signals for C-11, C-12, and C-13 provided additional evidence for the presence of this structural unit.[15,16]

At this point, all atoms of the molecular formula had been accounted for. Although no direct evidence for the ether linkage connecting C-9 and C-15 was provided by the HMBC data, chemical shift considerations and the need for one additional unsaturation required this linkage to be present, thereby completing the gross structure of malettinin A as shown in **1**. The chemical shift of C-9 is consistent with values reported for the ketal carbons of similar spiro-fused ring systems.[17,18]

NOESY data for malettinin A (**1**) did not enable conclusive assignment of the relative stereochemistry. Therefore, attempts were made to obtain crystals suitable for X-ray crystallographic analysis. Slow evaporation of a solution of **1** in MeOH after warming and cooling yielded crystals. However, these did not prove to be crystals of **1**. The final X-ray model (Fig. 1) revealed that the crystalline material was actually a product of addition of MeOH across the olefin unit of the furanone ring (**2**).[19] NMR data revealed that diastereomeric product **2** had selectively crystallized, as

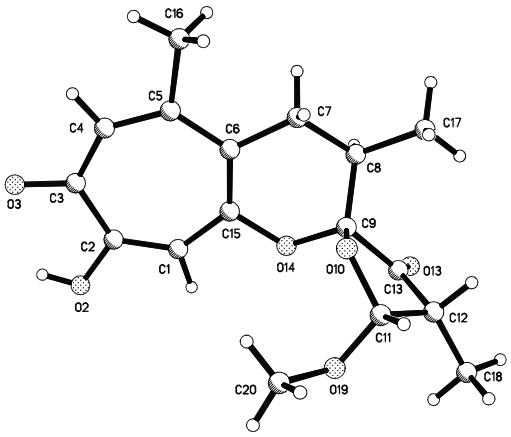
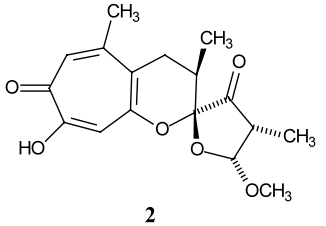


Figure 1. X-Ray crystal structure of the methanol adduct of malettinin A (**2**).

different diastereomeric product forms were evident in the reaction mixture. Despite this unexpected result, this experiment accomplished the original objective, as the X-ray structure of **2** enabled assignment of the relative stereochemistry of the precursor **1** as shown. To our knowledge, the ring system found in **1** and **2** has not been previously reported.



2

Tropolones display a variety of interesting properties.[20–26] Although tropolone itself and some simple analogs are known to exist as a rapidly interconverting equilibrium mixture of the two tautomers in solution,[22–24] only one tautomer structure is generally reported for bicyclic or more highly substituted fungal-derived tropolones.[10–13] The NMR data for **1** (e.g. the 10 ppm difference in δ-values for C-2 and C-3; the ca. 150 ppm δ-value for C-5) suggest that it exists in solution predominantly, if not exclusively, as the tautomer shown in **1**. The data did reveal the presence of a small amount of a closely related species that could not be removed by HPLC using any of four different stationary phases. However, analysis of the minor 2D NMR signals indicated that the species has the same gross structure as **1** (not a tautomer), with minor differences observed in the pyranone ring. It was not clear whether this material was an epimer or a minor conformer, although limited VT NMR experiments conducted up to 333°K showed no signal coalescence. The X-ray crystallographic model of **2** (Fig. 1) is consistent with the NMR data, as it showed a tropolone ring structure analogous to the tautomer depicted in **1**. This conclusion is based in part on the shorter C–O bond length at C-3, as well as the individual C–C bond lengths

throughout the tropolone ring. The bond length between the two oxygenated carbons in **2** was determined as 1.461 Å, while the average values for the other formal single and double C–C bonds in the ring were 1.414 and 1.368 Å, respectively. These data are in good agreement with those reported for 4-isopropyltropolone (β-thujaplicin),[25] for which the corresponding values were 1.469, 1.416, and 1.362 Å, and are also consistent with those reported for other tropolone X-ray crystal structures.[11] However, it is not clear why the tautomers represented by **1** and **2** would be preferred. The predominance of a given tropolone tautomeric form is seldom rationalized in literature reports of such compounds. Molecular modelling calculations were carried out in an effort to explore the energy difference between the two possible tautomers of malettinin A, and the results were only slightly in favor of tautomer **1** (ΔE calculated as 0.62 Kcal mol^{-1} using the PC Spartan PRO Ab-Initio package, version 6.0.4). Pycnidione and epolones A and B, previously isolated fungal metabolites containing tropolone units with a substitution pattern similar to that of malettinin A, also appear to exist in an analogous preferred tautomeric form.[13] Upon methylation, however, both tautomer types were trapped as methyl ether derivatives and isolated by chromatographic techniques. Methylation and acetylation experiments carried out on **1** were not conclusive, as each experiment gave a complex mixture of products, presumably due in part to the lability of the furanone ring noted above. Thus, the corresponding derivatives of **1** were not isolated.

Although a number of literature precedents are known, as cited above, the occurrence of the tropolone moiety in natural products is relatively rare. Malettinin A (**1**) is presumably biosynthesized from a polyketide precursor with some methylation and oxidative modifications, perhaps including an aromatic ring expansion, as appears to be the case for the fungal tropolone stipitatic acid.[27] Malettinin A (**1**) showed activity in standard disk assays against *Aspergillus flavus* (NRRL 6541), *Fusarium verticillioides* (NRRL 25457), and *Candida albicans* (ATCC 90029) at 250 μg/disk, causing a zone of inhibition of 31-32 mm in each assay. It exhibited an MIC value of ca. 5.9 μg/mL against *A. flavus*. Malettinin A also exhibited antibacterial activity, as inhibitory zones of 21 and 30 mm were observed in assays at 200 μg/disk when tested against *Staphylococcus aureus* (ATCC 29213), and *Bacillus subtilis* (ATCC 6051), respectively.

Acknowledgements

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7. A dark olive-colored *Mycelia sterilia* isolate (MYC-155) was collected from colonies of *Hypoxylon* stromata found on dead Aspen logs in Malette Meadow, New Mexico. A subculture was deposited in the National Center for Agricultural Utilization Research (NCAUR) culture collection and assigned the accession number NRRL 29110. Details of our general procedures for isolation of fungi from such sources have been described.[3]
8. Fermentation was carried out in eight 500-mL Erlenmeyer flasks each containing 50 g rice. Distilled water (200 mL) was added to each flask and the contents were soaked overnight before autoclaving at 15 lb/in^2 for 30 min. The flasks were cooled to rt and were inoculated with 3.0 mL of the culture inoculum and incubated at 25°C for 30 days. The fermented rice substrate was fragmented, then extracted with EtOAc (3×800 mL), and the combined EtOAc extracts were filtered and evaporated to yield 1.5 g of crude extract.
9. The crude extract was partitioned between hexane (3×100 mL) and CH_3CN (50 mL), and the CH_3CN-soluble portion (1.0 g) was fractioned by Sephadex LH-20 column chromatography (55×2.2 cm) eluting successively with 500 mL each of 1:4 hexane–CH_2Cl_2, 3:2 CH_2Cl_2–acetone, and 1:4 CH_2Cl_2–acetone. One fraction that eluted with 1:4 hexane–CH_2Cl_2 (230 mg) consisted of malettinin A (**1**): pale yellow amorphous solid; mp 186–188°C; $[\alpha]_D$ −15° (*c* 0.1 g/100 mL, $CHCl_3$, 25°C); UV λ_{max} (EtOH) 250 (ε 36000), 353 (ε 9300); IR ν_{max} ($CHCl_3$) 3208, 3030, 2928, 2870, 1719, 1620, 1540, 1451, 1275, 1156, 1052 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 1; NOESY correlations ($CDCl_3$, H-#H-#) H-4↔H_3-16; H-7_{eq}↔H-7_{ax}, 8, H_3-16, and H_3-17; H-7_{ax}↔H-7_{eq} and H_3-17; H-8↔H-7_{eq} and H_3-17; H-11↔H_3-17 and H_3-18; EIMS (70 eV) *m*/*z* 288 ($[M]^+$; rel. int. 100), *m*/*z* 273 ($[M-CH_3]^+$; rel. int. 69); FABMS (thioglycerol) obsd. *m*/*z* 289 $[M+H]^+$; HREIMS obsd. *m*/*z* 288.0996 $[M]^+$, calcd for $C_{16}H_{16}O_5$, 288.0998.
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19. A crystal of **2** (0.45×0.18×0.14 mm) obtained upon slow evaporation of a MeOH solution of **1** was subjected to analysis. Cell dimensions were determined from a least-squares analysis to be $a=6.8357(8)$, $b=8.613(2)$, $c=14.101(2)$ Å, $\alpha=90.50$, $\beta=99.87$, and $\gamma=105.90°$. Intensity standards were measured at 2 h intervals. Net intensities were obtained by profile analysis of the 5297 data and the calculated density is 1.355 mg/m^3. The systematic absence corresponded to space group P-1. Data were collected on an Enraf–Nonius CAD4 diffractometer (Mo K-α radiation, $\lambda=0.71073$ Å, graphite monochromator) at 210(2) K (N_2 cold gas stream) using ω–2ω scans (range 2.5–25.0°). Equivalent data were averaged, yielding 2726 unique data [$R_{int}=0.069$, 1483 $F>4\sigma(F)$]. Data were corrected for Lorentz and polarization effects, but not for absorption. No change in intensity standards was noted. Computer programs from the MoLEN package were used for data reduction. The structure was solved using XS, a direct methods program, and refined by full-matrix least-squares performed with the SHELXL-97 program. Illustrations were made with the XP program, and tables were made with the XCIF program. All are in the SHELXTL v5.1 package. Thermal ellipsoids are at the 35% level. All non-hydrogen atoms were refined with anisotropic thermal parameters, and the final refinement, based on 1483 reflections, gave $R_1=0.0564$, $R_2=0.1265$ standard deviation. Crystallographic data (excluding structure factors) for structure **2** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 214273. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk.
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