Acremine G, Dimeric Metabolite from Cultures of *Acremonium byssoides* A20[1]

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A new dimeric fungal metabolite, racemic acremine G, was isolated from cultures of the fungus *Acremonium byssoides* A20, along with the known acremines A−F. Its structure was elucidated by NMR spectroscopy and X-ray crystallography. A possible biosynthetic pathway is discussed. Acremine G mildly inhibited the growth of sporangia of *Plasmopara viticola.*

Endophytic fungi are one source for intelligent screening; they grow within their plant hosts without causing apparent disease symptoms, and growth in this habitat involves continual metabolic interaction between fungus and host.[2]

The genus *Acremonium* is an endophytic fungus and is a rich source of biologically active secondary metabolites including, for example, β-lactam antibiotics,[3] the immunosuppressant cyclosporins,[4] the tremorgenic indole diterpenoid lolitrem,[5] and prenylated phenol inhibitors of N-SMase.[6] A strain of *Acremonium bissoides*, named A20, was recently isolated from grapevine leaves artificially inoculated with *Plasmopora viticola.*[7] Cultures of this fungus, performed in Roux flasks containing sugar-rich media such as corn steep agar (CSA), led to the isolation of a series of structurally related metabolites, the acremines A (**1**), B (**2**), and C−F.[8]

We describe herein the isolation and structure determination of acremine G (**3**), a new dimeric derivative, produced by the same fungus.

The acremines are compounds derived biosynthetically from a monoterpene unit and a polyketide moiety; the structure and absolute configuration of acremine A (**1**) were determined through X-ray analysis and Mosher's method; a misassigned structure of **1** isolated from a plant was recently published[9] and subsequently corrected on the basis of synthetic evidence.[10]

Compound **3** was isolated as yellow needles, mp 130–132 °C, and was optically inactive; the IR spectrum exhibited hydroxy and conjugated carbonyl absorption bands at 3384 and 1699 cm^{-1}, respectively. The EIMS showed an $[M]^+$ at *m/z* 394 corresponding to the molecular formula $C_{24}H_{26}O_5$, confirmed by HREIMS measurement.

The structure of **3** was deduced first from analysis of ^1H and ^{13}C NMR spectra. Assignment was carried out through COSY, HMQC, and HMBC techniques, whereas the spatial arrangement was deduced from NOE experiments. The NMR data are reported in Table 1.

The ^{13}C NMR spectrum of **3** showed the presence of 14 sp^2-hybridized carbons attributable to two conjugated ketone groups, one tetrasubstituted aromatic ring, one disubstituted and two trisubstituted double bonds, and 10 sp^3-hybridized carbons attributable to five methyl, one methylene, one methine, and three quaternary carbons. Addition of D_2O to the sample caused the resonances at δ_H 6.50 and 2.00 to disappear, thus identifying one phenolic and one aliphatic hydroxy group. On the basis of the above and the following results three partial structures (A−C) could be constituted as shown in Figure 1.

The HMBC correlations observed between H-7 and C-1, C-2, and C-3 and between H_3-12 and C-4, C-5, and C-6 indicated that C-7 and C-12 are *para*-disposed as well as H-3 and H-6 since they exhibited a $^5J_{H,H} < 0.5$ Hz. The aromatic hydroxy group was placed at C-4 because H-3, which showed a mutual NOE with H-7, underwent a downfield shift by acetylation. Evidence for the linkage of C-7 with C-8 was provided by the vicinal coupling of 6.4 Hz observed between H-7 and H-8, while the allylic couplings observed between H-8 and H_2-10 and H_3-11 confirmed the connectivities of C-10, C-11, and C-9 (structure A).

Table 1. NMR Spectroscopic Data for Compound **3** ($CDCl_3$)

position	δ_C	δ_H	$^1H-^{13}C$ HMBC	NOE correlations
1	144.1			
2	124.7			
3	113.4	6.51	1,4,5,7	7
4	148.5			
5	123.9			
6	118.7	6.51	1,2,4,12	12
7	38.4	3.75	1,2,3,8,9,1′,2′,3′,4′	3,8,7′,8′
8	122.4	5.63	2′,10,11	
9	131.2			
10a	36.1	2.71	8,9	10b,7′,8′
10b		2.48	8,9,11,2′,3′	10a,7′,8′
11	22.6	1.67	8,9,10	8,10a, 10b,7′,8′
12	15.8	2.10	4,5,6	6
1′	196.6			
2′	54.9		2′,4′,12′	
3′	80.6			
4′	194.2			
5′	150.4			
6′	135.1	6.37		12′
7′	121.8	5.64	7,1′,2′,3′,8′,9′	
8′	142.9	5.76	2′,7′,9′,10′,11′	7,10′,11′
9′	70.7			
10′	29.7	1.21	8′,9′	7′,8′
11′	29.7	1.21	8′,9′	7′,8′
12′	16.9	2.11	4′,5′,6′	6′,7′,8′
4-OH		6.50		
9′-OH		2.00		

The presence of a hydroxydimethylallyl moiety (structure B) in which the vinylic protons are *trans*-disposed ($J_{H,H} = 15.8$ Hz) was revealed by comparison of the ^1H and ^{13}C NMR spectra with those of acremines **1** and **2**.[8]

The HMBC correlation observed between H-8′ and C-2′ permitted connection of C-2′ to C-7′, while the correlations observed between H-7′ and C-1′, C-3′, and C-7 permitted the attachment of C-2′ with C-1′, C-3′, and C-7. The correlations observed between H_2-10 and C-2′ and C-3′ defined the linkage between C-10 and C-3′.

The remaining C_4H_4O fragment contained a trisubstituted double bond (structure C). The mutual NOEs observed between H-6′ and H_3-12′ indicated their *cis* relationship, while the C-4′ carbonyl group was connected to C-5′ since it showed correlations with H-6′ and H_3-12′.

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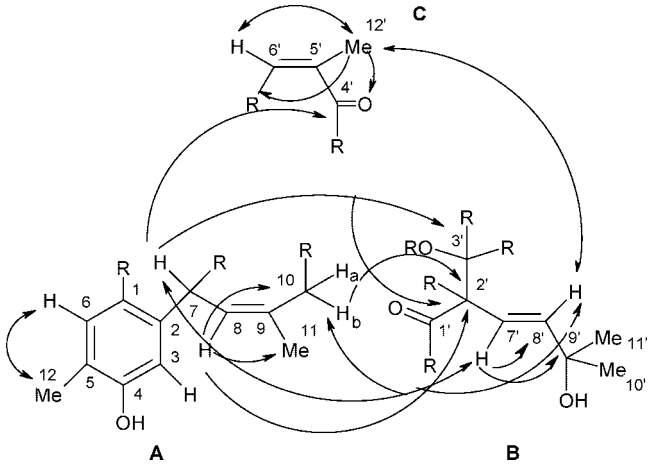


Figure 1. Partial structures for metabolite **3** and selected HMBC (→) and NOEs correlations (↔) of acremine G (**3**).

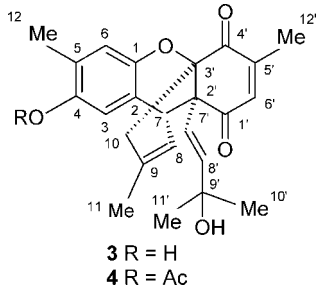


Figure 2. Compounds **3** and **4**.

^{13}C NMR shift criteria indicated that the oxygen atom at C-3′ must also be connected to C-1. In fact it cannot be linked to C-1′ or C-4′ because these carbons occurred at more downfield values than those expected for lactone functions, or to C-6′ because it occurred at a more upfield value than that expected for an enol ether moiety. Finally, C-4′ and C-6′ were linked to C-3′ and C-1′, respectively, to give a dihydrobenzoquinone ring in which the two carbonyl groups are 1,4 -disposed as in **1** and **2**.

The NOEs observed between H_3-11′ and H-7′ and H-8′ indicated that these protons are on the same face of the dihydrobenzopyran ring, this fact establishing the *S*-relative configuration at C-2′, having assumed as *R* those at C-7 and C-3′ (Figure 2).

Spectroscopic studies of the *O*-acetyl derivative **4** (Experimental Section) confirmed these assignments and provided evidence of the presence of only one phenolic OH group.

In order to obtain more detailed information about the molecular conformation of acremine G (**3**), an X-ray structure investigation was carried out. Single crystals suitable for X-ray analysis were obtained by recrystallization from benzene, and the compound was found to crystallize with this solvent in a 2:1 benzene to compound ratio.

Although the molecule contains three stereogenic centers (C-2′, C-3′, and C-7), it crystallizes as a racemic mixture, in a centrosymmetric space group ($P\bar{1}$).

The molecular structure, with atomic numbering scheme, is reported in Figure 3, where the arbitrarily chosen asymmetric unit shows an *R,S,R* configuration at C-7, C-2′, and C-3′, respectively. The main body of the molecule consists of four six-membered rings fused to each other by edge sharing. The C-2′−C-3′ edge is common to the three nonaromatic rings. These three nonaromatic rings adopt a more or less distorted envelope conformation. The C-1′/C-2′/C-3′/C-4′/C-5′/C-6′ ring has the C-3′ apex atom displaced by 0.570(4) Å from the plane of the other five atoms of the ring (maximum deviation from the mean plane of 0.029(3) Å). This five-atom ring is nearly coplanar with the planar phenyl C-1/C-6 ring. The C-2′/

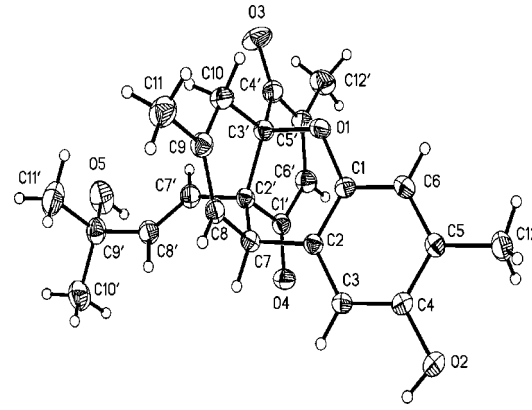


Figure 3. View of compound **3**, as obtained by X-ray analysis, with atom-numbering scheme. Displacement ellipsoids are drawn at the 30% probability level.

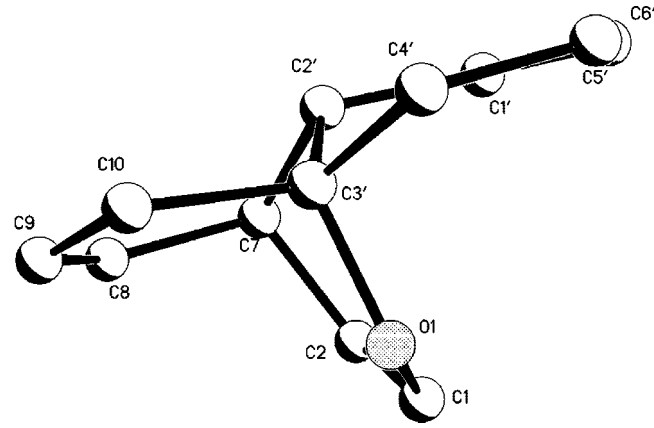


Figure 4. Skeleton of **3** showing the conformation of the three nonaromatic rings.

C-3′/C-7/C-8/C-9/C-10 carbocyclic ring is slightly distorted, with the C-2′ apex atom 0.737(4) Å out of the plane of the five remaining atoms (maximum deviation from the mean plane of 0.072(3) Å). The heterocyclic O-1/C-3′/C-2′/C-7/C-2/C-1 ring shows the largest distortion from the envelope conformation, with the C-2′ apex atom 0.783(2) Å out of the plane of the five remaining atoms of the ring (maximum deviation from the mean plane is 0.116(3) Å). The overall conformation of the three nonaromatic rings is illustrated in Figure 4, where it appears that the heterocyclic ring is roughly perpendicular to the other two, roughly parallel, rings. The connection of the rings around the C-2′−C-3′ bond may be described also by the torsion angles O-1−C-3′−C-2′− C-1′ [−64.7(3)°], C-4′−C-3′−C-2′−C-7′ [1567.0(2)°], and C-10−C-3′−C-2′−C-1′ [174.4(2)°]. The bond distances and angles within the four fused rings are in agreement with the values expected for sp^2- and sp^3-hybridized carbon atoms.

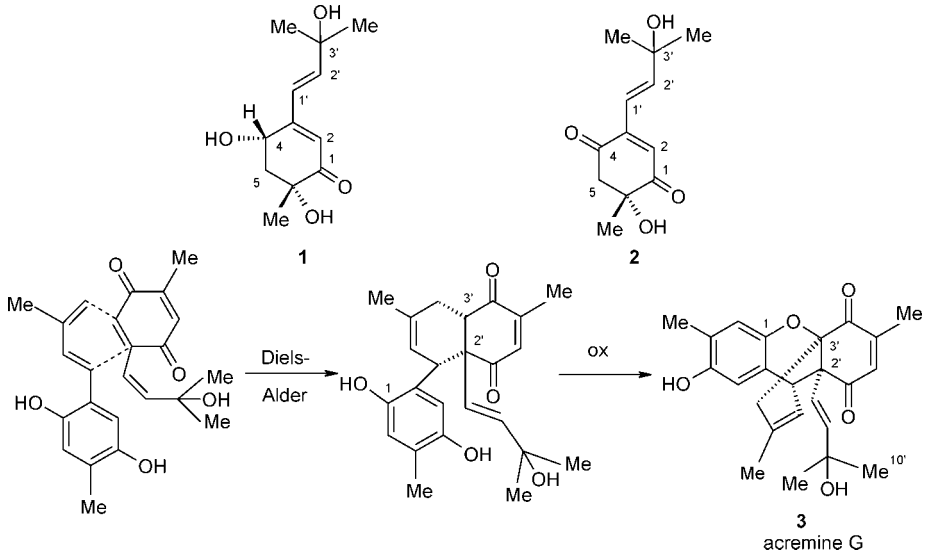
The configuration around the C-7′−C-8′ bond is *trans* with a torsion angle C-2′−C-7′−C-8′−C-9′ of 178.5(3)°.

In the crystal the molecules of opposite chirality are linked into centrosymmetric dimers by pairs of O-2−H···O4 hydrogen bonds [H···O-4 = 2.03(5) Å; O-2···O-4 = 2.892(4) Å; O-2−H···O-4 = 177(4)°].

A metabolite structurally similar to acremine G, allomicrophyllone, has been isolated from the aerial part of *Ehretia microphylla*,[11] a medicinal plant from the Philippines. Its structure was demonstrated only with NMR spectra in comparison with other dimeric prenylbenzoquinones isolated from the same plant; no monomeric units were found in the plant.

Several pathways can be envisaged to produce racemic acremine G (**3**); it could be generated probably by a nonenzymatic Diels–

Scheme 1. Probable Biosynthetic Pathway to Compound **3**



Alder-type cycloaddition.[12] One plausible path would involve a cycloaddition, possibly acid-catalyzed, of a dienophile-like structure deriving from unit **1** to a prenylbenzoquinone derivative of the unit **2**; a successive enzymatic oxidative coupling of the precursor between C-1(O) and C-3′ would give acremine G (Scheme 1). We believe that acremine G is a true natural compound, not an artifact, since we have not found in the extracts of the fungus the corresponding diene or dienophile. It is still debatable as to whether the biosynthesis of this kind of natural product proceeds enzymatically or not, but several authors have indicated the possibility of some plant species possessing *dielsalderase*-type enzyme systems that would be responsible for biosynthetic processes toward the racemic secondary metabolites.[12]

From a biological point of view, acremine G (**3**), like acremines A−F, inhibits the germination of sporangia of *Plasmopara viticola*;[8] at a concentration of 1 mM compound **3** shows a stronger inhibition of the sporangia (72.5%) with respect to acremines A (58.2%) and B (57.7%), respectively.[8] Compounds **1**, **2**, and **3** were tested for their cytotoxicity, *in vitro*, against the tumor cell line H460[13] and showed modest activity [IC_{50} (μM: >100 for **1**, 36 for **2**, and 48 for **3**)].

Experimental Section

General Experimental Procedures. UV spectra were measured for solutions in 95% EtOH. Mass spectra were obtained with a Finnigan-MATT-TSQ 70 eV, a Bruker Esquire 3000, and for HRMS a Bruker APEX-QZT ICR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DMX 500 or ARX 400 instrument at 305 K. The proton signals were assigned from the chemical shift correlation experiments (COSY) and the carbon nuclei from HSQC and HMBC experiments. The NOEs were determined by monodimensional NOE difference spectra.

HPLC analyses were performed using a LiChroCART column RP-18 250-4 (Merck) on an Agilent 1100 instrument; flow rate = 0.5 mL min^{-1}. Flash CC was performed on Merck Si gel; TLC and PLC with Merck HF_{254} Si gel. The purity of products was checked by TLC, NMR, and MS and deemed sufficient for the purpose of structural determination.

Fungal Material. The fungus was isolated and identified as *Acremonium byssoides*;[7] this fungus was deposited in the Culture Collection of CNR-ICRM-Milano, Italy.

Culture of *Acremonium byssoides*, Strain A20, and Extraction and Isolation of Acremine G (3). The fungal strain A20 was detected in young grape leaves cv. *Regina bianca* infected with *P. viticola*, collected in 1996 in an open-air vineyard at Mazara del Vallo (Trapani, Sicily). It was isolated with a lancet by the aid of a stereomicroscope, transferred to Petri dishes containing potato dextrose agar, and identified as an *Acremonium byssoides*, by conventional taxonomy. For chemical investigations, the fungus was grown in batches of 40 Roux flasks containing 100 mL of CSA (corn steep liquor 10 g L^{-1}, glucose 90 g L^{-1}, sucrose 100 g L^{-1}, yeast extract 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, and agar 15 g L^{-1}). After a 2-week growth period at 24 °C, the cultures were extracted twice with EtOAc/MeOH (100:1). The extracts (2.5 g) were dried over Na_2SO_4, evaporated to dryness, and chromatographed on a Si gel flash column eluted with *n*-hexane/EtOAc of increasing polarity. Collected fractions were further purified by means of PLC in CH_2Cl_2/MeOH (15:1) to give the pure metabolites in order of elution: acremine C, 230 mg, R_f 0.7 (*n*-hexane/EtOAc, 1:1); acremine D, 15 mg, 0.5; acremine E, 2 mg, 0.45; acremine B (**2**), 247 mg, 0.4; acremine G (**3**), 15 mg, 0.35, acremine A (**1**), 620 mg, 0.2; acremine F, 10 mg, 0.1.

Acremine G (3): crystallized from benzene; mp 130–132 °C; UV (ETOH) λ_{max} 211, 286 and 406 nm (ϵ 44 000, 8000, and 2000); IR (neat) ν_{max} 3384 (con. CO group), 1699, 1651, 1622, and 1515 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; CIMS *m/z* 394, 377, 255, 225, 207, and 191; EIMS *m/z* 394 [M]$^+$ (100%), 361, 255, 175, and 91; HREIMS *m/z* 394.1764 (calcd for $C_{24}H_{26}O_5$, 394.1780).

Acetylation of Compound 3. **3** (15 mg) was acetylated with pyridine/Ac_2O at rt; workup gave 8 mg of **4**; crystallized from CH_2Cl_2/hexane, mp 115–118 °C; UV (EtOH) λ_{max} 206, 248 sh, 275 sh, and 330 nm (ϵ 34500, 10 000, 4750, and 200); ^{1}H NMR ($CDCl_3$) δ 1.20 (6H, s, Me-10′ and Me-11′), 1.64 (1H, br signal, 9′-OH), 1.66 (3H, br ddd, J = 1.2, 1.1, 1.0 Hz, Me-11), 2.02 (3H, br d, J = 1.0 Hz, Me-12), 2.09 (3H, d, J = 1.8 Hz, Me-12′), 2.28 (3H, s, 4-OAc), 2.48 (1H, br ddq, J = 19.0, 1.2, 1.0 Hz, H-10b), 2.75 (1H, br ddq, J = 19.0, 2.3, 1.1 Hz, H-10a), 3.78 (1H, br d, J = 6.4 Hz, H-7), 5.62 (1H, br dddq, J = 6.4, 2.3, 1.2, 1.2 Hz, H-8), 5.63 (1H, d, J = 15.8 Hz, H-7′), 5.74 (1H, d, J = 15.8 Hz, H-8′), 6.36 (1H, q, J = 1.8 Hz, H-6′), 6.58 (1H, br q, J = 1.0, H-6), 6.72 (1H, br s, H-3); ^{13}C NMR ($CDCl_3$) δ 16.0 (C-12), 16.8 (Me-CO), 22.5 (C-11), 29.6 (C-10′, C-11′), 36.1 (C-10a), 38.3 (C-7), 54.5 (C-2′), 70.8 (C-9′), 80.8 (C-3′), 118.3 (C-6), 120.5 (C-3), 121.8 (C-7′), 122.7 (C-8), 124.8 (C-2), 129.5 (C-5), 131.3 (C-9), 135.2 (C-6′), 142.8 (C-8′), 143.0 (C-4), 148.5 (C-1), 149.3 (C-5′), 169.4 (Me-CO), 193.9 (C-4′), 195.3 (C-1′); EIMS *m/z* 436 [M$^+$] (68%), 394 (100), 361 (60), 361 (60), 297 (52), and 175 (68).

X-ray Crystallographic Study of Acremine G. The compound crystallizes in the triclinic system, $P\bar{1}$ space group, with cell parameters a = 9.040(2) Å, b = 9.384(2) Å, c = 14.263 (4) Å, α = 79.65(1)°, β = 97.980(1)°, γ = 88.36(1)°, V = 1156.1(5) Å^3, Z = 2, D_c = 1.245 g cm^{-3}, $F(000)$ = 262. The crystal suitable for X-ray analysis, with dimensions of 0.6 × 0.4 × 0.05 mm, was obtained upon slow recrystallization from benzene. Intensity data were collected, at room temperature, on a Siemens P4 diffractometer with graphite-monochromated Cu Kα radiation (λ = 1.54179 Å), using the $\theta/2\theta$ scan technique, voltage 40 kV, current 40 mA. Unit cell parameters were determined using 46 reflections in the range $12.6 \le 2\theta \le 46.0°$. A total of 6665 reflections (3575 unique, R_{int} = 0.1246) were collected up to 130° in 2θ and index range $-8 \le h \le 9$, $-10 \le k \le 9$, $-16 \le l \le 16$. Three standard reflections, monitored every 100 reflections, showed no

intensity decay. No empirical adsorption correction was deemed necessary. The structure was solved by direct methods using the SIR97 program,[14] which revealed the position of all non-H atoms. Refinement was carried out on F^2 by the full-matrix least-squares procedure with SHELXL97[15] for 325 parameters, with anisotropic temperature factors for non-H atoms. The final stage converged to $R = 0.0589$ ($R_w = 0.1248$) for 2290 observed reflections (with $I \geq 2\sigma(I)$) and $R = 0.0981$ ($R_w = 0.146$) for all unique reflections. The H atoms bonded to O-2, O-5, C-7, and C-8 were freely and isotropically refined; all other H atoms were placed in geometrically calculated positions and refined in a riding model.

Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 661285. Copies of the data can be obtained, free of charge, on application to the CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44-(0)12233336033 or e-mail: deposit@ccdc.cam.ac.uk).

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