Antifungal Diterpenoid Alkaloids from *Delphinium denudatum*

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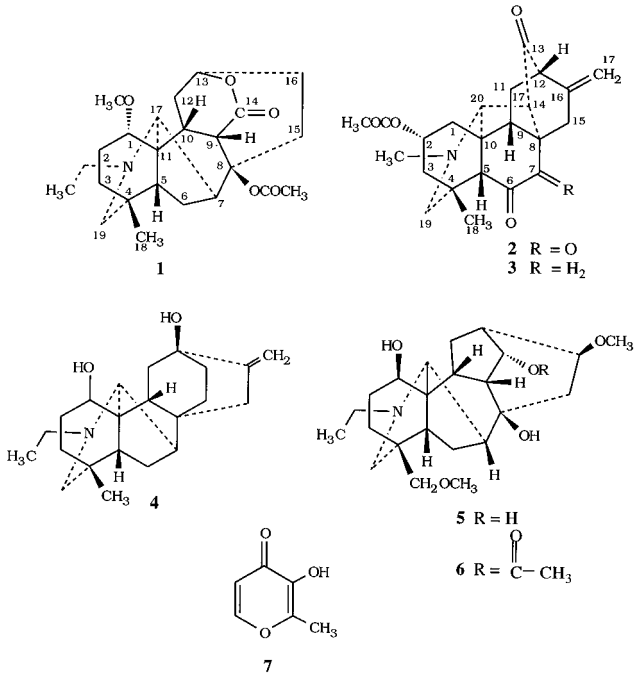
The roots of *Delphinium denudatum* have yielded a new diterpenoid alkaloid, 8-acetylheterophyllisine (**1**), in addition to the known alkaloids vilmorrianone (**2**), panicutine (**3**), denudatine (**4**), isotalatizidine (**5**), and condelphine (**6**), as well as 3-hydroxy-2-methyl-4*H*-pyran-4-one (**7**). Compounds **1**, **3**, and **7** have not been isolated from this plant previously. The structures of compounds **1**, **3**, and **7** were determined through spectral and X-ray diffraction analyses. Compounds **1**−**3** have shown antifungal activity against a number of human pathogenic fungi.

Delphinium species (Ranunculaceae) contain diterpenoid alkaloids that are generally of the veatchine or atisine type.[1] *Delphinium* species have also been used for the treatment of itches and other skin eruptions in folklore. *Delphinium denudatum* is widely distributed in the Western Himalayas and in Kashmir at altitudes of 2400−3650 m, especially on grassy slopes. The roots of this plant are bitter and used as a stimulant, alterative, and tonic.[2] Organic solvent extracts of the plant have been shown to have antimicrobial and immunomodulating properties.[3] The radioprotectant effect of the aqueous extract of *D. denudatum* against radiation-induced changes in rat myocardium has also been investigated.[4] The EtOH extracts of the roots of *D. denudatum* collected from Kashmir (Pakistan) have shown antifungal activity against *Stachybotrys atra*, *Trichophyton longifusus*, *Curvularia lunata*, *Drechslera rostrata*, *Epidermophyton flocosum*, *Microsporum canis*, *Nigrospora oryzae*, and *Ganoderma applanatum*,[5] and antibacterial activity against *Corynebacterium diphtheriae*, *Proteus vulgaris*, *Salmonella typhi*, and *Klebsiella pneumoniae*.[6]

Continuing our work on the important medicinal plants of Pakistan, we wish to report here the isolation of a new diterpenoid alkaloid, 8-acetylheterophyllisine (**1**), from the roots of *D. denudatum*. Other compounds isolated from this plant were vilmorrianone (**2**), panicutine (**3**), denudatine (**4**), isotalatizidine (**5**), condelphine (**6**), and 3-hydroxy-2-methyl-4*H*-pyran-4-one (**7**). Compounds **1** and **2** showed antifungal activity against *Allescheria boydii*, *E. floccosum*, and *Aspergillus niger*.[5] Panicutine (**3**) exhibited antifungal activity against *A. boydii*, *S. atra*, *Pleurotus ostreatus*, *N. oryzae*, *Dutarium rotatum*, and *A. niger*.[5]

The antifungal activity of compounds **1**−**3** was determined by the Agar tube diffusion method.[6] Test tubes charged with sterile Sabouraud dextrose agar were inoculated with test compounds at different concentrations and were kept in a slanting position at room temperature. On solidification the test fungal cultures were inoculated on the slant, and growth inhibitions were observed after an incubation period of 7 days. Nystatin and griseofulvin were used as standard antifungal drugs. MIC values of the antifungal compounds were determined by an agar tube dilution method[5] and are presented in Table 1.



8-Acetylheterophyllisine (**1**), a heteratisine-type diterpenoid base, was isolated by column and thin-layer chromatographies. The HREIMS showed a molecular ion at *m*/*z* 417.2506 corresponding to the molecular formula $C_{24}H_{35}NO_5$, which required eight degrees of unsaturation. Compound **1** exhibited only terminal UV absorption. The IR spectrum of the compound exhibited a strong absorption band at 1720 (ester C=O) cm^{-1}. The ^{1}H-NMR spectrum of **1** indicated the presence of NCH_2-CH_3 (δ 0.99 and 2.89), OCH_3 (δ 3.24), and acetyl methyl (δ 1.95) groups.

Structure **1** was confirmed by an X-ray diffraction method. A suitable crystal that formed in the orthorhombic space group $P2_12_12_1$ was selected for the study. Accurate lattice constants were a = 8.049(6) Å, b = 16.091(8) Å, and c = 16.964(7) Å. One molecule of composition $C_{23}H_{27}NO_5$ formed the asymmetric unit. All unique diffraction maxima with $2\theta \leq 45°$ were collected using 2θ:θ scans and graphite monochromated Mo K_α radiation (0.71069 Å). A total of 1686 unique reflections

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Table 1. Evaluation of Antifungal Activity of Compounds **1**, **2**, and **3** against Pathogenic Fungi (MIC values in μg/mL)[a]

compds tested	*A. boydii*	*A. niger*	*E. floccosum*	*P. ostreatus*
1	100	200	250	150
2	150	100	225	175
3	75	125	200	125

[a] Nystatin and griseofulvin were used as standards. Tests were carried out employing the agar tube dilution method.[5]

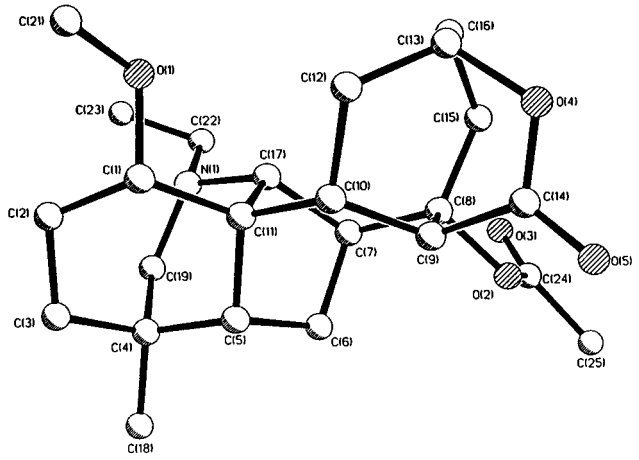

Figure 1. Computer-generated perspective drawing of the final X-ray model of 8-acetylheterophyllisine (**1**).

were collected, 1400 (83%) were judged observed [($|F_o|$) $\geq 6\sigma(|F_o|)$] and used in subsequent calculations. The structure was phased using direct methods and refined using full-matrix least-square techniques with anisotropic heavy atoms and isotropic riding hydrogens to a conventional crystallographic residual of 0.041 (R_W = 0.053) for the observed data.[7,8] A computer-generated drawing of the final X-ray model of 8-acetylheterophyllisine is given in Figure 1. Hydrogens are omitted for clarity, and no absolute configuration is implied.

Panicutine (**3**) (heterophylloidine), a hetidine-type base, was previously isolated from *Aconitum paniculatum* Lam. and *A. heterophylloides*.[9−11] We have now isolated this compound from *D. denudatum* as colorless crystals, and its molecular formula was derived as $C_{23}H_{29}NO_4$ from the HREIMS (M^+, *m*/*z* 383.21977). The structure was unimbiguously established by the single crystal X-ray diffraction technique. Panicutine (**3**) was recrystallized from MeOH−*iso*-octane, and a suitable crystal was selected for further study. The crystal formed in the orthorhombic space group $P2_12_12_1$, was a = 8.492(7) Å, b = 13.60(2) Å, c = 18.21(2) Å, with one molecule ($C_{23}H_{29}NO_4$) in the asymmetric unit. All unique diffraction maxima with $2\theta \leq 105°$ were collected using 2θ:θ scans and graphite monochromated with Cu Kα radiation (1.54178 Å). A total of 1600 unique reflections were collected. Of these 1400 (87.5%) were judged obtained observed [($|F_o|$) $\geq 3\sigma(F_o)$] and used in further calculations. The structure was solved by the direct method (SHELX) and refined by full-matrix least-squares techniques to a final discrepancy index of 0.073 (R_W = 0.096) for observed data.[7,8] A computer-generated perspective drawing of the final X-ray model is given in Figure 2.

3-Hydroxy-2-methyl-4*H*-pyran-4-one (**7**) was also isolated from this plant. This compound was earlier isolated from larch trees (*Larix decidua*), pine needles, chicory, wood tars, and a variety of other natural sources.[12,13] Compound **7** was obtained as large colorless crystals. Cell constants and an orientation matrix

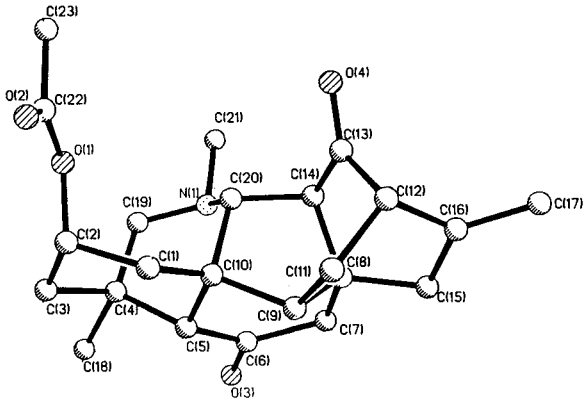

Figure 2. Computer-generated perspective drawing of the final X-ray model of panicutine (**3**).

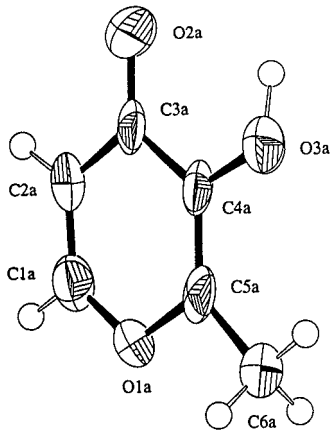

Figure 3. Computer-generated perspective drawing of the final X-ray model of 3-hydroxy-2-methyl-4*H*-pyran-4-one (**7**).

for data collection were obtained from a least-square refinement using the setting angles of 25 carefully centered reflections. This corresponded to a monoclinic $P2_1/a$ crystal with cell constants a = 7.075(2) Å, b = 36.010(11) Å, c = 7.168(2) Å, β = 109.47(2)° and three independent molecules in the asymmetric unit. A total of 3112 unique reflections were collected using Mo Kα radiations, of which 1205 (38.7%) were judged observed [($|F_o|$) $\geq 2\sigma(|F_o|)$] and used in further calculations. The structure was solved by direct methods (SIR92) and refined by the full-matrix least-square technique to a final discrepancy index of 0.076 (R_W = 0.077) for observed data.[7,8] Hydrogen atoms are included but not refined. A computer-generated perspective drawing of the final X-ray model is given in Figure 3.

Vilmorrianone (**2**), an atisine-type diterpenoid alkaloid, was previously isolated from *A. vilmorrianum*.[13] Denudatine (**4**) was earlier isolated from *A. jinyangense*[14] and *D. denudatum*,[15] while isotalatizidine (**5**) was also isolated from various *Aconitum* species[16−19] and *D. denudatum*.[20] Condelphine (**6**), an aconitine base, was reported from *A. delphinifolium*, *A. japonicum*,[21,22] and *D. denudatum*.[20] These four known compounds were isolated in the present investigation from the roots of *D. denudatum*.

Experimental Section

General Experimental Procedures. The purity of the samples was checked on precoated Si gel 254 plates. Solvents were freshly distilled before use. Melting points were determined on a Büchi 535 melting point apparatus. Optical rotations were determined on a

Polaritronic polarimeter. The UV spectra were recorded in CH_3OH on a Shimadzu UV 240 instrument. The IR spectra were recorded on JASCO IRA-1 IR spectrophotometer. The ^{1}H-NMR spectra were recorded in $CDCl_3$ on a Bruker AMX-400 NMR spectrometer at 400 MHz, while the ^{13}C-NMR spectra were recorded on the same instrument at 100 MHz, with TMS as internal standard. MS were measured on a JEOL HX-110 mass spectrometer.

X-ray diffraction studies (compounds **1**, **3**) were conducted on a R3M/V four-circled diffractometer (Siemens) connected with a MicroVax II computer (Digital) using Cu Kα or Mo Kα radiations. The X-ray structure of compound **7** was determined on a Rigaku AFCGS diffractometer with graphite monochromated Mo Kα radiation.

Plant Material. The roots of *D. denudatum* Wall. (2 kg) were collected from Azad Kashmir, Pakistan, in the months of July and August 1992.

Extraction and Isolation. A concentrated EtOH extract of the plant was dissolved in MeOH and filtered over a sintered funnel. The filtrate was again evaporated to a gum (4 g) and subjected to column chromatography using Si gel as stationary phase. The column was eluted with $CHCl_3$−MeOH mixtures of increasing polarity. The fraction obtained on elution with $CHCl_3$−MeOH (95:5) contained compounds **1**−**7**, which were separated through TLC.

8-Acetylheterophyllisine (1): recrystallized from *i*-C_8H_{18}−CH_2Cl_2 as brown crystals (3.4 mg); mp 152 °C; $[\alpha]_D$ 38.24° (MeOH), 0.0523 g; UV (MeOH) 314 (log ϵ 1.93), 338 (log ϵ 1.98) nm; IR ($CHCl_3$) 1714, 1727 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.03 (3H, br s, *N*-CH_2−C*H*$_3$), 1.95 (3H, s, COC*H*$_3$), 0.75 (3H, s, C*H*$_3$), 3.24 (3H, s, OCH_3), 4.69 (1H, br m, $W_{1/2}$ = 14.0 H_2, H-13), 3.25 (1H, d, J = 8 Hz, H-9), 3.35 (1H, δ, H-17); ^{13}C NMR ($CDCl_3$) δ 84.6 (C-1), 26.5 (C-2), 37.2 (C-3), 34.8 (C-4), 49.5 (C-5), 27.1 (C-6), 48.6 (C-7), 87.1 (C-8), 41.4 (C-9), 42.1 (C-10), 29.7 (C-12), 75.2 (C-13), 172.0 (C-14), 28.8 (C-15), 29.5 (C-16), 60.7 (C-17), 26.4 (C-18), 56.5 (C-19), 48.9 (*N*-*C*H_2-CH_3), 13.4 (*N*-CH_2-*C*H_3), 22.3 (OCO*C*H_3), 55.6 (OCH_3), 169.8 (*C*OCH_3); HREIMS *m*/*z* 417.2356 (M^+, $C_{24}H_{35}NO_5$, 6), 358.2374 ($C_{22}H_{32}NO_3$, 65), 326.2113 ($C_{21}H_{28}NO_2$, 15).

Crystal Data for 1: $C_{23}H_{27}NO_5$, MW = 417.453, orthorhombic, $P2_12_12_1$, a = 8.049(6) Å, b = 16.091(8) Å, c = 16.964(7) Å, V = 2197(2) Å^3, Z = 4, Dx = 1.262 mg/cm^3, Mo Kα (λ = 0.710 69 Å), F(000) = 904, T = 293 °K, R = 0.041, and Rw = 0.053, for 1400 unique observed reflections with $I > 2.00\sigma(I)$ (total = 1686), collected on a Rigaku AFC6S four-circuled diffracto meter using a crystal of approximate dimensions of 0.15 × 0.15 × 0.25 mm to a maximum 2θ value of 50.0°. The structure was solved by direct methods are refined by full-matrix least-squares calculations. The nonhydrogen atoms were refined anistropically.

Crystal Data for 3: $C_{18}H_{29}NO_4$, MW = 383.219, orthorhombic, $P2_12_12_1$ was a = 8.492(7) Å, b = 13.60(2) Å, c = 18.21(2) Å, V = 2102(4) Å^3, Z = 4, Dx = 1.211 mg/cm^3, Cu Kα (λ = 1.541 78 Å), F(000) = 824, T = 296 °K, R = 0.073, and RW = 0.096, for a total of 1600 unique reflections we judged od observed [$|F_o| \geq 3\sigma(F_o)$] and used in further calculations. All the data were collected on a computer-controlled R3M/V four-circle diffractometer, using a crystal of approximate dimensions of 0.25 × 0.22 × 0.25 mm to a maximum 2θ value of 50.0°. The structure was solved by direct methods (SHELX) and refined by full-matrix least squares calculations. The nonhydrogen atoms were refined anistropically.

Crystal Data for 7: $C_6H_6O_3$, MW = 126.11, monoclinic $P21/a$ was a = 7.075(2) Å, b = 36.010(11) Å, c = 7.168(2) Å, β = 109.47(2)°, V = 1721.8(a) Å^3, Z = 12, Dx = 1.459 mg/cm^3, Mo Kα (λ = 0.0710, 69 Å), F (000) = 792.00, T = −136.0 °K, R = 0.076, and R_W = 0.077, for 1205 unique observed reflections with $I > 2.00\sigma(I)$ (total 3112), collected on a Rigaku AFC6S diffractometer using a crystal of approximate dimensions of 0.40 × 0.40 × 0.33 mm. The structure was solved by direct methods (SIR 92) refined by full-matrix least-squares techniques.

Additional crystallographic details (atomic coordinates and equivalent isotropic displacement coefficients, lists of structure factors, interatomic distances, angles, anisotropic displacement coefficients for non-hydrogen atoms, hydrogen atom coordinates and their isotropic displacement co-efficients) of **1**, **3**, and **7** have been deposited at the Cambridge Crystallographic Data Centre. The coordinates can be obtained, upon request, from Dr. Olga Kennord, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, UK.

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