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MICROBIAL TRANSFORMATIONS OF 7α-HYDROXYFRULLANOLIDE

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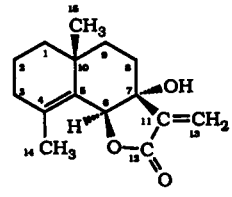
ABSTRACT.—Microbial transformations affected by *Aspergillus* species of 7α-hydroxyfrullanolide [**1**] have yielded 7α-hydroxy-11,13-dihydrofrullanolide [**2**] and 13-acetyl-7α-hydroxyfrullanolide [**3**], whose structures were determined spectroscopically.

Phytochemical investigations on *Sphaeranthus indicus* L. (Compositae) have resulted in the isolation of a number of terpenoidal constituents (1–4). 7α-Hydroxyfrullanolide [**1**], a sesquiterpene lactone, was particularly interesting since it showed pronounced cytotoxicity and antitumor activity against a number of human cancer cell lines. In vivo antitumor activity was not observed.

Microorganisms have been utilized to modify the structures of a number of naturally occurring bioactive compounds. Several of these transformed metabolites have shown interesting biological activities (5). Transformations by using microorganisms are also helpful to identify novel metabolic pathways that may also occur in mammalian metabolic systems.

It was decided to carry out microbial transformation on this abundantly present natural product (2.51×10^{-2}% yield) to convert it into potentially more useful metabolites. Nine fungal strains were screened for microbial transformations. Of these, *Aspergillus niger* and *A. quardilatus* (wild types) were found to be capable of transforming **1** into 7α-hydroxy-11,13-dihydrofrullanolide [**2**] and 13-acetyl-7α-hydroxyfrullanolide [**3**], respectively. The structures of these metabolites were fully elucidated on the basis of spectroscopic studies.

Compound **1** was transformed by *Aspergillus niger* into 7α-hydroxy-11,13-dihydrofrullanolide [**2**], which showed



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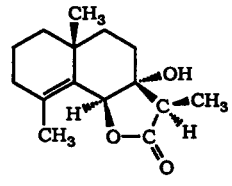

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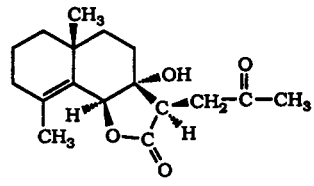

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terminal uv absorption indicating the lack of a chromophoric group. Its ir spectrum displayed intense absorptions at 1670 (C=C), 1757, 1752, 1745 (five-membered lactone), and 3600 (OH) cm^{-1}. The ^{1}H-nmr spectrum ($CDCl_3$, 300 MHz) featured a three-proton singlet at δ 1.03 due to the C-15 methyl group. A three-proton doublet centered at δ 1.28 ($J_{13,11}$=7.7 Hz) was assigned to the C-13 methyl protons, while another three-proton singlet at δ 1.74 was due to the allylic C-14 methyl protons. The C-11 methine

proton resonated as a quartet at δ 2.44 ($J_{11,13}$=7.7 Hz). A downfield one-proton singlet centered at δ 5.01 was assigned to H-6, geminal to the lactone moiety.

Two-dimensional nmr experiments (COSY-45° and HMQC) were very informative (6,7). The COSY-45° spectrum of **2** showed cross-peaks between the C-13 methyl protons (δ 1.28) and the C-11 methine proton (δ 2.44). The C-1 methylene protons (δ 1.41 and 1.85) showed vicinal couplings with the C-2 methylene protons (δ 1.70 and 1.60), which in turn showed vicinal couplings with the C-3 methylene protons (δ 2.15 and 2.20). The COSY-45° cross-peak between the C-8 (δ 1.63 and 1.90) and the C-9 methylene protons (δ 1.55 and 1.51) were also observed in the spectrum.

The ^{13}C-nmr spectrum of **2** exhibited resonances for all fifteen carbons. The multiplicity of each carbon was established with the help of DEPT experiments with the last polarization pulse angles θ=45°, 90°, and 135° (6–8). It established the presence of three CH_3, five CH_2, and two CH carbons (Table 1). The ^{13}C-nmr spectrum featured singlets at δ 10.6, 25.4, and 19.4 due to the C-13, C-14, and C-15 methyl carbons, respectively. The olefinic C-4 and C-5 signals resonated at δ 140.5 and 126.6, respectively. The complete ^{13}C-nmr chemical shift assignments for the various carbons are presented in Table 1. The two-dimensional HMQC (9) experiment helped to confirm the ^{1}H- and ^{13}C-nmr chemical shift assignments. The H-1 signals (δ 1.41 and 1.85) showed direct connectivity with C-1 (δ 39.6). Similarly, H-11 (δ 2.44) showed a one-bond correlation with C-11 (δ 47.6). The ^{1}H/^{13}C one-bond correlations are presented in Table 1. The β-stereochemistry of the C-13 methyl protons (δ 1.28) was deduced on the basis of nOe difference measurements. Irradiation of H-6α which resonated as a singlet at δ 5.01 resulted in a 19% enhancement of the C-11 methine proton which appeared as a singlet at δ 2.44.

TABLE 1. ^{13}C-Nmr Chemical Shifts and ^{1}H/^{13}C Single-Bond Correlations[a] in Compound **2**.

Carbon	^{13}C-nmr Chemical shift (δ)	Multiplicity[b] (DEPT)	^{1}H-nmr Chemical shift (δ)	Multiplicity and ^{1}H-^{1}H Coupling constant (Hz)
C-1	39.6	CH_2	1.41,	m
			1.85	m
C-2	18.2	CH_2	1.60,	m
			1.70	m
C-3	33.5	CH_2	2.15,	m
			2.20	m
C-4	140.5	-C-	—	—
C-5	126.6	-C-	—	—
C-6	80.3	CH	5.01	br s
C-7	79.1	-C-	—	—
C-8	31.5	CH_2	1.63,	m
			1.90	m
C-9	35.4	CH_2	1.51,	m
			1.55	m
C-10	33.2	-C-	—	—
C-11	47.6	CH	2.44	q, $J_{11,13}$=7.7 Hz
C-12	178.4	-C-	—	—
C-13	10.6	CH_3	1.28	d, $J_{11,13}$=7.7 Hz
C-14	25.4	CH_3	1.74	s
C-15	19.4	CH_3	1.03	s

[a]Determined by HMQC spectrum.
[b]Determined by DEPT experiment.

The hreims of **2** showed a molecular ion peak at *m/z* 250.1519 (calcd 250.1518) corresponding to the molecular formula $C_{15}H_{22}O_3$, indicating the presence of five degrees of unsaturation in the molecule. The peak at *m/z* 235.1349 ($C_{14}H_{19}O_3$) was due to the loss of a methyl group from the molecular ion. Other prominent ions were at *m/z* 217.1242 ($C_{14}H_{17}O_2$) and 189.1284 ($C_{13}H_{17}O$) due to the loss of H_2O and CO_2 from the $[M-CH_3]^+$ ion. On the basis of these spectroscopic studies, structure **2** was assigned to this transformed sesquiterpene lactone.

The second product, 13-acetyl-7α-hydroxyfrullanolide [**3**], $C_{17}H_{24}O_4$, transformed by *A. quardilatus*, also showed terminal uv absorption indicating the lack of conjugation in the molecule. Its ir spectrum displayed intense absorptions at 3610 (OH), 1757 (five-membered lactone), 1715 (ester carbonyl), and 1600 (C=C) cm^{-1}.

The ^{1}H-nmr spectrum ($CDCl_3$, 300 MHz) of **3** featured a three-proton singlet at δ 1.02 due to the C-15 methyl protons. Another three-proton singlet at δ 1.79 was assigned to the allylic C-14 methyl group. The acetyl methyl protons resonated as a sharp singlet at δ 2.29. Two AB double doublets resonating at δ 2.77 ($J_{13a,13b}$=19.5 Hz, $J_{13a,11}$=11.6 Hz) and 3.38 ($J_{13b,13a}$=19.5 Hz, $J_{13b,11}$=2.7 Hz) were assigned to the acetyl-bearing C-13 methylene protons. The C-11 methine proton appeared as a doublet of double doublets at δ 3.13 ($J_{11,13a}$=11.6 Hz, $J_{11,13b}$=2.7 Hz) exhibiting vicinal couplings with the C-13 methylene. The α-stereochemistry of the C-11 proton was assigned on the basis of nOe difference measurements. Irradiation of H-11α resulted in a 29% enhancement of H-6α which appeared at δ 5.02, thereby confirming their close proximity in space.

The hreims of **3** showed a molecular ion peak at *m/z* 292.1669, which is in agreement with the elemental formula $C_{17}H_{24}O_4$ (calcd 292.1674), indicating the presence of six degrees of unsaturation in the molecule. The ion at *m/z* 277.1426 ($C_{16}H_{21}O_4$) was due to the loss of a methyl group from the molecular ion. The ion at *m/z* 259.1332 ($C_{16}H_{19}O_3$) was due to the loss of a water molecule, while the ion at *m/z* 231.1356 ($C_{15}H_{19}O_2$) arose by the elimination of carbon monoxide and indicated the presence of a carbonyl moiety in the molecule. The other major ions at *m/z* 177.1256 ($C_{12}H_{17}O$) and 161.0988 ($C_{11}H_{13}O$) were also observed in the mass spectrum. Compound **3** showed a base peak at *m/z* 55.0494 (C_4H_7) resulting from the retro-Diels-Alder cleavage of ring A and suggested the presence of a double bond in the molecule. Another ion at *m/z* 249.1437 was due to loss of an acyl group from the molecular ion. These spectroscopic studies led to structure **3** for this transformed product.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H-nmr spectra were recorded on a Bruker AM-300 instrument at 300 MHz, while the ^{13}C-nmr spectra were recorded on the same instrument at 75.4 MHz. The ms were recorded on a Varian MAT 112S mass spectrometer connected to a DEC PDP 11/34 computer system. Hreims were recorded on a JEOL-JMS HX 110 mass spectrometer. Tlc experiments were performed on Si gel (GF_{254}) precoated plates (E. Merck 0.25 mm). The ingredients of each fermentation medium were made from components purchased from E. Merck and Difco.

FUNGAL MATERIAL.—Microorganisms were either purchased from the American Type Culture Collection (ATCC) or obtained (wild type) from the Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Five genera (7 species), *Aspergillus niger* (wild type), *A. quardilatus* (wild type), *A. alliaceus* (ATCC 1024), *Tricothecium roseum* (CMI 50600), *Gibberella fujikori* (ATCC 10704), *Cephalosporium aphidicola* (IMI 68689), and *Penicillium gladuii* (wild type) were used.

CULTURES AND FERMENTATION PROCEDURES.—The fungi used in this study were maintained on Sabouraud dextrose agar slants and stored at 4°. Microorganisms were grown in separate media. For *Aspergillus* species (*A. niger* and *A. quardilatus*), a selective medium consisting of tartaric acid (35%), sucrose (5%), KH_2PO_4 (2%), $MgSO_4$, H_2O (1%), NH_4NO_3 (2%), and $Zn(OAc)_2$ (0.03%) was used, and 0.04 N NaOH was used to

adjust to pH 6. For *Gibberella* species a selective medium, consisting of glucose (8%), NH_4NO_3 (0.48%), KH_2PO_4 (5.1%), $MgSO_4$. $7H_2O$ (1%), with trace elements (4%) was used. For *Tricothecium, Cephalosporium,* and *Penicillium* species a selective medium consisting of $NaNO_3$ (0.1%), KCl (0.025%), $MgSO_4$ (0.002%), $FeSO_4$ (0.005%), $CaSO_4$ (10.17%), and sucrose (15%) was employed, with 0.04 N NaOH being used to adjust pH 6.0.

Biotransformation experiments were performed in shake culture flasks by a two-stage fermentation procedure. For the preparation of stage I liquid culture, spore suspension from each slant was added to the conical flask (250 ml) containing 100 ml of liquid medium. Each flask was then incubated and placed on the shaker at 29° for 24 h at 200 rpm. Stage II cultures were prepared by transferring 10 ml of each stage I liquid culture into ten conical flasks (each containing 100 ml of medium).

The substrate (200 mg of 7α-hydroxyfrullanolide [**1**] was then transferred to five flasks as an EtOH solution. Culture controls consisted of fermentation blanks in which the organisms were grown under identical conditions but without the substrate. The substrate control contained the substrate in sterile media. Controls were incubated under the same conditions. Transformations were continued for 2–10 days (2 days for *A. niger* and 4 days for *A. quardilatus*) depending upon the period required for maximum formation of metabolites. The mycelium was filtered through a sintered funnel under vacuum. The filtrate was extracted with EtOAc and the EtOAc extract was dried over anhydrous Na_2SO_4. Fermentation flasks containing *A. niger* and *A. quardilatus* showed the transformation of **1**, while the untransformed substrate was recovered from other cultures. The transformed products **2** and **3** were separated by prep. tlc on Si gel precoated plates by using petroleum ether (40–60°)-$CHCl_3$ (4:6) as the solvent system.

7α-Hydroxyfrullanolide [**1**].—$[\alpha]^{20}D$ −39°, (*c*=0.4, MeOH); uv (MeOH) λ max 206 nm (log ε 4.23); ir ($CHCl_3$) ν max 3600 (OH), 1757 (C=O, γ-lactone system), 1674 (C=C), 1652 (=CH_2, exocyclic system) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 0.94 (3H, s, CH_3-15), 1.64 (3H, s, CH_3-14), 4.97 (1H, s, H-6), 5.71 (1H, s, C=CH), 6.06 (1H, s, C=CH); ^{13}C nmr ($CDCl_3$, 75.4 MHz) δ 38.2 (CH_2, C-1), 17.7 (CH_2, C-2), 32.6 (CH_2, C-3), 139.6 (-C-, C-4), 126.4 (-C-, C-5), 81.0 (CH, C-6), 75.2 (-C-, C-7), 30.8 (CH_2, C-8), 34.3 (CH_2, C-9), 32.1 (-C-, C-10), 144.4 (-C, C-11), 169.5 (-C-, C-12), 120.5 (CH_2, C-13), 25.6 (CH_3, C-14), 18.8 (CH_3, C-15); hreims *m/z* 248.1408 ($C_{15}H_{20}O_3$, calcd 248.1409, 22), 233.1171 ($C_{14}H_{17}O_3$, calcd 233.1177, 100), 215.1061 ($C_{14}H_{15}O_2$, calcd 215.1071, 38), 187.1120 ($C_{13}H_{15}O$, calcd 187.1122, 27), 178.1351 ($C_{12}H_{18}O$, calcd 178.1357, 16), 169.1014 ($C_{13}H_{13}$, calcd 169.1017, 37).

7α-Hydroxy-11,13-dihydrofrullanolide [**2**].—12 mg, $[\alpha]^{20}D$ −20° (*c*=0.5, $CHCl_3$); uv (MeOH) λ max 206 nm (log ε 4.23); ir ($CHCl_3$) ν max 3600 (OH), 1757, 1752, 1745 (5-membered lactone), 1670 (C=C) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz), see Table 1; ^{13}C nmr ($CDCl_3$, 75 MHz) see Table 1; hreims *m/z* 250.1519 (M^+, $C_{15}H_{22}O_3$, calcd 250.1518, 28), 235.1349 ($C_{14}H_{19}O_3$, calcd 235.1334, 100), 217.1242 ($C_{14}H_{17}O_2$, calcd 217.1230, 39), 189.1284 ($C_{13}H_{17}O$, calcd 189.2238, 10).

13-Acetyl-7α-hydroxyfrullanolide [**3**].—White gummy material (10 mg), $[\alpha]^{20}D$ −24° (*c*=0.5, $CHCl_3$); uv (MeOH) λ max 204 nm (log ε 4.23); ir ($CHCl_3$) ν max 3610 (OH), 1757 (5-membered lactone), 1715 (ester carbonyl), 1600 (C=C) cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 1.02 (3H, s, CH_3), 1.76 (3H, s, CH_3), 2.29 (3H, s, O=C-CH_3), 2.77 (1H, dd, $J_{13a,13b}$=19.5 Hz, $J_{13a,11}$=11.6 Hz, H-13a), 3.13 (1H, dd, $J_{11,13a}$=11.6 Hz, $J_{11,13b}$=2.7 Hz, H-11), 3.38 (1H, dd, $J_{13b,13a}$=19.5 Hz, $J_{13b,11}$=2.7 Hz, H-13b), 5.02 (1H, s, H-6α); Hreims *m/z* 292.1669 (M^+, $C_{17}H_{24}O_4$, calcd 292.1674, 19), 277.1426 ($C_{16}H_{21}O_4$, calcd 277.1439, 50), 259.1332 ($C_{16}H_{19}O_3$, calcd 259.1334, 38), 249.1437 ($C_{15}H_{21}O_3$, calcd 249.1490, 20), 231.1356 ($C_{15}H_{19}O_2$, calcd 231.1384, 30), 177.1256 ($C_{12}H_{17}O$, calcd 177.1279, 60), 161.0988 ($C_{11}H_{13}O$, calcd 161.0966, 45), 55.0494 (C_4H_7, calcd 55.0548, 100).

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