Microbial Transformations of Hypolipemic *E*-Guggulsterone

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Biotransformation of *E*-guggulsterone (pregna-4,17(20)-*cis*-diene-3,16-dione) (**1**) by *Aspergillus niger* resulted in the formation of four new hydroxyl derivatives identified as 7β-hydroxypregna-4,17(20)-*trans*-diene-3,16-dione (**2**), 7β-hydroxypregna-4,17(20)-*cis*-diene-3,16-dione (**3**), 7β-hydroxypregn-4-ene-3,16-dione (**4**), and 7β,15β-dihydroxypregn-4-ene-3,16-dione (**5**). The biotransformation of **1** with *Cephalosporium aphidicola* also resulted in the formation of four new steroidal derivatives as 11α-hydroxypregna-4,17(20)-*trans*-diene-3,16-dione (**6**), 11α-hydroxypregna-4,17(20)-*cis*-diene-3,16-dione (**7**), 11α,15β-dihydroxypregna-4,17(20)-*trans*-diene-3,16-dione (**8**), and 11α,15β-dihydroxypregna-4,17(20)-*cis*-diene-3,16-dione (**9**). The structures of these compounds were elucidated on the basis of 1D and 2D NMR spectroscopic techniques.

The pregnane-type steroid, *E*-guggulsterone (**1**) has been isolated from the gum resins of *Ailanthus grandis*[1] and *Commiphora mukul*.[2] The gum resin of *C. mukul* has been used in the treatment of epilepsy, ulcers, helminthus, rheumatoid arthritis, and hyperlipemia in the ancient Indian system of medicine.[3] The gum extract of *C. mukul*, called "gugulipid" has been found to be a safe and effective lipid-lowering agent comparable in efficacy to colifibrate.[4] Further pharmacological studies on the pure constituents of gum resin have revealed that compound **1** has pronounced hypolipemic and hypocholestremic activity.[5] It also exhibited cytotoxic activity against the human lung cancer cell line (NCI−H-226).

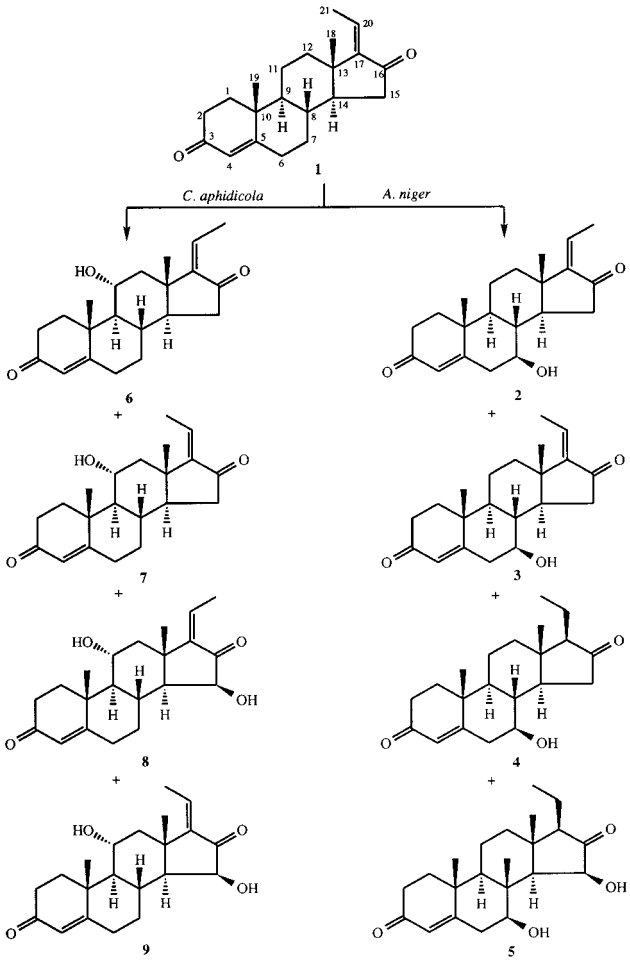
Microbial transformation of compound **1** with various fungi was undertaken in an effort to obtain new metabolites of **1**. This has resulted in the production of metabolites **2**−**5** by *Aspergillus niger* and metabolites **6**−**9** by *Cephalosporium aphidicola*. The metabolite **2** exhibited antibacterial activity against *Shigella boydii*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Corynebacterium diphtheriae*, although, interestingly, compound **1** lacks this activity.

Results and Discussion

The incubation of compound **1** with *A. niger* for 8 days produced a crude organic extract that was subjected to TLC to obtain more polar metabolites **2**−**5**, while fermentation of **1** with *C. aphidicola* for 3 days resulted in the formation of four new metabolites **6**−**9** (Scheme 1). Metabolites **2** and **3** were inseparable due to paucity of sample and exhibited the molecular ion at *m*/*z* 328 ($C_{21}H_{28}O_3$). The ^{1}H NMR spectrum of a mixture of **2** and **3** revealed that it was a *Z*- and *E*-isomeric mixture of the hydroxyl derivative of substrate **1**. The assignment of the stereochemistry of the hydroxyl group at δ 3.62 was based on the comparison of spectral data of metabolites **4** and **5**.

Compound **4** ($C_{21}H_{30}O_3$) exhibited the molecular ion at *m*/*z* 330.2199 determined by HREIMS. The IR spectrum exhibited the absorption band at 3400 cm^{-1}, indicating the presence of a free hydroxyl group. The ^{1}H NMR spectrum of **4** was remarkably different from

Scheme 1. Microbial Transformations of *E*-Guggulsterone (**1**) by *A. niger* and *C. aphidicola*



1 in several aspects. First, the signal at δ 6.45, corresponding to H-20 of exocyclic double bond in **1**, was not observed in **4**. The upfield 3H signal at δ 1.01, resonating as a triplet, was assigned to the C-21 methyl in **4** (which appeared as a doublet at δ 1.86 in **1**). In addition, the downfield signal at δ 3.40 (1H, ddd, J_1 =

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Table 1: ^{1}H NMR Data of Compounds **4**−**8** (δ in ppm)

proton	**4**	**5**	**6**	**7**	**8**
H-1	1.70 m H_a	1.65 m H_a	2.13 m H_a	2.06 m H_a	2.05 m H_a
	2.37 m H_b	2.08 m H_b	2.70 m H_b	2.20 m H_b	2.00 m H_b
H-2	2.21 m H_a	2.45 m H_a	2.22 m H_a	2.33 m H_a	2.55 m H_a
	2.40 m H_b	2.40 m H_b	2.40 m H_b	2.45 m H_b	2.60 m H_b
H-3					
H-4	5.74 br d (2.0)	5.70 br d (1.5)	5.71 br s	5.75 br s	5.75 br s
H-5					
H-6	2.49 m H_a	2.50 m H_a	2.30 m H_a	2.43 m H_a	2.50 m H_a
	2.51 m H_b	2.60 m H_b	2.49 m H_b	2.32 m H_b	2.32 m H_b
H-7	3.40 ddd (14.5, 9.5, 5.0)	3.60 m	1.18 m H_a	1.17 m H_a	1.24 m H_a
			1.85 m H_b	1.84 m H_b	2.15 m H_b
H-8	1.79 m	2.10 m	1.90 m	1.72 m	1.38 m
H-9	1.19 m	1.20 m	1.28 m	1.22 m	1.22 m
H-10					
H-11	1.60 m H_a	1.55 m H_a	4.08 ddd (15.5, 10.5, 5.0)	4.14 ddd (15.0, 10.5, 5.5)	4.14 ddd (15.5, 10.3, 5.0)
H-12	1.41 m H_a	1.30 m H_a	1.45 m H_a	1.68 m H_a	1.65 m H_a
	1.99 m H_b	1.90 m H_b	2.25 m H_b	2.65 m H_b	2.58 m H_b
H-13					
H-14	1.64 m	1.55 m	1.55 m	1.65 m	1.42 dd (6.0, 9.0)
H-15	1.80 m H_a	4.20 d (7.0)	2.10 m H_a	2.05 m H_a	3.90 dd (6.5)
	2.12 m H_b		2.15 m H_b	2.20 m H_b	
H-16					
H-17	1.65 m	1.85 m			
H-18	0.75 s	0.89 s	0.98 s	1.07 s	1.19 s
H-19	1.27 s	1.25 s	1.38 s	1.35 s	1.37 s
H-20	1.22 m H_a	1.35 m H_a	5.74 q (7.5)	6.52 q (7.5)	5.87 q (7.5)
	1.29 m H_b	1.65 m H_b			
H-21	1.01 t (7.5)	1.04 t (7.0)	2.05 d (7.5)	1.85 d (7.5)	2.09 d (7.5)

14.0, J_2 = 9.5, and J_3 = 5.0 Hz) was assigned to the methine H-7 geminal to the OH group. This assignment was supported by COSY-45° and HOHAHA experiments. HOHAHA spectra (60 and 100 ms) indicated a large spin system extending from H-4 and passing through the H-7 (which bears the hydroxyl group) to H-15 on the one side and to H-12 on the other side. Large coupling constants and peak pattern of H-7 indicated the β-stereochemistry (equatorial) of the C-7 hydroxyl group.[6] The position of the hydroxyl group at C-7 was also confirmed by ^{13}C NMR (DEPT) and HMBC experiments. The downfield signal at δ 75.0 was assigned to C-7 in **4** after comparison with that of **1** based on broad-band decoupled ^{13}C NMR experiments. The H-6 methylene protons (δ 2.49 and 2.51) and H-8 methine proton resonating at δ 1.79 exhibited heteronuclear interaction with C-7 (δ 75.0). Furthermore, the methyl protons resonated at δ 1.01 (C-21) and 0.75 (C-18), indicating the ^3J heteronuclear interactions with C-17 at δ 64.5. In addition, the C-20-methylene protons at δ 1.29 and 1.22 showed ^2J interactions with C-21 (δ 13.8) as well as with the carbonyl carbon resonating at δ 218.0 (C-16), hence exhibiting close proximity to the carbonyl carbon.

The HREIMS of compound **5** exhibited the M^+ at *m/z* 346.2158 corresponding to the molecular formula $C_{21}H_{30}O_4$. An IR absorption band was observed at 3510 cm^{-1} (OH). The ^{1}H NMR spectrum of **5** closely resembled that of compound **4** with an additional signal at δ 4.20, which could be assigned to H-15 on the basis of COSY and HOHAHA interactions. The configuration of the hydroxyl at C-15 was concluded to be β, on the basis of an observed ^{1}H NMR coupling pattern of H-15 (J = 7.0 Hz). The signal at δ 3.60 was due to H-7 geminal to OH group. Structure **5** was further investigated by the ^{13}C NMR, HMQC, and HMBC experiments. H-15 (δ 4.20) showed ^2J heteronuclear couplings with the C-16 carbonyl carbon resonating at δ 217.6 and a ^3J interaction with C-13 (δ 42.3). The H-20 methylene protons also showed heteronuclear correlations with the close lying C-16 (δ 217.6).

The HREIMS of compound **6** exhibited the molecular ion at *m/z* 328.2009, which was 16 mass units higher than that of the substrate. This suggested that compound **6** was an oxidized derivative of **1**. The IR absorption (3600 cm^{-1}) showed the presence of an OH group. The ^{1}H NMR spectrum of **6** was distinctly similar to that of substrate **1** but with the proton geminal to a new hydroxyl group that appeared as a doublet of double doublets at δ 4.08 (J_1 = 15.0, J_2 = 10.5, J_3 = 5.0 Hz). The magnitude of coupling constants indicated that the hydroxyl group must be equatorial and situated at C-7 or C-11. The C-11 position for the new hydroxyl group in an α-configuration was supported by coupling constant δ and COSY interactions. In the ^{1}H NMR spectrum the olefinic C-20 proton, which appears at δ 6.45 in **1**, had an upfield shift at δ 5.74, thus revealing that metabolite **6** is an 11-hydroxy *Z*-isomer of **1**. The ^{1}H NMR assignment was further supported by the ^{13}C NMR data (Table 1), which was assigned by comparing with the substrate and HMBC interactions.

The HREIMS of compound **7** showed the molecular ion at *m/z* 328.2033, which was again 16 amu greater than the substrate, thus suggesting that compound **7** was also a hydroxylated derivative of substrate **1**. The ^{1}H and ^{13}C NMR spectra were very similar to those of metabolite **6**, with the exception of a C-20 olefinic proton at δ 6.52 (J = 7.5 Hz) and a methyl group at δ 1.85 suggesting cis (*E*) configuration of metabolite **7** opposite to that of compound **6** and similar to that of the starting material.

The HREIMS of compound **8** showed the molecular ion at *m/z* 344.1952, which was 32 amu greater than that of substrate **1**, thus revealing that compound **8** is an oxidized derivative of compound **1**. In the ^{1}H NMR

Table 2: ^{13}C NMR Data of Compounds **4**−**8** (δ in ppm)

carbon	4	5	6	7	8
C-1	36.3	35.5	38.3	37.7	37.5
C-2	34.5	33.9	34.8	34.1	34.1
C-3	198.9	198.0	199.0	199.8	199.8
C-4	125.0	125.2	124.8	124.9	124.7
C-5	168.0	166.0	171.1	169.5	170.3
C-6	43.3	42.5	33.9	33.3	33.4
C-7	75.0	74.8	32.5	31.1	30.8
C-8	42.8	37.9	35.3	34.0	52.9
C-9	51.7	50.9	60.0	59.3	59.7
C-10	38.9	38.1	40.9	38.6	40.3
C-11	21.2	20.3	68.6	68.5	68.4
C-12	38.5	37.9	47.6	47.8	48.8
C-13	43.3	42.3	44.08	45.0	42.9
C-14	49.9	55.0	49.4	49.0	54.3
C-15	42.9	72.0	39.7	38.6	71.9
C-16	218.0	217.6	207.5	207.0	208.0
C-17	64.5	64.0	148.8	146.7	146.5
C-18	13.6	15.9	20.9	18.7	21.9
C-19	17.6	17.2	18.8	18.3	18.8
C-20	18.1	16.9	129.8	129.8	132.8
C-21	13.8	13.9	13.9	13.2	14.4

spectrum of **8**, two signals at δ 4.14 (1H, ddd, $J_1 = 15.5$ Hz, $J_2 = 10.3$ Hz, and $J_3 = 5.0$ Hz) and 3.90 (1H, d, $J =$ 6.0 Hz) indicated the presence of two new hydroxyl groups at positions C-11 and C-15, respectively, on the basis of COSY and HOHAHA interactions. The α- and β-configurations of the C-11 and C-15 hydroxyl groups in **8** were inferred from the observed coupling constants. The appearance of a vinyl proton resonating as a quartet at δ 5.87 ($J = 7.5$ Hz) and a doublet of three protons (C-21 methyl) at δ 2.09 ($J = 7.5$ Hz) in the ^1H NMR spectrum confirmed the trans (*Z*) configuration of the C-17 (20) double bond. This assignment was further confirmed by the ^{13}C NMR experiment and HMBC interactions.

Compound **9** is the *E*-isomer of **8**. The ^1H NMR spectrum was virtually identical to that of compound **8**, with differences in the chemical shift of the C-20 proton, which resonated downfield as a quartet at δ 6.61 ($J = 7.5$ Hz), and the C-21 methyl protons at δ 1.90.

The time-course studies of the transformations revealed that the *E*-isomer of **1** was converted to the *Z*-isomer. Under experimental conditions, *E*-isomer is converted to an enolate structure[7] in which free rotation around the 17/20 bond produces a mixture of *E*- and *Z*-isomers. In the time-dependent studies of these transformations, one flask was harvested daily, and the resulting metabolites were examined. In the transformation experiment with *A. niger*, the formation of polar metabolites **2** and **3** was detected on TLC after 12 h. After 24 h, TLC and spectral studies indicated the regiospecific formation of the less polar metabolite **4** from a mixture of **2** and **3**. Metabolite **4** then underwent hydroxylation at C-15 to yield metabolite **5**, which was detected on TLC after five days as the most polar compound.

The fungus *C. aphidicola* efficiently hydroxylated compound **1**, producing metabolites **6** and **7** after 5 h of incubation. After 72 h, the dihydroxylated guggulsterones **8** and **9** were major metabolites in the culture medium as detected by TLC.

Experimental Section

General Experimental Procedures. Optical rotations were determined in MeOH on a polarotronic D polarimeter. UV spectra were obtained in MeOH on a Hitachi U-3200 spectrophotometer. IR spectra were recorded in $CHCl_3$ on a JASCO IR A-302 spectrophotometer. The ^1H NMR spectra (δ ppm, J in Hz) were recorded in deuterated solvents (Me_2CO and $CHCl_3$) on Bruker AM-300 and 500 MHz spectrometers, while ^{13}C NMR spectra were recorded in the same solvents on a Bruker AM-500 MHz instrument at 125 MHz and on an AM-300 MHz instrument at 75 MHz. MS were recorded on Varian MAT-312 and JEOL HX-110 double-focusing mass spectrometers. The purity of the samples was checked on TLC (Si gel, precoated plates, Merck, PF_{254}, 20 × 20, 0.25 mm).

Isolation of Compound 1 from the Gum Resin of *Commiphora mukul*. The gum resin (5 kg) was purchased from the Karachi herbal market and dissolved in EtOAc. The extract was withdrawn, and the residue was repeatedly extracted with EtOAc. The EtOAc extracts were combined, and the solvent was removed *in vacuo* to furnish a dark brown extract. A part of the extract (400 g) was chromatographed on Si gel to effect preliminary separation. Elution was with increasing amounts of EtOAc in petroleum ether. The fractions eluted with 20% EtOAc in petroleum ether were combined, evaporated, and dissolved in EtOAc to furnish a crystalline solid (3.5 g): mp 230−234 $[\alpha]^{25}_D$ −66° (*c* 0.03; MeOH); ^1H NMR δ ($CDCl_3$, 500 MHz), 6.45 (1H, q, $J = 7.5$ Hz, H-20), 5.71 (1H, br s, H-4), 1.86 (3H, d, $J = 7.5$ Hz, H-21); EIMS *m/z* (rel int %) 312 M^+ (55), 298 (22), 297 (100), 270 (7), 255 (6), 227 (3), 189(10).

Fermentation Procedure. The culture of *A. niger* (ATCC no. 10549) was grown in a medium prepared with tartaric acid (35%), sucrose (5%), KH_2PO_4 (2%), $MgSO_4 \cdot 2H_2O$ (1%), NH_4NO_3 (2%), $Zn(OAc)_2$ (0.03%), and distilled H_2O (5 L). To adjust the pH to 6, 0.04 NaOH was used. The medium for *C. aphidicola* (IM, 68689) was prepared by mixing the following ingredients in distilled H_2O (5 L): glucose (250.0 g), KH_2PO_4 (25.0 g), $MgSO_4$ (10.0 g), KCl (5.0 g), glycine (10.0 g), *Gibberella* trace element solution (10.0 mL).

Time-Course Experiment. In each transformation experiment the fungus was grown as a shake culture at 29° C for 3 days in 8 conical flasks (250 mL), each containing sterile medium (100 mL). Compound **1** (100 mg) in Me_2CO (16 mL) was distributed equally among the flasks, and the organism was cultivated for 8 more days. Every day, one flask of the culture medium was harvested, filtered, saturated with NaCl, and extracted with EtOAc, and then the organic solvent was evaporated under vacuum. The crude concentrated extract was monitored by TLC.

Large-Scale Transformation. The following procedure was used for each fungus. The fungus was grown as a shake culture (100 mL of medium) in 50 conical flasks (250 mL) and incubated for 3 days at 29 °C. The substrate **1** (460 mg) was dissolved in Me_2CO (20 mL), and the solution was evenly distributed among 50 conical flasks. The incubation was allowed to continue for further 8 days. Then the broth was filtered and extracted with EtOAc. The mycelium was also washed with EtOAc, and the two extracts were combined and dried over anhydrous sodium sulfate and concentrated to a brown gum. The EtOAc extract

obtained from each fungus was purified by TLC using 60% EtOAc−petroleum ether as developing solvents.

Compounds 2 and 3: oil, 4 mg; UV (MeOH) λ_{max} (log ϵ), 279 (3.57); IR ($CHCl_3$) υ_{max} 3350, 1750 cm^{-1}; ^1H NMR δ ($CDCl_3$, 500 MHz), 5.61/6.52 (1H, q, J = 7.5 Hz, H-20), 5.72 (1H, br s, H-4), 3.62 (1H, m, H-7), 2.08/1.8 (3H, d, J = 7.5 Hz, H-21); EIMS *m/z* (rel int) 328 M^+ (92), 313 (72), 310 (100), 295 (24), 228 (60), 175 (72), 136 (77).

Compound 4: oil, 49 mg; $[\alpha]^{25}_D$ −50° (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (4); IR ($CHCl_3$) υ_{max} 3400, 2849, 1651 cm^{-1}; ^1H NMR δ (Me_2CO-d_6, 300 MHz), see Table 1; ^{13}C NMR δ (Me_2CO-d_6, 75 MHz), see Table 2; EIMS *m/z* (rel int) 330 M^+ (100), 312 (5), 315 (21), 231 (5), 177 (13), 124 (44), 55 (45).

Compound 5: oil, 35 mg; $[\alpha]^{25}_D$ −120° (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 237 (4); IR ($CHCl_3$) υ_{max} 3510, 1634 cm^{-1}; ^1H NMR δ ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR δ ($CDCl_3$, 125 MHz), see Table 2; EIMS *m/z* (rel int) 346 M^+ (22), 330 (12), 328 (9), 313 (7), 257 (6), 195 (6) 83 (100).

Compound 6: oil, 34 mg; $[\alpha]^{25}_D$ −9° (*c* 0.04, MeOH) λ_{max} (log ϵ) 240 (3.8), 195.2 (3.4); IR ($CHCl_3$) υ_{max} 3600, 2947, 1718, and 1659 cm^{-1}; ^1H NMR (Me_2CO-d_6, 500 MHz), see Table 1; ^{13}C NMR δ ($CDCl_3$ 125 MHz), see Table 2; EIMS *m/z* (rel int) 328 M^+ (90), 313 (100), 312 (11), 310 (5), 300 (3), 124 (98).

Compound 7: oil, 6.4 mg; $[\alpha]^{25}_D$ −27° (*c* 0.026, MeOH); λ_{max} (log ϵ) 240 (3.8), 199 (3.6); IR ($CHCl_3$) υ_{max} 3600, 2950, 1715, 1655 cm^{-1}; ^1H NMR δ ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR δ ($CDCl_3$, 125 MHz) see Table 2; EIMS *m/z* (rel int) 328 M^+ (33), 313 (11), 312 (4), 310 (25), 300 (12), 124 (100).

Compound 8: oil, 23 mg; $[\alpha]^{25}_D$ −28° (*c* 0.052, MeOH); λ_{max} (log ϵ) 241 (3.2), 202 (3.0), 193.2 (4.1); IR ($CHCl_3$) υ_{max} 3590, 2952, 1715, 1655 cm^{-1}; ^1H NMR δ ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR δ ($CDCl_3$, 125 MHz), see Table 2; EIMS (rel int) 344 M^+ (38), 330 (7) 329 (36), 328 (100), 326 (13), 83 (100).

Compound 9: oil, 4 mg, $[\alpha]^{25}_D$ −100° (*c* 0.006, MeOH); IR ($CHCl_3$) υ_{max} 3600, 2950, 1720, 1660 cm^{-1}; ^1H NMR δ ($CDCl_3$, 500 MHz), 6.59 (1H, q, J = 7.5 Hz, H-20), 5.75 (1H, br s, H-4), 4.17 (1H, H-11), 3.95 (1H, d, J = 6.0 Hz, H-15), 1.90 (3H, d, J = 7.5 Hz, H-21); EIMS (rel int) 344 M^+ (69), 330 (9), 329 (26), 328 (31), 326 (26), 315 (7), 311 (14), 83 (100).

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