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Novel reduction and hydroxylation products formed by *Aspergillus fumigatus* from Reichstein's Substance S

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Fermentation of Reichstein's Substance S with Aspergillus fumigatus *(AM-21) under aerobic conditions yielded 17α, 21-dihydroxy-5α-pregn-1-ene-3,20-dione, 17α,20α,21-trihydroxy-5α-pregn-1-en-3-one, 6β,17α,21-trihydroxypregn-4-en-3,20-dione, 15β,17α,21-trihydroxy-5α-pregnane-3,20-dione, and 15β,17α,20α,21-tetrahydroxy-5α-pregn-1-en-3-one. Each microbial metabolite was characterized by spectroscopic methods, including ^{13}C NMR chemical shifts. (Steroids* **62**:253–257, 1997) *© 1997 by Elsevier Science Inc.*

Keywords: Reichstein's Substance S; microbial transformation; *Aspergillus fumigatus;* metabolites

Introduction

Microbial transformations are often used as a general means to prepare steroid derivatives that are difficult to prepare by chemical methods, and specific microbial transformation steps have been introduced into numerous partial syntheses of new steroids wanted for evaluation as steroidal hormones and drugs.[1] In our studies on obtaining various physiologically important steroid derivatives by microbial transformations, we isolated a common strain of *Aspergillus fumigatus*[2] using progesterone as the sole source of carbon. In previous communications, the metabolism of progesterone,[3] testosterone,[4] androst-4-ene-3,17-dione,[5] and selective 1-dehydrogenation of progesterone[6] with this strain were reported. Further work on fermentation of Reichstein's Substance S(**1**) with the strain led to the formation of five derivatives, including three that appear to be novel. The characteristic transformations observed are 1-dehydrogenation, Δ^4-double bond hydrogenation, 20-keto reduction, 6β, and 15β-hydroxylations.

Experimental

Instrumental methods

Melting points were determined in open capillary tubes in a H_2SO_4 bath and are uncorrected. Ultraviolet spectra were recorded using methanol solutions on a Varian DMS-100S spectrometer. Infrared spectra were recorded using KBr discs on a Perkin-Elmer 177 spectrometer. Optical rotations were measured on solutions in 1 dm cells on a Perkin-Elmer 141 automatic spectropolarimeter. CD measurement was done on a JASCO J-20A automatic spectropolarimeter. ^1H NMR and ^{13}C NMR spectra were obtained with a JEOL FT-100 spectrometer at 100 and 25.05 MHz, respectively, with tetramethylsilane as an internal standard. Mass spectra were obtained with a MS-50 AE1 mass spectrometer operating at 70 eV by the direct insertion method. Thin-layer chromatography (TLC) and preparative TLC were performed, respectively, on 0.44-mm and 0.5-mm thick layers of silica gel G (BDH, Poole, UK). Layers prepared on glass plates were activated at 120°C for 1 h before use. Chromatograms were developed with chloroform/methanol/water in different proportions and were visualized by spraying with Lieberman–Burchard reagent and warming the plates at 110°C.

Materials

11-Deoxycortisol and corn steep liquor were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Inorganic salts were purchased from Sarabhai Chemical (Bombay, India). Silica gel for column chromatography and organic solvents were supplied by E. Merck (Bombay, India). Reagents and solvents were of analytical grade.

Microorganism, culture, and incubation conditions

The strain of *Aspergillus fumigatus* (AM-21) was isolated from soil by enrichment culture technique, using progesterone as the sole source of carbon. It is being maintained in Czapek Dox agar slant at the Culture Collection Unit of the Steroid and Terpenoid Division of the Institute of Chemical Biology, Calcutta.

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Received March 27, 1996; accepted September 12, 1996.

0039-128X/97/$17.00
PII S0039-128X(96)00205-0

The organism was cultivated in flasks containing a sterilized medium, composition (% w/v, sucrose 1, corn steep liquor 0.5, K_2HPO_4 0.05, pH 6.5). To each Erlenmeyer flask (1000 mL) containing 200 mL of the medium, 100 mg of Reichstein's Substance S was added, and the whole culture was sterilized by autoclaving at 121°C for 15 min. Inoculation of the sterilized medium was made with a cell suspension obtained from a 3-day-old culture maintained in Czapek Dox agar slants. A batch of 15 flasks thus inoculated was incubated at 37°C on a rotary shaker at 110 rpm in aerobic condition for 120 h.

Recovery and purification of biotransformation products

Fermentation media were harvested by filtration and extracted with *n*-butanol and filtered to separate the broth from the mycelium. This extract was washed with water and evaporated under reduced pressure. The mycelia were extracted with chloroform. TLC of this extract showed the presence of only unconverted 11-deoxycortisol. The broth extract was subjected to column chromatography over silica gel, eluting successively with petroleum ether/chloroform (1:1), chloroform, chloroform/methanol (99:1) and (98:2). The fractions thus obtained were further purified by rechromatography, followed by crystallization. Thus, four metabolites (**2**)(102 mg), (**3**)(85 mg), (**4**)(76 mg), (**5**)(40 mg), a mixture of two metabolites (55 mg), and unconverted (**1**) (900 mg including that of the mycelia extract) were isolated. The metabolite (**6**) (30 mg) was isolated as acetate by preparative TLC of the prepared acetate mixture of the two followed by crystallization. The recovered substrate **1** and its five purified metabolites accounted for 84% of the amount used for fermentation.

17α,21-Dihydroxy-5α-pregn-1-ene-3,20-dione (2). This metabolite apparently was crystallized from methanol to give needles; mp 241–242 C; $[\alpha]_D$+95.6 (*c* = 0.45 in MeOH); UV, λ_{max} 228 nm (ε 7600); IRγ_{max} 3444, 2924, 1700, 1668, 1444, 1390, 1370, 1271 cm^{-1}; MS m/z 346 (M^+, 75%), 287 (M^+–$COCH_2OH$, 71%), 269 (M^+–$COCH_2OH$–H_2O, 100%), 244 (25%), 229(30%); ^1H NMR (C_5D_5N) δ0.76 (s,3H,18–CH_3), 0.88 (s,3H,19–CH_3), 4.80 (d,1H, *J* = 19 Hz,21–H), 5.30 (d, 1H, *J* = 19 Hz, 21–H′), 5.96 (d, 1H, *J* = 10 Hz, 2–H), 7.04 (d, 1H, *J* = 10 Hz,1–H). Analysis calculated for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73%. Found: C, 72.75, H, 8.70.

Degradation of 2 and formation of 5α-androst-1-ene-3,17-dione. A mixture of **2** (70 mg) dissolved in acetic acid/water (1:1) (15 mL) with sodium bismuthate (1.5g) was shaken intermittently for 3 h and was left to stand overnight. The sodium bismuthate was filtered out, washed with 15 mL of acetic acid/water (1:1), and the combined filtrate was diluted with 75 mL water. This was then extracted with $CHCl_3$, and the $CHCl_3$ extract was washed free of acetic acid with water and dried ($MgSO_4$). The $CHCl_3$ was removed, and the residue was crystallized from acetone-hexane; mp 140–142C, $[\alpha]_D$ + 132 (*c* = 0.65 in MeOH) [Lit[9] mp 141–143C, $[\alpha]_D$ + 134], IR and ^1H NMR were comparable with those of an authentic sample (see Lunn[9]).

17α,20α,21-Trihydroxy-5 α-pregn-1-en-3-one(3). This novel steroid was crystallized from methanol as small prisms; mp 146–147 C; $[\alpha]_D$ + 70 (*c* = 0.55 in MeOH); UVλ_{max} 227 nm (ε 6980); IRγ_{max} 3370, 2928, 1665, 1546, 1444, 1377, 1275, 1160 cm^{-1}; MS *m/z* 348 (M^+, 5%), 330 (M^+–H_2O, 12%), 287 (M^+–CHOH · CH_2OH, 71%), 269 (M^+–$CHOHCH_2OH$–H_2O, 100%), 256 (12%); ^1H NMR (C_5D_5N) δ 0.80 (s, 3H, 18–CH_3), 1.00 (s, 3H, 19–CH_3), 3.48 (1H,m,20–H), 3.80(2H,m,21–H_2), 5.85 (d,1H, J = 10 Hz,2–H), 7.16 (d,1H,*J* = 10 Hz); λ_{max}230, Δε = +2.5 (Band I, π – π*), λ_{max}330, Δε –1.1(η – π*). Analysis calculated for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26%. Found: C, 72.44; H, 9.22.

6β, 17α, 21-Trihydroxypregn-4-ene-3,20-dione(4). It was crystallized from methanol as microneedles; mp 226–228 C; $[\alpha]_D$ + 58 (*c* = 0.65 in MeOH) [Lit[14] mp 230–232 C, $[\alpha]_D$ + 55 ($CHCl_3$)], IR, ^1H NMR and MS were identical with those of an authentic sample.[14]

Degradation of 3 with sodium bismuthate. The compound **3** (70 mg) was degraded to 5 α-androst-1-ene-3,17-dione(25 mg) as described for degradation of **2;** mp 141–142C, $[\alpha]_D$ + 130. Its ^1H NMR spectrum was identical with that of an authentic sample (see Lunn[9]).

20α, 21-Diacetoxy-17α-hydroxy-5 α-pregn-1-en-3-one(8). The compound **3** (45 mg) was acetylated with acetic anhydride (5 ml) and pyridine (0.5 ml) at 80°C for 1 h. The reaction mixture was dried under reduced pressure, and the acetate **8** was purified by chromatography over silica gel column followed by crystallization from MeOH, mp 103–104C, $[\alpha]_D$ + 24 (*c* = 0.8 in MeOH); UVλ_{max} 228 nm (ε 7670); ^1H NMR (C_5D_5N)δ0.85(s, 3H, 18–CH_3), 1.01 (s, 3H, 19–CH_3), 2.01 (s, 3H), 2.03 (s, 3H) (2 × OAc), 5.90 (d, 1H, J = 10 Hz, 2–H), 7.10 (d, 1H, J = 10 Hz, 1–H). Analysis calculated for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39% found: C, 69.35; H, 8.42.

15β, 17α, 21-Trihydroxy-5α-pregn-3,20-dione(5). This new compound crystallized from methanol as microneedles; mp 240–241 C; $[\alpha]_D$ + 38.5 (*c* = 0.58 in MeOH); IR γ_{max} 3530, 1712, 1450, 1418, 1091 cm^{-1}; MS *m/z* 364 (M^+, 33%), 346 (M^+–H_2O, 37%), 305 (M^+–$COCH_2OH$, 100%), 287 (M^+–$COCH_2OH$–H_2O, 75%); ^1H NMR (C_5D_5N) δ 0.88 (s,3H,19–CH_3), 1.28 (s, 3H, 18–CH_3), 2.56 (1H,dd,J = 8,16–H), 3.46 (1H dd, J = 1.5,16 Hz,16–H′), 4.56 (1H,m,15–H), 4.88 (d,1H,*J* = 18 Hz,21–H), 5.38 (d,1H,*J* = 18 Hz,21–H′). Analysis calculated for $C_{21}H_{32}O_5$: C, 69.20; H, 8.85% found: C, 69.25; H, 8.91.

15β,20α,21-Triacetoxy-17α-hydroxy-5α-pregn-1-en-3-one(7). This new compound crystallized from methanol as microneedles; mp, 100–101 C; $[\alpha]_D$ + 28.4 (*c* = 0.52 in MeOH); UV λ_{max} 228 (ε 7680); IR γ_{max} 3580, 1740, 1680, 1455, 1371, 1124, 1049 cm^{-1}; MS *m/z* 370 (M^+-2 AcOH, 100%), 328 (M^+–2 AcOH–C_2H_2O, 44%), 311 (22%), 285 (34%); ^1H NMR (C_5D_5N)δ0.90 (s,3H,19–CH_3), 1.16 (s,3H,18–CH_3), 2.00 (s,3H), 2.03 (s,3H), 2.10 (s,3H)(3 × OAc), 4.68 (2H,m,21–H_2), 5.41(1H,m,15–H),5.85(1H,m,20–H),5.95 (d,1H,*J* = 10 Hz, 2–H), 7.03 (d,1H,*J* = 10 Hz, 1–H); λ_{max} 228, Δε + 2.6 (Band I, π – π*), λ_{max} 330, Δε – 1.56(η – π*). Analysis calculated for $C_{27}H_{38}O_8$: C, 66.10; H, 7.81% found: C, 66.16; H, 7.76.

Hydrolysis of 7 to yield 6. Compound **7** (45 mg) was refluxed with 6% KOH in aqueous MeOH (8 mL) for 1 h. The product was poured into ice-water, worked up as usual, and purified by silica gel column chromatography eluting with petrol-EtOAc(7:3). The product was crystallized from MeOH to yield **6** (30 mg), mp 159–160 C,$[\alpha]_D$ + 86 (*c* = 0.85 in MeOH); UV λ_{max} 229 nm(ε 9230). MS *m/z* 364 (M^+, 4%), 346 (M^+–H_2O, 15%), 303 (M^+–CHOH · CH_2OH, 55%), 285 (M^+–CHOH · CH_2OH–H_2O, 100%) 267(35%); ^1H NMR(C_5D_5N) δ 0.91(s, 3H, 19–CH_3) 1.22(s,3H,18–CH_3), 3.46(1H, m, 20–H), 4.55(1H, m, 15–H), 5.90(1H, d, J = 10Hz, 2–H), 7.15 (1H, d, J = 10 Hz). Analysis calculated for $C_{21}H_{32}O_5$: C,69.20; H, 8.85% found: C, 69.15; H, 8.80.

Results and discussion

Five metabolites (2–6) of compound **1** were isolated from the semisolid residue obtained from the fermentation broth and were characterized by spectroscopic methods.

Metabolite **2** $C_{21}H_{30}O_4$ showed in its mass spectrum a discernible molecular ion at *m/z* 346, which is the same as that of the substrate **1.** However, its ultraviolet spectrum showed the absorption maximum at 228 nm, and the ^1H NMR spectrum displayed peaks at δ5.96 (d, 1H, J = 10 Hz) and 7.04 (d, 1H, J = 10 Hz) assignable to 2-H and 1-H, respectively, indicating it to be a Δ^1-isomer of 11-deoxycortisol (**1**). The characterization of the metabolite was ascertained by its ^{13}C NMR data (Table 1), which are consistent with the location of the Δ^1-double bond in metabolite **2.** All the carbon resonances were assigned by insensitive nuclei enhanced by polarization transfer [INEPT] experiments, known chemical shift rules, and comparison with the ^{13}C NMR data of Substance S **1** also measured in C_5D_5N. The ^{13}C NMR data of compound **1** in DMSO-d_6 are available.[7] In addition to this ^{13}C NMR evidence, the trans fusion of A/B ring was ascertained by degradation of the side chain of compound **2** with sodium bismuthate,[8] leading to the formation of 5 α -androst-1-ene-3, 17-dione characterized by comparison of its spectral properties with those of an authentic sample.[9] The compound as such, it seems, has not yet been reported; a preparation of its 21-acetate by chemical synthesis is found in the literature.[10]

Metabolite **3** $C_{21}H_{32}O_4$ showed in its mass spectra the molecular ion peak at *m/z* 348, indicating either hydrogenation of a double bond or reduction of a ketonic function. The presence of Δ^1-double bond, however, was suggested by the ^1H NMR spectrum, which was supported by its ultraviolet and IR spectra. However, the IR band attributable to the 20-ketone observed at about 1700 cm^{-1} was not noticeable in the IR spectrum. The mass spectrum, besides showing the molecular ion, displayed significant fragment ions at *m/z* 330, 287, and 269 ascribable to $[M^+-H_2O]^+$, $[M^+$–side chain$]^+$ and $[M^+$–side chain–$H_2O]^+$, respectively. The data clearly indicated the presence of 20-hydroxy group. The ^{13}C NMR spectral data (Table 1) were found to be in conformity with structure **3,** which were assigned with the help of INEPT experiments as well as by comparison with ^{13}C NMR data of known steroids.[7] The trans fusion of A/B rings was determined from the cotton effects observed in the CD spectrum of **3** as well as by its conversion to the known compound, 5α-androst-1-ene-3,17-dione. The configuration of the 20-hydroxyl group in **3** was indicated to be α by the characteristic negative shift in the molecular rotation (−140), which resulted upon acetylation, according to the conventions suggested by Fieser and Fieser[11] and Sarett.[12] Moreover, the 18-methyl group of **3** is found to be shifted downfield by 0.04 ppm in comparison to that of compound **2** in the ^1H NMR spectrum. This smaller de-shielding effect of the 20-hydroxyl group on the 18-methyl also indicated α configuration of the former. It has been reported[13] that the 18-methyl resonance frequency is subjected to greater de-shielding by 20 β-hydroxyl group.[13] It seems that compound **3** has not yet been reported in literature.

The mass spectrum of metabolite **4** showed the molecular ion at *m/z* 362, which suggested the addition of 16 mass units, as compared to that of the substrate. The significant fragment ions appeared at *m/z* 344, 326, 302, 285, and 267. The ^1H NMR spectrum showed the 18-, and 19-methyl singlets at δ0.89 and 1.50. The characteristic downfield shift of about 0.50 ppm of the 19-methyl group indicated 6 β-hydroxylation. The metabolite was identified as 6β, 17α, 21-trihydroxypregn-4-en-3,20-dione by direct comparison with an authentic sample.[14]

The metabolite **5** $C_{21}H_{32}O_5$ exhibited in its mass spectrum the molecular ion at *m/z* 364 and significant fragment ions at *m/z* 346, 305 and 287 attributable to $[M^+-H_2O]^+$, $[M^+-COCH_2OH-H_2O]^+$ and $[M^+-COCH_2OH-2H_2O]^+$, respectively. The ultraviolet spectrum did not show the presence of any en-3-one system, and the IR spectrum displayed bands for saturated ketones. The results indicated hydrogenation of the Δ^4-double bond and hydroxylation at a certain carbon of the substrate. The methyl singlets in the ^1H NMR spectrum at δ1.28 and 0.88 were assigned to 18-, and 19-methyl, respectively. The downfield shift of the C–18 methyl group by 0.52 ppm in comparison with that of substrate **1** strongly indicated the presence of 15β–OH, which was also supported by the ^1H NMR signals at δ 4.56 (1H, m) assignable to H–15, and at δ 2.56 (1H, dd, J = 8,16 Hz) and 3.46 (1H,dd,J = 1.5, 16Hz) attributable to 16-methylene group.[15] A comprehensive interpretation of ^{13}C NMR data of metabolite **5** (Table 1) by the application of known chemical shift rules, INEPT experiments, as well as by comparison with those of known steroids suggested the structure 15β, 17α,21-trihydroxy-5-pregn-3,20-dione with *trans*-fusion of A/B rings, as shown.

Metabolite **6** was isolated as its acetate **7.** The mass spectrum of compound **7,** $C_{27}H_{38}O_8$ did not show its molecular ion but exhibited a peak at *m/z* 370 as the base peak assignable to $[M^+-2xAcOH]^+$ and another significant peak at *m/z* 328 attributable to $[M^+-2xAcOH-C_2H_2O]^+$. The pres-

Table 1 ^{13}C NMR chemical shifts of deoxycortisol metabolites[a]

Carbon no.	Compounds				
	(1)	(2)	(3)	(5)	(7)
1	35.7[b]	158.8	158.4	38.6	157.9
2	34.6	127.5	127.4	38.3	127.6
3	198.1	198.3	199.1	210.3	198.9
4	124.1	41.2	41.4	44.8	41.2
5	170.4	44.5	44.5	46.9	44.6
6	32.4[c]	31.1[b]	31.9[b]	31.5	30.8
7	32.8[c]	31.7[b]	32.8[b]	32.9	33.4
8	35.9[b]	35.9	36.0	32.3	32.3
9	53.7	51.3	50.2	54.3	52.8
10	38.7	39.3	39.2	36.0	39.2
11	21.0	21.2	21.5	21.4	21.1
12	31.0	27.7	27.9	29.1	27.7
13	49.0	48.3	48.4	47.8	48.8
14	50.8	50.0	50.0	54.3	50.3
15	24.0	24.0	24.3	69.3	73.1
16	34.3	34.6	34.2	48.2	46.2
17	89.5	89.5	95.2	89.9	83.3
18	15.3	12.9	13.2	11.3	13.0
19	17.2	15.3	15.4	18.1	17.2
20	213.1	213.2	75.5	213.0	75.5
21	67.7	67.6	65.1	67.6	64.8

[a]Spectra were taken in C_5D_5N; The values are in ppm with respect to tetramethylsilane as internal standard.
[b,c] The resonances with the same subscript letters may be reversed.

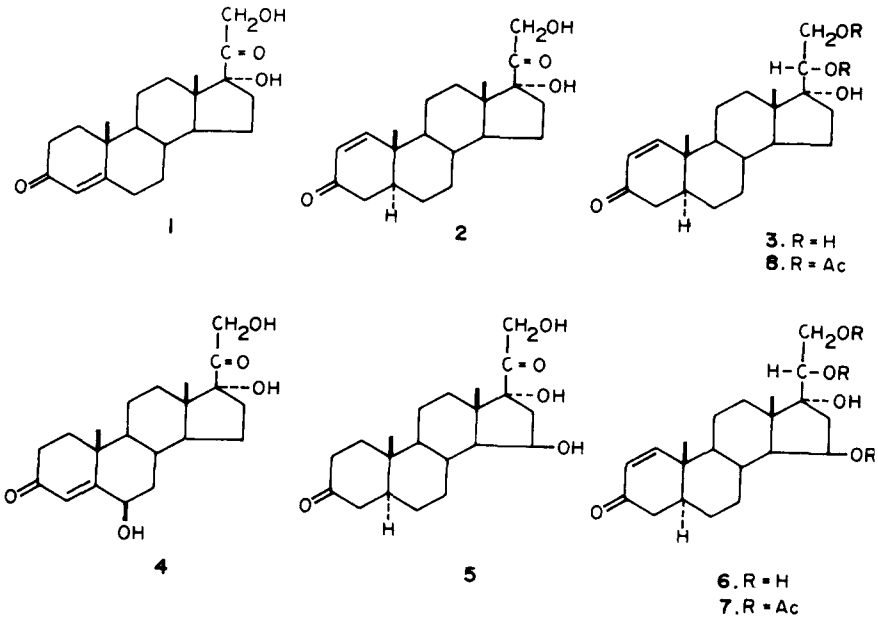

Figure 1 Structures of the substrate and metabolites.

ence of three acetate groups in **7** was evident from its ^{1}H NMR spectrum showing three acetoxy methyls at δ 2.00, 2.03, and 2.10. The ultraviolet spectrum displayed absorption maximum at 228 nm and ^{1}H NMR spectrum showed signals at δ 5.95 (*d*,1H, J = 10 Hz) and 7.03 (*d*,1H, J = 10 Hz) ascribable to 2-H and 1-H, respectively. The data supported the presence of Δ^1-double bond. The CD spectrum showed Cotton effects $\lambda_{max}330$, $\Delta\epsilon$ − 1.56 (η − Π^* transition) and $\lambda_{max}228$,$\Delta\epsilon$ + 2.6 (Band I, Π-Π^* transition). Taking into consideration the Cotton effects $\Delta\epsilon$ − 0.78/348 (ν - Π^*), + 11.9/226 (Band I, Π-Π^* reported for 17 β-acetoxy - 5α-androst-1-en-3-one and $\Delta\epsilon$ + 0.4/342 (ν - Π^*), +19.1/229 (Band I, Π-Π^*) for 17β-acetoxy-5β-androst-1-en-3-one,[16] the trans fusion of A/B rings of compound **7** was indicated. The configuration of the 20-hydroxyl group was suggested to be α by the negative shift (−173.8) in the molecular rotation of **7** when compared with that of compound **6** taking into consideration the convention suggested.[11,12] The α-orientation of the 20-hydroxyl group was also indicated by its smaller de-shielding effect on the 18-methyl group[13] when compared with that of compound **2** (see Experimental). The ^{1}H NMR values of compound **7** (see Experimental), as well as its ^{13}C NMR data (Table 1) assigned with the help of known chemical shift rules, distortionless enhancement by polarization transfer (DEPT) experiments as well as by comparison with the ^{13}C NMR data of known steroids suggested the structure, 15β, 20α, 21 -triacetoxy-17α-hydroxy-5α-pregn-1-en-3-one, as shown. To our knowledge, neither acetate **7** nor its parent compound **6** has yet been reported in literature.

The structures of the substrate and the isolated metabolites are shown in Figure 1. It is evident that the metabolites are formed by competitive enzymic reactions. However, it seems that 20-keto reduction follows 1-dehydrogenation and Δ^4-hydrogenation preceeds 1-dehydrogenation. The characteristic feature of this fungal strain of *A. fumigatus* is that it produces metabolites involving not only hydroxylation reactions but also 20-keto reduction, Δ^4-double bond hydrogenation, and 1-dehydrogenation.

Acknowledgments

The authors sincerely thank K. Yamasaki, Hiroshima University School of Medicine for the NMR spectra of compound **7**. Financial help was provided by CSIR, New Delhi in the form of a fellowship to one of us (SG) and in the Emeritus Scientist scheme to SBM.

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