Microbial Hydroxylation of Pregnenolone Derivatives

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Pregnenolone (1) and pregnenolone acetate (2) were incubated with the fungi *Cunninghamella elegans*, *Rhizopus stolonifer* and *Gibberella fujikuroi*. Incubation of 1 with *C. elegans* yielded metabolites, 3β,7β,11α-trihydroxypreg-5-en-20-one (3), 3β,6α,11α,12β,15β-pentahydroxypreg-4-en-20-one (4) and 3β,6β,11α-trihydroxypreg-4-en-20-one (5), while incubation with *G. fujikuroi* yielded two known metabolites, 3β,7β-dihydroxypregn-5-en-20-one (6) and 6β,15β-dihydroxypreg-4-ene-3,20-dione (7). Metabolites 4 and 5 were found to be new. Fermentation of 2 by *C. elegans* yielded four known oxidative metabolites, 1, androsta-1,4-diene-3,17-dione (8), 6β,15β-dihydroxyandrost-4-ene-3,17-dione (9) and 11α,15β-dihydroxypreg-4-ene-3,20-dione (10). Fermentation of 2 with *R. stolonifer* yielded two known metabolites, 11α-hydroxypreg-4-ene-3,20-dione (11) and 7. Compounds 1—11 were screened for their cholinesterase inhibitory activity in a mechanism-based assay.

Key words pregnenolone; pregnenolone acetate; fungal transformation; enzyme inhibition; acetylcholinesterase; butyrylcholinesterase

In continuation of our studies on the biotransformation of potential bioactive agents,[1—12] we subjected pregnenolone (**1**) to fungal metabolism with fungi reported to effect hydroxylation of steroids at various positions,[13,14] and as a result a series of transformed analogues **3**—**11** were obtained. Pregnenolone (**1**) is a major hormone in human nerve tissues.[15] Its therapeutic role in repairing defective neurons is well documented.[16] The most promising therapeutic strategy for activating the central nervous cholinergic functions has been the use of cholinomimatic agents. Hence AChE and BChE have long been an attractive target for rational drug design and discovery of mechanism-based inhibitors for the treatment of Alzheimer's disease.[17] The main function of AChE and BChE inhibitors is to boost the cholinergic function by increasing the endogenous level of acetylcholine. We have previously reported a number of natural and microbial transformed inhibitors of cholinesterase.[13] Compound **1**, on fermentation with *Cunninghamella elegans*, yielded a major metabolite **3**. The di-acetate derivative **3b** of compound **3** exhibited inhibitory activity against the butyrylcholinesterase (BChE) (IC_{50}=92.3 μM). The incubation of **2** with *C. elegans* yielded metabolites **7** and **9**, which showed some activity against the acetylcholinesterase (AChE) (IC_{50}=45 μM) and butyrylcholinesterase (BChE) (IC_{50}=99.5 μM), respectively.

Results and Discussion

Metabolism of pregnenolone (**1**) ($C_{21}H_{32}O_2$) by *C. elegans* for 6 d yielded a known metabolite **3**, and two new hydroxylated metabolites **4** and **5** (Fig. 1). The structure of known trihydroxylated metabolite **3** was identified as 3β,7β,11α-trihydroxypreg-5-en-20-one by comparison of its spectral data with the literature values.[18]

The new metabolite **4** was obtained as a white amorphous material. The IR spectral data displayed absorptions for free hydroxyl and ketonic moieties at 3374 and 1693 cm^{-1}, respectively, while the UV spectrum showed only the weak absorption at 204 nm. The HR-FAB-MS of **4** exhibited a quasi-molecular ion $[M-18+Na]^+$ at *m/z* 363.3780 corresponding to the formula of $C_{21}H_{30}O_5$ (Calcd for $C_{21}H_{30}O_5$+Na: 363.3808). The ^{1}H-NMR spectrum of **4** (Table 1) showed five hydroxyl-bearing methine proton signals at δ 3.57 (dt, *J*=10.9, 5.5 Hz), 3.75 (dd, *J*=14.4, 11.2 Hz), 3.66 (d, *J*=11.1 Hz), 3.83 (t, *J*=3.9 Hz) and 4.07 (ddd, *J*=15.0, 10.4, 5.0 Hz). The ^{13}C-NMR spectrum (broad-band decoupled, distortionless enhancement by polarization transfer (DEPT)) displayed resonances for 21 carbon atoms, including three methyls, four methylenes, ten methines, and four quaternary carbons. The OH-bearing methine proton signal at δ 3.57 was assigned to H-3, which showed homonuclear coupling (^{1}H–^{1}H COSY) with H_2-2 (δ 2.30, 1.70), while long-range ^{1}H–^{13}C correlation of H-2 (δ 2.30) with C-3 (δ 72.0) was observed in the heteronuclear multiple bond correlation (HMBC) spectrum of **4**. Migration of double bond from C-5/C-6 to C-4/C-5 of ring A was deduced from the downfield shift of the olefinic proton signal from δ 5.33 (H-6) to 5.64 (H-4) due to the anisotopic effect of C-3 and C-6 hydroxyl groups. The new position of the double bond was further deduced from the long-range ^{1}H–^{13}C correlations of H-2 (δ 2.30) with C-4 (δ 124.5) in the HMBC spectrum. A methine proton, which appeared at δ 3.83 as a triplet ($J_{6a,7e}$=3.9 Hz), was assigned to the H-6 based on its HMBC correlations with C-5 (δ 147.0) and C-4 (δ 124.0), while the homonuclear (^{1}H–^{1}H COSY) coupling was observed between H-4 (δ 5.64) and H-6. The stereochemistry at C-6 was deduced on the basis of nuclear Overhauser enhancement spectroscopy (NOESY) which showed coupling between H-6 and β-oriented Me-19 (δ 1.10) (Fig. 3). The OH-bearing methine proton resonating at δ 3.75 was assigned to H-11 on the basis of long-range HMBC correlation of H-11 (δ 3.75) with C-9 (δ 50.2). The newly oxygenated methine carbon which resonated at δ 64.0 (C-12) showed heteronuclear correlations with CH_3-18 (δ 0.63) and H-11 (δ 3.75) in the HMBC spectrum, thus indicating the vicinal hydroxyl groups at C-11 and C-12. The orientation of vicinal diols at C-11 and C-12 was inferred as equatorial through the larger coupling constant ($J_{12a,11a}$=11.1 Hz), representing diaxial interaction between the geminal H-11/H-12. A signal at δ 4.07 in the ^{1}H-NMR spectrum, was assigned to H-15 based on ^{1}H–^{1}H COSY correlations between H-15 and H_2-16 (δ 2.30, 1.50) and H-14 (δ 1.80), while the HMBC spectrum showed the correlation be-

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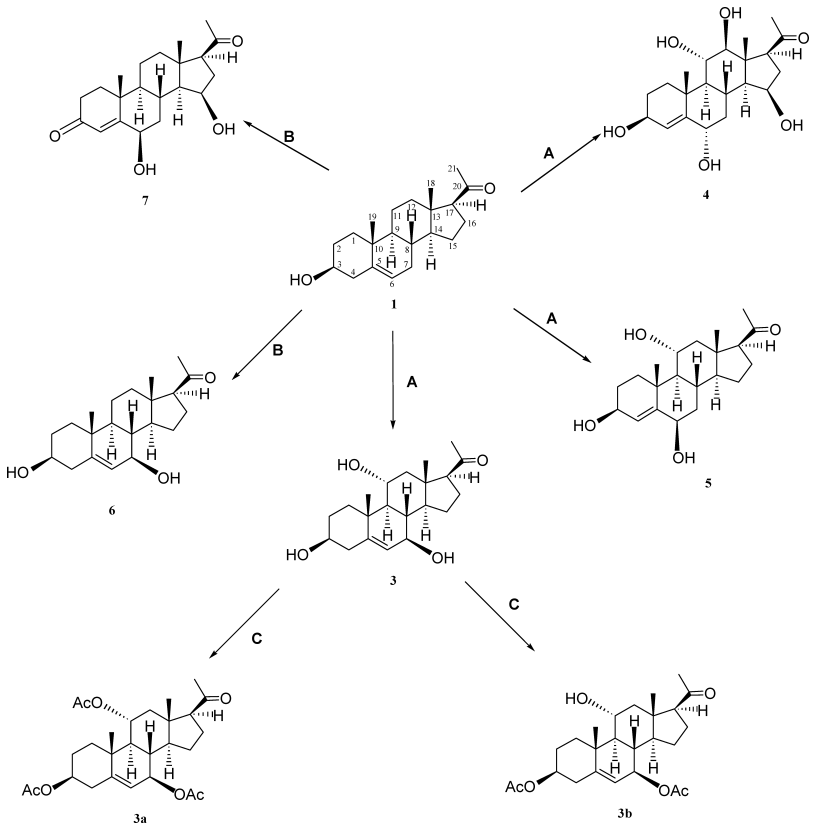

Fig. 1. Microbial Transformation of Pregnenolone (**1**)
A, *Cunninghamella elegans* (6 d); B, *Gibberella fujikuroi* (12 d); C, acetylation.

tween H_2-16 and C-15 (δ 69.0). The β-configuration of C-15 hydroxyl group was inferred from the multiplicity pattern of H-15 (δ 4.07, ddd, J=15.0, 10.4, 5.0 Hz) and NOESY correlations between H-15 and α-oriented H-14 (Fig. 3). The structure of this pentahydroxy compound **4** was finally deduced as 3β,6α,11α,12β,15β-pentahydroxypreg-4-en-20-one.

The HR-EI-MS of compound **5** showed an [M^+] at m/z 348.2591 corresponding to the formula of $C_{21}H_{32}O_4$ (Calcd 348.2664). The IR spectrum showed absorptions for hydroxyl and ketonic functionalities at 3422 and 1697 cm^{-1}, respectively, while the UV spectrum showed only weak absorption at 203 nm. The ^{1}H-NMR spectrum of **5** (Table 1) showed three downfield methine proton signals at δ 3.36 (bd, J=8.4 Hz), 3.50 (ddd, J=15.5, 10.9, 5.8 Hz) and 4.04 (dt, J=10.3, 5.2 Hz). The presence of three hydroxyl-bearing methine carbons was deduced from the HMQC experiment. An OH-bearing methine proton resonating at δ 3.50 (ddd, J=15.5, 10.9, 5.8 Hz) was similar to compound **1** and assigned to H-3 based on ^{1}H–^{1}H COSY spectrum which showed correlations of H_2-2 (δ 2.23, 1.50) with H-3. Migration of double bond from C-5/C-6 to C-4/C-5 in ring A was deduced from the downfield shift of the olefinic proton signal from δ 5.33 (H-6) to 5.45 (H-4), due to the anisotropic effect of C-3 and C-6 hydroxyl groups. The hydroxylation at C-6 position was inferred from the long-range ^{1}H–^{13}C correlations of H-6 (δ 3.36) with C-5 (δ 144.0) and C-4 (δ 121.0) in the HMBC spectrum, while the ^{1}H–^{1}H COSY spectrum showed allylic coupling between H-6 and H-4. The relative configuration of the hydroxyl group at C-6 was assigned to be β (pseudoax-

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **4** and **5** (400, 100 MHz, Respectively; $CDCl_3$+CD_3OD)

No.	**4** δ_H[a] (J=Hz)	**4** δ_C[b]	**5** δ_H[a] (J=Hz)
1	2.01 m	32.2 t	2.03 m
	1.50 m		1.48 m
2	1.70 m	25.0 t	1.50 m
	2.30 m		2.23 m
3	3.57 dt (10.9, 5.5)	72.0 d	3.50 ddd (15.5, 10.9, 5.8)
4	5.64 d (5.3)	124.5 d	5.45 s
5	—	147.1 s	—
6	3.83 t (3.9)	65.0 d	3.36 bd (8.4)
7	2.30 m	43.4 t	2.20 m
	1.42 m		1.40 m
8	1.50 m	38.6 d	1.55 m
9	2.30 m	50.2 d	1.75 m
10	—	40.0 s	—
11	3.75 dd (14.4, 11.2)	73.8 d	4.04 dt (10.3, 5.2)
12	3.66 d (11.1)	64.0 d	1.40 m
			2.20 m
13	—	45.0 s	—
14	1.80 m	43.0 d	1.67 m
15	4.07 ddd (15.0, 10.4, 5.0)	69.0 d	1.48 m
			1.76 m
16	2.30 m	39.7 t	1.56 m
	1.50 m		1.97 m
17	2.30 dd (10.7, 4.8)	50.1 d	2.47 t (9.4)
18	0.63 s	14.3 q	0.64 s
19	1.10 s	18.2 q	1.10 s
20	—	211.0 s	—
21	2.10 s	31.5 q	2.10 s

a) Assignments based on COSY and HMQC. *b*) Assignments based on HMQC and HMBC.

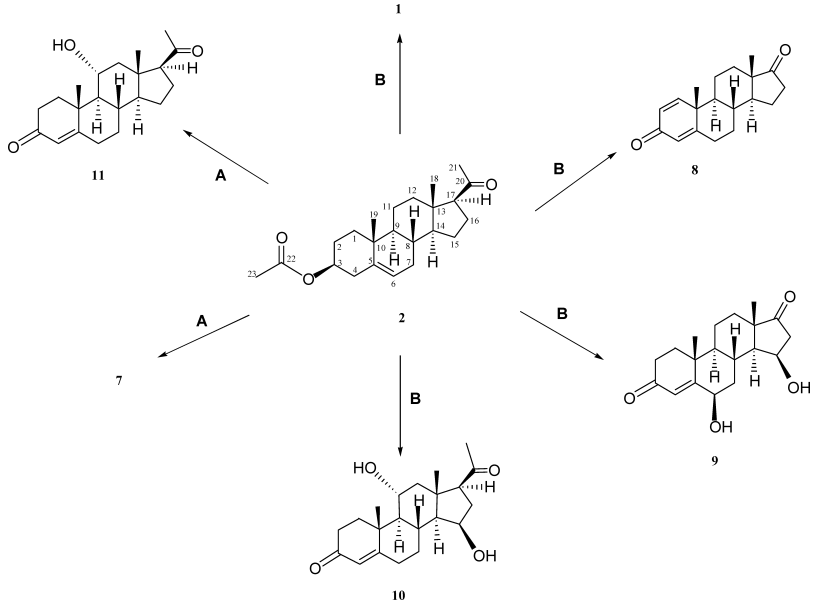

Fig. 2. Microbial Transformation of Pregnenolone Acetate (**2**)
A, *Rhizopus stolonifer* (3 d); B, *Cunninghamella elegans* (8 d).

ial), as the H-6 showed NOESY correlations with H-9 (δ 1.75) (Fig. 4). A signal at δ 68.0 was assigned to C-11, based on HMBC correlations between H_2-12 (δ 2.20, 1.40) and C-11. The stereochemistry of OH at C-11 was assigned to be α (equatorial) on the basis of NOESY couplings between H-11 and H -19 (δ 1.10) and H-18 (δ 0.64) (Fig. 4). Thus from the foregoing spectral studies, the structure of compound **5** was characterized as 3β,6β,11α-trihydroxypreg-4-en-20-one.

Fermentation of pregnenolone (**1**) by *Gibberella fujikuroi* for 12 d yielded two known metabolites **6** and **7** (Fig. 1). Comparison of the spectral data of metabolites **6** and **7** with the reported data led to their identification as known hydroxypregnenolones: 3β,7β-dihydroxypreg-5-en-20-one (**6**) and 6β,15β-dihydroxypreg-4-en-3,20-dione (**7**).[18,19]

Compound **2** when incubated with *Cunninghamella elegans* (TSY 0865) yielded four known oxidative metabolites, **1** and **8**—**10** (Fig. 2), while with *Rhizopus stolonifer* (TSY 0471) two known hydroxylated metabolites **11** and **7** (Fig. 2) were obtained. The structures of the known metabolites were identified as androsta-1,4-diene-3,17-dione (**8**), 6β,15β-dihydroxyandrost-4-ene-3,17-dione (**9**), 11α,15β-dihydroxypreg-4-ene-3,20-dione (**10**) and 11α-hydroxypreg-4-ene-3,20-dione (**11**) on the basis of spectral comparision.[20—22]

Compounds **1**—**11** were screened against the cholinesterase enzymes. Diacetate derivative **3b** of metabolites **3** and **9** exhibited inhibitory activity against butylcholinesterase with an IC_{50} value of 92.3±0.5 and 99.5±0.7 μM, respectively, while compound **7** inhibited AChE activity with an IC_{50} value of 45±0.4 μM. The galanthamine, a positive control was used in the assay, showed IC_{50} values of 0.5±0.001 and 8.5±0.01 μM against the AChE and BChE, respectively. Hydroxylations at allylic C-6 and C-15 positions apparently enhanced the inhibition potential against the enzymes (compounds **7**, **9**), while in compound **3b**, diacetate moieties seemed to be responsible for the enhanced inhibition of the enzyme.

Acetylcholinesterase is a key enzyme of cholinergic brain synapses and neuromuscular junctions. Its major biological

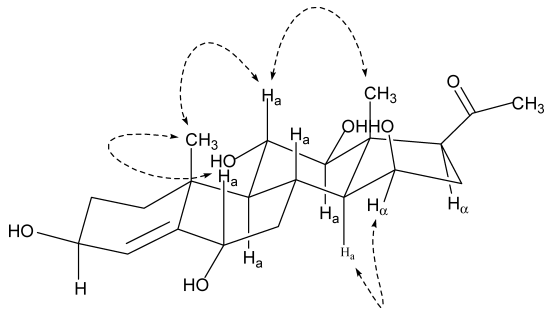

Fig. 3. Key NOESY Correlations of Compound **4**

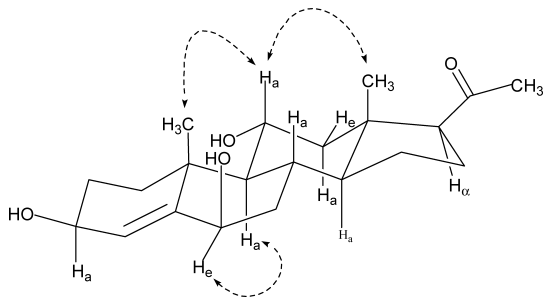

Fig. 4. Key NOESY Correlations of Compound **5**

role is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine.[23] According to the cholinergic hypothesis, the memory impairment in patients with senile dementia of Alzheimer's type results from a deficiency in cholinergic function in the brain.[17] The role of butyrylcholinesterase in normal aging and brain diseased persons is still elusive. Recently, it has been observed that BChE is found in significantly higher quantities in Alzheimer's patients than in normal age-related non-demented brains.[24]

Experimental

General Experimental Procedures Melting points were determined by a Buchi 535 melting-point apparatus. Optical rotations were measured on a Jasco DIP-360 digital polarimeter, and a Hitachi U-3200 spectrophotometer was used for UV spectrophotometry. The IR spectra were obtained on a FT-IR 8900 or FT-IR Bruker vector-22 spectrophotometer in $CHCl_3$. The ^{1}H- and ^{13}C-NMR spectra were recorded on a mixture of $CDCl_3$ and CD_3OD on a Bruker AM 400 NMR spectrometer using the UNIX data system at 400 and 100 MHz, respectively. 2D experiments in $CDCl_3$ were made on the Bruker AM 400 NMR spectrometer. The EI-MS and HR-EI-MS [ion source energy (70 eV), ion source temperature 250 °C] were recorded on a JEOL JMS 600 H mass spectrometer. FAB-MS spectra were obtained on a JEOL JMS-AX505WA. TLC was carried out on silica gel precoated plates (Merck, PF_{254}: 20×20 cm, 0.25 mm). Spots on TLC were detected by spraying ceric sulphate and vanillin solution, followed by heating. Column chromatography (CC) was performed on silica gel (70—230 mesh size). Compounds **1** and **2** were purchased from Fluka.

Fungi and Culture Conditions Cultures of *Cunninghamella elegans* (TSY 0865), *Rhizopus stolonifer* (TSY 0471), and *Gibberella fujikuruoi* (ATCC 10704) were grown on Sabouraud-4% glucose-agar (Merck) at 25 °C and stored at 4 °C. *C. elegans* broth media was prepared by mixing the following ingredients into distilled H_2O (3.0 l): glucose (30.0 g), glycerol (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g). *R. stolonifer* broth media was prepared by adding glucose (100 g), peptone (25 g), KH_2PO_4 (25 g) and yeast extract (15 g) to distilled water (4 l) and pH was maintained at 5.6. *G. fujikuroi* broth media was prepared by adding the following ingredients to distilled H_2O (3.0 l): glucose (80.0 g), KH_2PO_4 (5.0 g), $MgSO_4 \cdot 2H_2O$ (1.0 g), NH_4NO_3 (0.5 g) and *Gibberella* trace element solution (2 ml). The latter solution was prepared by mixing $Co(NO_3)_2 \cdot 6H_2O$ (0.01 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.161 g), $MnSO_4 \cdot 4H_2O$ (0.01 g) and NH_4 molybdate (0.01 g) into distilled water (100 ml).

General Fermentation and Extraction Conditions The fungal media were transferred into conical flasks (100 ml each) and autoclaved at 121 °C. Seed flasks were prepared from three-day old slants and fermentation was allowed for 2 d on a shaker at 25 °C. The remaining flasks were inoculated from the seed flasks. After 2 d, pregnenolone (**1**) and pregnenolone acetate (**2**) were dissolved in acetone and transferred into each flask (12 mg/ml) and flasks were placed on a rotatory shaker (128 rpm) at 25 °C for fermentation. Time course studies were carried out after 2 d and the transformation was analyzed on TLC. The culture media was filtered and extracted with CH_2Cl_2 and ethyl acetate. The extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and the brown gummy crude was analyzed by TLC.

Fermentation of Pregnenolone (1) with *Cunninghamella elegans* (TSY 0865) Compound **1** (370 mg) was dissolved in 20 ml acetone and placed in 30 flasks for fermentation. All the media were filtered after 6 d of fermentation and extracted with dichloromethane. The CH_2Cl_2 extract was evaporated under reduced pressure and a thick brown crude (1.02 g) was obtained, which was subjected to silica gel CC to afford compound **3** (104 mg) on elution with pet. ether–EtOAc (25 : 75), compound **4** (15 mg) on elution with pet. ether–EtOAc (22 : 78) and compound **5** (8.5 mg) on elution with pet. ether–EtOAc (30 : 70).

3β,7β,11α-Triacetoxypreg-5-en-20-one (**3a**): Metabolite **3** (20 mg) was treated with pyridine/Ac_2O (2 : 1) and stirred (5 h) at r.t. The reaction mixture was diluted with CH_2Cl_2 (18 ml) and shaken with 2% aqueous $NaHCO_3$ (15 ml) and 2% HCl (15 ml). The CH_2Cl_2 extract was then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude extract (17 mg) was then subjected to silica gel CC to afford compound **3a**. Crystalline solid (18%); mp 159—162 °C; $[\alpha]_D^{25}$ −8.8° (c=0.18, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 203 (4.3); IR (KBr) cm^{-1}: 2941 (C–H), 1732 (C=O), 1669 (C=C); ^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz): δ 2.01 (3H, s, Me-21), 1.13 (1H, s, Me-19), 0.70 (3H, s, Me-18), 5.23 (1H, m, H-11), 4.56 (1H, m, H-7), 5.1 (1H, m, H-3), 2.00 (9H, s, AcO).

3β,7β-Diacetoxy-11α-hydroxypreg-5-en-20-one (**3b**): Metabolite **3** (10 mg) was treated with pyridine/Ac_2O (2 : 1) and stirred (6 h) at r.t. The reaction mixture was diluted with CH_2Cl_2 (18 ml) and shaken with 2% aqueous $NaHCO_3$ (15 ml) and 2% HCl (15 ml). The CH_2Cl_2 extract was then dried over anhydrous Na_2SO_4, evaporated under reduced pressures and the crude extract was analyzed by TLC. The crude obtained (8 mg) was subjected to silica gel CC to afford compound **3b**. Crystalline solid (29.8%); mp 165—167 °C; $[\alpha]_D^{25}$ +2.8° (c=0.14, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 203 (4.1); IR (KBr) cm^{-1}: 3430 (OH), 1728 (C=O), 1668 (C=C); ^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz): δ: 2.00 (3H, s, Me-21), 1.12 (1H, s, Me-19), 0.69 (3H, s, Me-18), 3.8 (1H, m, H-11), 4.56 (1H, m, H-7), 5.2 (1H, m, H-3), 1.91 (6H, *s*, AcO).

3β,6α,11α,12β,15β-Pentahydroxypreg-4-en-20-one (**4**): Crystalline solid (4%); $[\alpha]_D^{25}$ −6.0° (c=0.3, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 204 (3.8); IR (KBr) cm^{-1}: 3374 (OH), 1693 (C=O); EI-MS *m/z* (rel. int., %): [M$^+$ missing], 348 [M−32]$^+$ (9), 330 (100), 312 (15), 257 (6), 145 (20), 55 (30); HR-FAB-MS *m/z*: 363.3780 [M−18+Na]$^+$ (Calcd for $C_{21}H_{30}O_5$+Na: 363.3808); ^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz) for δ, see Table 1. ^{13}C-NMR ($CDCl_3$+CD_3OD, 100 MHz) for δ, see Table 1.

3β,6β,11α-Trihydroxypreg-4-en-20-one (**5**): Crystalline solid (2.6%); $[\alpha]_D^{25}$ −1.11° (c=0.36, $CHCl_3$); UV λ_{max} (MeOH) nm (log ε): 203 (3.3); IR (KBr) cm^{-1}: 3422 (OH), 1697 (C=O); EI-MS *m/z* (rel. int., %): 348 (M$^+$) (21), 332 (94), 315 (11), 279 (18), 251 (5), 149 (83), 105 (21), 81 (26), 57 (35); HR-EI-MS *m/z*: 348.2591 (Calcd for $C_{21}H_{32}O_4$: 348.2664); ^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz) for δ, see Table 1.

Fermentation of Pregnenolone (1) with *Gibberella fujikuroi* (ATCC 10704) Compound **1** (300 mg) was dissolved in 20 ml acetone and equally distributed among 40 flasks and kept for fermentation. The media was filtered after 12 d, extracted with dichloromethane and evaporated under reduced pressure to obtain a thick brown crude (0.92 g). The crude obtained (0.92 g) was subjected to silica gel CC to obtain **6** (9.1 mg) on elution with pet. ether–EtOAc (60 : 40), and **7** (7.23 mg) on elution with pet. ether–EtOAc (50 : 50).

Incubation of Pregnenolone Acetate (2) with *Cunninghamella elegans* (TSY 0865) Four liters media of *Cunninghamella elegans* was prepared for incubation of pregnenolone acetate (**2**). Compound **2** (500 mg) was dissolved in acetone and fed into each flask (15 mg) and flasks were placed on a shaker for 8 d. A time-course study was also conducted after 2 d of incubation. The resulting mixtures were extracted with dichloromethane and transformations were detected through TLC. Fermentation was continued for further 6 d. After 8 d, all the flasks were filtered and extracted with dichloromethane. Crude extract thus obtained (0.9 g) was subjected to column chromatography (silica gel) to afford compound **1** (20 mg) on elution with pet. ether–EtOAc (70 : 30), **10** (11.5 mg) on elution with pet. ether–EtOAc (55 : 45) and two biodegraded products **8** (10.2 mg) on elution with pet. ether–EtOAc (65 : 35) and **9** (10.2 mg) on elution with pet. ether–EtOAc (60 : 40).

Incubation of Pregnenolone Acetate (2) with *Rhizopus stolonifer* (TSY 0471) Three liters media of *Rhizopus stolonifer* (TSY 0471) was prepared for incubation of pregnenolone acetate (**2**). Compound **2** (300 mg) was dissolved in acetone and fed into each flask (20 mg) and flasks were placed on a shaker for 12 d. After 12 d, all the flasks were filtered and extracted with dichloromethane and the resulting crude extract was analyzed by TLC. Crude extract (0.9 g) was subjected to column chromatography (silica gel) to afford two hydroxylated metabolites, **7** (11.2 mg) on elution with pet. ether–EtOAc (50 : 50) and **11** (12 mg) on elution with pet. ether–EtOAc (55 : 45).

***In Vitro* Cholinesterase Inhibition Assay** Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), eserine and galanthamine were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Cholinesterase inhibiting activities were measured by the spectrophotometric method developed by Ellman *et al.*[23] Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay AChE and BChE inhibition, respectively. The assay conditions and protocol were the same as described previously.[23]

Determination of IC_{50} Values The concentrations of test compounds that inhibited hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

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