# Microbial Hydroxylation of Hydroxyprogesterones and α-Glucosidase Inhibition Activity of Their Metabolites

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Microbial transformation of 11α-hydroxyprogesterone (**1**) with *Cunninghamella elegans*, *Gibberella fujikuroi*, *Fusarium lini*, and *Candida albicans* yielded 11α,15α,16α-trihydroxypregn-4-ene-3,20-dione (**3**), 11α-hydroxy-5α-pregnane-3,20-dione (**4**), 6β,11α-dihydroxypregn-4-ene-3,20-dione (**5**), 11α-hydroxypregna-1,4-diene-3,20-dione (**6**), 11α,17β-dihydroxyandrost-4-en-3-one (**7**), and 11α,15α-dihydroxypregn-4-ene-3,20-dione (**8**). On the other hand, microbial transformation of 17α-hydroxyprogesterone (**2**) with *Cunninghamella elegans* and *Fusarium lini* yielded 11α,17α-dihydroxypregn-4-ene-3,20-dione (**9**), and 17α-hydroxypregna-1,4-diene-3,20-dione (**10**). The structures of the metabolites **3** – **10** were deduced on the basis of spectroscopic methods. Compound **3** was identified as a new metabolite, which exhibited a promising inhibitory activity against the α-glucosidase enzyme.

*Key words:* 11α-Hydroxyprogesterone, 17α-Hydroxyprogesterone, Microbial Transformation, *Cunninghamella elegans*, *Candida albicans*, *Fusarium lini*, *Gibberella fujikuroi*, α-Glucosidase Inhibition

## Introduction

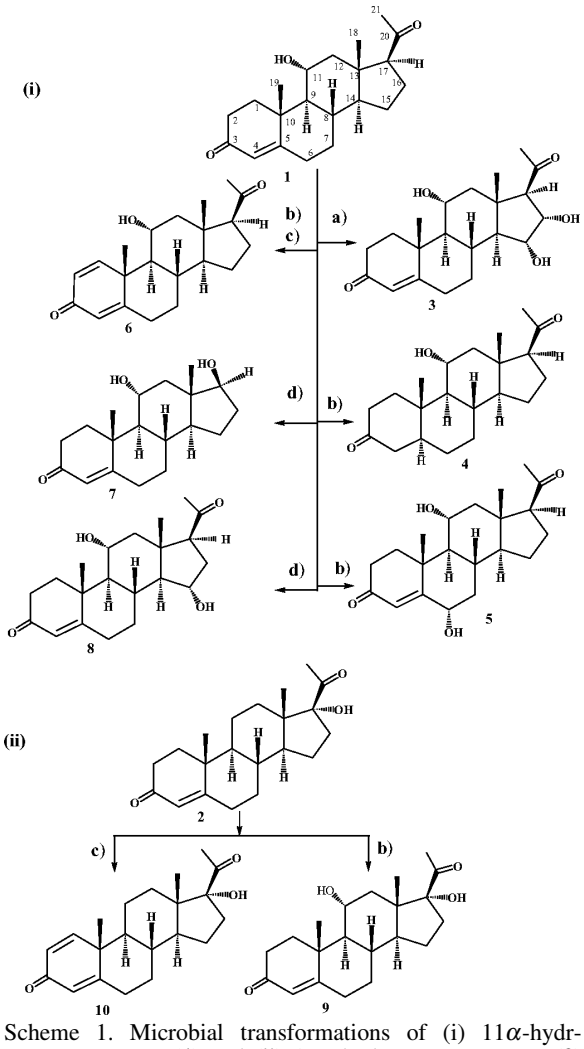
Microorganisms, particularly fungi, have been used successfully as *in vitro* models for the prediction of drug metabolism [1 – 3]. The structural transformations catalyzed by microorganisms have also been successfully employed to manufacture therapeutically important compounds at industrial levels.

The objective of our on-going efforts in this field has been to synthesize libraries of metabolites with interesting structural and biological properties. In this process, the inhibitory activity of the synthesized metabolites against α-glucosidase and their mode of the action were also studied. In continuation of this work [4 – 16], we incubated 11α-hydroxyprogesterone (**1**), and 17α-hydroxyprogesterone (**2**) separately with various fungi, which resulted in the reduction of the C-4/C-5 double-bond, introduction of a C-1/C-2 double-bond, and hydroxylations at C-6β, C-15α, C-16α, and C-11α positions. Compounds **1** and **2** are the known fungally transformed products of progesterone [17, 18]. Compound **1** is an antigonadotropic agent [19], widely used in *anti*-hair shedding lotions. Compound **2** possesses a pronounced gestagenic activity [20].

Metabolites **3** – **10** were screened for their α-glucosidase inhibitory activity, and compound **3** was found to be as potent as standard deoxynojirimycin. Glucosidase enzymes are involved in several biological processes such as intestinal digestion, biosynthesis of glucoproteins, and lysosomal catabolism of glucoconjugates. Intestinal α-glucosidase (AGH) catalyses the final step of the carbohydrate digestion by converting carbohydrates into monosaccharides, which are absorbed from the intestine. In this way, the inhibitors of AGH could suppress the postprandial hyperglycemia and thus can be useful for the management of type-II diabetes. The AGH inhibitors have been also used as inhibitors of tumor metastases, *anti*-obesity drugs, fungistatic compounds, insect antifeedants, antivirals, and immune modulators [21].

## Results and Discussion

The fermentation of compound **1** with *Gibberella fujikuroi* for 19 days yielded a new metabolite **3**, while incubation with *Cunninghamella elegans* for 10 days yielded the three metabolites **4** – **6**. Metabolite **6** was also formed in 15 days from the incubation of **1** with




Scheme 1. Microbial transformations of (i) 11α-hydroxyprogesterone (**1**) and (ii) 17α-hydroxyprogesterone (**2**) by a) *Gibberella fujikuroi*, b) *Cunninghamella elegans*, c) *Fusarium lini*, d) *Candida albicans*.

*Fusarium lini*. The fermentation of compound **1** with *Candida albicans* for 14 days yielded the metabolites **7** and **8** (Scheme 1).

The HREI MS of the new compound **3** exhibited the $M^+$ peak at *m/z* = 362.2080, corresponding to the formula $C_{21}H_{30}O_5$ (calcd. 362.2090), which was 32 a.m.u. higher than the substrate **1**. The IR spectrum of compound **3** showed absorptions for a hydroxyl group ($\nu$ = 3630 $cm^{-1}$) and a C=C bond ($\nu$ = 1672 $cm^{-1}$). The $^1H$ NMR spectrum showed two additional hydroxyl-bearing methine proton signals at $\delta$ = 3.76 (dd, $J_{15,14\alpha}$ = 12.2 Hz, $J_{15,16\beta}$ = 5.8 Hz), and 4.31 (dd, $J_{16,17\alpha}$ = 12.4 Hz, $J_{16,15\beta}$ = 5.8 Hz). The

Table 1. $^1H$ and $^{13}C$ NMR chemical shift data of the new metabolite **3**[a].

| Position | $\delta_C$ | $\delta_H$ |
|---|---|---|
| 1 | 33.5, t | 2.42; 2.25, m |
| 2 | 37.5, t | 2.0; 2.65, m |
| 3 | 200.0, s | – |
| 4 | 124.6, d | 5.72, br s |
| 5 | 170.1, s | – |
| 6 | 34.1, t | 2.25; 2.42, m |
| 7 | 31.5, t | 1.82; 1.15, m |
| 8 | 34.9, d | 2.31, m |
| 9 | 59.0, d | 1.14, m |
| 10 | 39.9, s | – |
| 11 | 68.8, d | 4.04, (td, *J* = 10.3, 10.3, 5.0 Hz) |
| 12 | 50.4, t | 2.3; 1.48, m |
| 13 | 44.8, s | – |
| 14 | 55.3, d | 1.25, s |
| 15 | 69.9, d | 3.76, (dd, *J* = 12.2, 5.8 Hz) |
| 16 | 84.0, d | 4.31, (dd, *J*= 12.4, 5.8 Hz) |
| 17 | 63.1, d | 2.51, m |
| 18 | 14.7, q | 0.67, s |
| 19 | 18.3, q | 1.29, s |
| 20 | 208.0, s | – |
| 21 | 31.3, q | 2.14, s |

[a] Carbon multiplicities were assigned by the DEPT experiment; s = quarternary, d = methine, t = methylene, q = methyl carbons.

$^{13}C$ NMR spectrum (Table 1) also showed two additional methine carbon signals at $\delta$ = 69.9 (C-15), and 84.0 (C-16). The positions of the newly introduced hydroxyl groups were deduced at C-15 and C-16 by the COSY 45° technique, which showed correlations of H-15 ($\delta$ = 3.76) and H-16 (4.31), with H-14 ($\delta$ = 1.25). The HMBC (heteronuclear multiple bond connectivity) spectrum further supported the position of the hydroxyl groups through correlations between H-14 ($\delta$ = 1.25) and C-15 ($\delta$ = 69.9) and C-16 ($\delta$ = 84.0), between H-15 ($\delta$ = 3.76), C-14 ($\delta$ = 55.3), C-16 ($\delta$ = 84.0) and C-13 ($\delta$ = 44.8), and between H-17 ($\delta$ = 2.51) and C-16 ($\delta$ = 84.0) and C-20 ($\delta$ = 208.0). The stereochemistry of the hydroxyl groups was determined by the NOESY correlations of H-15 ($\delta$ = 3.76) and H-16 ($\delta$ = 4.31) with H-8$\beta$ ($\delta$ = 2.31), which indicated that H-15 and H-16 were both $\beta$-oriented. The structure of the new metabolite **3** was thus deduced as 11$\alpha$,15$\alpha$,16$\alpha$-trihydroxypregn-4-ene-3,20-dione.

The HREI MS of the metabolite **4** showed an $M^+$ peak at *m/z* = 332.4025 (calcd. 332.4015 for $C_{21}H_{32}O_3$). The $^1H$ NMR spectrum indicated the reduction of the C-4/C-5 double-bond. The $\alpha$-stereochemistry of H-5 was deduced through the NOESY cross peaks between H-5 ($\delta$ = 1.65) and H-9$\alpha$ ($\delta$ = 0.89). Metabolite **4** (11$\alpha$-hydroxy-5$\alpha$-pregnane-3,20-dione) was previously reported as a hy-

drogenated product of 11α-hydroxyprogesterone by chemical methods [22, 23].

The HREI MS of metabolite **5** exhibited an $M^+$ peak at *m/z* = 346.2140 ($C_{21}H_{30}O_4$, calcd. 346.2153), and the $^1$H NMR spectrum an additional downfield methine signal at δ = 4.34 (brs, H-6), when compared with the substrate **1**. The $^{13}$C NMR spectrum also showed an additional methine carbon signal at δ = 73.2 indicating an OH-substitution. The location of the newly introduced OH at C-6 was deduced by the HMBC correlations between H-6 (δ = 4.34) and C-4 (δ = 126.1) and C-10 (δ = 40.1). The stereochemistry of the C-6 hydroxyl group was deduced to be β on the basis of the NOESY interaction between geminal H-6 and H-14α (δ = 1.28). The structure of the metabolite **5** was deduced as 6β,11α-dihydroxypregn-4-ene-3,20-dione by comparison with the reported data. This compound was previously obtained by hydroxylation of progesterone by *Cephalosporium aphidicola* [17].

The HREI MS of metabolite **6** showed an $M^+$ peak at *m/z* = 328.2030 (calcd. 328.2038 for $C_{21}H_{28}O_3$). A comparison between the NMR data of substrate **1** and its metabolite **6** revealed the presence of two additional olefinic signals at δ = 7.71 (d, $J_{1,2}$ = 10.7 Hz) and 6.16 (dd, $J_{2,1}$ = 10.7 Hz, $J_{2,4}$ = 1.7 Hz) in the $^1$H NMR spectrum of **6**, with the corresponding signals at δ = 161.6 and 125.5, respectively, in the $^{13}$C NMR spectrum. The position of the new double bond was assigned to be C-1/C-2 through COSY 45° and HMBC correlations. Compound **6** (11α-hydroxypregna-1,4-diene-3,20-dione) was previously obtained by the chemical derivatization of 1α,11α-epoxy-5-pregnene-3,20-dione [24].

The HREI MS of metabolite **7** exhibited an $M^+$ peak at *m/z* = 304.2048 (calcd. 304.2030 for $C_{19}H_{28}O_3$). The $^1$H NMR spectrum of **7** showed an additional hydroxyl-bearing methine proton signal at δ = 3.68 (t, $J_{17,16\alpha\beta}$ = 8.4 Hz), while the $^{13}$C NMR spectrum exhibited the corresponding methine carbon signal at δ = 81.4. The location of the new OH at C-17 was deduced through HMBC correlations of H-17 (δ = 3.68) with C-18 (δ = 12.2), and C-14 (δ = 48.8). The stereochemistry of C-17 OH was deduced as β by the NOESY correlation between H-17 (δ = 3.68) and H-14 (δ = 1.15). Metabolite **7** (11α,17β-dihydroxyandrost-4-en-3-one) was previously reported as the hydroxylated product of androsta-1,4-diene-3,17-dione by *Cephalosporium aphidicola* [15].

The HREI MS of metabolite **8** showed an $M^+$ peak at *m/z* = 346.2120 (calcd. 346.2140 for $C_{21}H_{30}O_4$). The $^1$H NMR spectrum displayed an additional methine signal at δ = 4.32 (dd, $J_{15,14\alpha}$ = 12.0 Hz, $J_{15,16\alpha\beta}$ = 6.4 Hz) with the corresponding methine carbon signal at δ = 70.3 (C-15). The position of the newly introduced hydroxyl group was deduced to be C-15 by $^3J_{CH}$ correlations between H-15 (δ = 4.32) and C-13 (δ = 43.5) and C-17 (δ = 63.5) in the HMBC experiment. The stereochemistry of the C-15 OH was inferred to be α on the basis of NOSEY correlation between H-8β (δ = 1.15) and H-15 (δ = 4.32). Compound **8**, (11α,15α-dihydroxypregn-4-ene-3, 20-dione), was previously reported as the microbial product of pregen-4-ene-3,12,15,20-tetraone by *Calonectria decora* [25, 26].

In another experiment, substrate **2** was subjected to incubation with *Cunninghamella elegans*, and *Fusarium lini*, which yielded metabolites **9** and **10**, respectively (Scheme 1).

The HREI MS of metabolite **9** showed an $M^+$ peak at *m/z* = 346.2164, corresponding to a formula $C_{21}H_{30}O_4$ (calcd. 346.2165). The $^1$H NMR spectrum of metabolite **9** showed an additional methine signal at δ = 3.99 (td, $J_{11,9\alpha}$ = 10.4 Hz, $J_{11,12\alpha}$ = 10.4 Hz, $J_{11,12\beta}$ = 5.2 Hz). The $^{13}$C NMR spectrum also showed an additional methine carbon signal at δ = 69.2. The stereochemistry of the newly introduced hydroxyl group at C-11 was deduced to be α on the basis of coupling constants and NOESY correlations of H-8β (δ = 1.15) and H-19β (δ = 1.29) with H-11 (δ = 3.99). The structure of metabolite **9** (11α,17α-dihydroxypregn-4-ene-3,20-dione) was further deduced by comparison with the reported data. This compound was previously obtained by the hydroxylation of progesterone by *Cephalosporium aphidicola* [17].

The HREI MS of metabolite **10** showed an $M^+$ peak at *m/z* = 328.6213, corresponding to the formula $C_{21}H_{28}O_3$ (calcd. 328.6223). The $^1$H NMR spectrum showed the presence of two additional olefinic signals at δ = 7.01 (d, $J_{1,2}$ = 10.7 Hz) and 6.23 (dd, $J_{2,1}$ = 10.6 Hz, $J_{2,4}$ = 1.8 Hz), indicating the presence of a double-bond. The position of the double bond at C-1/C-2 was deduced by comparison of the $^{13}$C NMR spectrum of compound **10** with the one of substrate **2**. Metabolite **10** was identified as 17α-hydroxypregna-1,4-diene-3,20-dione, and was previously obtained by chemical derivatization of 17α-hydroxypregn-4-ene-3,20-dione [27].

Compounds **1** – **10** were screened against the α-glucosidase enzyme. Compound **3** showed a potent inhibitory activity, as compared to the substrate **1** (Table 3), which is probably due to the hydroxylation

Table 2. Quantitative measurements of the inhibition of the α-glucosidase enzyme by substrates **1** and **2**, and their metabolites **3** – **10**.

| Compound | Concentration (μM) | % Inhibition | $IC_{50}$[a] (μM ±SEM) |
|---|---|---|---|
| **1** | 500 | NA | NA |
| **2** | 500 | 6 | – |
| **3** | 500 | 12 | 655 ± 26 |
| **4** | 500 | 21 | – |
| **5** | 500 | NA | NA |
| **6** | 500 | NA | NA |
| **7** | 1000 | 29 | NA |
| **8** | 500 | 21 | – |
| **9** | 500 | – | 797.2 ± 0.36 |
| **10** | 500 | NA | NA |
| Deoxynojirimycin[b] | – | – | 425 ± 8 |
| Acarbose[b] | – | – | 780 ± 12 |

[a] Concentration of the compound which inhibits 50 % of the enzyme; $IC_{50}$ values given are mean values of three assays; SEM: standard error mean; NA: not active; [b] standard.

Table 3. The kinetic parameters of inhibition of α-glucosidase (AGH) by compound **3**.

| $IC_{50}$ (μM) | $K_i$ (μM) | $K_m$ (μM) | Type of inhibition |
|---|---|---|---|
| 655 ± 26 | 333.33 ± 23 | 250 | competitive |

at the C-16 position. The results of the enzyme inhibition assay are presented in Table 2. The kinetic studies revealed that compound **3** inhibited the enzyme in a dose-dependent manner with a $K_i$ value of 333.33 μM. This value was calculated in three different ways: firstly, the slopes of each line in the Lineweaver-Burk plot were plotted against different concentrations of inhibitors; secondly, the $1/V_{maxapp}$ was calculated by plotting different fixed concentrations of AGH *versus* $\Delta V$ in the presence of different fixed concentrations of inhibitors. The $K_i$ value was then calculated by plotting different concentrations of inhibitor *versus* $1/V_{maxapp}$, which was the intercept on the *x* axis. Thirdly, $K_i$ was directly measured from the Dixon plot as an intercept on the *x* axis. Lineweaver-Burk and Dixon plots and their replots indicated that the active compound **3** exhibited a competitive-type of inhibition against AGH.

## Experimental Section

*General experimental procedures*

IR Spectra were recorded in $CHCl_3$ on a FT IR-8900 spectrophotometer. Melting points were determined on a Büchi 535 melting point apparatus. Optical rotations were measured on a Jasco DIP-360 digital polarimeter. UV spectra were recorded in $CHCl_3$ solution on a Hitachi U-3200 spectrophotometer. The $^1H$ and $^{13}C$ NMR spectra were recorded in $CDCl_3$ solution on a Bruker Avance-400 instrument at 400 and 100 MHz, respectively ($\delta$ in ppm, *J* in Hz). The EI and HREI MS data were obtained on a Jeol JMS 600H mass spectrometer. TLC were performed with precoated plates (Silica gel 60, $PF_{254}$, 0.2 mm, Merck). Compounds **1** and **2** were purchased from Fluka.

*Organism and media*

Microbial cultures were obtained from the Institute of Fermentation Osaka (IFO), and Northern Regional Research Laboratories (NRRL). All cultures were maintained on SDA and stored at 4 °C prior to use. The medium for *Cunninghamella elegans* (NRRL 1392) was prepared by mixing the following ingredients in distilled water (3.0 L): glucose (60 g), peptone (15 g), $KH_2PO_4$ (15 g), yeast extract (9 g), and glucose (30 g). The medium for *Candida albicans* (IFO 1594) was prepared by mixing the following ingredients in distilled $H_2O$ (3.0 L): peptone (15 g), yeast extract (9 g), and $KH_2PO_4$ (15 g). The pH of the solution was adjusted to 5.6. The medium (3.0 L) for *Fusarium lini* (NRRL 68751) was prepared as described for *Cunninghamella elegans*. The medium (3.0 L) for *Gibberella fujikuroi* (IFO 30336) was prepared by mixing glucose (240 g), $NH_4NO_3$ (3 g), $KH_2PO_4$ (15 g), $MgSO_4 \cdot 7H_2O$ (3 g), and *Gibberella* trace element solution (6 mL).

*General fermentation and extraction procedure*

The fermentation media thus obtained were distributed among 30 flasks of 250 mL capacity (100 mL each) and autoclaved. The fermentation was carried out according to a standard stage II protocol [28]. Substrates were dissolved in acetone and were evenly divided into 30 flasks (20 mg/0.5 mL in each flask), containing 24 h old stage II cultures and fermentation continued further for an additional time on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from flasks were taken daily and analyzed by TLC in order to determine the degree of transformation of the substrate. In all experiments, one control flask without biomass (for checking substrate stability) and one flask without exogenous substrate (for checking endogenous metabolites) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with $CH_2Cl_2$ (1.0 L) and the filtrate was extracted with $CH_2Cl_2$ (3 × 1.5 L). The combined organic extract was washed with brine and dried over anhydrous $Na_2SO_4$, evaporated under reduced pressure, and analyzed by thin-layer chromatography. Control flasks were also harvested and the content was compared by TLC to detect the biotransformed products.

Compound **1** (600 mg) was dissolved in 15 mL of acetone and the solution was evenly distributed among 30 flasks containing stage II culture of *Cunninghamella elegans*, *Candida albicans*, *Fusarium lini*, and *Gibberella fujikuroi* together

with the control flasks. The organic metabolites were extracted from the medium by solvent $CH_2Cl_2$ and evaporated to afford a brown gum (2.3 g). The crude residue was subjected to column chromatography. Elution with petroleum ether and EtOAc yielded compounds **4**–**6**, in 4.2, 5.0, and 2.8 % yield, respectively, from *Cunninghamella elegans* culturing. Incubation of substrate **1** with *Candida albicans* yielded metabolites **7** and **8** in 1.6 and 1.4 % yield, respectively, while *Fusarium lini* gave 1.1 % of metabolite **6**. The new metabolite **3** was obtained in 1.2 % yield from *Gibberella fujikuroi*.

Compound **2** (600 mg) was dissolved in 15 mL of acetone and the solution was evenly distributed among the flasks containing the stage II cultures of *Cunninghamella elegans* and *Fusarium lini* separately both fungi as above. The metabolites **9** and **10** were obtained in 2.2 and 3.1 % yield, respectively, by both the fungi.

*11α,15α,16α-Trihydroxypregn-4-ene-3,20-dione (**3**)* was obtained as a white powder. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 ($c$ = 0.8, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) = 238 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3417, 1718, 1662 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): see Table 1. – $^{13}C$ NMR ($CDCl_3$, 100 MHz): see Table 1. – MS (EI, 70 eV): *m/z* (%) = 362 (1) [$M^+$], 344 (6) [$M^+ - 18$], 242 (4), 225 (1), 205 (6), 163 (59), 129 (9), 122 (100), 77 (23), 55 (51). – MS (HREI): *m/z* = 362.2080 (calcd. 362.2090 for $C_{21}H_{30}O_5$).

*11α-Hydroxy-5α-pregnane-3,20-dione (**4**)* was obtained as a white amorphous solid. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 ($c$ = 0.02, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) = 202 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3484, 2927, 1700 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 4.01 (td, $J_{11,9\alpha}$ = 10.4 Hz, $J_{11,12\alpha}$ = 10.4 Hz, $J_{11,12\beta}$ = 5.2 Hz, 1H, H-11), 2.50 (m, 1H, H-17), 2.12 (m, 2H, H-4), 1.65 (m, 1H, H-5), 1.25 (m, 1H, H-14), 0.89 (m, 1H, H-9). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 200.1 (C-3), 45.1 (C-4), 47.3 (C-5), 59.5 (C-9), 68.1 (C-11), 63.3 (C-17). – MS (EI, 70 eV): *m/z* (%) = 332 (5) [$M^+$], 314 (100) [$M^+ - 18$], 299 (48), 271 (89), 229 (70), 121 (52), 81 (84), 91 (45), 71 (52), 55 (60). – MS (HREI): *m/z* = 332.4025 $M^+$ (calcd. 332.4015 for $C_{21}H_{32}O_3$).

*11α,6α-Dihydroxypregn-4-ene-3,20-dione (**5**)* was obtained as a white amorphous solid. – M. p. 241 – 242 °C. – $[\alpha]_D^{25}$ = +127 ($c$ = 0.01, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) = 245 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 4.34 (brs, 1H, H-6), 4.01 (td, $J_{11,9\alpha}$ = 10.4 Hz, $J_{11,12\alpha}$ = 10.4 Hz, $J_{11,12\beta}$ = 5.2 Hz, 1H, H-11), 2.51 (m, 1H, H-17), 5.80 (brs, 1H, H-4), 1.28 (m, 1H, H-14), 1.02 (m, 1H, H-9). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 200.5 (C-3), 126.1 (C-4), 167.7 (C-5), 73.2 (C-6), 40.1 (C-10), 68.5 (C-11), 63.3 (C-17), 59.5 (C-9). – MS (EI, 70 eV): *m/z* (%) = 346 (100) [$M^+$], 328 (7) [$M^+ - 18$], 239 (2), 225 (8), 207 (12), 161 (14), 147 (20), 122 (16), 107 (35), 91 (37), 81 (51), 55 (71). – MS (HREI): *m/z* = 346.2140 (calcd. 346.2153 for $C_{21}H_{30}O_4$).

*11α-Hydroxypregna-1,4-diene-3,20-dione (**6**)* was obtained as a white amorphous solid. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 ($c$ = 0.02, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) 242 nm (3.95). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 7.71 (d, $J_{1,2}$ = 10.6 Hz, 1H, H-1), 6.16 (dd, $J_{2,1}$ = 10.6 Hz, $J_{2,4}$ = 1.7 Hz, 1H, H-2), 6.08 (brs, 1H, H-4), 4.01 (td, $J_{11\beta,9\alpha}$ = 10.4 Hz, $J_{11\beta,12\alpha}$ = 10.4 Hz, $J_{11\beta,12\beta}$ = 5.2 Hz, 1H, H-11), 1.25 (m, 1H, H-14), 1.15 (m, 1H, H-9). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 161.6 (C-1), 125.5 (C-2), 186.5 (C-3), 124.9 (C-4), 167.5 (C-5), 68.5 (C-11). – MS (EI, 70 eV): *m/z* (%) = 328 [$M^+$] (7), 239 (3), 225 (11), 207 (22), 161 (9), 147 (17), 122 (100), 107 (8), 91 (14), 81 (7), 55 (4). – MS (HREI): *m/z* = 328.2030 (calcd. 328.2038 for $C_{21}H_{28}O_3$).

*11α,17β-Dihydroxyandrost-4-en-3-one (**7**)* was obtained as a white amorphous solid. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 ($c$ = 0.1, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) = 241 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1700, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 5.72 (brs, 1H, H-4), 4.04 (td, $J_{11,9\alpha}$ = 10.4 Hz, $J_{11,12\alpha}$ = 10.4 Hz, $J_{11,12\beta}$ = 5.2 Hz, 1H, H-11), 3.68 (t, $J_{17,16\alpha\beta}$ = 8.4 Hz, 1H, H-17), 1.15 (m, 1H, H-14), 1.05 (m, 1H, H-9). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 198.0 (C-3), 125.2 (C-4), 169.1 (C-5), 59.1 (C-9), 67.8 (C-11), 48.8 (C-14), 34.4 (C-16), 81.4 (C-17), 12.2 (C-18). – MS (EI, 70 eV): *m/z* (%) = 304 (71) [$M^+$], 286 (51) [$M^+ - 18$], 271 (12), 258 (11), 243 (10), 180 (31), 163 (100), 149 (32), 124 (81), 55 (26). – MS (HREI): *m/z* = 304.2048 (calcd. 304.2030 for $C_{19}H_{28}O_3$).

*11α,15α-Dihydroxypregn-4-ene-3,20-dione (**8**)* was obtained as a white amorphous solid. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 ($c$ = 0.02, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) = 241 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1700, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 5.73 (brs, 1H, H-4), 4.32 (dd, $J_{15,14\alpha}$ = 12.0 Hz, $J_{15,16\alpha\beta}$ = 6.4 Hz, 1H, H-15), 4.09 (td, $J_{11\beta,9\alpha}$ = 10.4 Hz, $J_{11\beta,12\alpha}$ = 10.4 Hz, $J_{11\beta,12\beta}$ = 5.2 Hz, 1H, H-11), 2.51 (m, 1H, H-17), 1.19 (m, 1H, H-14), 1.15 (m, 1H, H-8), 1.09 (m, 1H, H-9). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 169.5 (C-5), 125.5 (C-4), 70.3 (C-5), 68.8 (C-11), 63.1 (C-17). – MS (EI, 70 eV): *m/z* (%) = 346 [$M^+$] (6), 310 (10) [$M^+ - 36$], 314 (100), 225 (11), 207 (22), 161 (9), 147 (17), 122 (100), 107 (8), 91 (14), 81 (7), 55 (91). – MS (HREI): *m/z* = 346.2120 (calcd. 346.2140 for $C_{21}H_{30}O_4$).

*11α,17α-Dihydroxypregn-4-ene-3,20-dione (**9**)* was obtained as an amorphous solid. – M. p. 241 – 242 °C. – $[\alpha]_D^{25}$ = +127° ($c$ = 0.01, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ ($\lg \varepsilon$) 245 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1720, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 5.71 (brs, 1H, H-4), 3.99 (td, $J_{11,9\alpha}$ = 10.4 Hz, $J_{11,12\alpha}$ = 10.4 Hz, $J_{11,12\beta}$ = 5.2 Hz, 1H, H-11), 1.15 (m, 1H, H-8), 0.76 (s, 3H, H-18). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 170.5 (C-5), 127.6 (C-4), 89.1 (C-17), 69.2 (C-11), 58.9 (C-9), 16.5 (C-18). – MS (EI, 70 eV): *m/z* (%) = 346 (4) [$M^+$], 310 (4) [$M^+ - 36$], 285

(100), 225 (7), 207 (22), 161 (13), 147 (6), 122 (6), 107 (6), 91 (10), 81 (4), 55 (5). – MS (HREI): *m/z* = 346.2164 (calcd. 346.2165 for $C_{21}H_{30}O_4$).

*17α-Hydroxypregna-1,4-diene-3,20-dione (**10**)* was obtained as a white amorphous solid. – M. p. 199 – 200 °C. – $[\alpha]_D^{25}$ = +94 (*c* = 0.02, $CHCl_3$). – UV ($CHCl_3$): $\lambda_{max}$ (lg ε) = 245 nm (3.4). – IR ($CHCl_3$): $\nu_{max}$ = 3447, 1661 $cm^{-1}$. – $^1H$ NMR ($CDCl_3$, 400 MHz): δ = 7.01 (d, $J_{1,2}$ = 10.7 Hz, H-1), 6.23 (dd, $J_{2,1}$ = 10.6 Hz, $J_{2,4}$ = 1.8 Hz, H-2), 6.08 (brs, 1H, H-4), 2.28 (s, 3H, H-21), 1.27 (s, 3H, H-19), 0.77 (s, 3H, H-18). – $^{13}C$ NMR ($CDCl_3$, 100 MHz): δ = 154.5 (C-1), 185.9 (C-3), 127.6 (C-4), 168.6 (C-5), 43.4 (C-10), 89.5 (C-17). – MS (EI, 70 eV): *m/z* (%) = 328 [$M^+$] (7), 310 (5) [$M^+ - 18$], 239 (2), 225 (6), 207 (62), 161 (12), 147 (10), 122 (29), 107 (13), 91 (31), 81 (6), 55 (4). – MS (HREI): *m/z* = 328.6213 (calcd. 328.6223 for $C_{21}H_{28}O_3$).

*Enzyme inhibition assay*

α-Glucosidase (E.C.3.2.1.20) enzyme inhibition assay was performed according to the slightly modified method of Matsui [29]. α-Glucosidase from *Saccharomyces* species was purchased from Wako Pure Chemical Industries Ltd. (Wako 076-02841). The enzyme inhibition was measured spectrophotometrically through a continuous monitoring of the nitrophenyl produced by the hydrolysis of the substrate *p*-nitrophenyl α-D-glucopyranoside (PNP-G) (0.7 mM) and 500 m units/mL of the enzyme used.

The enzymatic reactions were performed at 37 °C for 30 min. The increment in absorption at 400 nm due to the hydrolysis of PNP-G by α-glucosidase was monitored continuously on a microplate spectrophotometer (SpectraMax, Molecular Devices, USA). Phosphate saline buffer (pH = 6.9) was used, which contained 50 mM sodium phosphate and 100 mM NaCl. 1-Deoxynojirimycin (425 μM) and acarbose (780 μM) were used as positive controls.

*Determination of kinetic parameters*

The different concentrations of test compound **3** inhibited the hydrolysis of the substrate *p*-nitrophenyl α-D glucopyranoside (PNP-G) in a dose-dependent manner. The $IC_{50}$ (inhibitor concentration that inhibits 50 % activity of the enzyme) values were calculated by using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

Dissociation/inhibition constants ($K_i$) were determined by the interpretation of Dixon [30], and Lineweaver-Burk plots and their secondary replots using initial velocities obtained over a substrate concentration range of 1 – 0.25 mM PNP-G. Non-linear regression equations were used to determine the values of $K_I$, $K_m$, and $V_{max}$ in the Lineweaver-Burk and Dixon plots. The dissociation/inhibition constant (*Ki*) of AGH-inhibitor complex into free AGH and inhibitor was determined graphically by Dixon and Lineweaver-Burk plots.

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