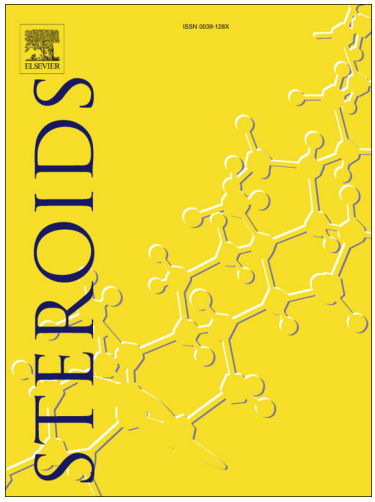

Fungal biotransformation of diuretic and antihypertensive drug spironolactone with *Gibberella fujikuroi, Curvularia lunata*, *Fusarium lini,* and *Aspergillus alliaceus*

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Fungal biotransformation of diuretic and antihypertensive drug spironolactone with *Gibberella fujikuroi, Curvularia lunata*, *Fusarium lini,* and *Aspergillus alliaceus*

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ABSTRACT

Fungal cells were used for the first time to synthesize derivatives of spironolactone (**1**), a diuretic and antihypertensive drug. Ten different fungi were screened for their ability to biotransform **1**, four of which were able to produce metabolites **2-8**. *Gibberella fujikuroi* produced canrenone (**2**), 1-dehydrocanrenone (**3**), *Curvularia lunuta* provided compound **2**, and 7α-thio-spironolactone (**4**), *Fusarium lini* yielded compounds **2**, **3**, 1β-hydroxycanrenone (**5**), 1α-hydroxycanrenone (**6**), 1-dehydro-15α-hydroxycanrenone (**7**), and 15α-hydroxycanrenone (**8**), while *Aspergillus alliaceus* was able to produce all the seven metabolites. Metabolites **5**, **6**, and **7** were identified as new compounds. Their structures were elucidated by using different spectroscopic techniques. Substrate **1** and its metabolites **2**, **3**, and **5-8** were also evaluated for α-glucosidase inhibitory activity *in vitro*. Substrate **1** was found to be strongly active with IC_{50} = 335 ± 4.3 μM as compared to the standard drug acarbose IC_{50} = 840 ± 1.73 μM, whereas all of resulting metabolites were found to be inactive.

Keywords: Spironolactone (Antihypertensive drug); *Aspergillus alliaceus*; *Curvularia lunuta*; *Fusarium lini*; *Gibberella fujikuroi*; α-Glucosidase inhibitory activity

1. Introduction

Spironolactone (**1**), a synthetic steroid, is a competitive antagonist of aldosterone. It increases the secretion of water and sodium, while decreasing the excretion of potassium by competing for the aldosterone sensitive Na^+/K^+ channel in the distal tubule of the nephrons. For decades, spironolactone (**1**) has been considered as an antagonist at the aldosterone receptors of the epithelial cells of the kidney, and is clinically used in the treatment of hyper aldosteronism, and occasionally as a potassium-sparing diuretic agent [1]. Spironolactone (**1**) is rapidly metabolized into a large number of derivatives by the liver [2]. The major metabolism of compound **1** results in the formation of canrenone (**2**), and 7α-methylthiospironolactone (**4**) [3], both of which are belived to mediate the therapeutic action of compound **1**.

Inspired by the extensive literature on the biotransformation of steroids, we decided to study the ability of the enzymatic systems of whole cell fungi to synthesize derivatives of spironolactone (**1**). Chemists have found widespread applications of biotransformation in the structural modifications of steroids with high regio- and stereo-selectivity [4]. Examples of hydroxylation, oxidation, reduction, isomerization, Michael addition, and reverse aldol reactions are well as documented in the literature [5].

Enzymes or whole cells biotransformation represent the most efficient catalytic systems known to carry out conventional chemical reactions [6, 7]. They play a vital role in the preparation of new oxygenated derivatives with different biological activities [8].

Previously we have reported several steroids having strong inhibitory activity against the enzyme α-glucosidase [9-12]. The discovery of the new α-glucosidase inhibitors provides an approach in the management of diabetes and hyperglycemia [13]. In this study, microbial transformation was used to synthesize derivatives of the diuretic and antihypertensive drug, spironolactone (**1**). These derivatives were then evaluated for their α-glucosidase inhibitory activity *in vitro*.

Initially, we screened spironolactone (**1**) against various bioactivities but it was found to be active against only α-glucosidiase enzyme. Therefore, we decided to evaluate transformed products of **1** against this enzyme.

2. Material and Methods

2.1 General

Spironolactone (**1**) ($C_{24}H_{32}O_4S$) was obtained from Jordan Pharmaceutical Manufacturer. Routine thin-layer chromatography was performed on Precoated TLC plates (silica gel, 20×20, 0.25 mm thick PF, Merck, Germany), and ceric sulfate solution was used as staining reagent. Column chromatography was performed on silica gel (70–230 mesh, Merck) for fractionation. Recycling preparative HPLC separation was performed on a JAI LC-908W instrument, equipped with YMC L-80 (4–5 μm, 20–50 mm i.d.) using MeOH-H_2O as the mobile phase, with UV detection at 254 nm. NMR spectra were recorded on Bruker Avance NMR spectrometers (Bruker, Spectrospin, Switzerland) at 400, 500 or 600 MHz in $CDCl_3$ or CD_3OD. HR-ESI mass spectra were recorded using a Bruker Daltonics Apex IV, 7.0T Ultra Shield Plus (Bruker Daltonics, Germany), and a JEOL JMS-600H (JEOL, Japan) mass spectrometer was used to record the electron impact mass spectra (EI-MS). Specific rotations were measured withMCP 200 polarometer (Germany). UV Spectra were measured in methanol on a Hitachi U-3200 spectrophotometer (Japan). Infrared (IR) spectra were measured using Shimudzu FT IR-8900 or Thermo-Nicolt Nexus 870FT-IR spectrophotometers (Japan). Melting points were measured without correction, using an Electrothermal (IA9300) digital melting point apparatus (Japan).

2.2 Microorganism

Fungi cultures were purchased from three international culture collection centers: American Type Culture Collection (ATCC), Northern Regional Research Laboratories (NRRL) and Tryptone Soya Yeast Extract (National Institute of Health Sciences, Tokyo-Japan) (TSY). The fungi used were *Aspergillus alliaceus* (ATCC 10060), *Aspergillus flavus* (ATCC 11489), *Aspegillus niger* (ATCC 1015), *Beauveriabassiana* (ATCC 7159), *Cunninghamella blakesleeana* (ATCC 8688A), *Cunninghamella elegans* (ATCC 36114), *Curvularia lunata* (ATCC 12017), *Fusarium lini* (NRRL 2204), *Gibberella fujikuroi* (ATCC 10704), and *Rhizopus stolonifer* (TSY 0471).

2.3 Preparation of media

Fungi were grown on Sabouraud dextrose agar (SDA), and preserved at 4 °C. The constituents of a suitable medium for each fungus (Table-1) were dissolved in 1.0 liter distilled water, and the pH was then adjusted by 0.1 M NaOH, and 0.1 M HCl solutions.

2.4 General screening protocol

Preparation of second generation culture: The media (100 mL) were transferred into conical flasks (250 mL), and autoclaved at 124 °C for 15 min. Three-day old slant fungal spores were added to the media under aseptic conditions, and incubated on a shaker (128 rpm) at 28 °C until a reasonable biomass was obtained. The mycelia thus formed were distributed into conical flasks containing 100 mL autoclaved media, and incubated on a shaker at 28 ^{o}C for a few days until a good biomass was obtained.

Addition of the substrate: The substrate was dissolved in 12 mL acetone (25 mg/mL), and added to flasks containing second stage culture, so that each flask contained about 25 mg of the substrate. For every screening study a positive control containing the substrate in the media without the fungus, and a negative control containing the fungus in the media without the substrate were prepared. The flasks were incubated in a shaker (128 rpm) at 28 °C.

Monitoring the biotransformation: A 10 mL sample of media was taken from the flask and filtered, and extracted with ethyl acetate. It was then evaporated, and reconstituted with 0.5 mL of chloroform, and tested by TLC. The TLC results were compared with the positive and negative controls, which were treated in the same way. The progress of the biotransformation was monitored every two days for a maximum of three weeks. Each of the ten fungi (Table-1) was screened for its ability to biotransform the three substrates under study. The results of the screening tests were re-produced on a larger scale to purify, and identify the biotransformation products.

2.5 Biotransformation of spironolactone (**1**)

Each of the ten fungi (Table-1) was screened for their ability to biotransform spironolactone (**1**) according to the above screening procedure. The results of the screening tests showed that four cultures, which include *Gibberella fujikuroi, Curvularialunata*, *Fusarium lini,* and *Aspergillus*

alliaceus, were suitable for biotransformation of compound **1**, and these tests were re-produced on a larger scale to separate, and identify the biotransformation products.

2.5.1 Biotransformation of spironolactone using *Gibberella fujikuroi*

Compound **1** (1,000 mg/ 20 mL) was distributed in 40 flasks, containing 3-day-old second generation culture of *G. fujikuroi,* and kept for 10 days. The mycelia were filtered and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The brown gummy material (1.1 g) obtained was subjected to silica gel column chromatography with gradient polarity of hexane: ethyl acetate mixtures of increasing polarity. Two main fractions were obtained and detected on thin layer chromatography (TLC). Fraction 1 (30 mg) yielded compound **1** (10 mg) by using preparative silica gel thin layer chromatography (precoated-TLC) (EtOAc: hexane 60:40 v/v) as a mobile phase. Fraction 2 (300 mg) yielded compounds **2** and **3** by using EtOAc: hexane (60:40, v/v) as a mobile phase. Compounds **2** (R_t: 49 min, 2 mg), and **3** (Rt: 41 min, 2 mg) were further purified by repeated RPHPLC (L-80, MeOH: H_2O = 70:30, 4 mL/min) (Figure 1).

2.5.2 Biotransformation of spironolactone (**1**) using *Curvularia lunata*

Compound **1** (750 mg/ 25 mL acetone) was distributed in 30 flasks containing 3 days old second generation culture of *C. lunata,* and kept for fermentation for 14 days. A crude brown gummy material (0.9 g) was obtained upon following the same steps mentioned above. The crude extract was then chromatographed over the silica gel column by elution with gradient hexanes-ethyl acetate solvent system which yielded three main fractions. Fraction 1 (300 mg) was purified to get spironolactone (**1**) (20 mg) using EtOAc: hexane 60:40 v/v) as a mobile phase, while fraction 2 (100 mg) was purified to yield metabolite **2** (1.5 mg) using EtOAc: hexane (60:40, v/v) as the mobile phase. Fraction 3 (100 mg) was stored in a freezer at -4 ^{o}C until it was finally purified by repeated RP-HPLC L-80, MeOH: H_2O = 70:30, 4 mL/min) with, 4 mL/ min) to obtained metabolite **4** (R_t: 19 min, 4 mg) (Figure 2).

2.5.3 Biotransformation of spironolactone using *Fusarium lini*

Spironolactone (**1**) (1,500 mg/ 30 mL acetone) and solution was distributed in 60 flasks containing three days old second generation culture of *F. lini*, and kept for fermentation for 14 days. The brown gummy material (1.1 g) obtained was subjected to silica gel column

chromatography with gradient polarity of hexane: ethyl acetate mixtures of increasing polarity. Four main fractions were obtained on the basis of TLC analysis. Each fraction was subjected to preprative-TLC. Fraction 1 (100 mg) was purified to obtain compound **1** (12 mg) using EtOAc: hexane (60:40, v/v) as a mobile phase, while fraction 2 was purified to obtain pure metabolites **2** (1 mg), and **3** (1 mg) using EtOAc: hexane 60:40 v/v as mobile phase. Fraction 3 (100 mg) yielded a mixture using MeOH: $CHCl_3$ (6: 94 with ammonia vapor, v/v), which was then subjected to repeated RP-HPLC (L-80, MeOH-H_2O = 70:30, 4 mL/min), to obtain metabolites **5** (R_t: 28 min, 3 mg), **6** (R_t: 23 min, 5 mg), and **7** (R_t: 21 min, 12 mg). Fraction 4 (35 mg) yielded metabolite **8**, using $CHCl_3$:CH_3OH (92:8 v/v with ammonia vapor) as a mobile phase, and further purified by RP-HPLC (L-80, MeOH-H_2O = 70:30, 4 mL/min), afforded metabolite **8** (R_t: 25 min, 5mg) (Figure 3).

2.5.4 Biotransformation of spironolactone (1) using *Aspergillus alliaceus*

Compound **1** (1,250 mg/ 25 mL of acetone) was distributed in 50 flasks containing 3-day-old second generation culture of *A. alliaceus*, and kept for fermentation for 10 days. The brown gummy material (1.5 g) was obtained after treating the mycelia, and media. Five main fractions were obtained on the basis of TLC analysis. Fraction 1 (50 mg), eluted from column at 30% ethyl acetate in hexane, consisted mainly of compound **1** (8 mg), while fraction 2 (100 mg), eluted from column at 35-40% ethyl acetate in hexane, consisted of metabolites **2** (1 mg) and **3** (1.5 mg). Fraction 3 (15 mg), eluted from column at 45% ethyl acetate in hexane, consisted of metabolite **4** (0.8 mg). Fraction 4 (75 mg), eluted from column at 60-65% ethyl acetate in hexane, was subjected to RP-HPLC using the solvent system MeOH: H_2O (70:30, v/v) on L-80 column, to obtain metabolites **5** (1 mg), **6** (1.3 mg), and **7** (1.4 mg). Fraction 5 (60 mg), eluted from column at 70% ethyl acetate in hexane, was also subjected to RP-HPLC, using the solvent system MeOH: H_2O (70:30, v/v) on L-80 column, to afford metabolite **8** (1.8 mg) (Figure 4).

2.6 Spectral data of the isolated compounds

The ^{1}H- and ^{13}C-NMR data of the isolated compounds are summerised in Tables-2 and -3, respectively. 2D-NMR experiments (HSQC, COSY, HMBC, and NOESY) were used to determine the structures of all metabolites. Other spectroscopic results are summerised as follows:

Spironolactone (**1**)

M.p. = 198–202 °C (lit. [14] 198-197 °C) $[\alpha]_D^{25}$ = –33.5°, **UV (MeOH) λ_{max} nm:** 240. **IR (KBr) ν_{max} cm^{-1}:**1768 (C=O), 1690 (C=C). ^{1}H-NMR ($CDCl_3$, 500 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 125 MHz): Table-4. **EI-MS (rel. int. %):** *m/z* 416.2 [M^+], $C_{24}H_{32}O_4S$. **HR-ESI-MS**: *m/z* 439.19123 [M+Na^+], ($C_{24}H_{32}O_4SNa$, calcd. 439.19135).

Canrenone (**2**)

M.p. = 148-150 ^{o}C (John, *et al* [15]148-150 °C). $[\alpha]_D^{25}$**:** -25.96^o ($CHCl_3$), **UV (MeOH) λ_{max} nm:** 283, **IR (KBr) ν_{max} cm^{-1}:** 1765 (C=O), 1654 (C=C).^{1}H-NMR ($CDCl_3$, 400 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 100 MHz): Table-3. **EI-MS (rel. int. %):** *m/z* 340.3 [M^+], $C_{22}H_{28}O_3$. **HR-ESI-MS:** *m/z* 363.19254 [M+Na]$^+$, ($C_{22}H_{28}O_3Na$, calcd. 363.19307).

1-Dehydrocanrenone (**3**)

M.p. = 135-139 ^{o}C (John, *et al* [15] 135-139 °C).$[\alpha]_D^{25}$**:** -27.11$^{o.}$ ($CHCl_3$), **UV (MeOH) λ_{max} nm:** 298 and 222. **IR (KBr) ν_{max} cm^{-1}:** 1769 (C=O), 1650 (C=C). ^{1}H-NMR ($CDCl_3$, 400 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 100 MHz): Table-3. **EI-MS (rel. int. %):** *m/z*338 [M^+], $C_{22}H_{26}O_3$.**HR-ESI-MS**: *m/z* 361.17708 [M+Na]$^+$, ($C_{22}H_{26}NaO_3$, calcd. 361.17742).

7α-Thio-spironolactone (**4**)

M.p. = 188-191 ^{o}C (Karim *et al* [16] 188-190 °C). $[\alpha]_D^{25}$**:** -26.96^o ($CHCl_3$). **UV (MeOH) λ_{max} nm:** 242. **IR (KBr) ν_{max} cm^{-1}:** 3416 (S-H), 1768 ((C=O), 1663 (C=C).^{1}H-NMR ($CDCl_3$, 600 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 150 MHz): Table-3. **EI-MS (rel. int. %):** *m/z*374.0 [M^+], $C_{22}H_{30}O_3S$. **HR-ESI-MS**: *m/z* 373.18420 [M-H]$^-$, ($C_{22}H_{29}O_3S$, calcd. 373.18429).

1β-Hydroxycanronene (**5**)

Amorphous, $[\alpha]_D^{25}$: -30.47^o ($CHCl_3$). **UV (MeOH) λ_{max} nm:** 283. **IR (KBr) ν_{max} cm^{-1}:** 3385 (O-H), 1738 (C=O), 1631(C=C). ^{1}H-NMR ($CDOD_3$, 500 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 125 MHz): Table-3. **EI-MS (rel. int. %):** *m/z* 356 [M^+]. **HR-ESI-MS**: *m/z* 379.18768 [M+ Na]$^+$, ($C_{22}H_{28}O_4Na$, calcd. 379.18798).

1α-Hydroxycanrenone (**6**)

Amorphous, $[\alpha]_D^{25}$: -27.52° ($CHCl_3$). **UV (MeOH) λ_{max} nm:** 283. **IR (KBr) ν_{max} cm^{-1}:** 3407 (O-H), 1750 (C=O), 1630 (C=C).^{1}H-NMR ($CDCl_3$, 400 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 100 MHz): Table-3. **EI-MS (rel. int. %):** *m/z* 356 [M^+]. **HR-ESI-MS:** *m/z* 379.18762 [M+ Na]$^+$, ($C_{22}H_{28}O_4Na$, calcd. 379.18798).**1-Dehydro-15α-hydroxycanrenone** (**7**)

Amorphous, $[\alpha]_D^{25}$: -26.24° ($CHCl_3$). **UV (MeOH) λ_{max} nm:** 298 and 222. **IR (KBr) ν_{max} cm^{-1}:** 3413 (C=O), 1766 (C=O),1652 (C=C)**.** ^{1}H-NMR ($CDOD_3$, 500 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 125 MHz): Table-3. **EI-MS (rel. int. %):** *m/z* 354 [M^+], $C_{22}H_{26}O_4$. **HR-ESI-MS**: *m/z* 377.17238 [M+ Na]$^+$, ($C_{22}H_{26}O_4Na$, calcd. 379.17233).

15α-Hydroxycanrenone (**8**)

M.p. = 234-238 °C.**$[\alpha]_D^{25}$:** -24.57° ($CHCl_3$). **UV (MeOH) λ_{max} nm:** 283.**IR (KBr) ν_{max} cm^{-1}:** 3396 (O-H), (1738C=O), 1646 (C=C).^{1}H-NMR ($CDCl_3$, 500 MHz): Table-2. ^{13}C-NMR ($CDCl_3$, 125 MHz): Table-3. **EI-MS (rel. int. %):** *m/z* 356.1 [M^+] , $C_{22}H_{28}O_4$. **HR-ESI-MS**: *m/z* 379.18762 [M+Na]$^+$, ($C_{22}H_{28}O_4Na$, calcd. 379.18798).

2.7 Assay for α-Glucosidase Inhibitory Activity

α-Glucosidase (E.C.3.2.1.20) enzyme inhibition assay was performed according to the slightly modified method of Matsui [17]. The activity was evaluated by using 0.1 M phosphate buffer (pH 6.8) at 37 °C. The enzyme (0.2 U/mL) in phosphate buffer saline was incubated with various concentrations of test compounds at 37 °C for 15 min. The substratep-nitrophenyl-α-d-glucopyranoside (0.7 mM) was added, and change in absorbance at 400 nm was monitored upto 30 min by multiplate reader. Test compound was replaced with DMSO (7% final) as control. Acarbose was used as the standard inhibitor. The IC_{50} value was calculated by using EZ- FIT5 software and the percent inhibition was calculated by the following formula:

% Inhibition = 100 - (OD test well / OD control) × 100.**3. Results and Discussion**

3.1 Biotransformation of spironolactone (**1**)

Ten different microorganisms (Table-1) were studied for their ability to transform spironolactone (**1**). The screening results showed that four fungi *A. alliaceus, G. fujikuroi, C. lunata*, and *F. lini,* were able to biotransform **1**. Large scale fermentation experiments were carried out in order to isolate metabolites **2-8**, produced as a result of whole cell biocatalysis of the enzymatic system of *Gibberella fujikuroi, Curvularia lunata*, *Fusarium lini,* and *Aspergillus alliaceus*.

Biotransformation of compound **1** with these four fungi resulted in the synthesis of three new (**5-7**), and four known metabolites **2**-**4**, and **8**.

Compound **1** was fully characterized by HR-ESI-MS, 1D- and 2D-NMR experimentsused as reference. The results of NMR assignments were in full agreement with the reported data [12].

HR-ESI-MS of **5** showed the $[M+Na]^+$ at *m/z* 379.18768 ($C_{22}H_{28}O_4Na$, calcd. 379.18798), indicating the presence of an additional oxygen, and removal of sulfur as compared to compound **1**. The IR spectrum showed absorption at 3385 cm^{-1}, indicating the presence of a hydroxyl group. The spectrum also showed the presence of carbonyls at 1738 cm^{-1}, and olefine C=C at 1630 cm^{-1}.

The ^{1}H- and^{13}C-NMR spectra of compound **5** showed, upon comparison with the spectra of **2**, the disappearance of a methylene signal and the appearance of a methine proton signal at δ 4.06 (dd, $J_{1ax,2ax}$= 12.5, $J_{1ax,2eq}$= 5.0 Hz) connected to a carbon resonating at δ 73.2, which indicated the presence of a hydroxyl group. The downfield shift of the methylene carbon at δ 43.9 (C-2) suggested that the hydroxyl group might be at C-1. Presence of OH group at C-1 was deduced from 2D-NMR. The COSY spectrum showed cross peaks between δ 4.06 (H-1) and 2.66, and 2.60 (H_2-2) (Figure 5). HMBC showed correlations of C-1 (δ 73.2) with 19-CH_3 (δ 1.14) and H_2-2 (δ 2.66, 2.60). In addition, in HMBC H_2-2 (δ 2.66, 2.60) also showed correlations with C-3 at δ 196.9 (Figure 5). The β (*equatorial*) stereochemistry of the newly introduced hydroxyl at C-1 in **5** was inferred from the multiplicity of H-1 at δ 4.06, which appeared as a dd with one larger coupling constant, $J_{1ax,2ax}$=12.5 Hz due to the *axial–axial,* and a small coupling constant ($J_{1ax,2eq}$= 5.0 Hz) due to the *axial-equatorial* coupling of protons. Finally, the stereochemistry of OH-1 was established from NOESY spectrum, which showed a correlation between H_{ax}-1(δ 4.06) and H_{ax}-9 (δ1.40) (Figure 6). The structure of metabolite **5** was thus deduced as 1β-hydroxycanrenone.

HR-ESI-MS of compound **6** showed the $[M+Na]^+$ at *m/z* 379.18762 ($C_{22}H_{28}O_4Na$, calcd. 379.18798) indicating the presence of an additional oxygen, and removal of sulfur compared to compound **1**. The IR spectrum showed absorption at 3407, 1750, and 1630 cm^{-1} due to the hydroxyl, carbonyl and olefinic functionalities, respectively. The ^{13}C-NMR ($CDCl_3$) spectrum of compound **6** showed 22 signals, similar to those of compound **5**. The ^{1}H-NMR spectrum showed an OH-bearing methine proton at δ 4.18 (1H, bs), connected to the carbon at δ 71.7. 2D-NMR experiments indicated the presence of the hydroxyl group at C-1. The COSY spectrum showed correlation of H-1 (δ 4.18) with H_2-2 (δ 2.58 and δ 2.88) (Figure 5). The HMBC spectrum

showed correlations of H-1 (δ 4.18) with C-19 (δ 17.8) and C-2 (δ 43.0). more H-2 (δ 2.58 and δ 2.88) also showed correlations with C-3 (δ 196.9) (Figure 5). The α-stereochemistry of OH-1 was inferred from the NOESY correlations between H_{eq}-1 (δ 4.18), and CH_3-19 (δ 1.13). This correlation was not seen in **5** due to different stereochemistry of OH-1 (Figure 6). Based on the above data, the structure of metabolite **6** was deduced as 1α-hydroxycanrenone.

Metabolite **7** showed the $[M+Na]^+$ at *m/z* 377.17238 ($C_{22}H_{26}O_4Na$, calcd 377.17233) in the HRESI-MS, indicating an additional oxygen and increased degree of unsaturation compared to compound **1**. IR spectrum showed absorptions at 3396, 1738, and 1646 cm^{-1} for hydroxyl, carbonyl, and olefinic functionalities, respectively. The ^{1}H-NMR spectrum showed a new resonance for an OH-bearing methine proton at δ 4.27 (1H, td, $J_{15ax,16ax/14ax}$= 10.0, $J_{15ax,16eq}$ =3.0 Hz) assigned to H-15. H-15 showed COSY correlation with H_2-16 (δ1.83 and 2.83) and H-14 (δ 1.40) (Figure 5), while in HMBC spectrum, the new hydroxylated methine carbon at δ 71.1 showed correlations with H-14 (δ 1.40) and H_2-16 (δ 1.83 and 2.83) (Figure 5). The α (*equatorial*) orientation of the newly introduced hydroxyl at C-15 was inferred from the multiplicity of H-15 signal at δ 4.40 as a triplet of doublet (td) with one large coupling constant due to coupling of the two di-*axial* coupling ($J_{15ax,16ax}$= 10.0 Hz) and a small coupling constant due to *axial–equatorial* coupling of protons ($J_{15ax,16eq}$=3.0 Hz). In addition to the multiplicity of H_{ax}-16 (δ 2.83) which appeared as a dd with two large coupling constants due to coupling of the two geminal protons ($J_{16ax,16eq}$=15.0 Hz), and due to coupling of the two di-*axial* protons ($J_{16ax,15ax}$ = 10.0 Hz). NOESY spectra showed correlations between H_{ax}-15 (δ 4.40) and H_3-18 (δ 1.06) and H_{ax}–8 (δ 2.52) indicated the α stereochemistry of H-15 (Figure 6). Based on the above mentioned spectral data, the structure of metabolite **7** was deduced as 1-dehydro-15α-hydroxycanrenone.

Compounds **2-4**, and **8** were characterized as known compounds based on detailed spectral analyses. Canrenone (**2**) was reported earlier by Chen *et al.* [18]. It is worth mentioning that canrenone (**2**) is the key intermediate in the synthesis of spironolactone, a widely used aldosterone antagonist diuretic in the clinic [19]; in addition, canrenone (**2**) is one of the main known metabolites of spironolactone in rat serum after oral administration of the drug [20]. 1-Dehydrocanrenone (**3**), a known synthetic steroid [14], is used to synthesize 2-oxasteroids, which are used to enhance the antiandrogenic activity [21]. Although 1-dehydrocanrenone (**3**) is known

as a synthetic steroid, but it is not known among the reported mammalian metabolites of spironolactone. Thus, this is the first report of conversion of **1** to 1-dehydrocanrenone using whole cell microbial transformation. The spectral data of this compound are reported for the first time, and compared with the values of similar compounds [22]. The ^{1}H- and ^{13}C-NMR chemical shifts of different hydrogen and carbon atoms of **4** are in full agreement with literature data [18]. 7α-Thio-spironolactone (**4**) is known as an intermediate steroid in synthesis of spironolactone (**1**). Moreover, spironolactone (**1**) and its metabolite 7α-thio-spironolactone (**4**) were also determined in pediatric plasma samples by HPLC method [23]. 15α-Hydroxycanrenone (**8**) was first reported previously as a biotransformation product of canrenone (**2**) using *Aspergillus ochraceus,* and by efficient hydroxylation of functionalized steroids by *Colletotrichum lini* ST-1 without determination of the exact stereochemistry of the OH-15 group [24, 25].

3.2 α-Glucosidase Inhibitory Activity

Spironolactone (**1**) and its metabolites **2**, **3**, and **5-8** were tested for α-glucosidase inhibitory activity *in vitro*. Results indicated that only **1** possess a strong activity with IC_{50} = 335 ± 4.3 µM, as compared to the standard drug acabose (IC_{50} =840±1.73 µM), while its metabolites were found inactive in this assay.

4.0 Conclusion

In conclusion, we report here an efficient method for the transformation of a diuretic and antihypertensive drug spironolactone (**1**) by using various fungal cultures. Biotransformation of compound **1** with these four fungi resulted in the production of three new **5-7**, and four known metabolites **2**-**4**, and **8**. Compounds were also evaluated for α-glucosidase inhibitory activity *in vitro*, as a result compound **1** was identified as a strong inhibitor with IC_{50} = 335 ± 4.3µM, as compared to the standard drug acabose IC_{50} = 840 ± 1.73 µM, while its metabolites were found to be inactive in this assay. This strategy can be used effectively for the synthesis of libraries of new derivatives of spironolactone (**1**).

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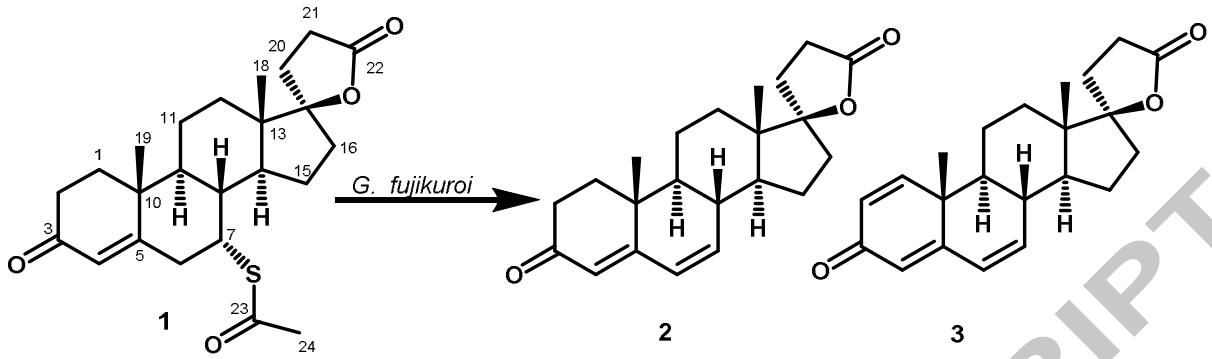


Figure 1: Biotransformation of spironolactone by *Gibberella fujikuroi.*

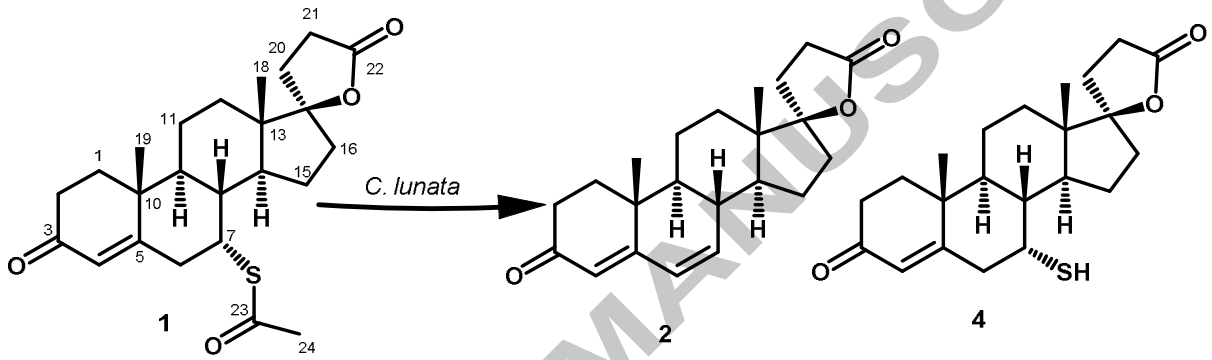


Figure 2: Biotransformation of spironolactone by *Curvularia lunata.*

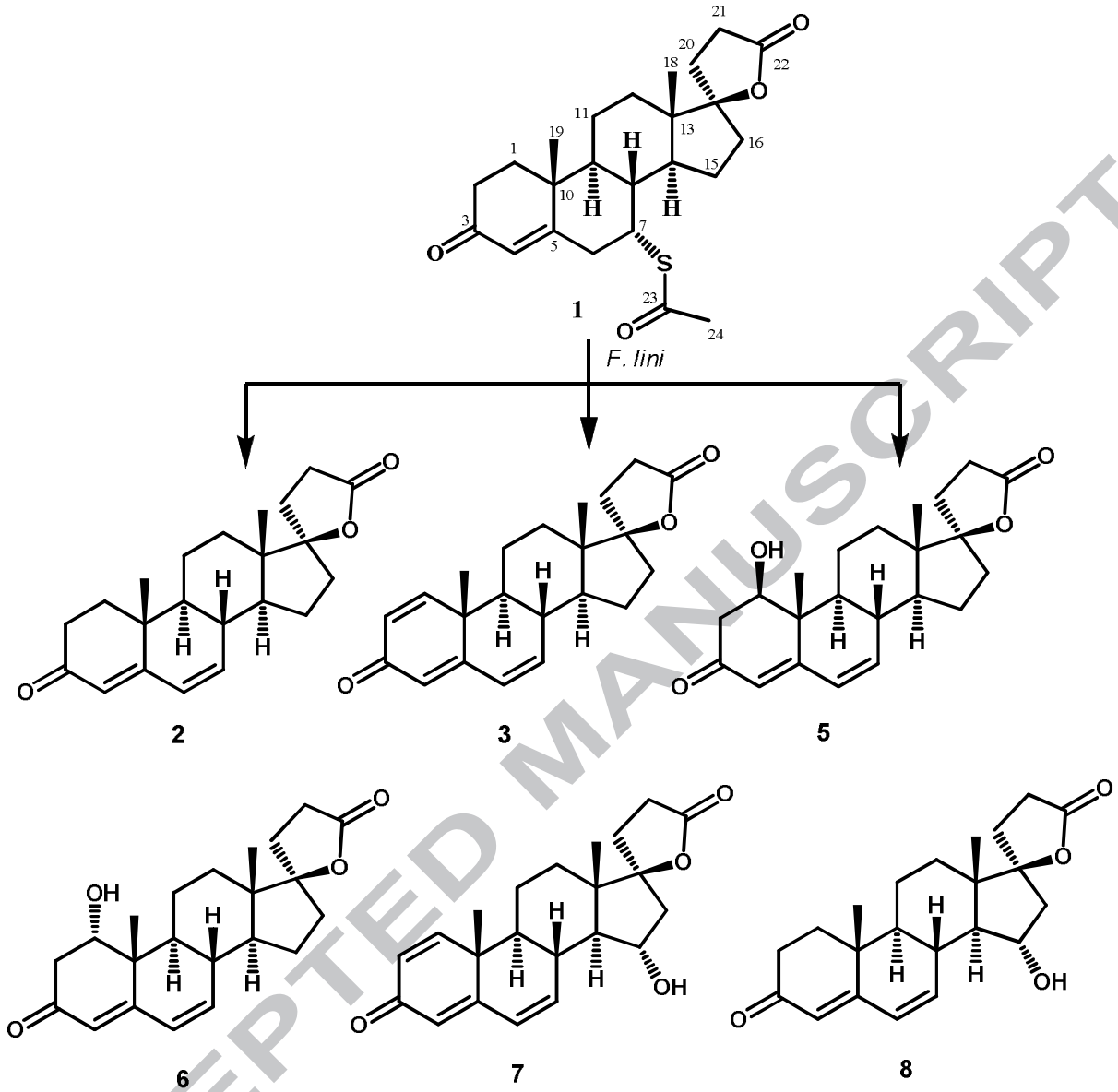
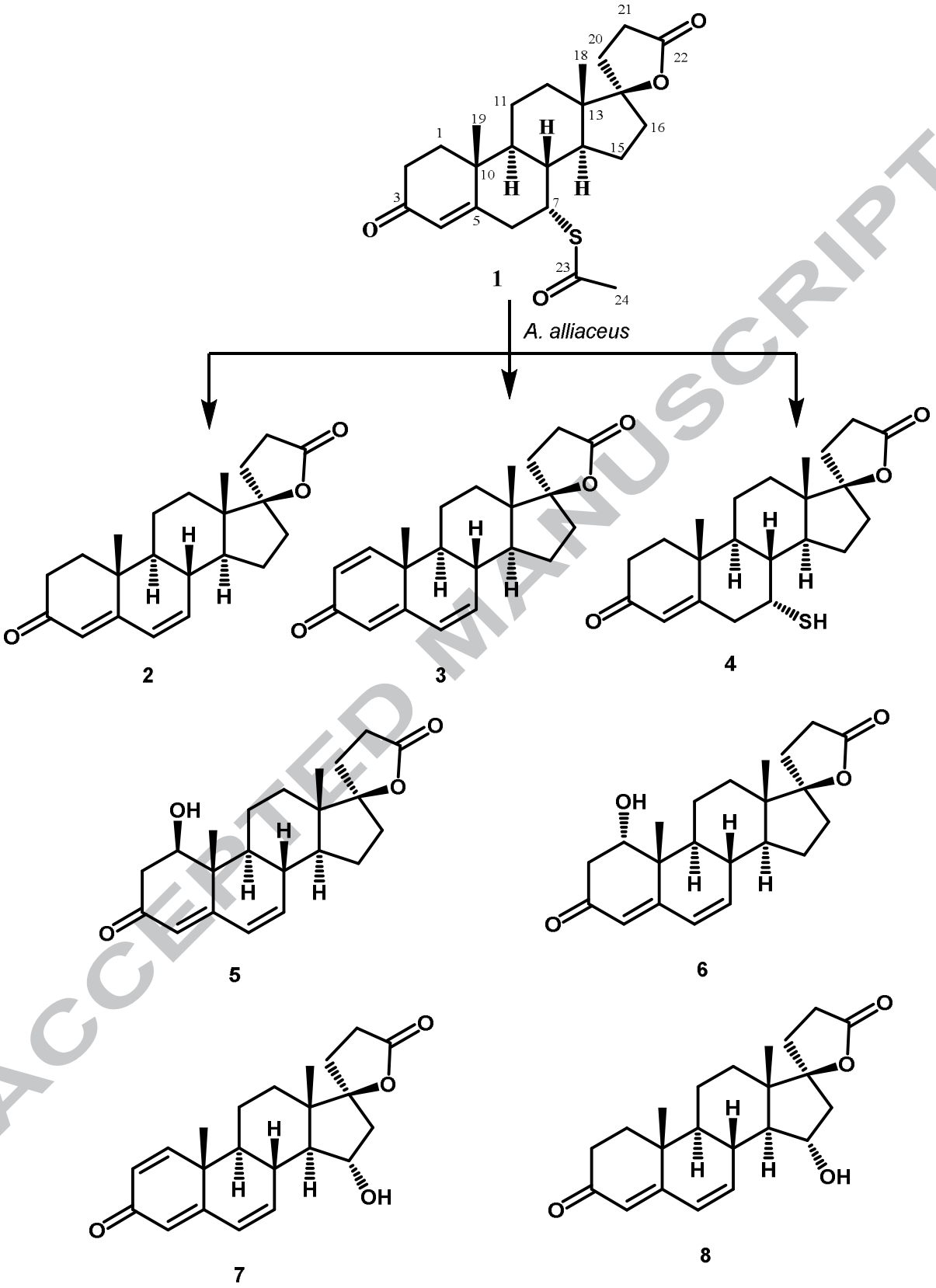


Figure 3: Biotransformation of spironolactone by *Fusarium lini*.



1

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Figure 4: Biotransformation of spironolactone by *Aspergillus alliaceus*.

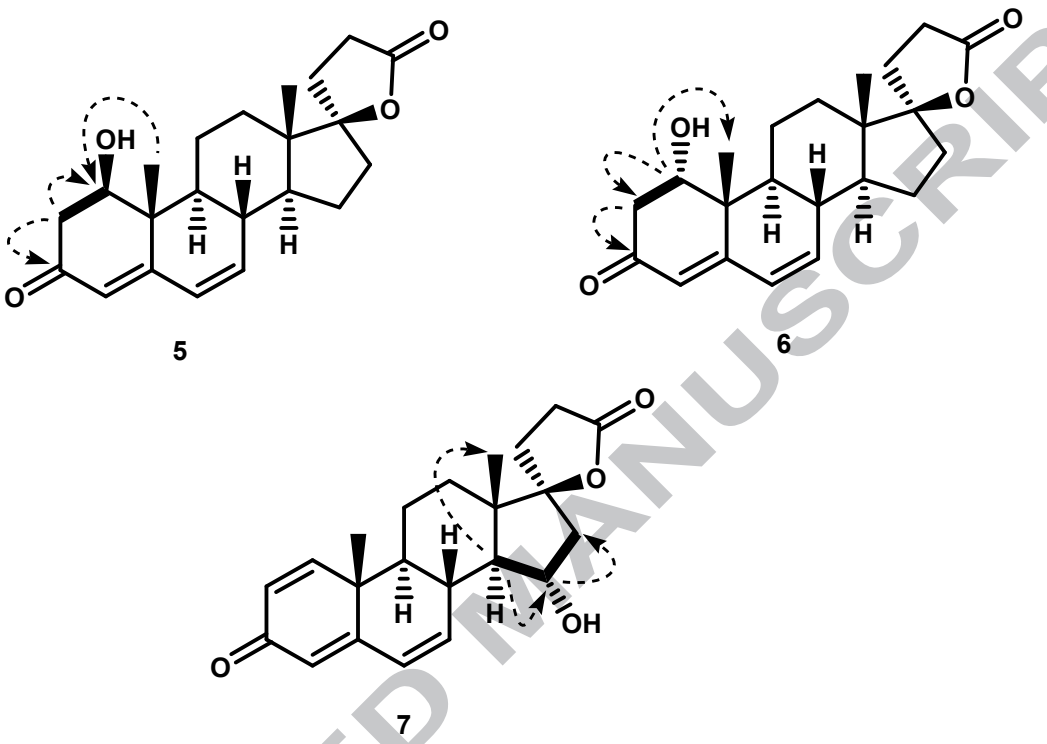


Figure 5: Key COSY and HMBC correlations in metabolites 5-7.

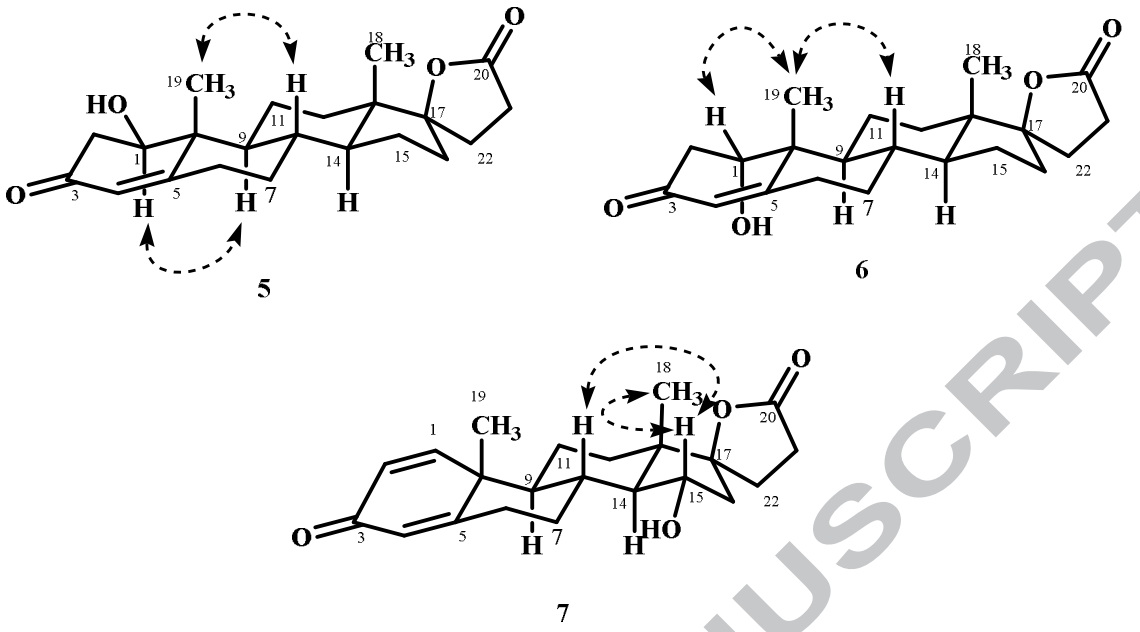


Figure 6: Key NOESY correlations in metabolites 5-7.

Table-1: Constituents of the media for each fungus.

Fungi	**Constituents of the media/ L solution**
Rhizopus stolonifer (TSY 0471)	Glucose (20.0 g), peptone (5.0 g), yeast extract (3.0 g), KH_2PO_4 (5.0 g), pH= 5.6**.**
Fusarium lini (NRRL 68751), *Cunninghamella blakesleeana* (ATCC 8688A), *Buvaria bassiana* (ATCC 7159), *Curvularia lunata* (ATCC 12017), *Cunninghamella elegans* (ATCC 36114)	Glucose (10.0 g), glycerol (10.0 mL), peptone (5.0 g), yeast extract (5.0 g), KH_2PO_4 (5.0 g), NaCl (5.0 g), pH= 7.6
Aspergillus alliaceus (ATCC 10060), *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 11489)	Glucose (10.0 g), glycerol (10.0 mL), peptone (5.0 g), yeast extract (5.0 g), KH_2PO_4 (5.0 g), NaCl (5.0 g), pH= 7.4.
Gibberella fujikuroi (ATCC 10704)	Glucose (30.0 g), glycerol (10.0 mL), peptone (5.0 g), Yeast extract (5.0 g), KH_2PO_4 (5.0 g), NaCl (5.0 g), pH= 6.4.

Table 2: ^{1}H-NMR chemical shift data of spironolactone (**1**) and its metabolites **2–8**.

Carbon	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[f]	8[e]
1	1.67, m; 2.03, m	1.71, m; 2.00, m	7.03, d, *J*=10.0	1.73, m; 2.03, m	4.06, dd, *J*=12.5,5.0	4.18, bs	7.07, d, *J*=10.0	1.71, m; 2.00, m
2	2.33, m; 2.37, m	2.42, m; 2.54, m	6.24, d, *J*=10.0	2.36, m; 2.43, m	2.60, m; 2.66, m	2.58, m; 2.88, m	6.27, dd, *J*=10.0, 1.5	2.42, m; 2.54, m
3	-	-	-	-	-	-	-	-
4	5.65, s	5.66, bs	5.99, bs	5.62, s	5.72, bs	5.77, bs	6.00, bs	5.70, bs
5	-	-	-	-	-	-	-	-
6	2.35, m; 2.80, m	6.12, dd, *J*=10.0, 2.4	6.25, d, *J*=10.0	2.42, m; 2.75, m	6.14, d, *J* =10	6.19, dd, *J*=10.0, 3.0	6.23, dd, *J*=10.0, 2.5	6.13, dd, *J*=10.0. 3.0
7	3.95,	6.06, dd, *J*=10.0,1.6	5.97, d, *J*=10.0	2.91, m	6.07, d, *J* =10	6.09, dd, *J*=10.0, 1.5	6.45, dd, *J*=10.0, 2.0	6.55, dd, *J*=10.0, 1.5
8	1.98, m	2.29, m	2.35, m	1.92, m	2.32, m	2.28, m	2.52, m	2.47, m
9	0.98, m	1.17, m	1.36, m	1.25, m	1.40, m	1.74, m	1.43, m	1.22, m
10	-	-	-	-	-	-	-	-
11	1.43, m; 1.61, m	1.45, m; 1.65, m	1.66, m; 1.87,	1.43, m; 1.61, m	1.64, m; 2.12, m	1.47, m; 1.77, m	1.66, m; 1.87, m	1.43, m; 1.67, m
12	1.28, m; 1.53, m	1.36, m;1.62, m	1.35, m; 1.65,	1.28, m; 1.54, m	1.60, m; 1.66, m	1.43, m; 1.63, m	1.45, m; 1.63	1.46, m; 1.57, m
13	-	-	-	-	-	-	-	-
14	1.37, m	1.34, m	1.42, m	1.65, m	1.35, m	1.40, m	1.40, m	1.38, m
15	1.36, m; 1.53, m	1.56, m; 1.84, m	1.57, m; 1.88,	1.36, m; 1.65, m	1.57, m; 1.88, m	1.57, m; 1.88, m	4.40, dd, *J*= 10.0, 3.0	4.33, m
16	1.78, m; 2.19, m	1.88, m; 2.32, m	1.88, m; 2.31, m	1.70, m; 2.24, m	1.85, m; 2.27, m	1.86, m; 2.31, m	1.83, m; 2.88, dd, *J*=15.0. 10.0	1.78dd, *J*=15.0. 3.5 2.84dd, *J*=15.0. 9.5
17	-	-	-	-	-	-	-	-
18	0.94, s	1.02, s	1.05, s	0.96, s	1.02, s	1.02	1.06, s	1.02, s
19	1.18, s	1.11, s	1.20, s	1.20	1.14, s	1.13	1.22, s	1.12, s
20	1.89, m; 2.33, m	1.89, m; 2.35, m	1.90, m; 2.37,	1.94, 2.29	1.90, m; 2.37, m	1.90, m; 2.37, m	2.07, m; 2.46, m	2.09, m; 2.49, m
21	2.45, m; 2.45, m	2.48, m; 2.54, m	2.45, m; 2.55,	2.42, 2.60	2.47, m; 2.58, m	2.48, m; 2.54, m	2.50, m; 2.57, m	2.50, m; 2.57, m
22	-	-	-	-	-	-	-	-
23- SCO	-	-	-	-	-	-	-	-
24- SCOCH	2.28, s	-	-	-	-	-	-	-

[a]500 MHz, CDCl3, [b]400 MHz, $CDCl_3$, [c]300 MHz, $CDCl_3$
[d]600 MHz, $CDCl_3$, [e]500 MHz, MeOD, [f]500 MHz, $CDCl_3$

Table-3: ^{13}C-NMR chemical shift data of spironolactone (**1**) and its metabolites **2–8**.

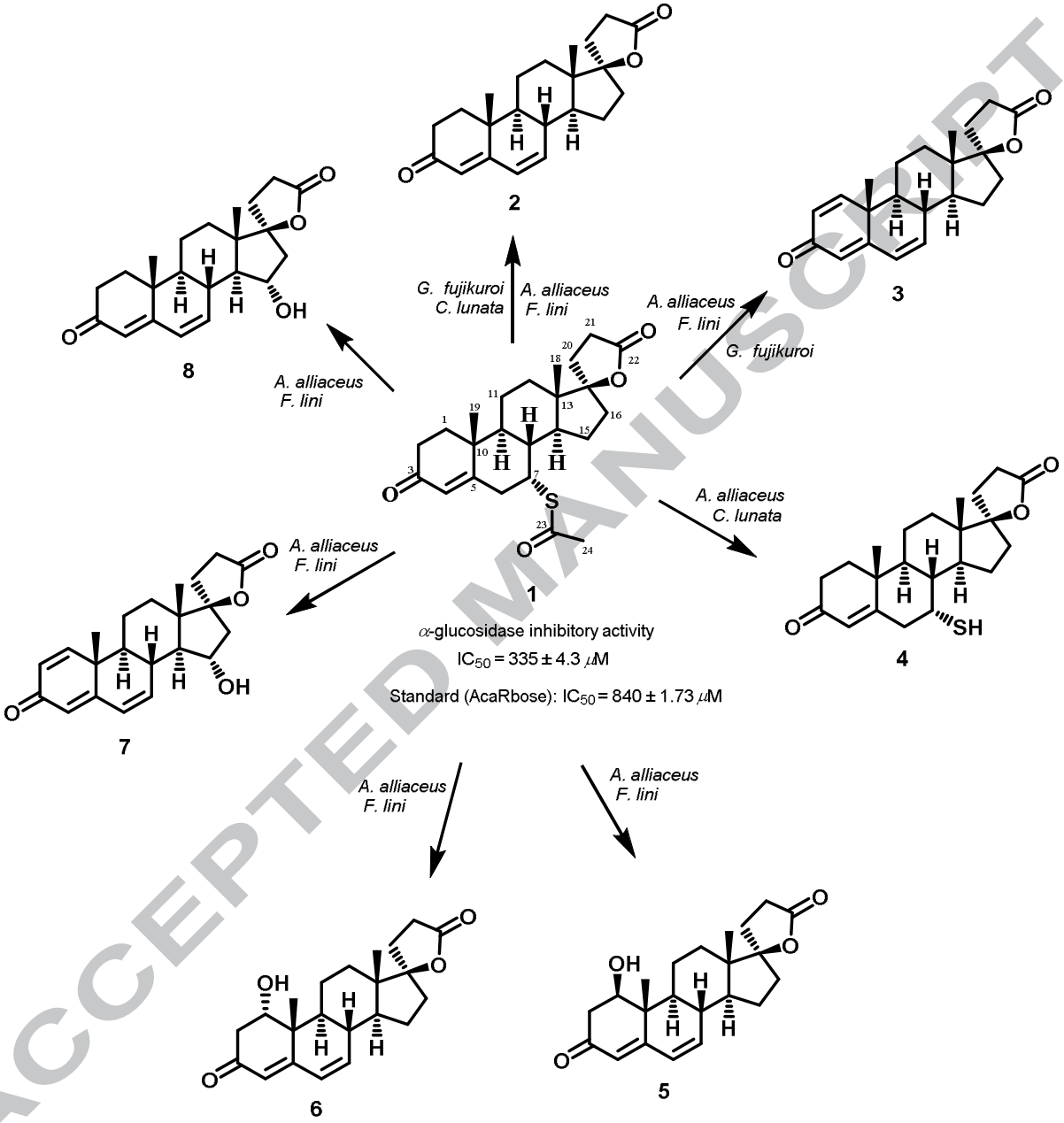
Carbon	1[b]	2[a]	3[a]	4[c]	5[b]	6[a]	7[c]	8[c]
1	35.5	34.0	152.5	35.7	73.2	71.7	152.4	34.0
2	33.8	33.9	128.3	34.0	43.9	43.0	127.8	33.9
3	198.5	199.3	186.2	198.5	196.9	196.4	186.2	199.4
4	126.9	124.0	124.2	127.0	124.3	123.4	123.9	124.0
5	165.6	163.0	161.8	166.8	162.0	159.2	161.8	162.7
6	39.8	128.3	128.2	39.7	128.3	128.8	128.3	128.0
7	45.0	139.3	136.7	45.1	139.7	139.9	137.3	139.9
8	38.8	37.8	38.3	40.2	38.3	37.7	37.7	37.3
9	49.2	50.4	47.2	46.9	51.2	43.6	48.0	50.3
10	38.4	36.0	41.0	38.6	42.0	41.0	41.0	36.0
11	20.4	20.1	21.3	20.3	22.7	19.6	21.2	20.0
12	31.0	31.1	31.1	31.0	31.9	31.5	31.6	31.7
13	45.4	46.	46.1	45.2	45.8	46.6	46.8	47.3
14	45.9	47.0	48.1	44.6	47.0	47.0	55.0	54.8
15	22.2	22.5	22.4	23.0	23.2	22.5	71.1	71.3
16	35.0	35.5	35.4	35.0	35.5	35.5	47.5	47.6
17	95.4	95.3	95.4	95.5	95.4	95.4	92.8	92.9
18	14.5	14.4	14.5	14.7	14.4	14.4	16.1	16.0
19	17.7	16.3	20.7	17.8	10.9	17.8	20.7	16.4
20	31.1	31.6	31.6	30.9	31.1	31.2	31.2	31.2
21	29.1	29.2	29.2	29.2	29.2	29.3	29.1	29.1
22	176.4	176.5	176.4	176.9	176.6	176.6	176.1	176.2
23- S<u>C</u>O	194.3	-	-	-	-	-	-	-
24- SCO<u>C</u>H$_3$	31.3	-	-	-	-	-	-	-

[a]100 MHz, $CDCl_3$
[b]125 MHz, $CDCl_3$
[c]150 MHz, $CDCl_3$

Table-4: α-Glucosidase Inhibitory Activity of Spironolactone (**1**) and its Metabolites.

Compounds	**% Inhibition**	**IC_{50}+SEM [µM]**
1	82.8	335±4.3
2	27.8	-
3	36.1	-
4	-	-
5	10.1	-
6	11.8	-
7	-31.8	-
8	-0.6	-
Standard (Acarbose)	59.1	840±1.73

Graphical Abstract



Highlights

- Biotransformation of spironolactone (**1**) yielded 3 new and 4 known metabolites.
- α-Glucosidase inhibitory activity of substrate **1** and metabolites were evaluated.
- Compound **1** was found to be potent α-glucosidase inhibitor (IC_{50} = 335±4.3 μM).
- The standard drug (acarbose) has shown IC_{50} =840±1.73 μM with α-glucosidase.