CHAPTER III

EXPERIMENTS

3.1 Plant Material.

The stem bark of *C. oblongifolius* (Plao-yai) was collected from Amphur Kuiburi, Prachuab Kirikhan Province, Thailand, in August 2003. The plant specimen was authenticated by comparison with a voucher specimen (No. BKF 084729) in the herbarium collection of the Royal Forest Department of Thailand [8]. Another specimen of this plant was collected in Loei Province, Thailand by Chaiyo Chaichantipyuth, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University [9].

3.2 Instruments and Equipments.

3.2.1. Fourier Transform Infrared Spectrophotometer (FT-IR).

The FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer and on Perkin-Elmer 399 spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet.

3.2.2. Optical rotation.

The optical rotations were measured on a Perkin-Elmer 341 and a Perkin-Elmer 241 spectropolarimeter, using a sodium lamp at wavelength 589 nm in chloroform and methanol.

3.2.3. Nuclear Magnetic Resonance Spectrometer (NMR).

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 200.13 and 50.32 MHz, respectively, on a Bruker Model AC-F200 Spectrometer and 400 and 100 MHz, respectively, on a Varian Mercury + 400 NMR Spectrometer. Moreover, ^{1}H-NMR spectra were acquired in $CDCl_3$ solution at 250 and 500 MHz on Bruker AC 250 FT or AM 500 FT spectrometers. ^{13}C-NMR spectra were determined at 125 MHz and assignments were made by polarization transfer using a DEPT135 sequence. Chemical shifts are expressed in parts per million (ppm) using residual protonated solvents as reference. COSY, NOESY, HSQC and HMBC experiments were performed on the Varian Mercury + 400 NMR Spectrometer and Bruker AM 500 FT spectrometer.

3.2.4. Mass Spectrometer (MS).

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV and a LC-MS system (Agilent 1100 HPLC-system connected to Aglient 1100 mass spectrometer) with electrospray ionization in positive mode (ESP+). The HRESIMS were performed on a micromass, Q-tof micro mass spectrometer. Incubation course was monitored by GC-MS on a Hewlett-Packard 5989B instrument, using a 25 m x 0.2 mm Ultra 2 capillary column (temperature programmed from 110 to 270 °C at 8 °C min^{-1}). High resolution mass spectrometry (HRMS) was performed on a JEOL MS700 spectrometry.

3.2.5. X-ray Diffractometer.

The X-ray diffractometer were obtained on a BRUKER SMART CCD diffractometer at Department of Physic, Faculty of Science and Technology, Thammasart University and on a SIEMENS SMART diffractometer at Department of Chemistry, Faculty of Science, Prince of Songkla University.

3.2.6. Melting point apparatus.

All melting points were determined with a Fisher-Johns melting point apparatus and a Reichert microscope and uncorr.

3.3 Chemicals.

3.3.1. Solvents.

All solvents used in this research such as hexane, ethyl acetate, methanol were commercial grade and were purified prior to use by distillation.

3.3.2. Other chemicals.

3.3.2.1 Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) and Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) was used as adsorbent for column chromatography.

3.3.2.2 Merck's TLC aluminium sheet, silica gel 60F 254 precoated 25 sheets, 20x20 cm^2, layer 0.2 mm. was used to identify the identical fractions and was developed using a suitable solvent system.

3.4 Culture media.

Potato Dextrose Agar (PDA, solid medium I) was used for storage of microorganism. In all transformation experiments, Soy bean Meal Glucose (SMG,

liquid medium I) was used in experimental section I. All fungi were maintained and sporulated on agar slants (solid medium II) and grown in Corn Steep Liquor Glucose (liquid medium II). All Streptomyces were grown in liquid medium III in experimental section II. The medium for growing tested bacteria was nutrient medium (agar and broth). Sabouraud medium (agar and broth) was used for growing tested yeast. The media's formula was shown in appendix A.

3.5 Microorganisms.

Absidia blakesleeana TISTR 3459, *Rhizopus oligosporus* TISTR 3001 and *Aspergillus ficuum* TISTR 3133 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand in experimental section I.

Microorganism strains obtained from various international collections and TISTR were maintained at the Laboratoire of Chimie and Biochimie Pharmacologiques and Toxicologiques, Paris, France. Forty microorganisms in experimental section II were used for the preliminary screening as follows:

1. *Aspergillus niger ATCC 9142*
2. *Rhizopus arrhizus ATCC 11145*
3. *Mucor plumbeus ATCC 4720*
4. *Cunninghamella elegans ATCC 36112*
5. *Cunninghamella echinulata NRRL 3655*
6. *Chaetomium indicum LCP98-4200*
7. *Absidia blakesleeana ATCC 6811*
8. *Verticillium lecanii IMI 068689*
9. *Septomyxa affinis ATCC 6768*
10. *Curvularia lunata NRRL 2380*
11. *Cunninghamella elegans TISTR 3370*
12. *Aspergillus niger TISTR 3254*
13. *Syncephalastrum racemosum TISTR 3457*
14. *Rhizopus oligosporus TISTR 3001*
15. *Absidia blakesleeana TISTR 3459*
16. *Cunninghamella elegans ATCC 26269*
17. *Cunninghamella echinulata ATCC 9245*
18. *Mortierella isabellina NRRL 1757*
19. *Rhizopus stolonifer ATCC 62276*

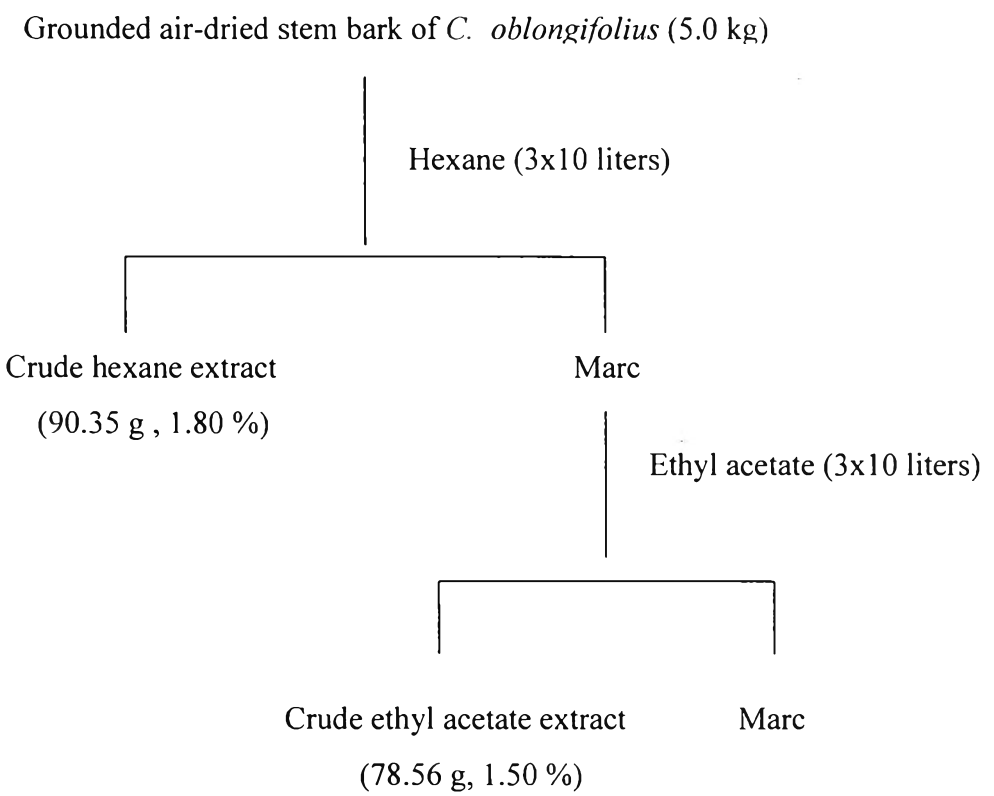
20. *Absidia corymbifera LCP 63-1800*
21. *Streptomyces albus ATCC 21838*
22. *Streptomyces albus DSM 41398*
23. *Streptomyces antibioticus MUCL 8167*
24. *Streptomyces argenteolus LMG 5967*
25. *Streptomyces aureofaciens ATCC 10762*
26. *Streptomyces candidus NRRL 5449*
27. *Streptomyces cinnamonensis ATCC 15413*
28. *Streptomyces coelicolor A (3)2*
29. *Streptomyces fradiae NRRL B 1195*
30. *Streptomyces griseolus ATCC 3325*
31. *Streptomyces griseus NRRL B 150*
32. *Streptomyces malachito ATCC 31547*
33. *Streptomyces clivaceus ATCC 1125*
34. *Streptomyces paucisporogenes LMG 5983*
35. *Streptomyces platensis NRRL 2364*
36. *Streptomyces punipalus NRRL 3529*
37. *Streptomyces rimosus NRRL 2234*
38. *Streptomyces sp. ATCC 55293*
39. *Streptomyces sp. ATCC 3351*
40. *Streptomyces violaceoruber DSM 40783*

3.6 Extraction and isolation.

The powdered, sun-dried stem bark (5.0 kg) of *C. oblongifolius* was collected from Amphur Kuiburi, Prachuab Kirikhan Province and was extracted with hexane (3x10 liters) and ethyl acetate (3x10 liters) at room temperature, respectively. The solution was filtered and evaporated under reduced pressure until dry to obtain crude hexane extract (90.35 g) and crude ethyl acetate extract (78.56 g), respectively. The crude extracts of the stem bark of *C. oblongifolius* are shown in Table 3.1 and the extraction procedures are shown in Scheme 3.1.

Table 3.1 The solvent extraction of the stem bark of *C. oblongifolius*.

Solvent extracted	Appearance	Weight (g)	%wt by wt of the dried stem bark
hexane	yellowish green oil	90.35	1.8
ethyl acetate	dark brown oil	78.56	1.5



Scheme 3.1 The procedure of extraction of the stem barks of *C. oblongifolius*.

3.7 Separation of crude extract of the stem bark of *C. oblongifolius* (Amphur Kuiburi, Prachuab Kirikhan Province).

3.7.1 Separation of hexane crude extract.

The crude hexane extract was obtained as yellowish green oil after evaporation. The crude hexane extract (90.0 g) was fractionated by silica gel column chromatography using Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) (300.0 g) as absorbent. The column was eluted with an increasing gradient of hexane,

hexane in ethyl acetate, ethyl acetate and finally methanol in ethyl acetate (approximately 50 ml per fraction) for 120 fractions. Each fraction was analyzed by TLC. The separation of crude hexane extract gave compound 1 (15.28 g).

3.7.2 Separation of ethyl acetate crude extract.

The ethyl acetate crude extract (70.0 g) was separated on silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) (300.0 g) using the column chromatographic technique. The column was eluted with an increasing gradient of hexane in ethyl acetate, ethyl acetate and finally methanol in ethyl acetate (approximately 50 ml per fraction) for 80 fractions. Each fraction was analyzed by TLC. The separation of crude ethyl acetate extract gave compound 1 (8.56 g).

Table 3.2 The results of separation of crude hexane and ethyl acetate extracts by column chromatography.

Crude extract	Weight Column (g)	Crystal (g) Compound 1	% wt/wt
Hexane	90	15.28	0.31
Ethyl acetate	70	8.56	0.17

3.8 Separation of crude extract of the stem bark of *C. oblongifolius* (Loei Province).

3.8.1 Separation of the partially crude hexane extract.

The crude hexane extract was obtained as white powder. The crude hexane extract (20.0 g) was fractionated by silica gel column chromatography using Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) (300.0 g) as absorbent. The column was eluted with an increasing gradient of hexane, hexane in ethyl acetate, and finally ethyl acetate (approximately 20 ml per fraction) for 100 fractions. Each fraction was analyzed by TLC. The separation of crude hexane extract gave compounds 2-4 as shown in Table 3.3.

Table 3.3 The results of separation of the crude hexane extract by column chromatography.

Compounds	Physical appearance	Weight (g)
2	white crystal	16.45
3	white solid	0.18
4	white solid	1.57

3.9 Purification and properties of the isolated compound from *C. oblongifolius*.

3.9.1 Purification and properties of compound 1.

The compound 1 was eluted with 10% ethyl acetate in hexane on silica gel chromatography. This compound was a white crystalline solid having melting point at 171-172 °C. It was soluble in various solvents such as hexane, ethyl acetate, chloroform, methanol and ethanol.

Compound 1 was colorless crystals (15.28 g, 0.31%), $[\alpha]_D^{25}$ -103.5° ($CHCl_3$; *c* 1.0).

FT-IR spectrum (KBr) (Fig. B1, Table 4.1) ν_{max} (cm^{-1}): 3500-2400, 2959, 2856, 1682, 1625, 1456, 1409, 1258, 1164 and 871.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B2, Table 4.2) δ (ppm): 4.82 (1H, s, H-17b), 4.76 (1H, s, H-17a), 2.65 (1H, s, H-13), 2.18 (1H, d, *J*=14, H-3b), 2.06 (1H, m, H-15a), 2.04 (1H, d, *J*=2, H-15b), 2.03 (1H, dd, *J*=2, 11, H-14b), 1.94 (1H, m, H-6b), 1.92 (1H, m, H-2b), 1.91 (1H, m, H-2a), 1.87 (1H, d, *J*=3, H-6a), 1.66 (1H, m, H-12b), 1.62 (1H, m, H-11a), 1.61 (1H, m, H-11b), 1.56 (1H, m, H-7a), 1.49 (1H, m, H-12a), 1.46 (1H, m, H-7b), 1.22 (3H, s, H-18), 1.16 (1H, dd, *J*=5, 11, H-14a), 1.12 (1H, d, *J*=5, H-3a), 1.09 (1H, d, *J*=7, H-5), 1.05 (1H, d, *J*=7, H-9), 1.01 (3H, s, H-20), 0.88 (1H, m, H-1a), 0.87 (1H, d, *J*=4, H-1b).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.) (Fig. B3, Table 4.3) δ (ppm): 184.9 (s), 155.4 (s), 102.3 (t), 56.8 (d), 55.1 (d), 48.7 (t), 44.2 (s), 43.8 (d), 43.0 (s), 41.1 (t), 40.7 (t), 39.4 (s), 39.4 (t), 37.8 (t), 32.8 (t), 28.1 (q), 21.7 (t), 18.9 (t), 18.0 (t), and 14.9 (q).

EI MS spectrum (Fig. B4) *m/z*: 302 [M^+], 275 (40), 259 (42), 243 (40), 213 (30), ,187 (20), 158 (40), 131 (80), 116 (90), 91 (100), 79 (55), and 55 (30).

3.9.2 Purification and properties of compound 2.

The compound 2 was eluted with 20% ethyl acetate in hexane on silica gel chromatography. This compound was a white crystalline solid having melting point at 147-148 °C. It was soluble in various solvents such as hexane, ethyl acetate, chloroform, methanol and ethanol.

Compound 2 was colorless crystals (16.45 g, 82.25%), $[\alpha]_D^{25}$ -53.6° ($CHCl_3$; *c* 1.0).

FT-IR spectrum (KBr) (Fig. B5, Table 4.4) ν_{max} (cm^{-1}): 2930, 2875, 1655, 1615, 1460, 1380, 1260, 1120 and 920.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B6, Table 4.5) δ (ppm): 7.10 (1H, d, *J* = 10, H-1), 5.89 (1H, dd, *J* = 17, 10, H-14), 5.86 (1H, d, *J* = 10, H-2), 5.16 (1H, dd, *J* = 17, 1, H-15b), 4.94 (1H, dd, *J* = 10, 1, H-15a), 1.92 (1H, m, H-7b), 1.90 (1H, m, H-12b), 1.84 (1H, m, H-11b), 1.78 (1H, m, H-5), 1.72 (1H, m, H-12a), 1.70 (2H, m, H-6b, H-11a), 1.62 (1H, m, H-9), 1.53 (1H, m, H-7a), 1.52 (1H, m, H-6a), 1.38 (3H, s, H-17), 1.32 (3H, s, H-16), 1.16 (3H, s, H-18), 1.08 (3H, s, H-19) and 1.05 (3H, s, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.) (Fig. B7, Table 4.6) δ (ppm): 205.7 (s), 157.9 (d), 147.8 (d), 126.2 (d), 111.1 (t), 75.4 (s), 74.1 (s), 53.7 (d), 50.4 (d), 45.0 (t), 42.7 (s), 39.8 (s), 35.8 (t), 29.0 (q), 28.0 (q), 26.0 (q), 21.7 (q), 20.6 (t), 15.9 (t), and 15.9 (q).

EI MS spectrum (Fig. B8) *m/z*: 302 [M^+], 287 (100), 269 (10), 204 (25), 149 (25), 135 (35), 108 (26), 91 (35) and 55 (37).

3.9.3 Purification and properties of compound 3.

The compound 3 was eluted with 40% ethyl acetate in hexane on silica gel chromatography. This compound was a white solid having melting point at 124-125 °C. It was soluble in various solvents such as hexane, ethyl acetate, chloroform, methanol and ethanol.

Compound 3 was colorless crystals (0.18 g, 0.90%), $[\alpha]_D^{25}$ -66.8° ($CHCl_3$; *c* 1.0).

FT-IR spectrum (KBr) (Fig. B9, Table 4.7) ν_{max} (cm^{-1}): 3500-3200, 2938, 2867, 1712, 1508, 1258 and 1117.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B10, Table 4.8) δ (ppm): 5.91 (1H, dd, *J* = 18, 11, H-14), 5.18 (1H, dd, *J* = 18, 2, H-15b), 4.96 (1H, dd, *J* = 11, 2, H-15a), 3.92 (1H, t, *J* = 6, H-1), 2.95 (1H, dd, *J* = 15, 8, H-2b), 2.39 (1H, dd, *J* = 15, 5, H-2a), 2.20 (1H, m, H-11b), 1.59 (1H, m, H-12b), 1.58 (1H, m, H-11a), 1.57 (2H, m, H-6b, H-12a), 1.55 (1H, m, H-9), 1.53 (2H, m, H-6a, H-7b), 1.52 (1H, m, H-5), 1.38 (1H, m, H-7a), 1.36 (3H, s, H-17), 1.31 (3H, s, H-16), 1.10 (3H, s, H-18), 1.07 (3H, s, H-19) and 0.89 (3H, s, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.) (Fig. B11, Table 4.9) δ (ppm): 215.1 (s), 147.8 (d), 110.7 (t), 78.0 (t), 74.7 (s), 73.6 (s), 55.3 (d), 51.2 (d), 47.3 (s), 45.1 (t), 42.7 (s), 45.1 (t), 35.6 (t), 28.8 (q), 27.4 (q), 25.1 (q), 20.7 (t), 20.4 (q), 17.8 (t) and 10.9 (q).

EI MS spectrum (Fig. B12) *m/z*: 320 [M^+], 305 (100), 287 (50), 269 (15), 217 (12), 199 (12), 175 (15), 149 (60), 135 (20), 108 (95), 93 (55), 81 (60) and 55 (70).

3.9.4 Purification and properties of compound 4.

The compound **4** was eluted with 50% ethyl acetate in hexane on silica gel chromatography. This compound was a white crystalline solid having melting point at 141-142 °C. It was soluble in various solvents such as hexane, ethyl acetate, chloroform, methanol and ethanol.

Compound **4** was colorless crystals (1.57 g, 7.85%), $[\alpha]_D^{25}$ -54.1° ($CHCl_3$; *c* 1.0).

FT-IR spectrum (KBr) (Fig. B13, Table 4.10) ν_{max} (cm^{-1}): 3600-3200, 2931, 2857, 1712, 1660, 1448, 1376, 1285, 1117 and 1093.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B14, Table 4.11) δ (ppm): 7.13 (1H, d, *J* = 10, H-1), 5.87 (1H, d, *J* = 10, H-2), 5.82 (1H, dd, *J* = 17, 11, H-14), 5.44 (1H, dd, *J* = 18, 2, H-15b), 5.26 (1H, dd, *J* = 11, 2, H-15a), 3.80 (1H, dd, *J* = 7, 4, H-12), 2.09 (1H, dd, *J* = 9, 6, H-9), 1.98 (1H, m, H-11b), 1.94 (1H, m, H-11a), 1.92 (1H, m, H-7b), 1.85 (1H, dd, *J* = 12, 3, H-5), 1.72 (1H, m, H-6b), 1.58 (1H, m, H-7a), 1.62 (1H, m, H-9), 1.52 (1H, m, H-6a), 1.39 (3H, s, H-16), 1.38 (3H, s, H-17), 1.16 (3H, s, H-18), 1.09 (3H, s, H-19) and 1.04 (3H, s, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.) (Fig. B15, Table 4.12) δ (ppm): 205.0 (s), 157.4 (d), 142.6 (d), 126.0 (d), 115.9 (t), 76.8 (s), 75.4 (s), 70.1 (d), 53.3 (d), 44.7

(s), 43.6 (d), 41.6 (t), 38.8 (s), 27.7 (q), 27.5 (q), 25.4 (q), 23.0 (t), 21.3 (q), 20.2 (t), and 19.0 (q).

EI MS spectrum (Fig. B16) *m/z*: 318 [M^+], 305 (18), 287 (10), 248 (25), 205 (70), 189 (100), 161 (48), 134 (35), 107 (30), 91 (45), 71 (45) and 55 (48).

3.10 Preparation microorganism for biotransformation in experimental section I.

3.10.1 Storage of microorganisms.

Fungi were streaked by needle on PDA agar slant and incubated at room temperature for 5 days. Stock cultures were maintained at 4°C in a refrigerator.

3.10.2 Preparation of spore suspension.

Stock cultures were suspended in 1% tween 80 in distilled water and filtered by a sterile filter cloth. Fungal spores were counted by Haemacytometer and adjusted spore suspension of 2.5×10^6 - 2.5×10^7spores/ml.

3.10.3 Growing filamentous fungi.

One ml of preparation spore suspension was suspended in 1% tween 80 in distilled water, obtained by transferring to a SMG medium (Appendix A) 50 ml in erlenmeyer flask 250 ml. The cultures were cultivated on rotary shaker at 150 rpm at room temperature for 72 hours.

3.10.4 Biotransformation.

Preliminary screenings were conducted with erlenmeyer flasks (250 ml) containing 50 ml of medium. For each fungus three flasks were inoculated with a suspension of the corresponding microorganism. The incubation was maintained at room temperature on a rotary shaker (150 rpm) for 72 h. An aliquot (5 ml) was used to inoculate similar flasks that were incubated under the same conditions. At the end of the first 24 h. of incubation, a solution of substrate (**1**, 20 mg in 0.4 ml EtOH) was evenly distributed in one flask while the other was used as a control. An additional flask was prepared to be used as substrate control, containing only the medium and the substrate.

3.10.5 Determination of transformation time.

The incubation mixtures were harvested from shake flasks everyday for two weeks and analyzed by TLC method. Samples of 5 ml were extracted with EtOAc (5 ml) for three times. The organic layer was spotted onto a TLC plate and developed with 100% EtOAc as the solvent system. On the 7^{th} day, a preliminary

TLC analysis of the residue showed no remaining of starting material and new more polar spots were seen with the highest intensity. No transformation products were found in the controls.

3.11 Extraction and isolation of biotransformation products.

All flasks were cultivated for seven more days at room temperature after the mycelium was filtered off and washed with EtOAc. The broth was extracted with the same volume of EtOAc for three times and the organic layers were combined and dried over anh. Na_2SO_4. Solvent was removed by evaporation under reduced pressure using a rotary evaporator to furnish a syrupy residue. The residues from the experimental flasks were spotted and compared with those of the controls by silica gel TLC plate which was developed by EtOAc-MeOH (9:1). The spot was detected by UV 254 nm and visualized by spraying with Vanillin-EtOH-H_2SO_4, followed by heating at 100 °C. TLC chromatography showed that fungi had the ability to biotransformation substrates. Transformed products were more polar than the substrate. No transformation products were found in the controls.

Preparative experiments were conducted in 250 ml erlenmeyer flasks in which each containing 50 ml of the liquid medium. The procedure and conditions were the same as for the preliminary experiments.

3.12 Biotransformation of substrate by some fungi.

3.12.1 Biotransformation of Compound 1 by *Absidia blakesleeana*.

Twenty-six flasks with 50 ml of medium were used for 72 h culture of the fungus. The compound **1** (500 mg) in EtOH (20 ml) was evenly distributed among 25 flasks and one was kept as control. After 7 days of the incubations, the fermentation was harvested. The cultures were filtered and the broth was extracted with EtOAc (5 × 500 ml). The extracts were combined and dried over anhydrous Na_2SO_4. The crude extract (485 mg) was chromatographed on silica gel column in a gradient of hexane-EtOAc to give four metabolites, metabolite **1a** (33 mg, 6.8%), metabolite **1b** (75 mg, 15.5%), metabolite **1c** (45 mg, 9.3%) and metabolite **1d** (65 mg, 13.4%).

3.12.2 Purification and properties of biotransformed products.

3.12.2.1 Purification and properties of metabolite 1a

The metabolite **1a** was eluted with 30% ethyl acetate in hexane on silica gel chromatography. This compound was a white colorless needle having melting point at 161-163 °C. It was soluble in various solvents such as methanol and ethanol.

Metabolite **1a** (33.0 mg, 6.8%) was as colorless needles, $[\alpha]_D^{20}$ -35° (c 0.10 , CH_3OH).

FT-IR spectrum (KBr) (Fig. B17, Table 4.13) ν_{max} (cm^{-1}): 3486, 3369, 2937, 2867, 2579, 1688 , 1462 and 1248.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B18, Table 4.14) δ (ppm): 4.85 (1H, s, H-17b), 4.82 (1H, s, H-17a), 3.63 (1H, s, H-7), 3.08 (1H, d, *J* = 17, H-15b), 2.69 (1H, s, H-13), 2.18 (1H, d, *J* = 10, H-3b), 2.22 (1H, m, H-6b), 2.06 (2H, m, H-6a, H-15a), 2.05 (2H, m, H-1b, H-5), 1.96 (1H, m, H-14b), 1.94 (1H, m, H-11b), 1.71 (1H, ddt , *J* = 2, 6, 12, H-12b), 1.58 (1H, m, H-12a), 1.51 (1H, m, H-11a), 1.49 (2H, m, H-2a, H-2b), 1.34 (1H, m, H-14a), 1.31 (1H, m, H-1b), 1.24 (3H, s, H-18), 1.14 (3H, s, H-20), 1.08 (1H, m, H-3a).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B19, Table 4.14) δ (ppm): 180.6 (s), 154.8 (s), 102.4 (t), 78.9 (s), 78.0 (d), 51.2 (s), 43.8 (s), 43.1 (s), 42.4 (d), 42.0 (d), 41.2 (t), 38.4 (t), 37.7 (t), 33.2 (t), 31.9 (t), 29.0 (t), 28.1 (q), 27.4 (t), 18.8 (t), and 17.1 (q).

LC-MS spectrum (Fig. B24), *m/z* (rel.int.): 357 $[M+Na]^+$ and HRESIMS m/z: 357.2039 $[M+Na]^+$.

3.12.2.2 Purification and properties of metabolite 1b

The metabolite **1b** was eluted with 40% ethyl acetate in hexane on silica gel chromatography. This compound was a colorless crystal having melting point at 258-260 °C. It was soluble in various solvents such as methanol and ethanol.

Metebolite **1b** (75 mg, 15.5%) was as colorless crystal, $[\alpha]_D^{20}$ -24° (c 0.10, CH_3OH).

FT-IR spectrum (KBr) (Fig. B25, Table 4.15) ν_{max} (cm^{-1}): 3416, 2933, 2863, 3245, 1696, 1474, 1376, 1232, 1162 and 1053.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B26, Table 4.16) δ (ppm): 4.87 (1H, s, H-17b), 4.76 (1H, s, H-17a), 4.16 (1H, m, H-11), 3.56 (1H, s, H-7), 2.78 (1H, d, *J*=13, H-12b), 2.71 (1H, s, H-13), 2.25 (2H, m, H-15a, H-15b), 2.16 (2H, m, H-3a, H-3b), 2.12 (1H, m, H-6b), 2.06 (1H, m, H-1b), 2.00 (1H, m, H-6a), 1.96 (1H, d, *J*=7, H-9), 1.93 (1H, m, H-2b), 1.90 (1H, m, H-14b), 1.88 (1H, d, *J*=12, H-5), 1.77 (1H, m, H-14a), 1.38 (1H, d, *J*=14, H-2a), 1.20 (3H, s, H-18), 1.19 (3H, s, H-20), 1.12 (1H, dd, *J* = 4, 13, H-1a), 1.11 (1H, m, H-12a).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B27, Table 4.16) δ (ppm): 180.4 (s), 155.5 (s), 102.2 (t), 76.2 (d), 69.0 (d), 53.1 (d), 49.8 (s), 47.9 (d), 45.3 (t), 43.4 (s), 43.0 (d), 42.5 (s), 40.8 (t), 40.7 (t), 38.8 (t), 38.2 (t), 28.9 (t), 28.8 (q), 19.2 (t) and 15.2 (q).

LC-MS spectrum (Fig. B33), *m/z* (rel.int.): 357 $[M+Na]^+$ and HRESIMS m/z: 357.2036 $[M+Na]^+$.

3.12.2.3 Purification and properties of metabolite 1c

The metabolite **1c** was eluted with 50% ethyl acetate in hexane on silica gel chromatography. This compound was a white colorless crystal having melting point at 279-281 °C. It was soluble in various solvents such as methanol and ethanol.

Metabolite **1c** (45 mg, 9.3%) was as colorless crystal, $[\alpha]_D^{20}$ -80° (c 0.10 , CH_3OH).

FT-IR spectrum (KBr) (Fig. B35, Table 4.17) ν_{max} (cm^{-1}): 3400, 2960, 2914, 1688, 1446, 1384, 1244, 1182 and 1069.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B36, Table 4.18) δ (ppm): 4.80 (1H, s, H-17b), 4.77 (1H, s, H-17a), 3.51 (1H, s, H-7), 3.37 (1H, dd, *J* = 5 and 12, H-1), 2.88 (1H, d, *J* = 3, H-11a), 2.85 (1H, d , *J* = 4, H-11b), 2.64 (1H, s, H-13), 2.25 (1H, m, H-15b), 2.17 (1H, m, H-6b), 2.14 (1H, m, H-3b), 2.00 (1H, m, H-2b), 1.93 (1H, m, H-6a), 1.88 (1H, br s, H-12b), 1.74 (1H, d, *J* = 7, H-9), 1.71 (1H, m, H-15a), 1.68 (2H, br s, H-5, H-12b), 1.54 (1H, m, H-2a), 1.44 (1H, m, H-12a), 1.20 (1H, m, H-14a), 1.18 (1H, m, H-3a), 1.18 (3H, s, H-18), 1.10 (3H, s, H-20).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B37, Table 4.18) δ (ppm): 180.2 (s), 155.4 (s), 102.0 (t), 82.1 (d), 76.6 (d), 49.2 (d), 48.6 (s), 45.2 (s),

45.1 (d), 44.3 (s), 43.9 (d), 42.4 (s), 39.3 (t), 35.9 (t), 33.5 (t), 29.3 (t), 28.6 (t), 27.8 (q), 19.8 (t), and 10.7 (q).

LC-MS spectrum (Fig. B42), *m/z* (rel.int.): 357 $[M+Na]^+$ and HRESIMS m/z: 357.2036 $[M+Na]^+$.

3.12.2.4 Purification and properties of metabolite 1d

The metabolite **1d** was eluted with 40% ethyl acetate in hexane on silica gel chromatography. This compound was a white colorless needle having melting point at 290-292 °C. It was soluble in various solvents such as methanol and ethanol.

Metabolite **1d** (65 mg, 13.4%) was as colorless needles, $[\alpha]_D^{20}$ -69° (c 0.10 , CH_3OH).

FT-IR spectrum (KBr) (Fig. B43, Table 4.19) ν_{max} (cm^{-1}): 3431, 2949, 2886, 1696, 1470, 1388, 1221, 1162, 1038 and 886.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B44, Table 4.20) δ (ppm): 4.99 (1H, s, H-17b), 4.85 (1H, s, H-17a), 3.57 (1H, s, H-7), 2.40 (1H, d, *J* = 17, H-15b), 2.23 (1H, d, *J* = 18, H-15a), 2.18 (1H, m, H-3b), 2.13 (1H, m, H-6b), 2.02 (1H, m, H-6a), 1.96 (2H, m, H-2b, H-14b), 1.88 (1H, m, H-1b), 1.82 (2H, m, H-11b, 12b), 1.78 (1H, d, *J* = 14, H-9), 1.57 (1H, m, H-11a), 1.53 (1H, m, H-12a), 1.48 (1H, m, H-2a), 1.43 (1H, d, *J* = 8, H-5), 1.35 (1H, m, H-14a), 1.20 (3H, s, H-18), 1.01 (3H, s, H-20), 0.96 (1H, dt, *J* = 4, 13, H-3a), 1.20 (3H, s, H-18), 1.01 (3H, s, H-20).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B45, Table 4.20) δ (ppm): 180.5 (s), 155.3 (s), 102.2 (t), 79.3 (s), 76.2 (d), 48.0 (d), 47.2 (d), 45.2 (t), 45.1 (s), 43.8 (t), 42.9 (s), 40.4 (t), 39.7 (t), 38.9 (s), 37.8 (t), 29.0 (t), 27.9 (q), 19.6 (t), 19.0 (t), and 14.8 (q).

LC-MS spectrum (Fig. B49), *m/z* (rel.int.): 357 $[M+Na]^+$

3.12.3. Biotransformation of compound 1 by *Rhizopus oligosporus.*

Twenty-six flasks with 50 ml of medium were used for 72 h culture of the fungus. The compound **1** (500 mg) in EtOH (20 ml) was evenly distributed among 25 flasks and one was kept as control. After 7 days of the incubations, the fermentation was harvested. The cultures were filtered and the broth was extracted with EtOAc (5 × 500 ml). The extracts were combined and dried over

anhydrous Na_2SO_4. The crude extract (432 mg) was chromatographed on silica gel column in a gradient of hexane-EtOAc to give three metabolites, metabolite **1e** (35 mg, 8.1%), metabolite **1a** (45 mg, 10.4%) and metabolite **1f** (23 mg, 5.3%).

3.12.4. Purification and properties of biotransformed products.

3.12.4.1 Purification and properties of metabolite 1e

The metabolite **1e** was eluted with 30% ethyl acetate in hexane on silica gel chromatography. This compound was a white colorless crystal having melting point at 238-241 °C. It was soluble in various solvents such as methanol and ethanol.

Metabolite **1e** (35.0 mg, 8.1%) was as colorless crystal, $[\alpha]_D^{20}$ -60° (c 0.10 , CH_3OH).

FT-IR spectrum (KBr) (Fig. B50, Table 4.21) ν_{max} (cm^{-1}): 3459, 2925, 2890, 1688, 1450, 1279, 1193 and 1038.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B51, Table 4.22) δ (ppm): 4.83 (1H, s, H-17b), 4.79 (1H, s, H-17a), 3.56 (1H, s, H-7), 2.67 (1H, m, H-13), 2.27 (2H, br s, H-15a, 15b), 2.17 (1H, d, *J* = 12, H-3b), 2.15 (1H, d, *J* = 12, H-6b), 2.02 (1H, m, H-6a), 1.91 (1H, m, H-14b), 1.89 (2H, m, H-2b, H-14b), 1.88 (1H, m, H-1b), 1.82 (2H, m, H-11b, 12b), 1.78 (1H, d, *J* = 14, H-9), 1.57 (1H, m, H-11a), 1.53 (1H, m, H-12a), 1.48 (1H, m, H-2a), 1.43 (1H, d, *J* = 8, H-5), 1.35 (1H, m, H-14a), 1.20 (3H, s, H-18), 1.01 (3H, s, H-20), 0.96 (1H, dt , *J* = 4, 13, H-3a), 1.18 (3H, s, H-18), 1.10 (3H, s, H-20).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B52, Table 4.22) δ (ppm): 180.0 (s), 155.1 (s), 103.7 (t), 77.1 (d), 49.1 (d), 48.3 (s), 47.2 (d), 45.3 (t), 43.7 (d), 43.2 (s), 40.4 (t), 39.4 (s), 38.7 (t), 37.8 (t), 33.5 (t), 29.7 (t), 28.7 (q), 19.1 (t), 17.9 (t), and 15.5 (q).

LC-MS spectrum (Fig. B53), *m/z* (rel.int.): 318 $[M+Na]^+$

3.12.4.2 Purification and properties of metabolite 1f

The metabolite **1f** was eluted with 70% ethyl acetate in hexane on silica gel chromatography. This compound was a white solid having melting point at 169-170 °C. It was soluble in various solvents such as methanol and ethanol.

Metabolite **1f** (23.0 mg, 5.3%) was as white solid, $[\alpha]_D^{20}$ -34° (c 0.10 , CH_3OH).

FT-IR spectrum (KBr) (Fig. B57, Table 4.17) ν_{max} (cm^{-1}): 3330, 3194, 3055, 3023, 2933, 1731, 1680, 1450, 1240, 1209, 1026 and 847.

^{1}H-NMR spectrum (CD_3OD, 400 MHz) (Fig. B58, Table 4.18) δ (ppm): 3.46 (1H, d, *J* = 11, H-17b), 3.35 (1H, d, *J* = 11, H-17a), 2.27 (1H, m, H-7b), 2.22 (1H, m, H-15b), 2.11 (1H, m, H-1b), 2.07 (1H, d, *J* = 3, H-13), 1.96 (1H, dt, *J* = 5, 10, H-11b), 1.93 (1H, m, H-12b), 1.85 (1H, m, H-14b), 1.84 (1H, m, H-3b), 1.82 (2H, m, H-6b, H-6b), 1.71 (2H, m, H-11a), 1.70 (1H, m, H-5), 1.56 (1H, m, H-12a), 1.52 (1H, m, H-14a), 1.43 (2H, m, H-2a, H-2b), 1.23 (1H, m, H-7a), 1.21 (3H, s, H-18), 1.15 (4H, s, H-3a, H-20), 1.08 (1H, m, H-15a), 1.05 (1H, m, H-1a).

^{13}C-NMR spectrum (CD_3OD, 100 MHz) (Fig. B59, Table 4.18) δ (ppm): 181.2 (s), 81.1 (s), 79.2 (s), 70.5 (d), 50.7 (d), 49.5 (s), 47.0 (t), 45.2 (s), 44.8 (s), 41.0 (d), 40.2 (t), 38.9 (t), 37.8 (t), 33.6 (t), 30.3 (t), 29.6 (q), 29.0 (t), 22.7 (t), 20.9 (t), and 18.1 (q).

LC-MS spectrum (Fig. B65), *m/z* (rel.int.): 357 $[M+Na]^+$ and HRESIMS m/z: 375.2138 $[M+Na]^+$.

3.13 Preparation microorganism for transformation in experimental section II.

3.13.1 Storage of microorganisms.

Microorganisms were streaked by needle on agar slant (solid medium II) and incubated at room temperature for 5-10 days. When the fungi were grown, they were kept in a refrigerator at 4°C.

3.13.2 Preparation of spore suspension.

After addition of glucose (5g/l), the stock cultures were initiated by inoculating with a suspension of the surface growth from agar slants in 1 ml of sterile medium (25% glycerol).

3.13.3 Growing filamentous fungi.

Suspended spores (2-4 drops) were obtained by transferring to a liquid medium (Appendix A) 100 ml in erlenmeyer flask 250 ml. The cultures were cultivated on rotary shaker at 200 rpm at 27 °C for 48 hours.

3.13.4 Biotransformation.

Preliminary screenings were conducted with erlenmeyer flasks (250 ml) containing 100 ml of medium. For each fungus one flask was inoculated with a suspension of the corresponding microorganism. Cultures were grown with shaking on a rotatory shaker operating at 200 rpm and 27 °C. After 48-60 h, the substrate was added to each flask as an ethanolic solution (2 ml each containing 50 mg of substrate) to give a final concentration of 0.5 mg/ml.

3.13.5 Determination of transformation time.

The incubation mixtures were sampled for TLC analysis at 24, 48, 72, 92 and 116 h. Samples of 0.5 ml were saturated with sodium chloride and extracted with EtOAc (1.5 ml) for three times. The organic layer was spotted onto a TLC plate and developed with EtOAc-cyclohexane (5:5) as the solvent system and further analyzed by GC/MS. On the 4^{th} day, a preliminary TLC analysis of the residue showed new more polar spots with the highest intensity.

3.14 Extraction and isolation of biotransformation products.

In the screening experiments, three fungal strains, *Rhizopus oligosporus* TISTR 3001, *Mucor plumbeus* ATCC 4720 and *Rhisopus stolonifer* ATCC 62276 were found to produce metabolites of substrate **2** reproducibly, and were used for preparative experiments.

Preparative experiments were conducted in procedure and conditions which were the same as for the screening experiments. Fungi were grown in ten 250 ml-conical flasks containing 100 ml of liquid medium. A total of 500 mg of the substrate (in 20 ml of ethanol) was evenly distributed among the flasks. The cultures were incubated on a rotatory shaker (200 rpm) at 27 °C for 4 days.

All flasks were cultivated for four days at 27 °C after the mycelium was filtered off and washed with acetone. The broth was extracted with the same volume of CH_2Cl_2 for three times and the organic layers were combined and dried over anh. Na_2SO_4. Solvent was removed by evaporation under reduced pressure using a rotary evaporator to furnish a syrupy residue. The residues from the experimental flasks were spotted and compared to those of the controls by silica gel TLC plate which was developed by EtOAc-cyclohexane (5:5) and further analyzed by GC/MS. The spot was detected by UV 254 nm and visualized by spraying with phosphomolybdic acid-

H_2SO_4, followed by heating at 100 °C. TLC chromatography showed that fungi had the ability to biotransformation substrates. Transformed products were more polar than the substrate.

3.15 Biotransformation of substrates by some fungi.

3.15.1 Biotransformation of compound 2 by *Rhizopus oligosporus.*

Ten flasks with 100 ml of medium were used for 48 h culture of the fungus. The compound **2** (500 mg) in EtOH (20 ml) was evenly distributed among 10 flasks. After 4 days of the incubations, the fermentation was harvested. The incubation mixtures were pooled and then filtered to remove mycelium. The filtrate was extracted three times with equal volumes of CH_2Cl_2. The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated at 40 °C in vacuum. The mycelium was extracted three times with acetone with magnetic stirring and the solvent evaporated. The broth extract and mycelium extract obtained were separately flash-chromatographed on a silica gel column using increasing amounts of EtOAc in cyclohexane as the eluent. From 500 mg of **2**, a mycelium crude extract (350 mg) and a broth crude extract (401 mg) were obtained, which was fractionated by flash column chromatography on silica gel. Elution with cyclohexane-EtOAc (8:2) of mycelium crude extract resulted in the recovery of unchanged **2** (53 mg). The broth crude extract was chromatographed on silica gel column in a gradient of hexane-EtOAc to give two metabolites, metabolite **2a** (230 mg, 46.0%) and metabolite **2b** (8 mg, 1.6%).

3.15.2 Purification and properties of biotransformed products.

3.15.2.1 Purification and properties of metabolite 2a

The metabolite **2a** was eluted with 20% ethyl acetate in hexane on silica gel chromatography. This compound was a white crystal having melting point at 84-85 °C. It was soluble in various solvents such as ethyl acetate, chloroform, methanol and ethanol.

Metabolite **2a** (230.0 mg, 46.0%) was as white crystal, $[\alpha]_D^{20}$ +15.4° (c 0.50 , $CHCl_3$).

FT-IR spectrum (KBr) (Fig. B66, Table 4.25) ν_{max} (cm^{-1}): 3486, 3369, 2937, 2867, 2579, 1688 , 1462 and 1248.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B67, Table 4.25) δ (ppm): 7.10 (1H, d, *J* = 10, H-1), 5.89 (1H, dd, *J* = 18, 12, H-14), 5.86 (1H, d, *J* = 10, H-2), 5.16 (1H, dd, *J* = 17, 1, H-15b), 4.94 (1H, dd, *J* = 11, 1, H-15a), 1.92 (1H, m, H-7b), 1.90 (1H, m, H-12b), 1.84 (1H, m, H-11b), 1.78 (1H, m, H-5), 1.72 (1H, m, H-12a), 1.70 (2H, m, H-6b, H-11a), 1.62 (1H, m, H-9), 1.53 (1H, m, H-7a), 1.52 (1H, m, H-6a), 1.38 (3H, s, H-17), 1.32 (3H, s, H-16), 1.16 (3H, s, H-18), 1.08 (3H, s, H-19) and 1.05 (3H, s, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.) (Fig. B68, Table 4.25) δ (ppm): 205.1 (s), 157.6 (d), 147.4 (d), 125.8 (d), 110.7 (t), 75.0 (s), 73.7 (s), 53.2 (d), 49.9 (d), 44.6 (t), 42.3 (s), 39.4 (s), 35.4 (t), 28.6 (q), 27.6 (q), 25.6 (q), 21.3 (q), 20.2 (t), 18.6 (q), and 15.5 (q).

EI MS spectrum (Fig. B73) *m/z*: 318 [M^+], 285 (6), 233 (10), 215 (5), 192 (10), 149 (25), 124 (100), 96 (75) and 55 (30).

3.15.2.2 Purification and properties of metabolite 2b

The metabolite **2b** was eluted with 50% ethyl acetate in hexane on silica gel chromatography. This compound was a white solid having melting point at 104-105 °C. It was soluble in various solvents such as ethyl acetate, chloroform, methanol and ethanol.

Metabolite **2b** (8 mg, 1.6%) was as white solid, $[\alpha]_D^{20}$ +6° (c 0.50, $CHCl_3$).

FT-IR spectrum (KBr) (Fig. B74, Table 4.27) ν_{max} (cm^{-1}): 3416, 2933, 2863, 3245, 1696, 1474, 1376, 1232, 1162 and 1053.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B75, Table 4.28) δ (ppm): 7.10 (1H, d, *J* = 10, H-1), 5.89 (1H, dd, *J* = 18, 11, H-14), 5.86 (1H, d, *J* = 10, H-2), 5.16 (1H, dd, *J* = 17, 1, H-15b), 4.94 (1H, dd, *J* = 11, 1, H-15a), 1.92 (1H, m, H-7b), 1.90 (1H, m, H-12b), 1.84 (1H, m, H-11b), 1.78 (1H, m, H-5), 1.72 (1H, m, H-12a), 1.70 (2H, m, H-6b, H-11a), 1.62 (1H, m, H-9), 1.53 (1H, m, H-7a), 1.52 (1H, m, H-6a), 1.38 (3H, s, H-17), 1.32 (3H, s, H-16), 1.16 (3H, s, H-18), 1.08 (3H, s, H-19) and 1.05 (3H, s, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz.) (Fig. B76, Table 4.28) δ (ppm): 205.1 (s), 157.6 (d), 147.4 (d), 125.8 (d), 110.7 (t), 75.0 (s), 73.7 (s), 53.2 (d),

49.9 (d), 44.6 (t), 42.3 (s), 39.4 (s), 35.4 (t), 28.6 (q), 27.6 (q), 25.6 (q), 21.3 (q), 20.2 (t), 18.6 (q), and 15.5 (q).

EI MS spectrum (Fig. B82) *m/z*: 355, 334 (2), 301 (5), 291 (70), 233 (22), 149 (25), 124 (100), 96 (85) and 55 (25).

3.15.3. Biotransformation of compound 2 by *Rhizopus stolonifer*

The procedure was similar to the one previously described for *R. oligosporus*. From 500 mg of **2**, a mycelium crude extract (460 mg) and a broth crude extract (175 mg) were obtained, which was fractionated by flash column chromatography on silica gel. Elution with cyclohexane-EtOAc (8:2) of mycelium crude extract resulted in the recovery of unchanged **2** (228 mg). For broth crude extract gave two metabolite, metabolite **2a** (64 mg, 12.8%) and metabolite **2c** (40 mg, 8.0%).

3.15.4 Purification and properties of biotransformed products.

3.15.4.1 Purification and properties of metabolite 2c

The metabolite **2c** was eluted with 30% ethyl acetate in hexane on silica gel chromatography. This compound was a white solid having melting point at 95-96 °C. It was soluble in various solvents such as ethyl acetate, chloroform, methanol and ethanol.

Metabolite **2c** (40.0 mg, 8.0%) was as white solid, $[\alpha]_D^{20}$ +18° (c 0.10 , $CHCl_3$).

FT-IR spectrum (KBr) (Fig. B83, Table 4.29) ν_{max} (cm^{-1}): 3415, 2946, 2890, 1695, 1659, 1450, 1383, 1282, 1178, 1106 and 1075.

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig. B84, Table 4.30) δ (ppm): 6.05 (1H, dd, *J* = 17, 11, H-14), 5.42 (1H, dd, *J* = 10, 1, H-15b), 5.10 (1H, d, *J* = 10, 1, H-15a), 4.03 (1H, m, H-11a), 2.59 (1H, m, H-2b), 2.40 (1H, m, H-12b), 2.37 (1H, m, H-2a), 2.10 (1H, m, H-1b), 1.94 (1H, m, H-12a), 1.91 (1H, m, H-7b), 1.65 (1H, m, H-1a), 1.57 (1H, m, H-7a), 1.54 (1H, m, H-9), 1.51 (1H, m, H-5), 1.36 (3H, s, H-17), 1.26 (3H, s, H-16), 1.14 (3H, s, H-18), 1.07 (6H, s, H-19, H-20).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig. B85, Table 4.30) δ (ppm): 216.7 (s), 148.1 (d), 112.5 (t), 74.2 (s), 73.4 (s), 64.9 (d), 61.9 (d), 55.6 (d),

47.5 (s), 43.5 (t), 42.8 (t), 38.5 (t), 37.4 (s), 34.0 (t), 31.5 (q), 27.3 (q), 26.2 (q), 21.4 (t), 20.7 (q), and 15.7 (q).

EI MS spectrum (Fig. B90) *m/z*: 320 [M^+], 305 (100), 287 (50), 269 (25), 235 (20), 215 (18), 149 (28), 135 (30), 109 (26), 91 (18), 69 (70) and 55 (100).

3.15.5 Biotransformation of compound 2 by *Mucor plumbeus*

The procedure was similar to the one previously described for *R. oligosporus*. From 500 mg of **2**, a mycelium crude extract (160 mg) and a broth crude extract (640 mg) were obtained, which was fractionated by flash column chromatography on silica gel. Elution with cyclohexane-EtOAc (8:2) of mycelium crude extract resulted in the recovery of unchanged **1** (48 mg). For broth crude extract gave two metabolite, metabolite **2a** (50 mg, 10.0%) and metabolite **2b** (72 mg, 14.4%)

3.16 Determination of biological activity

3.16.1 Antimicrobial activity of pure metabolites

Evaluation of the antimicrobial activity of pure compounds was determined by the antimicrobial susceptibility test microdilution method [34]. Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231 and *S. cerevisiae* TISTR 5169.

A. Preparation of pure compounds

Two milligrams of pure compounds and antibiotic drug standards were dissolved in 1 ml of 10% DMSO in sterile distilled water and kept in a refrigerator at 4 °C for bioassay. Antibiotic drug standards (Tetracycline HCl and Amoxicillin) were used as positive controls.

B. Preparation of bacterial and yeast tested inoculum

Test bacteria were grown in Nutrient Broth (NB) 37 °C for 2-6 h, depending on the growth rate. The bacterial cultures were adjusted with nutrient broth to match with that of 0.5 McFarland standard (OD 0.1 at 625 nm).

Yeast was grown in Sabouraud Glucose Broth (SGB) for 24-48 h at room temperature (25-30°C). With a sterile wire loop, the tops of four or five isolated colonies of a similar morphologic type were transferred to a tube containing 4 to 5 ml

of SGB and incubated at room temperature for 6-8 h. The turbidity of the yeast suspension was adjusted with SGB to match the turbidity of 0.5 McFarland.

The final inoculum was diluted with NB to obtain a cell suspension containing approximately 10^6 CFU/ml. The final inoculum was approximately 10^5 CFU/ml.

C. Assay procedure

Solutions of pure metabolites and antibiotic drug standards were diluted with Mueller-Hinton Broth (MHB) and SGB for assays of antibacterial and antifungal (yeast form) activity respectively. Fifty µl of pure metabolites were dispensed into each well in sterile microtiter plates (96-well bottom wells). Fifty µl of the final adjusted microbial suspension was inoculated into each well (Final inoculum size of bacterial and yeast was approximately 2.5 $x10^5$ and 2.5 x 10^4 CFU/ml, respectively). One hundred µl of medium was used as the sterility control. A100 µl volume of medium and microbial inoculum mixture acted as the growth control. Microbial microtiter plates were incubated at 37 °C and room temperature for bacterial and yeast, respectively.

D. Reading of microtiter plates assays

Antibacterial and antifungal (yeast form) activities were determined by measuring the turbidity of each well in microtiter plates by using the Sunrise microplate reader (TECAN, AUSTRIA) before and after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).