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M.H. Haroon [a b c], S.R. Premaratne [a], M. Iqbal Choudhry [c] & H.R.W. Dharmaratne [a d]

[a] Natural Products Programme, Institute of Fundamental Studies, Kandy, Sri Lanka

[b] Faculty of Applied Sciences, South Eastern University of Sri Lanka, Oluvil, Sri Lanka

[c] H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

[d] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA

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A new β-glucuronidase inhibiting butyrolactone from the marine endophytic fungus *Aspergillus terreus*

M.H. Haroon[abc], S.R. Premaratne[a], M. Iqbal Choudhry[c] and H.R.W. Dharmaratne[ad]*†

[a]*Natural Products Programme, Institute of Fundamental Studies, Kandy, Sri Lanka;* [b]*Faculty of Applied Sciences, South Eastern University of Sri Lanka, Oluvil, Sri Lanka;* [c]*H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan;* [d]*National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA*

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An endophytic fungus *Aspergillus terreus*, *var. boedijnii* (Blochwitz) was isolated from red marine alga *Laurencia ceylanica,* J. Agardh, cultured in large scale and extracted with EtOAc. The above-mentioned extract yielded a new butyrolactone, 3-hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl) -2,5-dihydro-2-furanone (**1**), along with the previously reported nine compounds, butyrolactone-1 (**2**), 6-hydroxymellin (**3**), (3 *R*, 4 *R*)-6,7-dimethoxy-4-hydroxy-mellin (**4**), (+)-territonin (**5**), (+)-territonin-A (**6**), (+)-asterrelenin (**7**), (+)-terrein (**8**), oleic acid (**9**) and glucopyranosyl-β-sitosterol (**10**), on column and preparative thin-layer chromatography. Compounds **1–8** were subjected to β-glucuronidase inhibitory activity test, and **1** showed a remarkable activity, while **2** and **7** showed moderate activity.

Keywords: *Aspergillus terreus*; *Laurencia ceylanica*; butyrolactone; 3-hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl)-2,5-dihydro-2-furanone; β-glucuronidase

1. Introduction

Endophytes are mostly fungi or bacteria that, for all or part of their life cycle, invade the tissues of living plants and cause asymptomatic infections entirely within the plant tissues. The same organism may also be pathogen at other times (Strobel, 2002). Endophytes are increasingly being identified as a group of organisms capable of producing secondary metabolites with interesting biological activities (Hussain, Krohn, Draeger, Meier, & Schulz, 2009). During the present investigation, we isolated a fungal strain *Aspergillus terreus* from the marine red algae *Laurencia ceylanica,* collected from the east coast of Sri Lanka. The *A. terreus* strain was cultivated on large scale and extracted with EtOAc. By using various chromatographic techniques, we isolated and characterised a new natural product, **1**, along with nine known compounds: butyrolactone-1 (**2**), 6-hydroxymellin (**3**), (3 *R*, 4 *R*)-6,7-dimethoxy-4-hydroxymellin (**4**), (+)-territonin (**5**), (+)-territonin-A (**6**), (+)-asterrelenin (**7**), (+)-terrein (**8**), oleic acid(**9**), and gucopyranosyl-β-sitosterol(**10**). Of them,

*Corresponding author. Email: rwdharma@olemiss.edu
†Present address: National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA

2 and **4–8** have been previously reported from *A. terreus* (Kiriyama, Nitta, Sakaguchi, Taguchi, & Yamamoto, 1977; Li et al., 2005; McIntyre, Reed, Sadler, & Simpson, 1989; Rao et al., 2000; Shimada et al., 2004). Compound **3** was reported from dogwood anthracnose fungus *Discula* sp. (Venkatasubbaiah & Chilton, 1991) and compound **8** was reported from *Phoma* sp. (Dunn, Entwistle, & Johnstone, 1975). Compounds **1–8** were tested for β-glucuronidase inhibitory activity and **1** showed a promising inhibitory activity.

β-Glucuronidase is an acid hydrolase that catalyses the hydrolysis of glucuronides to yield their respective aglycones and free glucuronic acid (Freeman et al., 1999; Levy & Conchi, 1966). Deficiency of this enzyme in humans leads to the development of lysosomal storage diseases known as mucopolysaccharidoses. However, the over-expression of β-glucuronidase of intestinal bacteria in humans and rats is related to colon cancer. In addition, β-glucuronidase of bacteria, present in the biliary tract is associated with gallstone formation (Pineda, Goldbarg, Banks, & Rutenburg, 1959). Therefore, specific inhibitors of β-glucuronidase could be developed as drugs in the treatment of relevant diseases.

2. Results and discussion

The EtOAc extract of *A. terreus* was subjected to repeated column chromatography (CC) on flash silica gel giving 10 compounds. Compounds **1** and **2** were isolated as pale yellow powders. The IR, UV and NMR data of **1** and **2** suggested that they have similar structures, and spectroscopic analysis and comparison with the literature data suggested compound **2** to be butyrolactone-1 (Figure 1), reported from *A. terreus* (Kiriyama et al., 1977; Rao et al., 2000). However, when NMR spectra of compounds **1** with **2** were compared, a difference was noted in the substitution pattern of the tri-substituted aromatic ring of **2**. A ^{13}C NMR resonance at δ 196.9, a downfield ^{1}H NMR singlet at δ 9.76 (1H) and IR absorbance at 2854 cm^{-1} indicated the presence of an aldehyde moiety (–CHO) in **1**. Comparison of the ^{13}C NMR spectra of **1** and **2** (Kiriyama et al., 1977) revealed that the isopentenyl group of compound **2** at C-18 was replaced by an aldehyde group in compound **1**. A ^{13}C NMR resonance at δ 171.3, ^{1}H NMR singlet at δ 3.77 (3 H) and strong IR absorbance at 1736 cm^{-1} indicated the presence of a methyl ester moiety (–COOMe) in compound **1**, as was found in **2**. Further, in the ^{1}H NMR spectrum of **1**, signals due to seven aromatic protons and one methylene group was detected as in the case of **2**. Positive CI MS spectrum of **1** showed $[M+H]^+$ at 385, indicating its molecular weight as 384. Accordingly, the molecular formula of **1** was proposed as $C_{20}H_{16}O_8$. Considering the above evidence, a tentatative structure of **1** was proposed to the new compound (Figure 1). The *para*-disubstituted aromatic ring protons that resonated at δ 6.85 (2H, d, $J = 8.5$ Hz) and δ 7.60 (2H, d, 8.6 Hz) were assigned to H-6/H-2 and H-3/H-5, respectively. The tri-substituted aromatic ring protons that resonated at δ 7.0 (1H, d, $J = 8.5$ Hz), 6.69 (1H, d, $J = 8.5$ Hz) and 7.14 (1H, s) were assigned to H-15, H-16 and H-19, respectively (Figure 1). In the HMQC spectrum of **1**, the aromatic protons that resonated at δ 6.85 (H-2/H-6), 7.60 (H-3/H-5), 7.0 (H-15), 6.70 (H-16) and 7.14 (H-19) were found to be coupled with carbon atoms resonating at δ 116.6 (C-2/C-6), 130.3 (C-3/C-5), 139.9 (C-15), 117.6 (C-16), and 135.8 (C-19), respectively. Similarly, protons resonating at δ 9.67 (H-20), 3.54 (H-13) and 3.77 (H-12) were found to be coupled with 196.9 (C-20), 39.1 (C-13) and 53.9 (C-12), respectively. COSY correlations between H-5/3 and H-6/2 indicted the presence of a *para*-disubstituted benzene ring. Similarly, strong coupling ($J = 8.1$ Hz) between H-16 and H-15 and a single peak of H-19 indicated the presence of a tri-substituted benzene ring. In the HMBC experiment of **1**, correlation of methylene protons (H_2-13) with C-14 (126.3), C-10 (86.4), C-19 (135.8), C-15 (139.7) and C-7 (129.5) were observed. Similarly, aldehydic

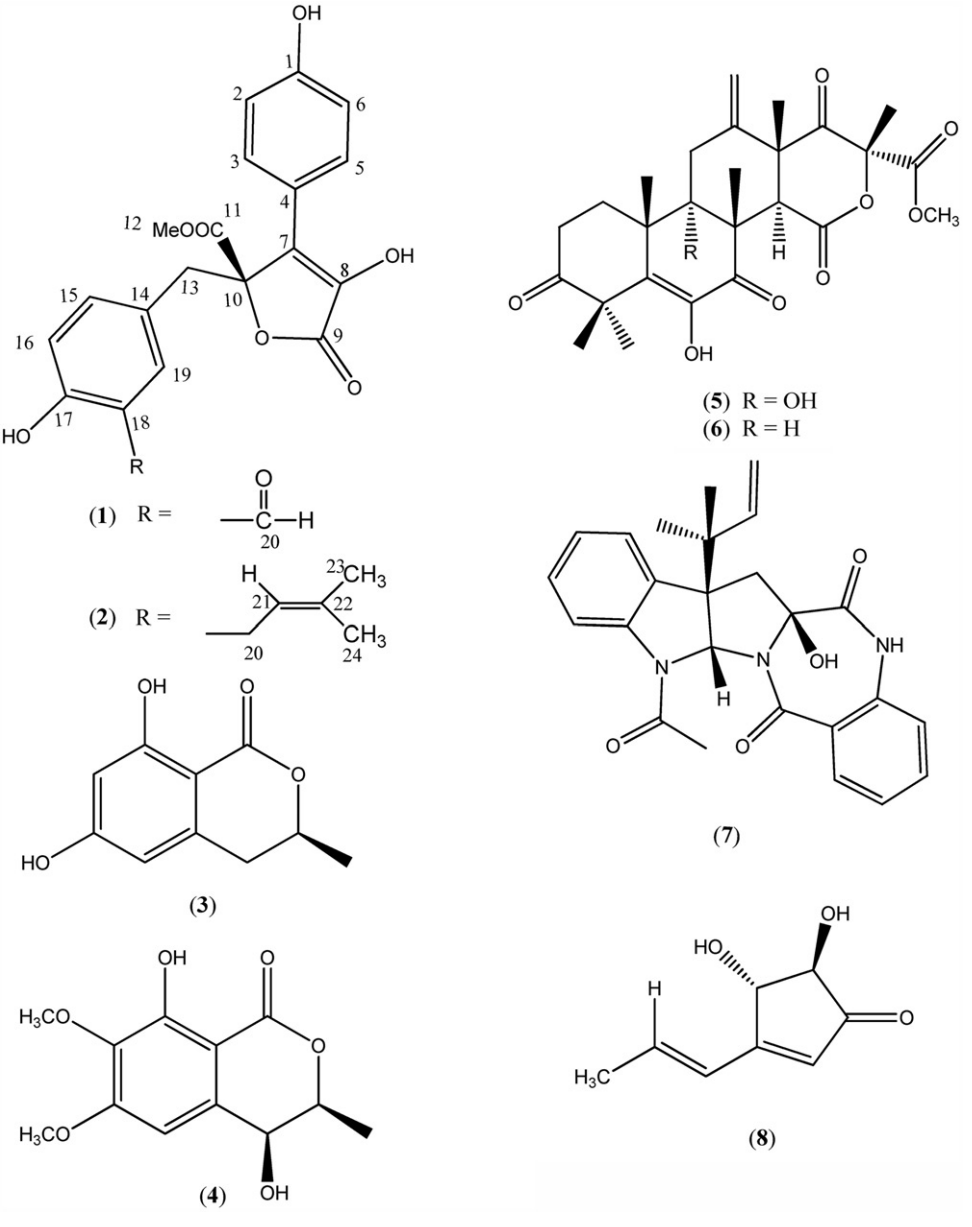

Figure 1. Structures of compounds **1**–**8**.

proton (H-20, δ 9.76) showed HMBC correlations with C-18 (121.9), C-19 (135.8), and C-17 (161.6), suggesting that the aldehyde group was attached to the aromatic ring (Figure 2). This was confirmed by ^{13}C and DEPT NMR spectra of **1**.The sign of optical rotation of **1** was found to be the same as that reported for (+)-butyrolactone-1 (Kiriyama et al., 1977). On the basis of the above spectroscopic evidence, the structure of compound **1** was deduced as 3-hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl)-2,5-dihydro-2-furanone, which is a new butyrolactone.

The structures of compounds **3–10** were elucidated by the spectroscopic analysis and by comparing with the literature data (Dunn et al., 1975; Kiriyama et al., 1977;

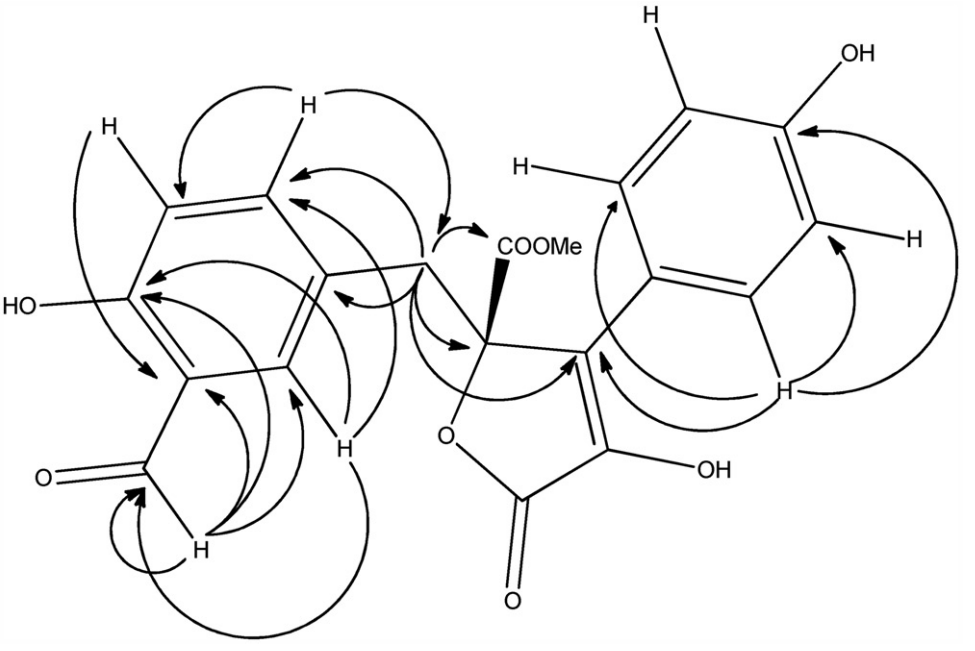

Figure 2. Key HMBC correlation of compound 1.

Table 1. β-Glucuronidase inhibition activity.

Compound	**1**	**2**	**7**	Glucosaccharo-(1,4)-lactone
IC_{50} (μM)	6.2 ± 2.49	96.7 ± 0.74	126 ± 2.49	48.4 ± 1.25

Li et al., 2005; McIntyre et al., 1989; Rai, 2007; Rao et al., 2000; Shimad, et al., 2004; Venkatasubbaiah & Chilton, 1991).

Compounds **1**–**8** were subjected to β-glucuronidase inhibitory activity test, using glucosaccharo-(1,4)-lactone as the positive control. Compound **1** showed considerable β-glucuronidase inhibitory activity, with an IC_{50} value of 6.2 μM, when compared with the positive control (Table 1). Compounds **2** and **7** showed moderate β-glucuronidase inhibitory activity with IC_{50} values of 96.7 and 126 μM, respectively, while compounds **3**–**6** and **8** showed no activity. The significant inhibitory activity of compound **1** is probably due to the acceptance of a proton from the carboxylic acid at the active site of the enzyme. A number of naturally occurring compounds having β-glucuronidase inhibitory potential have been reported from other plants; and these compounds can be used in the treatment of medical conditions related to colon cancer and gallstone formation (Kawasaki, Hayashi, Arisawa, Morita, & Berganza, 1988).

3. Experimental

3.1. *General procedure*

Melting points were determined on a Büchi 535 melting point apparatus (Buchi UK Ltd, United Kingdom). Optical rotations were measured in MeOH on a Jasco DIP-360 digital

polarimeter (JASCO International Co. Ltd, Tokyo, Japan). IR spectra were recorded on a JASCO FT IR-8900 spectrophotometer (JASCO International Co. Ltd, Tokyo, Japan). UV spectra were obtained on a Hitachi UV 3200 spectrophotometer (Hitachi, Japan). 1D and 2D spectra were recorded on Bruker Avance AV 400 MHz, Bruker Avance AV 500 MHz and Bruker Avance AV 600 MHz NMR spectrometers (Basel, Switzerland), and ^{13}C NMR experiments were conducted on the same instruments at 75.45, 125 and 150 MHz, respectively. Chemical shifts (δ) were given in ppm, relative to TMS, as the internal standard and coupling constants J in Hz. EI and HREI MS were measured on a Jeol JMS HX 600 mass spectrometer (Tokyo, Japan). Column chromatography was carried out by using silica gel (E. Merck, 230–400 mesh size). TLC separations were performed on E. Merck (Darmstadt, Germany) pre-coated silica gel 60, PF254, 0.2 mm aluminium foil, and spots were detected using ceric sulphate as the spray reagent.

3.2. *Isolation and identification of the fungal strain*

The endophytic fungi were isolated from the blades of the seaweeds using the method developed by Petrini (Fokkema & Heuvel, 1986) to isolate endophytes from higher plants with certain modifications. Algal material (*L. ceylanica*) was collected at Arugam Bay on the East coast of Sri Lanka. Algal samples were placed in EtOH (70%), and then they were transferred into sodium hypochlorite solution (NaOCl, 5%), and again transferred into EtOH (96%) for 1–2 min in each solution for surface sterilisation. Finally, all the algal samples were rinsed thrice with sterile deionised water to remove EtOH. Sterilised algae were then cut into pieces ($0.5 \times 0.5\,cm^2$) and placed in petri dishes containing a Standard Nutrient Agar (SNA) medium under sterile conditions; they were then kept at room temperature for incubation. The SNA medium was prepared by mixing the following ingredients ($g\,L^{-1}$) in distilled water and autoclaved at 121°C for 30 min: KH_2PO_4 (1.0 g), KNO_3 (1.0 g), $MgSO_4 \cdot 7$ H2O (0.5 g), KCl (0.5 g), glucose (0.2 g), sucrose (0.2 g), agar (20 g), benzylpenicillin ($250\,mgL^{-1}$) and streptomycin sulphate ($250\,mgL^{-1}$). The above strain was identified as *A. terreus*. A subculture has been deposited at the Natural Products Laboratory, Institute of Fundamental Studies, Kandy, Sri Lanka.

3.3. *Cultivation of Aspergillus terreus*

The growth medium (Czpex Dox) for *A. terreus* was prepared by mixing the following chemicals ($g\,L^{-1}$) in filtered (No. 3 Whatman filter paper) natural seawater ($500\,mL\,L^{-1}$) and distilled water ($500\,mL\,L^{-1}$): $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), sugar (30 g), peptone (30 g). Culture medium (6.0 L) was prepared and distributed (250 mL, per flask) among 24 Erlenmeyer flasks of 1000 mL capacity and autoclaved at 121°C for 30 min. Then agar blocks inoculated with mycelia were added to a 1000 mL flask containing 250 of mL culture medium and incubated at 28°C with a control (without fungal strain) for 14 days.

3.4. *Extraction and isolation of pure compounds*

After 14 days, 250 mL of EtOAc was added to each flask containing a culture medium and mycelium, and mixed thoroughly using a sonicator. The EtOAc layer was carefully separated from each flask and evaporated using a rotary evaporator at 50°C. The EtOAc extract was dried in a vacuum oven to afford a brown gummy residue (4.2 g). This EtOAc extract was subjected to CC on flash silica gel (230–400 mesh size) to yield compounds **1**–**10**. The above-mentioned column was initially eluted with gradient polarities of hexane and EtOAc, which afforded eight fractions: (F-1 to F-8). Repeated flash silica gel (SiO_2) CC of F-1 (5% EtOAc : hexane) yielded pure compound **9** (20 mg, 0.47%). Fraction F-2

(8% EtOAc : hexane), when subjected to CC, yielded two fractions – F-21 (30 mg) and F-22 (5 mg). Further CC of F-21 and F-22 yielded **3** (7 mg, 0.16%, 7% acetone : hexane) and **4** (1.5 mg, 0.03%, 6% acetone : hexane), respectively, as pure compounds. Further, Fraction F-3 (15% EtOAc : hexane) on CC afforded two fractions (F-31 and F-32), which were further purified by repeated CC to afford pure compounds **5** (15 mg, 0.35%, 8.5% acetone : hexane) and **6** (2.5 mg, 0.05%, 7.5% acetone : hexane). Subjection of fractions F-4 (30% EtOAc : hexane) and F-5 (35% EtOAc : hexane) to CC yielded two pure compounds **7** (15 mg, 0.35%, 15% acetone : hexane) and **1** (2 mg, 0.04%, 17% acetone in hexane). Similarly, fractions F-6 (40% EtOAc : hexane) and F-7 (45% EtOAc : hexane), when subjected to CC, yielded pure compounds **2** (20 mg, 0.47%, 21% acetone : hexane) and **8** (15 mg, 0.35%, 24% acetone : hexane) as pure compounds and F-8 (60% EtOAc : hexane) on CC yielded 10 (2.5 mg, 0.05%, 2.5% MeOH : $CHCl_3$).

3.5. *β-Glucuronidase inhibition assay*

The β-glucuronidase inhibition assay was performed according to a slight modification of the method developed by Collins, Ng, Fong, Wan, and Yeung (1997). β-Glucuronidase (E.C.3.2.1.31) from bovine liver (G-0251) and *p*-nitrophenyl-β-D-glucuronide (N-1627) were purchased from Sigma Chemical Co. (USA). Sodium carbonate (anhydrous) was from Fluka, and all other reagents were obtained from E. Merck and were of analytical grade. By the spectrophotometric method, β-glucuronidase inhibitory activity was determined by measuring the absorbance at 405 nm of *p*-nitrophenol formed from substrate. The total reaction volume was 250 µL. The reaction mixture, containing 185 µL of 0.1 M acetate buffer (pH 7.0), 5 µL of the test compound solution and 10 µL of enzyme, was incubated at 37°C for 30 min. The plates were read on a multiplate reader (SpectraMax plus 384, Molecular Devices, CA, USA) at 405 nm after the addition of 50 µL of 0.4 mM *p*-nitrophenyl-β-D-glucuronide. The results are expressed with the Standard Error of the Mean (S.E.M.), which equals standard deviation $/\pm\sqrt{n}$, where n represents the number of replicates for the IC_{50} value. Three replicates were used for all compounds.

3-Hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl)-2,5-dihydro-2-furanone (**1**): Pale yellow powder (2 mg, 0.04%,); m.p. = 86–88°C (acetone : hexane); $[\alpha]_D^{25} = +128^\circ$ ($c = 0.05$, MeOH; UV (MeOH), λ_{max} (nm): 203, 224, 257.8, 306; IR (KBr), ν_{max} (cm^{-1}): 841.0, 1068.8, 1122, 1176.5, 1278, 1382.9, 1438.4, 1609.7, 1654.8, 1739.2, 2854, 2925.8, 3445; ^1H NMR (500 MHz, CD_3OD): δ 6.85 (2H, d, $J = 8.5$, H-2, H-6), 7.60 (2H, d, $J = 8.6$, H-3, H-5), 3.77 (3 H, s, H_3-12), 3.54 (2H, s, H_2-13), 7.0 (1H, $J = 8.5$, H-15), 6.69 (1H, d, $J = 8.5$, H-16), 7.14 (1H, s, H-19), 9.76 (1H, s, H-20); ^{13}C NMR (125 MHz, CD_3OD): δ 39.1(C-13), 53.9 (C-12), 86.4 (C-10), 116.8 (C-2 and C-6), 117.6(C-16), 121.9 (C-18), 122.9 (C-4), 126.3 (C-14), 129.5 (C-7), 130.3 (C-3 and C-5), 135.8 (C-19), 139.9 (C-15), 140.1 (C-8), 159.5 (C-1), 161.6 (C-17), 170.1 (C-9), 171.3 (C-11), 196.9 (C-20). CI-MS (positive mode) m/z 385 $[M + H]^+$ (10), 341 (93), 309 (100).

Butyrolactone-1 (**2**): Yellow powder (20 mg, 0.47%); m.p. = 86–90°C (acetone : hexane) [lit. m.p. = 94–96°C]; $[\alpha]_D^{25} = +70^\circ$ ($c = 0.08$, MeOH); UV (MeOH), λ_{max} (nm): 226.8, 308; IR (KBr), ν_{max} (cm^{-1}): 837, 1036.3, 1115.1, 1179.3, 1261.4, 1385.1, 1610.1, 1739.7, 2925.2, 3411.5; ^1H NMR (500 MHz, CD_3OD): δ 1.56 (3 H, s, H_3-23), 1.65 (3 H, s, H_3-24), 3.06 (2H, d, $J = 7.2$ Hz, H_2-20), 3.41 (2H, d, $J = 3.4$ Hz, H_2-13), 3.77 (3 H, s, H_3-12), 6.41 (1H, d, $J = 1.4$ Hz, H-19), 6.48 (1H, d, $J = 8.1$ Hz, H-16), 6.85 (2H, d, $J = 8.8$ Hz, H-2/H-6), 7.56 (2H, d, $J = 8.8$ Hz, H-3/H-5); ^{13}C NMR (125 MHz, CD_3OD) δ 17.7 (C-23), 25.9 (C-24), 28.7 (C-20), 39.6 (C-13), 53.8 (C-12), 86.8 (C-10), 115.1 (C-16), 116.6 (C-2/6), 123.2 (C-4), 123.6 (C-21), 125.1 (C-14), 128.5 (C-18), 128.9 (C-7), 129.7 (C-15), 130.3 (C-3/

5), 132.4 (C-19), 132.9 (C-22), 139.9 (C-8), 155.1 (C-17), 159.3 (C-1), 170.7 (C-9), 171.6 (C-11); FAB-MS (positive mode) m/z 425 $[M+H]^+$ (55), 369 (55), 175 (100).

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