

### **Cassane Diterpenes from *Caesalpinia bonduc***

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CDRI communication no. 7533

**Abstract**—Three new cassane diterpene hemiketals, caesalpinolide-C (**1**), caesalpinolide-D (**2**), caesalpinolide-E (**3**) and one new cassane furanoditerpene (**4**) were isolated from *Caesalpinia bonduc*. Molecular structure of new compounds were elucidated using NMR spectroscopy in combination with IR, UV and mass spectral data and relative stereochemistries were determined through ROESY correlation. The isolated compounds were tested for their antiproliferative activity in MCF-7 (breast adenocarcinoma), DU145 (prostate carcinoma), C33A (Cervical carcinoma) and Vero (African green monkey kidney fibroblast) cells.

#### **Keywords**

*Caesalpinia bonduc*, Fabaceae, phytochemistry, cassane diterpenes, butenolides, furanoditerpenes, antiproliferative activity.

#### **1. Introduction**

*Caesalpinia bonduc* L. Roxb., (Fabaceae), was collected from the intertidal zone of south Andaman Islands, India as a marine creeper of the mangrove plants (Bennet, 1987). In traditional system of medicine different parts of the plant are used to treat asthma, chronic fever, cough, headache and stomach upset (Nandkarni, 1976; Satyavati et al., 1956; Chopra et al., 1956). Different parts of the plant have shown variety of pharmacological activities such as antimicrobial, adaptogenic, contractile activity in smooth muscles and skeletal muscles and antifilarial activity (Simin et al., 2001; Kannur et al., 2006; Datté et al., 2004; Rastogi et al., 1996).

We previously described the isolation of two epimeric cassane butenolide hemiketals and their antiproliferative activity against different cancer cell lines (Yadav et al., 2007, Kanojiya and Yadav, 2008). Continuing with our earlier studies on *Caesalpinia bonduc*, here we report the isolation and structure elucidation of four new cassane diterpenes caesalpinolide-C (**1**), caesalpinolide-D (**2**), caesalpinolide-E (**3**) and cassane furanoditerpene (**4**) (figure 1) alongwith other known compounds. *Caesalpinia* sp. has reputation of producing cassane furanoditerpenes (Kinoshita et al., 2005; Kaluni et al., 2005; Lyder et al., 1998<sup>a</sup>; Jiang et al., 2001; Linn et al., 2005; Ragasa et al., 2002; Jiang et al., 2002; Roengsumran et al., 1998). Among cassane diterpenes production of the cassane butenolides or butenolide hemiketals is possibly via photooxygenation of the furan ring and these compounds have very limited distribution (Lyder et al., 1998<sup>b</sup>; Torres-Mendoza et al., 2004; Roach et al., 2003; Kinoshita et al., 2005; Jiang et al., 2002).

These compounds were isolated following fractionation of the ethanolic extract where hexane fraction was found to be active against MCF-7 cell lines (Yadav et al., 2007). The isolated compounds were tested for their antiproliferative activity against MCF-7 (breast adenocarcinoma), DU145 (prostate carcinoma), C33A (Cervical carcinoma) and Vero (African green monkey kidney fibroblast) cell lines and were found to show low to moderate activity profile. The structure elucidation of new compounds and their activity profile are discussed in the following sections.

## 2. Results and Discussions

The EtOH extract of the plant showed inhibition of breast cancer cell lines and was fractionated in to n-hexane, chloroform, n-butanol and water soluble fractions. Active n-hexane fraction was further subjected to repeated chromatographic purifications to yield new cassane butenolide hemiketals named as caesalpinolide-C-E (**1-3**) and one cassane furanoditerpene (**4**).

The compound **1** was isolated as white crystals, mp. 216-218 °C,  $[\alpha]_D^{25}$  -95.0 (c 0.12, MeOH), gave a pseudomolecular ion  $[M+H]^+$  peak at  $m/z$  335.2222 in DART-HRMS spectrum consistent with the molecular formula of C<sub>20</sub>H<sub>30</sub>O<sub>4</sub> (calc. for 334.21441). It exhibited IR maxima at 3420 and 1725 cm<sup>-1</sup>, indicative of hydroxyl and  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone functionalities, respectively. The <sup>1</sup>H NMR spectrum (Table 1) together with the COSY, gHSQC, and gHMBC data revealed the presence of a cassane butenolide skeleton with an  $\alpha/\beta$ -unsaturated  $\gamma$ -lactone moiety. The presence of  $\alpha$ - $\beta$ -unsaturated  $\gamma$ -lactone moiety was substantiated by the signals for butenolide olefin H/C at  $\delta$  5.87/ 113.4, downfield quaternary olefin carbon at  $\delta$  175.1, lactone carbonyl at  $\delta$  171.8 and hemiketal carbon at  $\delta$  107.5. <sup>13</sup>C NMR spectrum showed presence of twenty carbons accounting for four methyl, one olefin methine, five sp<sup>3</sup> methine, five methylene, three sp<sup>3</sup> quaternary, one olefinic quaternary and one carbonyl carbon. <sup>1</sup>H NMR showed signals for four methyl groups at  $\delta$  1.08(s), 1.37(s), 1.51(s) and 1.35(d, 7.2). Signal at  $\delta$  1.35 corresponds to CH<sub>3</sub>-17 of cassane skeleton as indicated by its HMBC correlation with the quaternary olefinic carbon at  $\delta$  175.1. Appearance of the CH<sub>3</sub>-17 as doublet indicated C-14 of cassane skeleton to be a methine appearing at  $\delta$  2.87 (*m*) which in turn showed correlations in HMBC spectrum with signals at 175.1(C-13), 113.4(C-15), 107.5(C-12) of the butenolide framework. The other two methyl signals at  $\delta$  1.08 and 1.51 showed common HMBC correlations which indicated them to be the CH<sub>3</sub>-18 and CH<sub>3</sub>-19 methyl

of cassane skeleton. The remaining methyl at  $\delta$  1.37(CH<sub>3</sub>-20) along with CH<sub>3</sub>-18 and CH<sub>3</sub>-19 showed HMBC correlations with the deshielded angular methine at  $\delta$  56.8 indicating an unsubstituted C-5 position.

Among other distinct proton signals are signals for H-5 at  $\delta$  0.97 which showed HSQC with the C-5 at  $\delta$  56.8. Spectrum also revealed the presence of an oxymethine at  $\delta$  4.68/66.3 which was assigned to C-6 based on its COSY correlation with characteristic signal for H-5 at  $\delta$  0.97. Other resolved signals were those for H-8 at  $\delta$  2.36 (*tt*,  $J = 12.2, 4.2$  Hz), H-7 $\beta$  and H-9 at  $\delta$  1.80 (*m*) and H-11 $\alpha$  at  $\delta$  2.76 (*dd*,  $J = 12.6, 3.0$  Hz). Unambiguous assignments were made by analysing 2D NMR correlations (DQF-COSY, HSQC and HMBC). Most of the methylene signals appeared as complex multiplets overlapped with other signals in <sup>1</sup>H NMR spectrum and were assigned based on their correlations in the COSY, HSQC and HMBC spectrum. To further simplify the spectrum and assign the relative stereochemistry at the hemiketal C-12 position, **1** was acetylated with acetic anhydride and pyridine at room temperature to afford the monoacetylated product **1a** and diacetylated product **1b** (major), whereas at elevated temperatures afforded diacetylated product **1b** exclusively. Monoacetylation at hemiketal hydroxyl in **1a** was confirmed by the appearance of C-12 ketal at  $\delta$  105.1, relatively upfield to  $\delta$  107.5 in **1** whereas diacetylated product **1b** has similar shift in the signals for C-12 in addition to downfield shift in the signal for H-6 which appeared at  $\delta$  5.51 as compared to  $\delta$  4.68 in the parent compound. The <sup>1</sup>H NMR spectrum of diacetate **1b** showed resolved signals for the H-8 at  $\delta$  1.89 (*m*), H-11 $\beta$  at  $\delta$  2.69 (*dd*,  $J = 13.2, 3.0$  Hz), H-11 $\alpha$  at  $\delta$  1.31 (*t*,  $J = 13.2$  Hz), H-7 $\alpha$  at  $\delta$  1.53 (*m*) and H-9 at  $\delta$  1.46 (*m*) partially overlapped with signals for H-3, along with appearance of singlet for two acetate methyls at  $\delta$  2.04. Relative stereochemistries were assigned based on ROESY correlations for **1b** (fig. 2). ROESY correlations among signals for CH<sub>3</sub>-20 with CH<sub>3</sub>-19, H-8 and COCH<sub>3</sub>-6 and from CH<sub>3</sub>-18 with H-5, H-6 indicated for the relative stereochemistry of the ring A and B and the  $\alpha$ -orientation of H-5/6. ROESY correlations from H-14 with the signals for H-15 and from CH<sub>3</sub>-17 with the signals for COCH<sub>3</sub>-12 clearly revealed the orientation of the ketal hydroxyl to be  $\alpha$ -oriented (figure 2). Based on the above experimental observations the structure of **1** was assigned as 6 $\beta$ -hydroxy-12 $\alpha$ -hydroxycassa-(13)15-en-16,12-olide.

The compound **2** was isolated as white fluffy crystals, mp. 220-222 °C,  $[\alpha]_D^{25} -44.0$  (c 0.05, MeOH), showed a pseudomolecular ion [M+H]<sup>+</sup> peak at m/z 335.2222 in DART-HRMS spectrum consistent with the molecular formula of C<sub>20</sub>H<sub>30</sub>O<sub>4</sub> (calc. for 334.2144). It exhibited IR maxima at 3382 and 1728 cm<sup>-1</sup>, indicative of hydroxyl and  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone functionalities, respectively. The presence of  $\alpha,\beta$ -butenolide hemiketal ring was characterized by signals at  $\delta$  6.48 (H-15) in <sup>1</sup>H NMR spectrum and a hemiketal carbon at  $\delta$  107.1 alongwith lactone carbonyl at  $\delta$  171.4 and olefinic carbons of butenolide at  $\delta$  113.2(C-15), 179.4(C-13). Resonances for four tertiary methyl groups appeared at  $\delta$  0.75, 0.79, 0.88 and 1.81. Singlet at  $\delta$  1.81 corresponds to CH<sub>3</sub>-17 of cassane skeleton as indicated by its <sup>2</sup>J/<sup>3</sup>J HMBC correlation with quarternary oxygenated carbon C-14 ( $\delta$  74.5), C-13 ( $\delta$  179.4) and C-8 ( $\delta$  49.8). The other two methyl signals at  $\delta$  0.88 and 0.79 showed common HMBC correlations which indicated them to be the CH<sub>3</sub>-18 and CH<sub>3</sub>-19 methyl of cassane skeleton. The remaining methyl at  $\delta$  0.75(CH<sub>3</sub>-20) along with CH<sub>3</sub>-18 and CH<sub>3</sub>-19 showed HMBC correlations with the deshielded angular methine at  $\delta$  55.6 indicating an unsubstituted C-5 position. Signal for H-5 at  $\delta$  0.92 (*brd*, 10.8 Hz) showed

HSQC with the C-5 at  $\delta$  55.6 and indicated for an unsubstituted C-6 position. Other resolved signals were those for H-8 at  $\delta$  1.91 (*dt*,  $J = 11.4, 3.6$  Hz), H-9 at  $\delta$  1.63 (*m*), H-7 $\beta$   $\delta$  2.44 (*dd*,  $J = 12.2, 3.6$  Hz) and H-11 $\alpha$  at  $\delta$  2.74 (*d*, 10.2 Hz). Unambiguous assignments were made by analysing DQF-COSY, HSQC and HMBC experiments. Most of the methylene signals appeared as complex multiplets overlapped with other signals in  $^1\text{H}$  NMR spectrum and were assigned based on their correlations in the COSY, HSQC and HMBC spectrum. To further simplify the spectrum and assign the relative stereochemistry at the hemiketal C-12 position, **2** was acetylated with acetic anhydride and pyridine at room temperature to afford the monoacylated product **2a**. Monoacetylation at hemiketal hydroxyl in **2a** was confirmed by the appearance of C-12 ketal at  $\delta$  105.1, relatively upfield to  $\delta$  107.1 in **2**. The  $^1\text{H}$  NMR spectrum of **2a** showed signals for the H-8 at 1.55 (*m*, partially overlapped with signals for H-2), H-9 at  $\delta$  1.21 (*m*, partially overlapped with signals for H-3), H-11 $\beta$  at  $\delta$  2.66 (*dd*,  $J = 13.2, 2.4$  Hz), and H-7 $\alpha$  at  $\delta$  2.04 (*m*) along with appearance of singlet for acetate methyl at  $\delta$  2.05. Relative stereochemistries were assigned based on ROESY correlations (fig. 3) observed among signals for CH<sub>3</sub>-20 with CH<sub>3</sub>-19, H-8, H-11 $\beta$ , COCH<sub>3</sub>-12, and from CH<sub>3</sub>-18 to signals for H-5, H-6 which characterizes the relative stereochemistry of A and B rings. ROESY correlation of the acetate methyl singlet at  $\delta$  2.05 with signals for CH<sub>3</sub>-17 at  $\delta$  1.28 clearly revealed that the ketal hydroxyl was  $\alpha$ -oriented (figure 2). Based on the above experimental observations the structure of **2** was assigned as 12 $\alpha$ ,14 $\beta$ -dihydroxycassa-(13)15-en-16,12-olide.

The compound **3** gave a  $[\text{M}+\text{H}]^+$  molecular ion peak at  $m/z$  377.23280,  $[\text{M}+\text{H}_2\text{O}]^+$  at  $m/z$  394.25667 in DART-HRMS spectrum consistent with the molecular formula of C<sub>22</sub>H<sub>32</sub>O<sub>5</sub>. It exhibited IR maxima at 3403 and 1742 cm<sup>-1</sup>, indicative of hydroxyl and  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone functionalities, respectively. The presence of  $\alpha,\beta$ -butenolide hemiketal ring was characterized by signals at  $\delta$  5.72 (H-15) in  $^1\text{H}$  NMR spectrum and a hemiketal carbon at  $\delta$  105.7 along with lactone carbonyl at  $\delta$  170.5 and olefinic carbons of butenolide at  $\delta$  113.8(C-15), 172.7(C-13).  $^{13}\text{C}$  NMR and phase sensitive gHSQC spectrum showed presence of twenty carbons accounting for four methyl, one olefin methine, five  $sp^3$  methine, five methylene, three  $sp^3$  quaternary, one olefinic quaternary and one carbonyl carbon.  $^1\text{H}$  NMR showed signals for four methyl groups at  $\delta$  0.98(*s*), 1.00(*s*), 1.10(*s*) and 1.16(*d*, 7.2) and one acetate methyl at  $\delta$  2.04(*s*). Signal at  $\delta$  1.16 corresponds to CH<sub>3</sub>-17 of cassane skeleton as indicated by its HMBC correlation with the quaternary olefinic carbon at  $\delta$  172.7. Appearance of the CH<sub>3</sub>-17 as doublet indicated C-14 of cassane skeleton to be a methine appearing at  $\delta$  2.92 (*m*) which in turn showed correlations in HMBC spectrum with signals at 172.7(C-13), 113.8(C-15), 105.7(C-12) of the butenolide framework. The other two methyl signals at  $\delta$  1.08 and 1.51 showed common HMBC correlations which indicated them to be the CH<sub>3</sub>-18 and CH<sub>3</sub>-19 of cassane skeleton. The remaining methyl at  $\delta$  1.37(CH<sub>3</sub>-20) along with CH<sub>3</sub>-18 and CH<sub>3</sub>-19 showed HMBC correlations with the deshielded angular methine at  $\delta$  55.4 indicating an unsubstituted C-5 position. Comparison of the NMR data of **3** with **1a** suggested them to be isomeric with the only difference in the position of the acetate substitution. In **1a** acetate was at C-12 position whereas in **3** it was found to be at C-6 position as indicated by HMBC correlation of the H-6 with the acetate carbonyl at  $\delta$  170.5. Acetylation of **3** with acetic anhydride and pyridine at room temperature afforded the same compound as

**1b** as characterized with its TLC profile and  $^1\text{H}$  and  $^{13}\text{C}$ NMR data. Recently isolation of  $6\alpha$ -acetoxy isomer of the compound **3** has been reported from *Bowdichia nitida* (Matsuno et al., 2008). The report showed antiperiplaner orientation for H-5 ( $\delta$  1.31, *d*, 11.0 Hz) and H-6 ( $\delta$  5.12, *ddd*, 11.0, 11.0, 4.1Hz) whereas in our case H-5 and H-6 has  $\alpha$ -orientation with very low order of coupling observed in COSY spectrum. Based on the above experimental observations the structure of **3** was assigned as  $6\beta$ -acetoxy-12 $\alpha$ -hydroxycassa-(13)15-en-16,12-olide.

The compound **4** gave a  $[\text{M}+\text{H}_2\text{O}]^+$  molecular ion peak at  $m/z$  358.22966 in DART-HRMS spectrum consistent with the molecular formula of  $\text{C}_{22}\text{H}_{28}\text{O}_3$ . It exhibited IR maxima at 3403 and  $1742\text{ cm}^{-1}$ , indicative of acetate and furan ring systems.  $^{13}\text{C}$  NMR spectrum showed presence of twenty two carbons accounting for three aromatic methine, five aromatic quaternary, five methyl, one oxymethine, four methylene, two  $\text{sp}^3$  quaternary and one carbonyl carbon. The  $^1\text{H}$  NMR showed signals for one acetate methyl at  $\delta$  2.01 (*s*), one aromatic methyl at  $\delta$  2.33 (*s*), and other three methyls at  $\delta$  1.61(*s*), 1.10(*s*), and 1.07(*s*). The presence of trisubstituted benzofuran moiety was characterized by doublets for 1,2 substituted furan at  $\delta$  7.53 (*d*,  $J=2.2$  Hz) and 6.72 (*dd*,  $J=2.1, 0.7$  Hz), one proton singlet at  $\delta$  7.39 and aromatic methyl at  $\delta$  2.33. From the HSQC and HMBC correlations it was evident that the aromatic methyl was present at the C-14 position and signal for H-11 appeared as an isolated broad singlet. Two methyl signals at  $\delta$  1.07 and 1.10 showed common HMBC correlations which indicated them to be the  $\text{CH}_3$ -18 and  $\text{CH}_3$ -19 and the remaining methyl at  $\delta$  1.61 was assigned as  $\text{CH}_3$ -20 of the cassane skeleton.  $\text{CH}_3$ -18 and  $\text{CH}_3$ -19 showed HMBC correlations with the deshielded angular methine at  $\delta$  55.4 indicating an unsubstituted C-5 position which was further substantiated by its HSQC correlation with the proton at  $\delta$  1.55. That the acetoxymethine  $\delta$  5.84 (*brd*, 5.5) was present at the C-6 position was confirmed by its HMBC correlations with the C-5, C-10 and C-8. Relative stereochemistry of the acetoxymethyl function was established on ROESY correlations observed for H-6 with signals for  $\text{CH}_3$ -18, and for acetate methyl with the signals for  $\text{CH}_3$ -19 and  $\text{CH}_3$ -20. Based on the above experimental results the structure of **4** was characterized as  $6\beta$ -acetoxy-17-methylvoucapane-8(14),-9(11)-diene.

Other known compounds isolated from the plant are furanoditerpene (**5**) (Jadhav et al., 2003), friedelin (Ageta et al., 1995), lupeol (Burns et al., 2000), mixture of  $\alpha$  and  $\beta$ -amyirin (Mahato and Kundu, 1995) and mixture of  $\beta$ -sitosterol and stigmasterol (Rubinstein et al., 1976). Structure of known compounds was elucidated based on their spectroscopic comparison to the data reported in the literature.

Isolated compounds were tested for their antiproliferative activity against MCF-7 (breast adenocarcinoma), DU145 (prostate carcinoma), C33A (Cervical carcinoma) and Vero (African green monkey kidney fibroblast) cancer cell lines and were found to show moderate to low order  $\text{IC}_{50}$  ( $\mu\text{M}$ ) values (Table 3). Among the tested compounds, **2** was found to show better activity profile in cervical and prostate and breast carcinoma cell lines whereas compound **5** was found most active in breast and cervical carcinoma cell lines.

As pointed out earlier (Kinoshita et al., 2005) there has been enormous confusion of botanical names because of enormous morphologic variations (and possibly chemical

variations as well) at the infra-specific level in some *Caesalpinia* species. The isolation of furanoditerpene **5** from species of *Caesalpinia* i.e. *C. crista* has been reported as chemotaxonomic marker for that species to differentiate it from the synonymous *C. bonduc* (Jadhav et al., 2003). Whereas, isolation of the **5** from *C. bonduc* L. Roxb. in our report revealed that the *C. crista* in the earlier report was actually *C. bonduc* L. Roxb. Further isolation of the cassane butenolide hemiketals in present study may serve as distinguishing feature for *C. bonduc* from other related species of genus.

### 3. Experimental

#### 3.1. General experimental procedure

Melting points were recorded on a Complab melting point apparatus and are uncorrected. IR spectra (KBr) were recorded on a Perkin-Elmer RX-1 spectrophotometer. UV spectra were obtained on a Perkin Elmer  $\lambda$ -15 UV spectrophotometer. Optical rotation was measured on Rudolf Autopol III polarimeter, NMR spectra were run on 600 MHz Varian Inova Spectrometer, Electrospray mass spectra were recorded on MIMASS QUATTRO II triple quadrupole mass spectrometer. DART-HRMS were recorded on JEOL AccuTOF-DART instrument. Column chromatography was performed using silica gel (60-120 mesh) and flash silica gel (230-400 mesh).

#### 3.2. Plant material

The whole plant of *Caesalpinia bonduc* were collected from intertidal zone of south Andaman Islands, India, and identified by Dr. M. N. Srivastava, Botany Division, Central Drug Research Institute. A voucher specimen (No. 464) is kept in the herbarium of the Institute.

#### 3.3. Extraction and isolation

Air dried and powdered plant material (4 Kg) were extracted at room temperature with EtOH. The concentrated EtOH extract (230 g) was then fractionated successively into four fractions: *n*-Hexane (38 g), CHCl<sub>3</sub> (20 g), *n*-BuOH (58 g), and aqueous (80 g). The *n*-Hexane (32 g) fraction was subjected to column chromatography over silica gel, eluted with EtOAc:Hexane gradient (0 to 100%) to afford eighteen fractions (F-1 to F-17). Fraction F-4 was subjected to repeated chromatographic purification over silica gel eluting with gradient of EtOAc:Hexane (0% to 2 %) to give pure compounds **5** (25 mg) and friedelin (15mg). Fraction F-5 was subjected to repeated chromatographic purification over silica gel using isocratic elution with Me<sub>2</sub>CO: CHCl<sub>3</sub> (2%). Slightly impure compound was obtained which was further purified by preparative TLC (MERCK PTLC plates concentration zone silica gel 0.5mm) using Me<sub>2</sub>CO: CHCl<sub>3</sub> (15%) as eluent to afford compound **4** (4mg). F-7 was chromatographed over flash silica gel eluting with gradient of EtOAc:Hexane (20% to 30 %) to give **3**(6mg). Fraction F-8 upon chromatography over silica gel eluting with gradient of Me<sub>2</sub>CO: CHCl<sub>3</sub> (0.2%) to give compound which was found to be a mixture of at least two compounds in <sup>13</sup>C NMR spectrum. TLC was run over silver nitrate (10%) impregnated silica gel which showed two distinct spots. Further chromatographic purification over AgNO<sub>3</sub> impregnated (10%) silica gel using Me<sub>2</sub>CO: CHCl<sub>3</sub> (0.5%) afforded pure lupeol (20 mg) and mixture of  $\alpha$  and  $\beta$ -amyrin (25 mg). Fraction F-9 after chromatographic purification over silica gel eluting with EtOAc:Hexane (15%) afforded mixture of  $\beta$ -sitosterol and stigmasterol

(40mg). Fraction F-13 was subjected to repeated chromatographic purification over silica gel eluting with gradient of MeOH: CHCl<sub>3</sub> (0.5 to 6%) to give pure compounds **1**(12 mg) and **2**(10 mg).

**1**: M.p. 216-218 °C;  $[\alpha]_D^{25}$  -95.0 (*c* 0.12, MeOH); UV  $\lambda_{\max}^{\text{MeOH}}$  nm: 215; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3493, 2929, 1731, 1650, 1228, 1029, 934; for <sup>1</sup>H NMR (600 MHz, Pyr-*d*<sub>5</sub>) and <sup>13</sup>C NMR (150 MHz, Pyr-*d*<sub>5</sub>) spectroscopic data see table 1; ESIMS (pos.): *m/z* 357 [M+Na]<sup>+</sup>, 335 [M+H]<sup>+</sup>, 316 [M-H<sub>2</sub>O]<sup>+</sup>; DART HRMS: [M+H]<sup>+</sup> 335.2097; calc. for C<sub>20</sub>H<sub>31</sub>O<sub>4</sub>; 335.2222. **1** (3mg) was acetylated with acetic anhydride (1ml) and pyridine (0.2ml) at room temperature, kept overnight and was concentrated under reduced pressure and purified by column chromatography using gradient of Me<sub>2</sub>CO:CHCl<sub>3</sub> (0.5 to 4%) to afford pure monoacylated product **1a**(2.0mg) and a very minute amount of diacetylated product **1b**; Again 3mg of **1** was acetylated by acetic anhydride (1ml) and pyridine (0.4ml) at elevated temperature of 70 °C to afford exclusively **1b**(1.8 mg). **1a**; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3447, 2930, 1752, 1643, 1461, 1378, 1160, 1030. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150 MHz); see table 1. ESIMS (pos.): *m/z* 399 [M+Na]<sup>+</sup>, 394 [M+H<sub>2</sub>O]<sup>+</sup>, 377 [M+H]<sup>+</sup>, 316 [M-CH<sub>3</sub>COOH]<sup>+</sup>. **1b**; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 2928, 1745, 1643, 1459, 1370, 1204, 1157, 1024, 966. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150 MHz); see table 1. ESIMS (pos.): *m/z* 441 [M+Na]<sup>+</sup>, 359 [M-CH<sub>3</sub>COOH]<sup>+</sup>.

**2**: M.p.: 220-222 °C;  $[\alpha]_D^{25}$  -44.0 (*c* 0.05, MeOH); UV  $\lambda_{\max}^{\text{MeOH}}$  nm: 216; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3401, 2927, 1729, 1659, 1593, 1216, 931; for <sup>1</sup>H NMR (600 MHz, Pyr-*d*<sub>5</sub>) and <sup>13</sup>C NMR (150 MHz, Pyr-*d*<sub>5</sub>) spectroscopic data see table 1; ESIMS (pos.): *m/z* 357 [M+Na]<sup>+</sup>, 335 [M+H]<sup>+</sup>, 316 [M-H<sub>2</sub>O]<sup>+</sup>; DART HRMS: [M+H]<sup>+</sup> 335.2200; calc. for C<sub>20</sub>H<sub>31</sub>O<sub>4</sub>; 335.2222. **2** (3mg) was acetylated with acetic anhydride (1ml) and pyridine (0.2ml) at room temperature, kept overnight. The reaction mixture was concentrated under reduced pressure and purified by column chromatography using gradient of Me<sub>2</sub>CO:CHCl<sub>3</sub> (0.5 to 4%) to afford pure monoacylated product **2a**(2.3mg); **2a**; white solids; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3447, 2930, 1770, 1745, 1651, 1461, 1430, 1378, 1245, 1165, 1030. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150 MHz); see table 1. ESIMS (pos.): *m/z* 399 [M+Na]<sup>+</sup>, 377 [M+H]<sup>+</sup>, 316 [M-CH<sub>3</sub>COOH]<sup>+</sup>.

**3**:  $[\alpha]_D^{25}$  -44.82 (*c* 0.029, MeOH); UV  $\lambda_{\max}^{\text{MeOH}}$  nm: 214; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup>: 3490, 2930, 1730, 1656, 1590, 1216, 929; for <sup>1</sup>H NMR (600 MHz, Pyr-*d*<sub>5</sub>) and <sup>13</sup>C NMR (150 MHz, Pyr-*d*<sub>5</sub>) spectroscopic data see table 2; DART HRMS: [M+H]<sup>+</sup> 377.2292; calc. for C<sub>22</sub>H<sub>33</sub>O<sub>5</sub>; 377.2328.

**3** (2mg) was acetylated with acetic anhydride (1ml) and pyridine (0.2ml) at room temperature, kept overnight. The reaction mixture was concentrated under reduced pressure and purified by column chromatography using gradient of Me<sub>2</sub>CO:CHCl<sub>3</sub> (0.5 to 4%) to afford pure compound **3a** (1mg); It was found to be similar to **1b** from its TLC mobility and NMR data.

4: oily,  $[\alpha]_D^{25}$  -45.0 (*c* 0.02, MeOH); UV  $\lambda_{\max}^{\text{MeOH}}$  nm: 212, 266, 282, 294; IR  $\nu_{\max}^{\text{KBr}}$   $\text{cm}^{-1}$ : 3436, 2929, 1729, 1624, 1458, 1134, 1033, 765, 1386, 1696, 756 for  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ) spectroscopic data see table 2; ESIMS (pos.): *m/z* 363  $[\text{M}+\text{Na}]^+$ , 280  $[\text{M}-\text{CH}_3\text{COOH}]^+$ ; DART HRMS:  $[\text{M}+\text{H}_2\text{O}]^+$  358.2288; calc. for  $\text{C}_{22}\text{H}_{30}\text{O}_4$ ; 358.2144.

### 3.3. Antiproliferative assays:

Antiproliferative activity of these compounds was measured by using the MTT-micro culture tetrazolium assay described by Mosmann (1983) using staurosporine (a broad spectrum inhibitor of protein kinases) as a positive control. Briefly, cells at the exponential growth phase were harvested and seeded into a flat bottom 96 well plate. A total of 180  $\mu\text{l}$  volume containing  $10^4$  cells were added to each well of the plate. After 24 h incubation in a 5% humidified  $\text{CO}_2$  incubator at 37 °C, 20  $\mu\text{l}$  of the test agent was added in triplicates to give final concentration of 150, 75, 37.5, 18.75 and 9.375  $\mu\text{M}$ . After 48 h of incubation at 37 °C, 25  $\mu\text{l}$ /well MTT (stock solution 5 mg/ml PBS) was added and the plate was again incubated at 37 °C for 4 h. Supernatant was removed carefully by aspiration and DMSO (150  $\mu\text{l}$ ) was added to each well to dissolve the formazan dye by gentle shaking for 15 minutes. The plates were read immediately in a plate reader operating at 540 nm. Mean values were calculated and the  $\text{IC}_{50}$  values for each cell line were interpolated from the dose response curve.

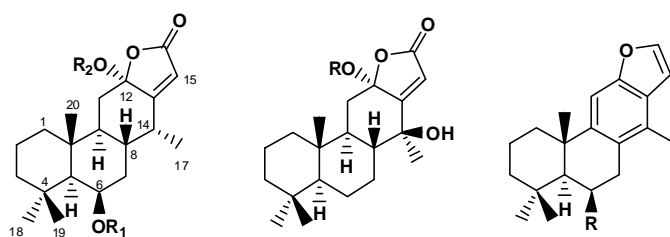
**Acknowledgment:** Thanks are due to MoES for financial assistance and Dr. R. Maurya for his constant encouragement and critical suggestions throughout the course of work.

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**Figure 1.** Structure of compounds.

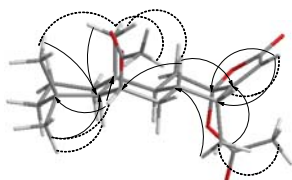
1  $R_1=R_2=H$   
 1a  $R_1=H, R_2=COCH_3$   
 1b  $R_1=R_2=COCH_3$   
 3  $R_1=COCH_3, R_2=H$

2  $R=H$   
 2a  $R=COCH_3$

4  $R=OCOCH_3$   
 5  $R=H$

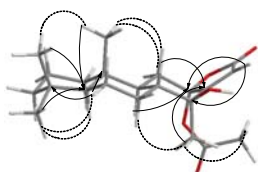
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Kanojija, Sudhir Sinha, M. N. Srivastava, Ram Raghubir



**Figure 2.** Selected ROESY (.....) and HMBC (→) correlations for **1b**.

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**Figure 3.** Selected ROESY (.....) and HMBC (→) correlations for **2a**.

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**Table 1.** NMR Data for Compounds **1**, **1a**, **2**, **2a** and **2b**.

Position	<b>1</b> (Pyr- <i>d</i> <sub>5</sub> ) <sup>b</sup> δH [mult., J(Hz)]	<b>1</b> (Pyr- <i>d</i> <sub>5</sub> ) <sup>a</sup> δC	<b>1a</b> (CDCl <sub>3</sub> ) <sup>a</sup> δH [mult., J(Hz)]	<b>1a</b> (CDCl <sub>3</sub> ) <sup>a</sup> δC	<b>1b</b> (CDCl <sub>3</sub> ) <sup>a</sup> δH [mult., J(Hz)]	<b>1b</b> (CDCl <sub>3</sub> ) <sup>a</sup> δC	<b>2</b> (Pyr- <i>d</i> <sub>5</sub> ) <sup>a</sup> δH [mult., J(Hz)]	<b>2</b> (Pyr- <i>d</i> <sub>5</sub> ) <sup>a</sup> δC	<b>2a</b> (CDCl <sub>3</sub> ) <sup>a</sup> δH [mult., J(Hz)]	<b>2a</b> (CDCl <sub>3</sub> ) <sup>a</sup> δC
1	1.66 [ <i>m</i> ]* 1.03 [ <i>brt</i> , 13.2 Hz]	42.4	1.73 [ <i>brd</i> , 12.6 Hz] 1.03 [ <i>m</i> ]*	42.1	1.76 [ <i>m</i> ]* 1.06 [ <i>m</i> ]*	42.2	1.59 [ <i>m</i> ]* 0.96 [ <i>dd</i> , 13.2, 3.0 Hz]	40.1	1.72 [ <i>m</i> ]* 1.00 [ <i>dt</i> , 12.9, 3.6 Hz]	39.8
2	1.66 [ <i>m</i> ]* 1.37 [ <i>m</i> ]*	19.7	1.65 [ <i>m</i> ]* 1.42 [ <i>m</i> ]*	18.8	1.62 [ <i>m</i> ] 1.49 [ <i>m</i> ]	18.9	1.45 [ <i>m</i> ]* 1.32 [ <i>m</i> ]*	19.5	1.55 [ <i>m</i> ]* 1.48 [ <i>m</i> ]*	18.9
3	1.37 [ <i>m</i> ]* 1.20 [ <i>brt</i> , 12.6 Hz]	44.3	1.42 [ <i>m</i> ]* 1.19 [ <i>m</i> ]*	43.7	1.42 [ <i>m</i> ]* 1.21 [ <i>dt</i> , 13.8, 3.6 Hz]	43.8	1.32 [ <i>m</i> ]* 1.12 [ <i>dt</i> , 13.2, 3.6 Hz]	42.4	1.43 [ <i>m</i> ]* 1.16 [ <i>brdt</i> , 13.3, 3.9 Hz]	41.9
4	-	34.6	-	34.1	-	34.1	-	33.7	-	33.4
5	0.97 [ <i>brs</i> ]*	56.8	0.96 [ <i>brs</i> ]	56.1	1.06 [ <i>brd</i> , 1.02 Hz]	55.4	0.92 [ <i>brd</i> , 10.8 Hz]	55.6	0.91 [ <i>m</i> ]*	55.3
6	4.68 [ <i>brs</i> ]	66.3	4.51 [ <i>brs</i> ]	67.1	5.51 [ <i>brs</i> ]	69.3	1.66 [ <i>m</i> ]* 1.23 [ <i>dq</i> , 12.6, 3.0 Hz]	22.1	1.72 [ <i>m</i> ]* 1.27 [ <i>m</i> ]*	21.2
7	1.80 [ <i>m</i> ]* 1.66 [ <i>m</i> ]*	41.0	1.65 [ <i>m</i> ]* 1.57 [ <i>m</i> ]*	39.2	1.76 [ <i>m</i> ]* 1.53 [ <i>m</i> ]	35.5	2.44 [ <i>dd</i> , 12.2, 3.6 Hz] 1.45 [ <i>m</i> ]*	27.4	2.04 [ <i>m</i> ] 1.27 [ <i>m</i> ]*	26.4
8	2.36 [ <i>tt</i> , 12.2, 4.2 Hz]	36.5	2.08 [ <i>tt</i> , 12.0, 4.8 Hz]	35.5	1.89 [ <i>m</i> ]	36.2	1.91 [ <i>dt</i> , 11.4, 3.6 Hz]	49.8	1.55 [ <i>m</i> ]*	49.0
9	1.80 [ <i>m</i> ]*	46.5	1.42 [ <i>m</i> ]*	44.8	1.46 [ <i>m</i> ]*	44.7	1.63 [ <i>m</i> ]*	48.2	1.21 [ <i>m</i> ]*	47.0
10	-	38.2	-	37.4	-	37.95	-	37.9	-	37.5
11	2.76 [ <i>dd</i> , 12.6, 3.0 Hz] 1.66 [ <i>m</i> ]*	39.5	2.67 [ <i>dd</i> , 13.8, 3.0 Hz] 1.30 [ <i>brt</i> ,	37.8	2.69 [ <i>dd</i> , 13.2, 3.0 Hz] 1.31 [ <i>t</i> ,	37.93	2.74 [ <i>d</i> , 10.2 Hz] 1.59 [ <i>m</i> ]*	39.6	2.66 [ <i>dd</i> , 13.2, 2.4 Hz] 1.27 [ <i>m</i> ]*	37.8

	13.2 Hz]				13.2 Hz]					
12	-	107.5	-	105.1	-	105.2	-	107.1	-	105.1
13	-	175.1	-	169.9	-	169.9	-	179.4	-	172.5
14	2.87 [m]	37.3	3.00 [m]	36.0	2.98 [m]	36.0	-	74.5	-	74.4
15	5.87 [s]	113.4	5.86 [s]	115.0	5.87 [s]	115.5	6.48 [s]	113.2	6.06 [s]	114.8
16	-	171.8	-	169.7	-	169.7	-	171.4	-	169.1
17	1.35 [d, 7.2 Hz]	13.8	1.03 [d, 7.2 Hz]	11.6	1.03 [d, 7.2 Hz]	11.8	1.81 [s]	22.1	1.28 [s]	20.2
18	1.51 [s]	34.5	1.01 [s]	33.8	0.98 [s]	33.9	0.88 [s]	34.2	0.89 [s]	33.8
19	1.08 [s]*	24.5	1.24 [s]	24.3	1.00 [s]	23.5	0.79 [s]	22.5	0.84 [s]	22.2
20	1.37 [s]	18.1	1.13 [s]	17.5	1.10 [s]	17.3	0.75 [s]	14.8	0.82 [s]	14.4
COCH <sub>3</sub> -6	-	-	-	-	2.04 [s]	21.9	-	-	-	-
						170.6				
COCH <sub>3</sub> - 12	-	-	2.04 [s]	21.7	2.04 [s]	22.0	-	-	2.05 [s]	-
				168.6		168.8				

\* Overlapped signals, <sup>a</sup>Data recorded at 600 MHz for <sup>1</sup>H and 150 MHz for <sup>13</sup>C.

**Table 2.** NMR Data for Compounds **3** and **4**.

Position	<b>3</b>		<b>4</b>	
	(Pyr- <i>d</i> <sub>5</sub> ) <sup>a</sup> δH [mult., J(Hz)]	(Pyr- <i>d</i> <sub>5</sub> ) <sup>b</sup> δC	(CDCl <sub>3</sub> ) <sup>a</sup> δH [mult., J(Hz)]	(CDCl <sub>3</sub> ) <sup>a</sup> δC
1	1.76 [m]* 1.07 [m]*	42.2	2.31 [brd, 12.6] 1.49 [brdd, 13.5, 1.4 Hz]	43.2
2	1.61 [m] 1.49 [m]	19.0	1.88 [tt, 13.8, 3.3] 1.65 [td, 13.9, 3.4 Hz]	19.9
3	1.41 [brd, 12.6 Hz] 1.20 [dt, 13.2, 3.6 Hz]	43.9	1.45 [dd, 13.0, 3.1] 1.25 [m]*	43.3
4	-	34.1	-	34.3
5	1.07 [brs]*	55.4	1.55 [brs]	51.8
6	5.51 [brs]	69.5	5.84 [brd, 5.5]	67.9
7	1.76[m]* 1.56 [m]*	35.7	3.06 [dd, 18.1, 5.4] 2.98 [d, 18.0]	35.3
8	1.87 [m]	36.1	-	123.9
9	1.56 [m]	45.3	-	146.3
10	-	37.9	-	38.7
11	2.43 [dd, 13.2, 3.6 Hz] 1.44 [t, 13.2 Hz]	38.0	7.39 [brs]	105.4
12	-	105.7	-	154.0
13	-	172.7	-	125.8
14	2.92 [m]	36.2	-	128.6
15	5.72 [s]	113.8	6.72 [dd, 2.1, 0.7]	105.1
16	-	170.5	7.53 [d, 2.2]	144.6
17	1.16 [d, 7.2 Hz]	13.1	2.33 [s]	16.2
18	0.98 [s]	33.9	1.07 [s]	33.7
19	1.00 [s]	23.6	1.10 [s]	23.3
20	1.10 [s]	17.4	1.61 [s]	27.7
COCH <sub>3</sub> -6	2.04 [s]	22.0	2.01 [s]	22.0
		170.7		171.2

\* Overlapped signals, <sup>a</sup>Data recorded at 600 MHz for <sup>1</sup>H and 150 MHz for <sup>13</sup>C.

**Table 3:** In-vitro antiproliferative activity of compounds.

Compounds	IC <sub>50</sub> (μM)			
	MCF-7	DU145	C33A	Vero
<b>1</b>	89.82	89.21	77	>150
<b>2</b>	75.23	68.52	58.2	>150
<b>3</b>	97.90	113.50	101.03	>133
<b>5</b>	64.03	119.72	75.83	166.44
Staurosporine*	9.4	5.4	1.5	9.0

\* Staurosporine was used as positive control in anti-proliferative assay