Antiproliferative Trihydroxyalkylcyclohexenones from *Pleiogynium timoriense*

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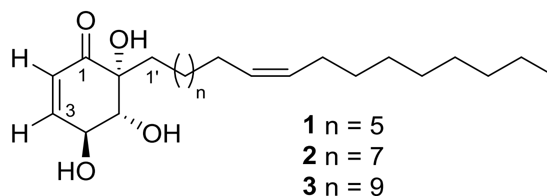
ABSTRACT: Investigation of a DCM extract of the bark of *Pleiogynium timoriense* from the former Merck collection of natural product extracts for antiproliferative activity indicated that it was active with an IC_{50} value of 1.3 μg/mL against the A2780 ovarian cancer cell line. Bioassay-directed fractionation of this extract yielded the three new bioactive trihydroxyalkylcyclohexenones **1**-**3**. Their structures were determined by a combination of spectroscopic and chemical methods. Compounds **1-3** exhibited submicromolar antiproliferative activity against the A2780 human ovarian cancer cell line, with IC_{50} values of 0.8, 0.7, and 0.8 μM, respectively.

As part of an investigation of the former Merck natural products extract library for antiproliferative constituents, now maintained by the Natural Products Discovery Institute,[1] we identified a DCM fraction of the ethanol extract of the bark of *Pleiogynium timoriense* (Anacardiaceae) as a promising extract with an IC_{50} value of 1.3 μg/mL against the A2780 ovarian cancer cell line. *P. timoriense,* also known as the Burdekin plum, is a tree found in northeast Australia and Malaysia as well as locations in the south-central Pacific and southwestern Pacific.[2] Its fruit is used to make jam,[3] and its leaves have been reported to be a source of antioxidants. Twelve compounds, including kaempferol, gallic acid, various kaempferol, quercitin, and myricetin glycosides, and three galloyl derivatives have been identified from the ethanolic extract of the leaves.[4] It has also been reported that cyanidin 3-glucoside can be found in the fruit of *P. timoriense*.[5,6] This DCM fraction was selected for fractionation based on its antiproliferative activity and the lack of reported antiproliferative compounds from the species.

The DCM fraction (0.30 g) was fractionated using Sephadex LH-20 column chromatography, and two rounds of C_{18} HPLC to yield the three active compounds **1** – **3**. Compound **2** was obtained in the largest amount and was investigated first.



^{13}C NMR and HRESIMS data indicated that compound **2** had the molecular formula of $C_{25}H_{44}O_4$ ($[M+H]^+$ *m/z* 409.3291, calcd for $C_{25}H_{45}O_4^+$ 409.3312). Its ^{1}H NMR spectrum indicated the presence of an α,β–unsaturated carbonyl group (H-2, δ 6.1, 1H, dd, J = 10.2, 0.7 Hz; H-3 δ

6.8, 1H, ddd, J = 10.1, 3.9, 1.3 Hz) which was confirmed by its ^{13}C NMR spectrum (C-1, δ 200.2; C-2, δ 126.4; C-3, δ 145.9). A large peak for methylene protons in the ^{1}H NMR spectrum (δ 1.22 – 1.34) as well as a triplet at δ 0.88 (H-19′, 3H, J = 6.9 Hz) indicated the presence of a long alkyl chain in **2**. This was consistent with the ^{13}C NMR data which showed 10 signals at approximately δ 29 as well as signals at δ 23.0 (C-2′), δ 22.7 (C-18′), and δ 14.7 (C-19′). The NMR spectroscopic data are comparable to those of other known trihydroxyalkylcyclohexenone derviatives.[7-10] The presence of a double bond within the alkyl chain was indicated by a signal at δ 5.34 (2H, m) in the ^{1}H NMR spectrum as well as by COSY correlations from δ 5.34 (H-10′, H-11′) to δ 2.00 (H-9′, H-12′).

COSY correlations between H-3 and H-4 as well as H-4 and H-5 were used to establish the positions of C-4 and C-5 (Figure 1). The lack of other correlations in the COSY spectra indicated that C-5 must be attached to an oxygenated tertiary carbon (C-6). HMBC correlations of H-2 to C-6, H-4 to C-6, and H-5 to C-1 (Figure 1) indicated that the structure contained a cyclic moiety, which is consistent with the calculated hydrogen deficiency index of four.

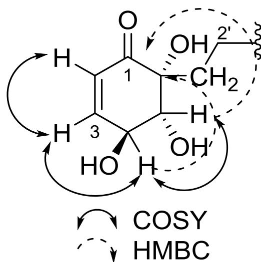


Figure 1. Selected 2D NMR correlations of **2**.

The configuration of the double bond in the alkyl chain was assigned as Z based on the shifts of the adjacent carbon atoms (δ 27.2 C-9′ and C-12′) which would have been more shielded in the case of an E-configuration (δ ~32).[11,12] The connectivity of the alkyl chain at C-6 was

determined from the HMBC spectrum, which showed long range correlations from H-1′ to C-6. The remaining ^{13}C NMR signals were assigned using HSQC and HMBC spectroscopy. Complete NMR assignments of all carbons and protons for **2** are reported in Table 1.

The location of the double bond in the alkenyl chain was determined through MS analysis of the products resulting from derivatization with dimethyl disulfide, following the method of Mansour[13] and Roumy.[9] The LC-MS of the dimethyl disulfide derivative of **2** contained a strong fragment ion at *m/z* 329.19 (calcd. $[C_{17}H_{29}O_4S]^+$ 329.18), indicating a $\Delta^{10',11'}$ double bond.

The ^{1}H NMR spectra of compounds **1** and **3** were similar to those of compound **2** (Table 1). The structures of **1** and **3** were assigned by comparison of NMR and MS data with those of **2**. ^{1}H and ^{13}C NMR spectroscopic data indicated that the structure of the cyclic moiety was identical based on chemical shifts and coupling constants. The only differences in the structures were due to the length of the alkyl chain and the location of the double bond within the chain. HRESIMS and ^{13}C NMR data were used to determine that **1** contained two fewer methylene groups than **2** ($[M+H]^+$ *m/z* 381.2972, calcd for $C_{23}H_{41}O_4^+$ 381.2999) and that **3** contained two additional methylene groups ($[M+H]^+$ *m/z* 437.3604, calcd for $C_{27}H_{49}O_4^+$ 437.3625).

LC-MS analysis of the dimethyl disulfide derivative of **1** showed a strong fragment ion at *m/z* 301.12 (calcd for $[C_{15}H_{25}O_4S]^+$ 301.15), and that of the same derivative of **3** showed a strong fragment ion at 357.20 (calcd for $[C_{19}H_{33}O_4S]^+$ 357.21). These results indicated $\Delta^{9',10'}$ and $\Delta^{12',13'}$ double bonds in **1** and **3**, respectively.

Determination of the configurations of the stereogenic centers on the cyclohexenone ring of **2** proved to be a challenge. Comparison of ^{1}H NMR shifts and coupling constants with those of similar known trihydroxyalkylcyclohexenones was not definitive in determining the relative configuration.[7,8,10] However, the observed coupling constants of H-2 and H-3 in its ^{1}H NMR spectrum (H-2, dd, J = 1.1, 10.2 Hz; H-3, ddd, J = 1.1, 3.7, 10.2 Hz) are consistent with those of similar alkylcyclohexenones with the same relative configuration as proposed.[14] In order to further support our proposed relative configuration, the cyclic double bond of **2** was selectively reduced with diphenylsilane in the presence of $ZnCl_2$ and $Pd(PPh_3)_4$ to give the substituted cyclohexanone **4**. Irradiation of H-3 in a selective NOE experiment indicated correlations to H-1′ and H-4, but no correlation was observed to H-5b (δ 1.84) which indicated that H-3 was equatorial (Figure 2). The small coupling constants observed for H-3 in the ^{1}H NMR spectrum of **4** (1H, dd, J = 4.5, 1.1 Hz) allowed the assignment of equatorial orientation for H-4 in **4**, and thus the corresponding pseudo-equatorial orientation of H-4 in **2**. A correlation was observed from H-1' (δ 1.7) to H-6a (δ 2.7) in a 2D NOESY spectrum, indicating that the alkyl chain was in an axial orientation. The conformational preference for three axial substituents and only one equatorial substituent in **4** is presumably due to hydrogen bond formation between the carbonyl and the C-2 hydroxy groups.

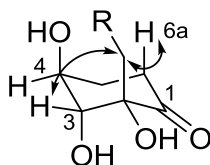


Figure 2. Selected NOE correlations of **4**

The absolute configuration of **1** was determined by application of the dibenzoate chirality rule.[15,16] Surprisingly, acylation of **1** with *p*-bromobenzoyl chloride yielded the tribenzoate **5**. The corresponding J couplings observed in the ^{1}H NMR spectrum of **5** (H-2, dd, J = 10.5, 2.2 Hz; H-3, dd, J = 10.5, 2.3 Hz; H-4, ddd, J = 7.5, 2.3, 2.2 Hz; H-5, d, J = *7.5*) are significantly different

from those of **1-3**, indicating a change to a major conformation with the C-4 and C-5 benzoate groups and the C-6 alkenyl side chain equatorial and only the C-6 benzoate group axial (Figure 3). The resulting ECD spectrum showed a positive Cotton effect at 253 nm as predicted by the Newman projections of the C-4/C-5 and C-5/C-6 bonds. The expected weaker negative second Cotton effect is presumably buried in the strong positive background ellipticity. Thus, the absolute configuration of **1** is assigned as 4*S*,5*R*,6*R*. Additionally, since **2** ($[\alpha]^{22}_{D}$ +21) and **3** ($[\alpha]^{22}_{D}$ +19)

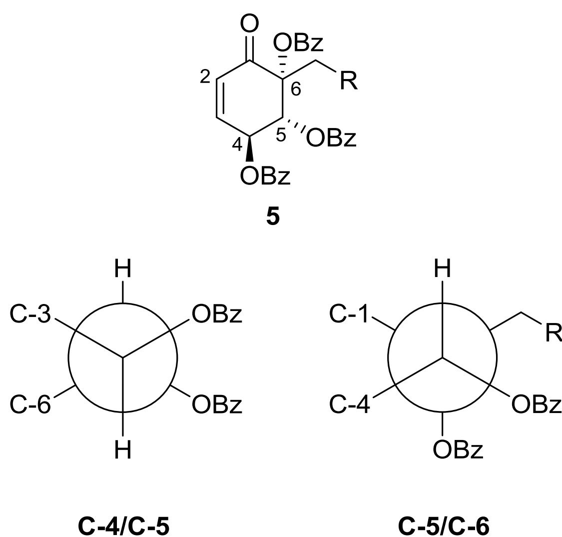


Figure 3. Structure of **5** and Newman projections of the C-4/C-5 and C-5/C-6 bonds of its major conformer

have similar values of optical rotation to **1** ($[\alpha]^{22}_{D}$ +23), their absolute configurations must also be 4*S*,5*R*,6*R*.

The three isolated compounds are similar in structure to other known hydroxyalkylcyclohexenones that are found from *Tapirira obtusa, T. guianesnsis,* and *Lannea edulis* in the family Anacardiaceae.[9,11,17] Furthermore, they contain the same oxygenation pattern as the zeylenones, many of which have been isolated from various members of the *Uvaria* genus (Annonaceae).[18-20]

The antiproliferative activities of the three new compounds were determined against the A2780 ovarian cancer cell line. All three compounds exhibited moderate antiproliferative activity, with IC_{50} values of 0.8 ± 0.4, 0.7 ± 0.3, and 0.8 ± 0.5 μM, respectively.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO P-2000 polarimeter, and UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. ECD analysis was performed on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, band width 2.0 nm. ^{1}H and ^{13}C NMR spectra were recorded using either Bruker Avance 500 or 600 spectrometers. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS or a Thermo Electron TSQ LC-ESI-MS. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Bidentate C_{18} column (250 x 10 mm) or a Varian Lichrosorb 5 Diol column (250 x 10 mm).

Antiproliferative Bioassay. The assay was performed at Virginia Tech according to specifications previously described.[21] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[22]

Plant Material. Bark of *Pleiogynium timoriense* (DC) Leenh. was collected by Dr. Paul Cox under the auspices of the New York Botanical Garden (NYBG) from a seaward-facing forest on the island of Eua, Tonga in July 1987; a voucher specimen, PC01113 (ID number 40077), is on deposit at the NYBG.

Extraction and Isolation. An EtOH extract of the bark of *P. timoriense* was subjected to liquid−liquid partitioning to give active hexanes, DCM, and aqueous MeOH fractions with IC_{50}

values to the A2780 ovarian cancer cell line of 3.0, 1.3, and 6.2 μg/mL, respectively. The active DCM fraction 23050-C6 (0.30 g) was fractionated using Sephadex LH-20 column chromatography (1:1 DCM/MeOH) to generate an active fraction (222 mg, IC_{50} 0.5 μg/mL). This fraction was further purified utilizing C_{18} HPLC (MeOH/H_20 gradient) to yield 3 semi-pure active fractions. These fractions were further purified using C_{18} HPLC (MeCN/H_2O gradient) to yield the active compounds **1** (5.4 mg), **2** (6.9 mg), and **3** (1.4 mg)

*Compound **1***: $[\alpha]^{22}_D$ +23 (*c* 0.5 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.59) nm; ECD (MeOH) $[\Delta\varepsilon]_{330\text{ nm}}$ -0.17, $[\Delta\varepsilon]_{242\text{ nm}}$ +1.72, $[\Delta\varepsilon]_{209\text{ nm}}$ -1.07; ^1H NMR ($CDCl_3$, 500 MHz) see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz) see Table 1; HRESIMS $[M+H]^+$ *m/z* 381.2972 (calcd for $C_{23}H_{41}O_4^+$ 381.2999), $[M+Na]^+$ *m/z* 403.2805 (calcd for $C_{23}H_{40}NaO_4^+$ 403.2819).

*Compound **2***: $[\alpha]^{22}_D$ +21 (*c* 0.7 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.44) nm; ^1H NMR ($CDCl_3$, 500 MHz) see table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 1; HRESIMS $[M+H]^+$ *m/z* 409.3291 (calcd for $C_{25}H_{45}O_4^+$ 409.3312), $[M+Na]^+$ *m/z* 431.3125 (calcd for $C_{25}H_{44}NaO_4^+$ 431.3132).

Compound **3:** $[\alpha]^{22}_D$ +19 (*c* 0.1 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.25) nm; ^1H NMR ($CDCl_3$, 500 MHz) see table 1; ^{13}C NMR ($CDCl_3$, 150 MHz) see Table 1; HRESIMS $[M+H]^+$ *m/z* 437.3604 (calcd for $C_{27}H_{49}O_4^+$ 437.3625), $[M+Na]^+$ *m/z* 459.3431 (calcd for $C_{27}H_{48}NaO_4^+$ 459.3445).

Reduction of Compound 2. Compound **2 (**5.8 mg, 0.014 mmol) was dissolved in $CHCl_3$ (3 mL) and Ph_2SiH_2 (5.3 μL, 5.3 mg, 0.028 mmol), $ZnCl_2$ (0.5 mg, 0.0037 mmol), and $Pd(PPh_3)_4$ (0.3 mg, 0.00026 mmol) were added. The reaction mixture was stirred for 4 h at rt. The solvent

was removed under reduced pressure and the residue was purified utilizing silica gel column open column chromatography (7:3 hexanes/EtOAc) to yield 2.3 mg of **4** (0.0056 mmol, 39%).

Compound 4: ^{1}H NMR (methanol-d$_4$, 600 MHz) δ 5.38 (2H, m, H-10′, H-11′), 4.03 (1H, q, *J* = 4.3 Hz, H-4), 3.71 (1H, dd, *J* = 4.5, 1.1 Hz, H-5), 2.70 (1H, ddd, *J* = 13.4, 11.7, 5.6 Hz, H-2a), 2.37 (1H, dt, *J* = 13.4, 5.2 Hz, H-2b), 2.16 (2H, m, H-3a, H-1′b), 1.97 (4H, brs, H-9′, H-12′), 1.84 (1H, m, H-3b), 1.70 (1H, ddd, *J* = 13.9, 12.4, 4.3 Hz, H-1′a), 1.29 (24H, brs, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-13′, H-14′, H-15′, H-16′, H-17′, H-18′) 1.08 (2H, m, H-2′), 0.90 (3H, t, *J* = 7.0 Hz, H-19′); HRESIMS $[M+H]^+$ *m/z* 411.3472 (calcd for $C_{23}H_{43}O_4^+$ 411.3479), $[M+Na]^+$ *m/z* 433.3288 (calcd for $C_{23}H_{42}NaO_4^+$ 433.3288).

***p*-Bromobenzoylation of Compound 1.** Compound **1** (0.4 mg, 0.001 mmol) was dissolved in DCM (2 mL), and 44.9 mg (0.37 mmol) of DMAP and 80.7 mg (0.37 mmol) of *p*-bromobenzoyl chloride were added. The reaction mixture was stirred for 1.5 h at rt. The solution was diluted with DCM (5 mL) and washed with H_2O (5 mL x 3), 3M HCl (5 mL), and brine (5 mL). The organic layer was dried with anhydrous $MgSO_4$ and the solvent was removed under vacuum. The resulting residue was purified utilizing diol HPLC (hexanes/EtOAc gradient) to yield compound **5** (0.8 mg, 82%).

Compound 5: $[\alpha]^{22}_{D}$ +9 (*c* 0.07 MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.12) nm, 248 (4.17); ECD (MeOH) $[\Delta\varepsilon]_{253\ nm}$ +23.84, $[\Delta\varepsilon]_{234\ nm}$ 0.00, $[\Delta\varepsilon]_{202\ nm}$ +7.94; ^{1}H NMR ($CDCl_3$, 500 MHz) 7.92 (2H, d, *J* = 8.6 Hz), 7.85 (2H, d, *J* = 8.6 Hz), 7.77 (2H, d, *J* = 8.6 Hz), 7.64 (2H, d, *J* = 8.6 Hz), 7.58 (2H, d, *J* = 8.6 Hz), 7.53 (2H, d, *J* = 8.6 Hz), 6.86 (1H, dd, *J* = 10.5, 2.3 Hz, H-3), 6.37 (1H, dd, *J* = 10.5, 2.2 Hz, H-2), 6.30 (1H, ddd, *J* = 7.5, 2.2, 2.2 Hz, H-4), 6.05 (1H, d, *J* = 7.5 Hz, H-5), 5.34 (2H, m, H-11′, H-12′), 2.35 (1H, m, H-1′a), 2.07 (1H, m, H-1′b), 1.98 (4H, m,

H-10′, H-13′), 1.29 (20H, brs, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′, H-14′, H-15′, H-16′), 1.23 (2H, m, H-2′), 0.88 (3H, t, $J = 7.0$ Hz); HRESIMS $[M+H]^+$ *m/z* 927.1055 (calcd for $C_{44}H_{50}Br_3O_7^+$ 927.1057), $[M+H]^+$ *m/z* 929.1048 (calcd for $C_{44}H_{50}Br_3O_7^+$ 929.1038), $[M+H]^+$ *m/z* 931.1082 (calcd for $C_{44}H_{50}Br_3O_7^+$ 931.1020), $[M+H]^+$ *m/z* 933.1018 (calcd for $C_{44}H_{50}Br_3O_7^+$ 933.1001).

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C and 2D NMR spectra of compounds **1-3**, ^{1}H NMR spectra of **4** and **5**, COSY, NOESY, selective TOCSY and NOE spectra of **4**, and ECD spectra of **1** and **5** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table 1. NMR Spectroscopic Data for Compounds 1 – 3.

	1[a]		**2**[b]		**3**[a]	
position	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type[i]	δ_H, (*J* in Hz)
1	200.2, C		200.2, C		201.6, C	
2	126.4, CH	6.10, dd (10.1, 0.8)	126.4, CH	6.10, dd (10.2, 0.7)	126.3, CH	6.10, dd (10.2, 0.8)
3	145.8, CH	6.80, ddd, (10.1, 3.9, 1.4)	145.9, CH	6.80, ddd, (10.1, 3.9, 1.3)	145.5, CH	6.80, ddd, (10.1, 4.0, 1.5)
4	68.6, CH	4.62, brs	68.5, CH	4.62, brs	68.5, CH	4.63, brs
5	75.4, CH	3.98, brs	75.4, CH	3.98, brs	75.3, CH	3.98, dd, (3.0, 1.5)
6	78.1, C		78.1, C		77.8, C	
1′	36.1, CH_2	1.83,m	36.1, CH_2	1.83,m	36.1, CH_2	1.83,m
2′	23.0, CH_2	1.13, m	23.0, CH_2	1.13, m	22.7, CH_2	1.13, m
3′	29.8, CH_2[c]	1.26, brs[g]	29.8, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
4′	29.8, CH_2[c]	1.26, brs[g]	29.8, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
5′	29.7, CH_2[c]	1.26, brs[g]	29.7, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
6′	29.5, CH_2[c]	1.26, brs[g]	29.5, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
7′	27.2, CH_2[d]	2.00, m[h]	29.5, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
8′	129.9, CH[e]	5.34, m[i]	29.5, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
9′	129.8, CH[e]	5.34, m[i]	27.2, CH_2[d]	2.00, m[h]	29.4, CH_2[c]	1.25, brs[g]
10′	27.2, CH_2 [d]	2.00, m[h]	129.9, CH[e]	5.34, m[i]	29.4, CH_2[c]	1.25, brs[g]
11′	29.3, CH_2[c]	1.26, brs[g]	129.9, CH[e]	5.34, m[i]	27.1, CH_2[d]	2.00, m[h]
12′	29.2, CH_2[c]	1.26, brs[g]	27.2, CH_2[d]	2.00, m[h]	129.8, CH[e]	5.35, m[i]
13′	29.3, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.26, brs[g]	129.8, CH[e]	5.35, m[i]
14′	29.3, CH_2[c]	1.26, brs[g]	29.3, CH_2[c]	1.26, brs[g]	27.1, CH_2[d]	2.00, m[h]
15′	31.9, CH_2	1.26, brs[g]	29.3, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
16′	22.7, CH_2	1.26, brs[g]	29.3, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
17′	14.1, CH_3	0.88, t (6.9)	31.9, CH_2	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
18′			22.7, CH_2	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
19′			14.2, CH_3	0.88, t (6.9)	31.8, CH_2	1.25, brs[g]
20′					22.6, CH_2	1.25, brs[g]
21′					14.0, CH_3	0.88, t (6.9)
OH		3.60[f]		3.59[f]		exchanges
OH		2.94[f]		2.90[f]		exchanges
OH		2.41[f]		2.37[f]		exchanges

[a]$CDCl_3$, 500 MHz, 150 MHz
[b]$CDCl_3$, 500 MHz, 125 MHz
[c, d, e, f]Interchangeable assignment within a column
[g, h, i]Overlapping Signals
[j]Obtained from HMBC and HSQC spectra

Graphical Abstract

Pleiogynium timoriense
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