New Pyrano-Pyrone from *Goniothalamus tamirensis* Enhances the Proliferation and Differentiation of Osteoblastic MC3T3-E1 Cells

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The genus *Goniothalamus* comprises about 160 species of shrubs and trees growing in tropical and subtropical Asia.¹ Many of these species are used for timber, as fiber sources, as mosquito repellant, and most interestingly in folk medicine in several countries.²³ Phytochemical studies of *Goniothalamus* species have led to the isolation and characterization of a large number of styryl-lactones. These can be classified into several main subtypes: styryl-pyrones, furano-pyrones, furando-furones, and pyrano-pyrones.⁴ Styryl-lactones possess significant cytotoxic activity against several human tumor cell lines such as A-549 (lung carcinoma), HT-29 (colon adenocarcinoma), MCF-7 (breast carcinoma), RPMI (melanoma), U251 (brain carcinoma), and to induce apoptosis in HL-60 leukemic cell.⁵ Some of styryl-lactones have been synthesized via total synthesis.⁶⁻¹⁰ Goniopyrone, the first pyrano-pyrone compound, was first isolated in 1990 from *G. giganteus*.¹¹,¹² It was high bioactive, showing ED₅₀ values of ca. 0.67 μg/ml in the cytotoxicity of human tumor cell lines (A-549, MCF-7, HT-29), high toxicity to the brine shrimp (BS), and significant inhibition of the formation of crown gall tumors on potato discs (PD). Typically, up to date, pyrano-pyrene compounds are found in some *Goniothalamus* species.

The formation of bone involves a complex series of events, including the proliferation and differentiation of osteoprogenitor cells. Eventually, this results in the formation of a mineralized extracellular matrix. Most effective osteoporosis therapies reduce bone loss but do not restore lost bone mass and strength. Therefore, it is desirable to discover bone-building (anabolic) agents that stimulate new bone formation and correct the imbalance of microarchitecture characteristic of established osteoporosis.¹³ As new bone formation is primarily a function of the osteoblasts, agents that regulate bone formation act either by increasing the proliferation of cells in the osteoblastic lineage or inducing osteoblast differentiation.¹⁴ Early intervention is now possible with the help of some effective medications, which may reduce the risk of first and recurrent fractures.

In our investigation of phytochemistry of the leaves of *G. tamirensis*, 9-deoxygoniopyrone (2) and a new pyrano-pyrene, (+)-8-epi-9-deoxygoniopyrone (1), were isolated and identified using spectral methods. To investigate whether these compounds could stimulate the function of osteoblasts, their effects on cell growth, collagen content, alkaline phosphatase (ALP) activity, and calcium deposition were assessed in in vitro osteoblastic target cell line, MC3T3-E1 cells and caused a significant elevation of collagen content, alkaline phosphatase activity, and nodule mineralization in the cells (*p*<0.05). Our data suggest that the enhancement of osteoblast function by 1 and 2 may result in the prevention of osteoporosis.

**Key words** (+)-8-epi-9-deoxygoniopyrone; pyrano-pyrene; *Goniothalamus tamirensis*; MC3T3-E1 cell; osteoporosis

**Results and Discussion**

Compound 1 was obtained as white needle-like crystal, [α]D²° +93.3°, mp 131—132 °C. The molecular formula of 1 was deduced to be C₁₃H₁₄O₄ from the Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) ion

![Image](https://via.placeholder.com/150)

Fig. 1. (+)-8-Epi-9-deoxygoniopyrone and Known Goniopyrone Compounds from *Goniothalamus* Species

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Deoxygoniopypyrone, and (−)-8-Epi-9-deoxygoniopypyrone

The long range 1H–13C heteronuclear multiple bond correlation (HMBC) and 1H–1H correlation spectroscopy (COSY) spectra of 1 also confirmed the assignment of the pyrone ring. Four methine proton signals at δH 4.93, 4.47, 4.44 and 3.59 ppm were correlated, as showed in the 1H–13C HSQC spectrum, respectively, to four methine carbon signals at δC 76.8, 65.8, 74.3 and 72.6, indicating the presence of four oxygen-bearing carbons of the pyrano ring in 1. The presence of a mono-substituted phenyl group was also confirmed by five proton signals appeared around δH 7.35—7.42 and six carbon signals at δC 127.4, 128.6, 128.7, and 137.9 ppm.

Tables 1 and 2 showed that compound 1 has 1H- and 13C-NMR parameters similar to those of compound 5. However, a careful examination of 2D-NMR spectral data of 1 including COSY, HMBC, HSQC, especially nuclear Overhauser effect spectroscopy (NOE), and further examination using Mosher’s method suggested that 1 is a chiral isomer of 5, a new gonioppyrane-type isomer, named (+)-8-epi-9-deoxygoniopypyrone. The presence of the cross peaks between protons H-1 and H-5 in the COSY spectra specifically illustrate the “W-type” interaction in the bicyclic structural skeleton of pyrano-pyrones, as described by Lan et al. (Fig. 3). The coupling constant between protons H-7 and H-8 (J=10.0 Hz), was evidence of the axial-trans configuration between H-7 and H-8 protons. As a result, the two substituted groups at C-7 and C-8 were in the equatorial configuration. The axial methine proton signal at δH 3.59 (H-8) had NOE correlation to the axial-oriented methylene proton signal at δH 2.23 (H-9), while the methylene proton signals (H-4a, H-4b) did not have correlation to methylene proton signals (H-9). Furthermore, the NOE spectrum of 1 showed cross peaks between the axial methine proton signal at δH 4.46 (H-7) to the axial methylene proton signal at δH 2.88 (H-4a). These above evidences showed that in 1 the pyrano ring had the chair form and the lactone ring had the boat form. The chair-boat form of 1 was also confirmed by interactions of protons, i.e., H-7 and H-4a, H-8 and H-9b on the NOE spectrum of both 1 and acetylated product (1a) (Fig. 2, Table 3) indicated the “S” configuration for this carbon atom. As a result, we concluded that 1 is a new pyrano-pyrene and its absolute configuration is described as 1S, 5S, 7R, 8S, 3exo, 7endo. This was also confirmed by its physical properties, such as melting point and optical rotation of 1 in comparison with 5 (Table 1).

Compound 2 was also isolated as white powder, and its
molecular weight was indicated by a peak in the APCI-MS at \[ m/z \text{ 235 } [M+H]^+ \], as in the case of 1, corresponding to a molecular formula of \[ C_{13}H_{14}O_4 \]. Like 1, compound 2 also is a pyrano-pyrone compound. Comparison of the 1H- and 13C-NMR spectral data of 2 and 1 and other known pyrano-pyrones (Tables 1, 2) showed that 2 was 9-deoxygoniopyrone, a known compound isolated from the leaves of \( G. \) giganteus.\(^{12} \) In addition, the absolute stereostructure of 2 was confirmed by 2D-NMR spectral data and its optical rotation in comparison with 9-deoxygoniopyrone. The presence of the long range correlations through four bonds in the 1H–1H COSY spectrum between H-1 and H-5, H-8 and H-9b, H-4a and H-9a of 2 are due to “W-type” interaction of those protons in pyrano-pyrone structure (Fig. 3). In addition, the NOESY spectrum of 2 showed the cross peaks between H-4b and H-9b, and between the axial proton H-7 and H-4a. This evidence confirmed the chair–chair form of 2 (Fig. 2), and this is the first report about chair–chair conformation of this compound.

In this study, we investigated the effects of 1 and 2 on the osteoblast function using pre-osteoblastic target cell line, MC3T3-E1, as an in vitro model of osteoblast differentiation. At the range of concentration of 2.67—10.68 \( \mu M \), the compounds significantly increased MC3T3-E1 cell growth (Fig. 4). Given that they significantly increased osteoblast growth, we then investigated the effect of these compounds on collagen synthesis using Sirius Red-based colorimetric assay. Both 1 and 2 increased collagen synthesis at a concentration of 2.67 \( \mu M \) (Fig. 5). Next, the effects of 1 and 2 on the osteoblast differentiation were assessed by measuring the ALP activity, a major osteoblast differentiation marker. Both 1 and 2 increased the ALP activity up to 125% at concentration of 2.67 \( \mu M \) (Fig. 6). Finally, we examined the effects of 1 and 2 on mineralization, another important process in differentiation of MC3T3-E1 cells, by measuring the calcium deposition by Alizarin Red staining. Consistent with the effects on ALP activity and collagen synthesis, 1 and 2 (2.67 \( \mu M \)) significantly stimulated mineralization (Fig. 7). At this concentration, both 1 and 2 did not show any cytotoxicity.

Table 3. 1H-NMR Data of (R)-MTPA Ester (1b), (S)-MTPA Ester (1c) of 1

<table>
<thead>
<tr>
<th>Position</th>
<th>1b (( \delta_H ))</th>
<th>1c (( \delta_H ))</th>
<th>( \Delta \delta = \delta_2 - \delta_8 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.149</td>
<td>5.142</td>
<td>-0.007</td>
</tr>
<tr>
<td>4a</td>
<td>3.007</td>
<td>3.002</td>
<td>-0.005</td>
</tr>
<tr>
<td>4b</td>
<td>2.893</td>
<td>2.901</td>
<td>+0.008</td>
</tr>
<tr>
<td>5</td>
<td>4.511</td>
<td>4.51</td>
<td>-0.001</td>
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<tr>
<td>7</td>
<td>4.698</td>
<td>4.633</td>
<td>-0.065</td>
</tr>
<tr>
<td>8</td>
<td>5.058</td>
<td>5.037</td>
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</tr>
<tr>
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<td>2.368</td>
<td>-0.003</td>
</tr>
<tr>
<td>9b</td>
<td>2.236</td>
<td>2.245</td>
<td>+0.009</td>
</tr>
</tbody>
</table>
more, 17β-estradiol (E2) used as a positive control significantly increased cell growth, collagen content, ALP activity, and mineralization at 10.0 μM. The greater activity of 1 and 2 compared with the positive control demonstrated that 1 and 2 increased the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

In conclusion, the new pyrano-pyrone, (+)-8-epi-9-deoxygonioppyrone (1) and 9-deoxygonioppyrone (2) were isolated from a chloroform extract of G. tamirensis leaves. Their absolute stereostructures are discussed and confirmed using IR, MS, and NMR spectra. The effects of the isolated compounds on the proliferation and differentiation of osteoblastic MC3T3-E1 cells were also investigated. We showed that both 1 and 2 at concentration of 2.67 μM stimulated the growth and differentiation of osteoblastic MC3T3-E1 cells. Therefore, 1 and 2 may be beneficial in stimulating the osteoblastic activity, resulting in bone formation.

**Experimental**

**General Experimental Procedures** Melting points were taken on a Boetius melting point apparatus and the optical rotations were determined on an ATAGO POLAR-2X polarimeter. FT-IR spectra were measured on an IMPAC-410 (Niccollet America) spectrometer. APCl-MS was performed on a LC-MSD-Trap-SL Agilent 1100 series spectrometer and NMR spectra were recorded in CDCl3 with a Bruker Avance 500 (Germany) spectrometer using tetramethylsilane (TMS) as the internal standard. The high resolution mass spectra were obtained using a Variant 910 FTICR mass spectrometer. Column chromatography (CC) was performed on silica gel (70—230, 230—400 mesh, Merck). Thin layer chromatography (TLC) was carried out on precoated DC Alufolien 60 F254 plates (Merck). Spots were detected under UV radiation (254, 365 nm) and by spraying the plates with 10% H2SO4 followed by heating with a heat gun.

**Plant Material** Leaves of G. tamirensis were collected in Huong Son District, Ha Tinh Province, Vietnam, and identified by an experienced botanist at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). A voucher of specimen (No: VN-1087) was deposited at the Institute of Ecology and Biological Resources, VAST.

**Isolation and Purification** Dried powdered leaves of G. tamirensis (760 g) were extracted with methanol at room temperature three times. After removing the solvent under reduced pressure, the crude extract (90 g) was dissolved in 11 of water to form a suspension. This was partitioned successively with n-hexane, chloroform, and n-butanol to give n-hexane (7.3 g), chloroform (37.5 g) and n-butanol (39.0 g) residues, respectively. Then, the chloroform extract was subjected to a flash silica gel column chromatography and eluted with a gradient of dichloromethane—methanol (0, 2, 4, 8, 16, 32, 64, 100% MeOH by volume) to give eight fractions (C1—8). Fraction C5 was chromatographed repeatedly on a silica gel column and eluted with an isocratic solvent system of n-hexane/ethyl acetate (EtOAc) (7/3, v/v). Based on thin-layer chromatography (TLC) monitoring, it was then recrystallized from the mixture of n-hexane/ethyl acetate (7/3, v/v) to yield compound 1 (1.1650 g, 0.1533% from dried leaves). Next, the fraction C4 was also subjected to a silica gel column chromatography and eluted with n-hexane/EtOAc (4/1, v/v) to obtain 54 mg (7.1053×10−3 %) of compound 2. The purity of 1 and 2 exceeded 97% by high-performance liquid chromatography (HPLC) analysis.

Compound 1 was obtained as white needle-like crystals; 1H- and 13C-NMR data are provided in Tables 1 and 2. IR (KBr) cm−1: 3397, 3290, 3039, 1737, 1082. FT-ICR-MS m/z: 234.0852 (Calcd for C12H12O4: 234.0886). APCl-MS m/z: 235 [M+H]+. [α]D20 +93.3° (c=0.4, CHCl3), mp 131—132 °C.

Acetylation A mixture of 3 ml anhydride acid acid and 30 mg of 1 was placed in a 10 ml round-bottomed flask. The reaction mixture was stirred well with a magnetic stirrer and refluxed for 5 h in an oil bath. Then, the solvent was removed under low pressure. The residues were washed several times with saturated NaHCO3 and distilled water and dried to obtain a crude product. Finally, an acetylated (1a) product of 1 (25 mg) was obtained by column chromatography with n-hexane/ethyl acetate (3/1, v/v) as eluent.

Acetylated product of 1 was obtained as white amorphous powder; 1H-NMR (CDCl3) δ: 1.94 (3H, s), 2.21 (1H, dd, Jd=4.5, 14.0 Hz), 2.32 (1H, dt, J=2.5, 14.0 Hz), 2.90 (1H, dd, J=5.0, 19.0 Hz), 3.02 (1H, dd, J=19.0 Hz, 5.0 Hz), 4.51 (1H, brs), 4.67 (1H, d, J=10.0 Hz), 4.84 (1H, dd, J=2.5, 10.0 Hz, 5.0 Hz) (1H, brs), 7.34 (5H, m). 13C-NMR (CDCl3) δ: 20.64 (q), 32.58 (t), 36.41 (t), 66.07 (d), 71.20 (d), 73.03 (d), 73.95 (d), 127.16 (d), 128.48 (d), 128.81 (d), 136.98 (s), 168.56 (s), 169.70 (s).

**Methoxytrifluoromethylphenylacetic Acid (MTPA) Reaction** A solution of 1 (2.5 mg) in 300 μl of dehydrated CH2Cl2 was reacted with (R)-MTPA-Cl (4 μl) in the presence of N,N-dimethyl-4-aminopyridine (4-DMAP) (2.5 mg); the mixture was stirred occasionally at room temperature for 1 h. After adding 300 μl of CH2Cl2, the solution was washed with H2O (300 μl), 5% HCl (300 μl), successively. The organic layer was evaporated and the residue was purified by preparative TLC silica gel (0.25 mm thickness), developed with n-hexane/ethyl acetate/methanol (2/1/0.1, v/v/v) to furnish the ester, 1b (4 mg, 88%). In a similar manner, 1e (3.5 mg, 73%) was prepared from 1 (2.5 mg) using (S)-MTPA-Cl (4 μl), and 4-DMAP (3.5 mg). The 1H-NMR data of compounds 1b and 1e are provided in Table 3.

**Cell Culture and Materials** Murine osteoblastic MC3T3-E1 cells were cultured at 37 °C in 5% CO2 atmosphere in α-modified minimal essential medium (α-MEM; GibcoBRL, Grand Island, NY, USA). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. When cells reached confluence, cells were subcultured using 0.02% EDTA—0.05% trypsin solution.
5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

**Alkaline Phosphatase Activity** The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid, to initiate differentiation. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. The cell number was determined microscopically and then calculated as number of living cells (*i.e.*, those not stained with trypan blue). The cells were lysed with 0.2% Triton X-100, and the lysate was centrifuged at 14000×g for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

**Collagen Content** The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and compounds for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Collagen content was quantified by Sirius Red dye assay kit (Asan Co., Korea). The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Axan Co., Korea).

**Calcium Deposition Assay** The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and compounds for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Calcium deposition was quantified by Sirius Red-based colorimetric assay. Cultured osteoblasts were washed with PBS, followed by fixation with Bouin’s fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained with Sirius Red dye reagent for 10 min with gentle shaking. To quantify the bound dye, the stain was removed and the cultures were washed with 0.01N HCl to remove non-bound dye. The stained material was dissolved in 0.1N NaOH and absorbance was measured at 550 nm.

**Statistics** The results are expressed as the mean±S.E.M. (*n*= 5). Statistical analysis was performed using a one-way ANOVA (*p*<0.05) with the SAS statistical software.

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**References**