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STRUCTURE OF ORGANIC COMPOUNDS

Absolute Configuration of Andrographolide and Its Proliferation of Osteoblast Cell Lines[1]

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Abstract—Andrographolide, $C_{20}H_{30}O_5$, is a labdane diterpenoid which was isolated from the leave of *Andrographis paniculata*. Its crystal structure is determined by single crystal X-ray diffraction: monoclinic, sp. gr. $P2_1$, $Z = 2$. Absolute configuration is determined by the refinement of the Flack parameter to 0.21(19). In the crystal, molecules are linked by O–H···O hydrogen bonds and C–H···O interactions into two dimensional network parallel to the (001) plane. Its proliferation of osteoblast cell lines is reported.

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INTRODUCTION

Osteoporosis means porous bone, which is a metabolic bone disease due to loss of bone mass resulting in the increased risk of skeleton fracture such as fracture of hip, wrist, humerus, spine or pelvis that is usually found in elderly person. Osteoporotic fractures are more common than heart attack, stroke and breast cancer combined [1]. Moreover, fragility may result in the chronic pain [2] and also affect the quality of life.

Estrogen, such as estradiol as shown in Fig. 1, is a common treatment to conserve bone mass and prevent osteoporosis in post-menopausal women that is a way to up-regulate of bone resorption in the body, but mode of action is not clear. Osteoporosis patients have to take a steroid type medicine in the long term, which increasing the risk of side-effects such as breast cancer, hypertension and/or stroke. It led us to search for a molecule that being isosteres for estrogen, which can be used to prevent the osteoporosis like an estrogen but with less side effects. In this work, we will focus on the isolation of andrographolide from the leaves of *Andrographis paniculata*, which is a good source of natural molecules having a similar size or containing the atom like estrogen. Herein we report the isolation, characterization, proliferation of osteoblast cell lines, and absolute configuration of andrographolide (**1**).

[1] The article is published in the original.

EXPERIMENTAL

Reagents and Techniques

All the chemical reagents and solvents were of analytical grade, purchased commercially, and used without further purification. Melting points were determined on a Fisher-John melting point apparatus. Infrared spectra were recorded on a Perkin-Elmer FTS 165 FT-IR spectrometer. Ultraviolet–Visible

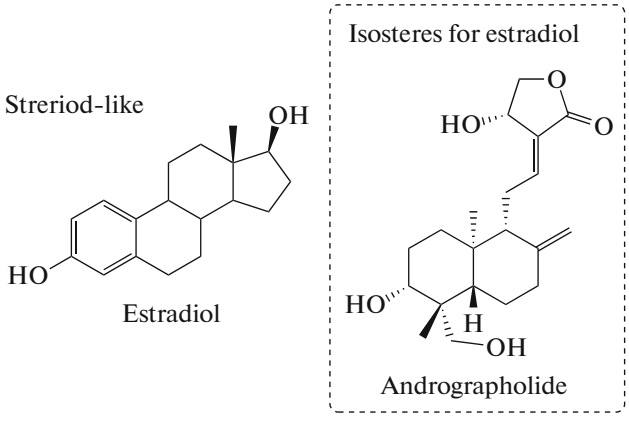

Fig. 1. Chemical structures of estradiol and andrographolide.

Table 1. Crystal data and structure refinement parameters of $C_{20}H_{30}O_5$ (**1**)

System, sp. gr., Z	Monoclinic, $P2_1$, 2
a, b, c, Å	6.55846(8), 8.01925(10), 18.0105(2)
β, deg	97.3729(3)
V, Å^3	939.41(2)
D_x, g cm^{-3}	1.239
Radiation, λ, Å	CuK_α, 1.54178
μ, mm^{-1}	0.711
T, K	294(2)
Sample size, mm	0.47 × 0.37 × 0.12
Diffractometer	APEX2
Scan mode	ω/2θ
Absorption correction, T_{min}, T_{max}	Multi-scan, 0.7294, 0.9202
θ_{max}, deg	70.03
h, k, l ranges	$-7 \le h \le 7$, $-7 \le k \le 9$, $-21 \le l \le 21$
Number of reflections: measured/unique ($N1$), R_{int}/with $I > 2\sigma(I)$ ($N2$)	9459/2851, 0.0231/2842
Refinement method	Full-matrix least-squares on F^2
Number of refined parameters	238
$R1/wR2$ relative to $N1$	0.0381/0.1074
$R1/wR2$ relative to $N2$	0.0380/0.1073
S	1.073
$\Delta\rho_{max}/\Delta\rho_{min}$, e/Å^3	0.20/−0.23
Programs	APEX2 [4], SAINT [4], SADABS [4], SHELXTL [5], PLATON [6], Mercury [7]

(UV–Vis) absorption spectra were recorded on a Shimadzu UV-2450 spectrometer. The ^{1}H NMR spectra were recorded on 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometer in $CD_3OD + CDCl_3$ with TMS as the internal standard. Chemical shifts reported in ppm are expressed in hertz. Crystallographic data were collected on a Bruker SMART APEX2 CCD area-detector diffractometer.

Plant Material

Young leaves were collected from Na-Mhom, Songkhla province in the southern part of Thailand in June 2016 and identified as *Andrographis paniculata* (Acanthaceae).

Isolation and Extraction

Ground-dried leaves of *A. paniculata* (780 g) were extracted with $CHCl_3$ (for 3 days) at room temperature; further evaporation under reduced pressure afforded a greenish crude $CHCl_3$ extract, which was defatted with *n*-hexane and then subjected to QCC (Quick column chromatography) eluting with increasing polarities of CH_3OH in $CHCl_3$ to yield five fractions (F1–F5). Fraction F2 was further separated by column chromatography with a gradient of CH_3OH–$CHCl_3$ to afford six subfractions (F2*A*–F2*F*) and andrographolide (137.1 mg) [3] which was recrystallized from $CHCl_3$: CH_3OH (8 : 2 v/v) solution to give colorless plate-shaped single crystals.

X-ray Crystallography

Single crystals of **1** suitable for X-ray diffraction study were obtained by recrystallization from $CHCl_3$: CH_3OH (8 : 2 v/v) solution by slow evaporation at room temperature after several days. The sample for diffraction study was selected optically. The crystal data of **1** are summarized in Table 1.

The structure was solved by direct methods Non-hydrogen atoms were refined anisotropically. Hydroxy H atoms were located from the difference maps and refined isotropically. The remaining H atoms were placed in calculated positions with d(C–H) = 0.93 Å for cyclohexane, 0.98 Å for dihydrofuran, 0.97 Å for CH_2 and 0.96 Å for CH_3. The U_{iso}(H) values were constrained to be $1.5U_{eq}$ of the carrier atom for methyl H and $1.2U_{eq}$ for the remaining H atoms. A rotating group model was used for the methyl groups.

CCDC 1503128 contains the supplementary crystallographic data for this article. These data can be obtained free of charge at www.ccdc.cam.ac.uk or by e-mailing data_request@ccdc.cam.ac.uk, or by contacting the Cambridge Crystallo-graphic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk.

RESULTS AND DISCUSSION

*Spectroscopic Data of **1***

Colorless crystals, mp 218–222°C; UV (CH_3OH) λ_{max} 238 nm; FT-IR (neat) ν_{max} 3299, 1727, 1673, and 908 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD+$CDCl_3$) : δ 6.78 (*dt*, J = 1.8, 11.7 Hz, 12-H), 4.84 (*d*, J = 6.0 Hz, 14-H), 4.34 (*dd*, J = 10.5, 6.0 Hz, 15β-H), 4.11 (*dd*, J = 10.2, 2.1 Hz, 15α-H), 4.77 (*brs*, 17α-H), 4.51 (*brs*, 17β-H), 4.06 (*d*, J = 10.8 Hz, 19α-H), 3.19 (*d*, J = 11.1 Hz, 19β-H), 3.30 (*t*, J = 8.4 Hz, 3β-H), 2.45 (*brt*, J = 6.6 Hz, 11-H_2), 2.32 (*brt*, J = 3.6 Hz, 9-H_2), 2.27 (*m*, 7-H), 1.86 (*m*) 1.77–1.60 (*m*), 1.25–1.10 (*m*), 1.11 (*s*, 20-CH_3), 0.58 (*s*, 18-CH_3). ^{1}H NMR spectrum is shown in Fig. 2.

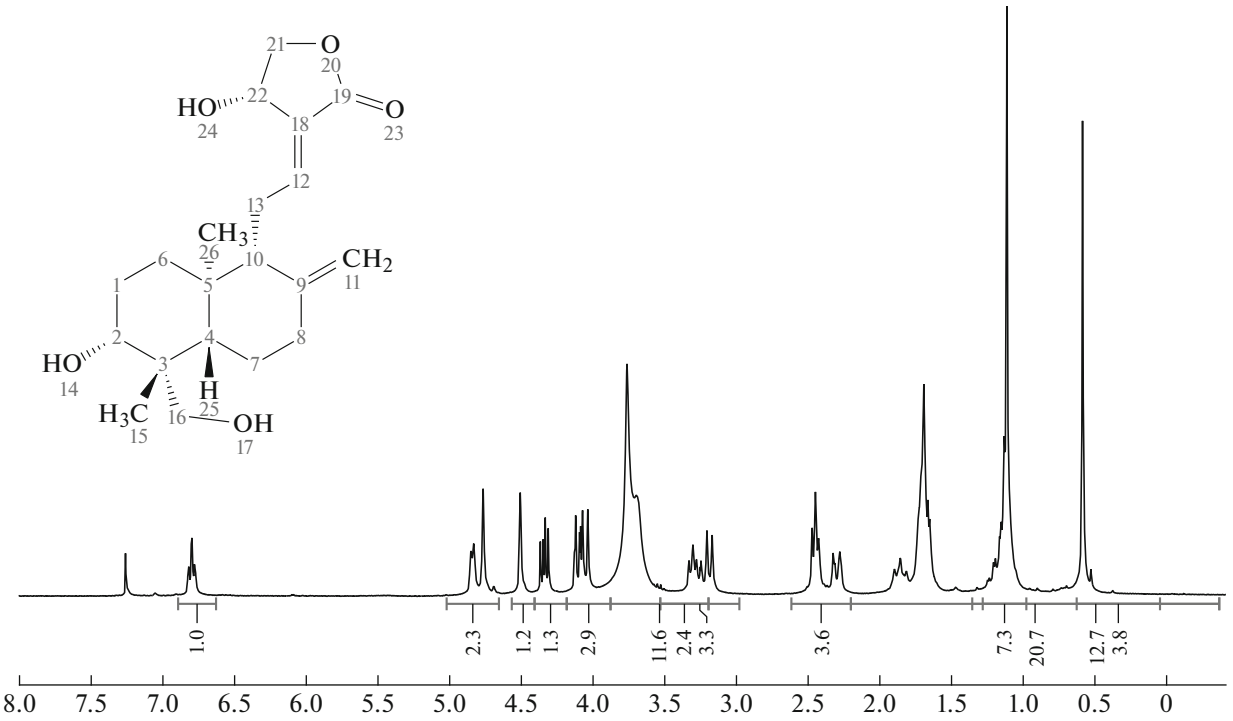

Fig. 2. ^{1}H NMR (300 MHz, CD_3OD + $CDCl_3$) spectrum of andrographolide (1).

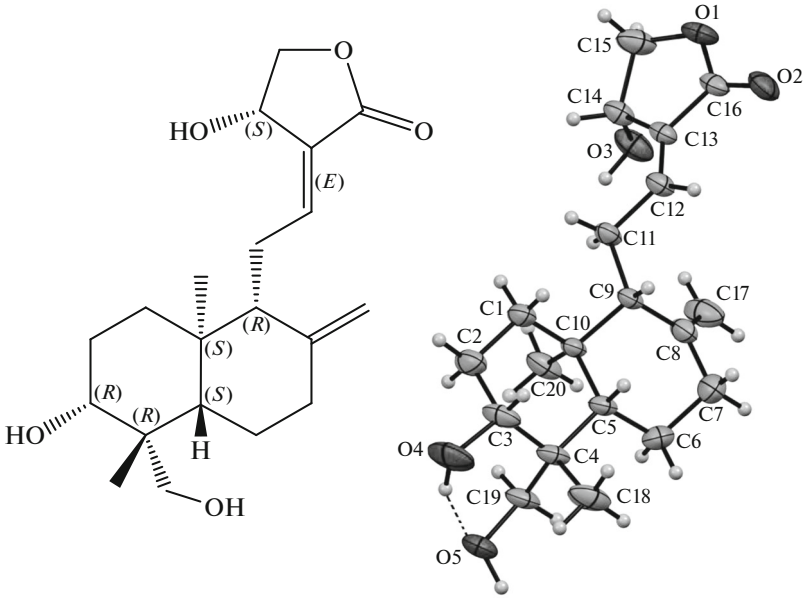

Fig. 3. Absolute configuration and molecular structure of **1** drawn with 50% probability displacement ellipsoids.

*Crystal Structure of **1***

The molecular structure of angrographolide **(1)** [systematic name: (4*S*,*E*)-4-hydroxy-3-(2-((1*R*,5*R*,6*R*,8a*S*)-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl)ethylidene)dihydrofuran-2(3*H*)-one], is shown in Fig. 3. All bond distances are within normal ranges [8], the selected bond lengths, angles and torsion angles are listed in Table 2. The molecule of **1** has two fused cyclohexane rings in standard chair conformations and the dihydrofuran ring (C13–C16/O1) in an envelope conformation with the puckering atom C14 having deviations of 0.126(2) Å from the C13/C15/C16/O1 plane, with Q = 0.209(2) Å and θ = 60.8(5)° [9]. The orientations of the

Table 2. Selected bond lengths *d* (Å) and bond angles ω (deg) for $C_{20}H_{30}O_5$ (**1**)

Bond	*d*	Bond	*d*	Bond	*d*
O1–C16	1.348(2)	O4–H1O4	0.90(5)	C8–C17	1.325(3)
O1–C15	1.454(3)	O5–C19	1.444(2)	C9–C11	1.538(2)
O2–C16	1.202(2)	O5–H1O5	0.95(4)	C10–C20	1.528(2)
O3–C14	1.413(2)	C4–C19	1.536(2)	C11–C12	1.493(2)
O3–H1O3	0.91(3)	C4–C18	1.540(2)	C12–C13	1.329(2)
O4–C3	1.442(2)				
Bond angle	ω	Bond angle	ω	Bond angle	ω
C16–O1–C15	110.14(13)	C9–C8–C7	113.21(18)	C13–C12–C11	126.34(14)
O2–C16–O1	121.45(15)	C17–C8–C7	122.3(2)		

ethylidene and dihydrofuran moieties can be described by the torsion angles C9–C11–C12–C13 = 158.18(18)°, C11–C12–C13–C16 = 175.59(19)°, and C11–C12–C13–C14 = –3.8(3)°. The bond angles around C8 and C12 atoms are indicative of sp^2 hybridization for these atoms and the bond length of 1.325(3) Å confirms the C8=C17 double bond whereas 1.329(2) Å confirms the C12=C13 double bond (Table 2). Absolute configuration was determined by the refinement of the Flack parameter to 0.21(19), the configurations at atoms C3, C4, C5, C9, C10, and C14 are in *R*, *R*, *S*, *R*, *S,* and *S*, respectively.

In the crystal packing, the molecules are linked into two dimensional network parallel to the (001) or

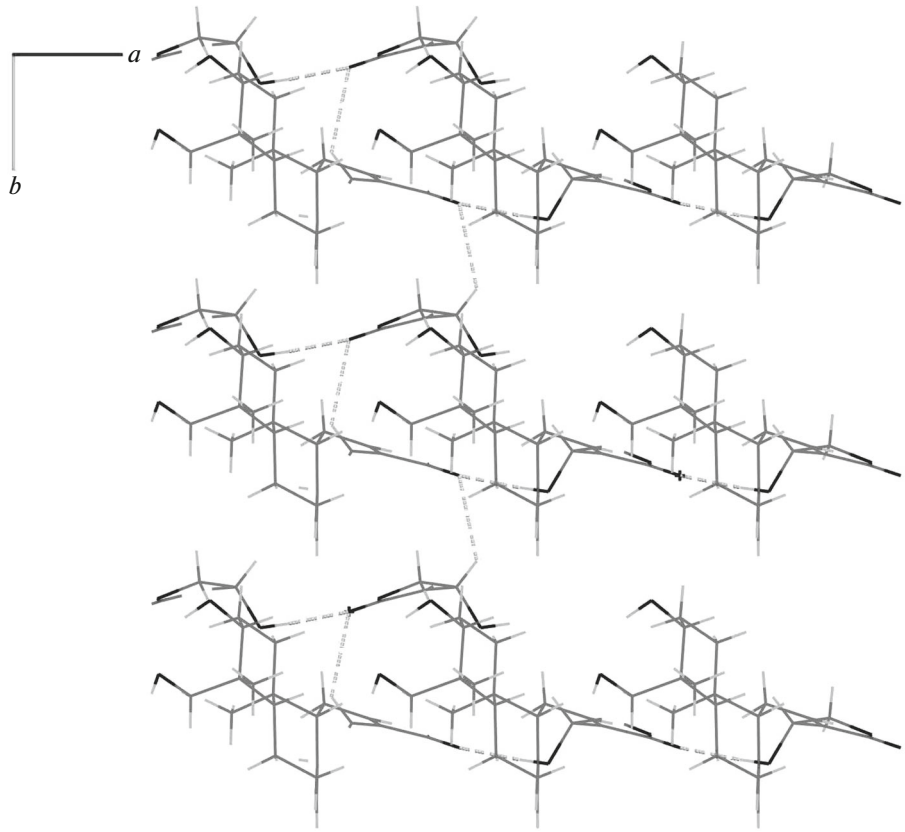

Fig. 4. Molecular sheets of **1**, parallel to the *ab* plane. O–H···O hydrogen bonds and C–H···O interactions are drawn as dashed lines.

Table 3. Hydrogen bond geometry

D–H···*A*	*D*–H, Å	H···*A*, Å	*D*···*A*, Å	*D*–H···*A*, deg
O3–H1O3···O2^{i}	0.91(3)	1.90(3)	2.8123(19)	177(3)
O4–H1O4···O5	0.90(5)	1.85(5)	2.667(3)	150(4)
O5–H1O5···O4^{ii}	0.95(4)	1.79(4)	2.709(3)	162(3)
C14–H14*A*···O2^{iii}	0.98	2.58	3.303(3)	130

Symmetry codes: ^{i} $-1+x, y, z$; ^{ii} $-1-x, 1/2+y, -z$; ^{iii} $1-x, -1/2+y, 1-z$.

ab plane (Fig. 4) by O–H···O hydrogen bonds and C–H···O interactions which are listed in Table 3.

Cell Culture and Cell Proliferation Assay

Mouse osteoblastic MC3T3-E1 cells were a generous gift from Dr. Kanokwan Panyayong, Faculty of Dentistry, Prince of Songkla University. Mouse osteoblastic MC3T3-E1 cells were cultured in α–minimum essential medium (α–MEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin. MC3T3-E1 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Cell proliferation and toxicity of andrographolide on MC3T3-E1 cells were determined by 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (**MTT**) assay. MC3T3-E1 cells were seeded on 96-well plates (at a density of 5.000 cells/well in triplicate) and grown to ≈70–80% confluence, and then the culture medium was changed to a fresh normal medium containing of 0.001, 0.01, 0.1, 1, 10, 100, and 1000 μg/mL andrographolide and incubated for 24 h. At the end of incubation, a solution of 5 mg/mL MTT was added and incubated for 3 h at 37°C to allow forming of crystal formazan which then solubilised in a 40 μL of acid isopropanol with a gentle shaking. Absorbance of formazan dye was measured at λ = 510 nm using a microplate reader. The relative percentage of cell survival was calculated by the mean absorbance of the treated cell (OD_T) and the mean absorbance of control cell (OD_C) with formula:

$$\text{Cell viability, \%} = (OD_T/OD_C).$$

Statistical Analyses

The data were represented as mean ± SEM (standard error of mean). Statistical analyses were conducted using SPSS (statistical package for the social sciences). Significance was determined by one-way ANOVA (analysis of variance) followed by Duncan's multiple range tests for multiple comparisons. Differences were considered significant at *P*-values less than 0.05.

Osteoblast Proliferation

MTT assay was used to determine cell viability of osteoblast cell lines. Table 4 shows the effect of andrographolide (**1**) on cell viability of osteoblast cell lines. After treatment with **1** at various concentrations (0.01 to 1000 μg/mL), the result shows that **1** at concentration in range of 0.01 to 100 μg/mL can induce cell proliferation of osteoblast cell lines in dose-dependent manner. Interestingly, **1** with 10 μg/mL shows the highest cell proliferation with 152.88 ± 23.63% of cell viability which is much better than the control (100.00 ± 7.08%). Surprisingly that **1** possesses no cytotoxic against osteoblast cell lines even at high concentration as 100 μg/mL.

Table 4. The effect of andrographolide (**1**) on cell viability of osteoblast cell lines

	Concentration (μg/mL)						
	0.01	0.1	1	10	100	1000	control
Cell viability[a]	116.23 ±5.44	128.80 ±17.49	126.70 ±9.47	152.88 ±23.63	109.95 ±7.20	27.22 ±2.40	100.00 ±7.08

[a] After 24 h of treatment.

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