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Miliutine A acid, a new cyclofarnesane sesquiterpene from the stems of *Miliusa velutina*

Hoang Khang Le[a,b], Thanh Tung Phan[a,b], Thi Kieu Loan Vo[a,b], Thi Thuy Duong Ngo[a,b], Hoang Long Ngo[c], Poul Erik Hansen[d] and Quang Ton That[a,b]

[a]Faculty of Chemistry, University of Science, Ho Chi Minh City, Vietnam.
[b]Vietnam National University, Ho Chi Minh City, Vietnam.
[c]NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam.
[d]Department of Science and Environment, Roskilde University, Roskilde, Denmark.

Corresponding Author Email: ttquang@hcmus.edu.vn

ABSTRACT

Two new cyclofarnesane sesquiterpenes such as miliutine A acid (**1**), miliutine A methyl ester (**2**) and four known phenol derivatives (**3**-**6**) were isolated from the ethyl acetate extract of the stems of *Miliusa velutina*. NMR spectroscopic and mass spectrometry were used for identifying relative configurations. The assignments of the absolute configurations were determined based on electronic circular dichroism (ECD) and NOESY spectra analysis. All six compounds were screened for their *in vitro* cytotoxic activities against HepG2 cell line using the SRB assay and they showed weak or none activities.

Keywords: *Miliusa velutina*, cyclofarnesane, ECD spectrum, HepG2, miliutine A acid.

1. Introduction

In Vietnam, *Miliusa velutina* is the traditional medicinal plant, which was used to cure cancer, parasitic disease, and used as a natural pesticide (Vo Van Chi. 1991). Besides, it is widely distributed in the Asian countries India, Laos, Myanmar, Cambodia and Thailand. Recent studies revealed that the chemical constituents of *M. velutina* showed the presence of acetogenins (Jumana et al. 2000; Wongsa et al. 2011), alkaloids (Hasan et al. 2000; Jumana et al. 2000; Nguyen et al. 2017), flavonoids (Wongsa et al. 2017), phenylpropanoids (Jumana et al. 2000), steroids and terpenoids (Hasan et al. 2000). Bioactivities of these types of compounds were also studied in case of anti-*Plasmodium falciparum*, antifungal, anticancer effects and two compounds, acetogenins A, B showed antibacterial activity by the disk diffusion assay and cytotoxicity by the brine shrimp lethality bioassay (Jumana et al. 2000).

There no many results are available so research on *M. velutina* seems very timely. Our research showed that sesquiterpene is one of main chemical constituents of *M. velutina.* As a continuous result of our research from the stems of *M. velutina* (Nguyen et al. 2022), six more compounds (**1**-**6**) were isolated and elucidated structures for the first time. Among them, there are two new cyclofarnesane sesquiterpenes, miliutine A acid (**1**), miliutine A methyl ester (**2**), along with four known phenol derivatives such as 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (**3**), protocatechuic acid (**4**), methyl gallate (**5**) and syringaldehyde (**6**) (Figure 1). All six compounds were screened for their *in vitro* cytotoxic activities against HepG2 cell line using the SRB assay. In 1985, from *Cleistopholis patens* collected in Ghana, compound **2** was first isolated and elucidated chemical structure just by 1D NMR ($CDCl_3$, 250MHz) and up today there no more any data of its (Waterman et al. 1985). So the identifying of absolute configurations of compounds **1** and **2** become more important and necessary.

The absolute configuration of compound **1** was determined based on the ECD Cotton effects in combination with NOESY spectrum.

[**Figure 1**. near here]

2. Results and discussion

2.1. Identification

Compound **1** was isolated as a white amorphous powder. Its HR-ESI-MS showed the ion at *m/z* 251.1649 $[M-H]^-$ (calcd. for $C_{15}H_{23}O_3$, 251.1653) and be assigned the molecular formula as $C_{15}H_{24}O_3$, with four degrees of unsaturation.

The ^1H NMR spectrum showed the presence of three olefinic protons at δ_H 5.69 (1H, *q*, *J* = 1.3 Hz), 4.59 (1H, *s*), 4.90 (1H, *s*) and one oxymethine proton at δ_H 3.43 (1H, *dd*, *J* = 9.2, 4.1 Hz). In addition, there are three methyl signals at δ_H 2.17 (3H, *d, J* = 1.3 Hz), 1.03 (3H, *s*) and 0.76 (3H, *s*).

The ^{13}C NMR spectrum showed the presence of 15 carbon resonances, consisting of one carboxyl carbon at δ_C 171.1 (C-1), four olefinic carbons at δ_C 114.9 (C-2), 163.7 (C-3), 147.1 (C-2') and 109.0 (C-9'), one oxymethine carbon at δ_C 77.1 (C-5′), three methyl carbons at δ_C 19.4 (C-6), 16.4 (C-7′) and 26.2 (C-8′).

The main HMBC correlations from H-1′ to C-4, C-5, C-2′, C-3′, C-5′, C-6′, C-7′, C-8′, and C-9′; from H-5′ to C-1′, 3′, 6′, 7′, 8′ and from H-9′ to C-1′, 2′, 3′ established the cyclization between C-1′ and C-6′, including two methyl groups at C-6', methylidene group at C-2'. The signal at δ_H 3.43 (1H, *dd*, *J* = 9.2, 4.1 Hz, H-5′) with coupling constants 9.2 Hz (J_{aa}) and 4.1 Hz (J_{ae}) suggested that H-5′ was axial. The HMBC correlations between H-2/C-1,4,6; H-6/C-1,2,3,4; H-4/C-2,3,5,6 and H-5/C-1′,2′ showed that the *α*,*β*-unsaturated acid moiety attached to the ring (**Figure S2**).

Moreover, the relative configuration of **1** was established by analyzing the NOESY spectrum (Figure S3). The NOESY experiment showed correlations between H-1′/H-3’/H-5′/CH_3-8′ which indicated that these protons are the same side. The signal at δ_H 3.43 (1H, *dd*, *J* = 9.2, 4.1 Hz, H-5′) with coupling constants 9.2 Hz (J_{aa}) and 4.1 Hz (J_{ae}) suggested that H-5′ was axial. Besides, the NOESY correlation between H-1’/H-5’ indicated that both protons H-1’ and H-5’ are located at axial positions and determined that hydroxy group at C-5’ is located at equatorial position. And the correlations between H-2/H-4 identified the configuration *E* of C2=C3 double bond (**Figure S3**) . Based on all the aforementioned analysis, compound **1** was identified as (2*E*)-5-(5′-hydroxy-6′,6′-dimethyl-2’-methylidenecyclohexyl)-3-methyl-2-pentenoic acid which was named miliutine A acid. The absolute configuration of compound **1** was determined by the combination between ECD and NOESY spectra.

First, the absolute configuration of compound **1** was determined by the ECD spectrum. There is only one of positive Cotton effect at λ_{max} (nm) 220 (+6.51) on the ECD spectrum of **1** (**Figure S4**), determined that the absolute configuration of chiral carbon center C-1’ as (*S*), similar to the compound of (2*E*, 4*E*, 1’*S*, 3’*S*)-5-(3′-hydroxy-6′,6′-dimethyl-2’-methylidenecyclohexyl)-3-methyl-2, 4-pentadienoic acid [Synonym: (4*S*, 6*S*)-4-hydroxy-γ-ionylideneacetic acid; ECD (c 80.0 μM, MeOH) λ_{max} (nm) 208 (+17.83)] (Rajachan et al. 2020). Besides, the NOESY correlation between H-1’/H-5’ indicated that the proton H-5’ is located at axial position and determined that hydroxy group is located at equatorial position. That allows to identify that the absolute configuration of chiral carbon center C-5’ as (*S*).

Hence, the structure of **1** was determined to be (2*E*, 1’*S*, 5’*S*)-5-(5′-hydroxy-6′,6′-dimethyl-2’-methylidenecyclohexyl)-3-methyl-2-pentenoic acid (**Figure S5**).

Compound **2** was isolated as a colourless oil. The HR-ESI-MS showed the adduct ion at *m/z* 267.1962 $[M+H]^+$ (calcd. for $C_{16}H_{27}O_3$, 267.1955) suggested the molecular formula

$C_{16}H_{26}O_3$ and four degrees of unsaturation. The NMR data of **2** were similar to those of **1**, except for the present of a methoxy signal at δ_H 3.69 (3H, *s*) and δ_C 50.9 ppm. Consequently, compound **2** is the methyl ester of compound **1.** The chemical shift of H6 (-CH_3) at δ_H 2.16 (3H, *d, J* = 1.3 Hz) indicated that the C2=C3 double bond was assigned the configuration *E.* The compound was named miliutine A methyl ester. Besides, the specific rotations ($[\alpha]_D^{25}$) of compounds **1** and **2** are positive values of + 32.5 (c 0.01, MeOH), + 66.7 (c 0.01, MeOH), respectively. Based on all above data, both the compounds could be determine the same of the absolute configuration. Hence, the IUPAC nomenclature of compound **2** was identified as methyl (2*E*, 1'*S*, 5'*S*)-5-(5′-hydroxy-6′,6′-dimethyl-2'-methylidenecyclohexyl)-3-methyl-2-pentenoate.

The known compounds were identified as 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (**3**) (Baderschneider et al. 2001), protocatechuic acid (**4**) (Teng et al. 2005), methyl gallate (**5**) (Lubis et al. 2018) and syringaldehyde (**6**) (Yi et al. 2010), by comparison of their NMR data with those reported in the literature.

2.2. Biological tests

Camptothecin (0.07 μg/mL; 0.20 μM) was used as a positive control, with a cytotoxic value of 52.20% and DMSO as a negative control. From the ethyl acetate extract, six compounds (**1-6**) were isolated, elucidated structures and tested for the cytotoxicity against the cancer cell line HepG2 at 100 μg/mL. The results indicated that all six compounds had no or weak activities with cytotoxic percent values lower than 50% (**Table S15**).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on an A. Krüss Optronic polarimeter P8000 (Krüss Optronic GmbH, Hamburg, Germany). Electronic Circular Dichroism (ECD) spectrum was recorded on a Chirascan qCD spectrometer (Applied Photophysics Ltd.). The 1D, 2D NMR spectra

were recorded by a Bruker AM500 FT-NMR spectrometer. HR-ESI-MS data were acquired on Bruker micrOTOF QII (Bruker Singapore Pte., Ltd.) mass spectrometer. Column chromatography (CC) was performed on silica gel (Merck) type 0.063-0.200 mm ASTM, silica gel (Himedia) type 37-63 *μm* GRM7484-500G, and LiChroprep® RP-18, 40–63 μm (Merck KGaA, Darmstadt, Germany). Analytical and preparative TLC was carried out on pre-coated Kieselgel 60F-254 or RP-18 plates (Merck KGaA, Darmstadt, Germany). Optical density values were determined with a 96-well microtiter plate reader (Synergy HT, Biotek Instruments). Other chemicals were of the highest grade available.

3.2. Plant material

The stems of *Miliusa velutina* were collected in September 2021 in An Giang province, Vietnam and were authenticated by Botanist Viet Hoang, Department of Ecology - Evolutionary Biology, Faculty of Biology - Biotechnology, VNUHCM–University of Science, Ho Chi Minh City. A voucher specimen (MVE 2021) has been deposited at the Department of Organic Chemistry, Faculty of Chemistry, VNUHCM–University of Science.

3.3. Extraction and isolation

The stems of *Miliusa velutina* were ground into powder. The plant material (10.5 kg) was then extracted under reflux successively with *n*-hexane (HE), ethyl acetate (EA), and methanol (ME) to obtain three corresponding extracts: *n*-hexane (45.3 g), ethyl acetate (121.0 g) and methanol (593.7 g). The ethyl acetate extract was chromatographed on silica gel column and eluted using solvents with *n*-hexane:ethyl acetate (stepwise, 10-100% EA), ethyl acetate:methanol (stepwise, 0-100% ME) to afford 17 fractions (B1-B17). Fraction B5 (5.9 g) was subjected to a silica gel column chromatography (CC), eluted with solvents system of *n*-hexane:ethyl acetate (stepwise, 0-100% EA) to give three sub-fraction (B5.1-B5.3). Refined B5.1 (59.8 mg) fraction by silica gel CC, eluted with solvents system *n*-hexane:isopropanol (9:1), to yield compound **1** (10.2 mg). Fraction B2 (605.0 mg) was separated by CC on silica

gel and eluted with *n*-hexane:ethyl acetate (stepwise, 30-100% EA), to afford two sub-fractions (B2.1 and B2.2). Sub-fraction B2.1 (218.6 mg) was chromatographed over silica gel column with *n*-hexane:ethyl acetate (stepwise, 20-100% EA), to afford two sub-fractions (B2.1.1 and B2.1.2). Sub-fractions B2.1.1 (57.0 mg) was purified by preparative TLC with *n*-hexane:isopropanol (99:1) to yield compound **2** (4.4 mg). Fraction B9 (8.6 g) was subjected to silica gel column and eluted with *n*-hexane:ethyl acetate (stepwise, 30-100% EA), and ethyl acetate:methanol (stepwise, 0-10% ME), to afford 9 fractions (B9.1-B9.9). Sub-fractions B9.2 (2.8 g) was subjected to silica gel column and eluted with *n*-hexane:ethyl acetate (stepwise, 30-100% EA), and ethyl acetate:methanol (stepwise, 0-10% ME), to afford 10 fractions (B9.2.1-B9.2.10). Sub-fractions B9.2.7 was subjected to silica gel CC eluted with chloroform:methanol solvents system (stepwise, 1-5% ME) to give compound **3** (5.5 mg) and compound **6** (5.0 mg). Sub-fractions B9.5 (637.9 mg) was separated over a Sephadex LH-20 column with chloroform:methanol (50:50), to afford four sub-fractions (B9.5.1–B9.5.4). Sub-fractions B9.5.2 (78 mg) was subjected to silica gel CC eluted with chloroform:methanol solvents system (90:10) to give two compounds **4** (10.5 mg) and **5** (18.0 mg).

3.3.1. Miliutine A acid (1)

White amorphous powder; $[\alpha]_D^{25}$ + 32.5 (*c* 0.01, MeOH); ECD (c 15.04 mM, MeOH) λ_{max} nm (θ, mdeg) 220 (+6.51); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see supplementary **Table S1**; HR-ESI-MS *m/z* 251.1649 $[M-H]^-$ (calcd. for $C_{15}H_{23}O_3$, 251.1653) (**Figure S11**).

3.3.2. Miliutine A methyl ester (2)

Colourless oil; $[\alpha]_D^{25}$ + 66.7 (*c* 0.01, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see supplementary **Table S1**; HR-ESI-MS 267.1962 $[M+H]^+$ (calcd. for $C_{16}H_{27}O_3$, 267.1955) (**Figure S14**).

3.4. Cell Lines and Cell Culture

HepG2 cells were purchased from the American Type Culture Collection (Manassas, Rockville). Cells were cultured at 37 °C and 5 % CO_2 in Eagle's Minimal Essential Medium (EMEM) supplemented with 10 % (v/v) FBS (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich), 0.025 μg mL^{-1} amphotericin B (Sigma-Aldrich), 100 IU mL^{-1} penicillin G (Sigma-Aldrich), and 100 μg mL^{-1} streptomycin (Sigma-Aldrich).

3.5. Cytotoxic activity evaluated by SRB assay

The assay was performed as previously described with some modifications (Vistica et al. 1990). Cells were seeded at a density of 10,000 cells/well (HepG2) in 96-well plates. Cells were cultured for 24 h before being incubated with isolated compounds at different concentrations for 48 h. Treated cells were fixed with cold 50 % (w/v) trichloroacetic acid (Merck KGaA) for 1–3 h, washed and stained with 0.2 % (w/v) SRB (Sigma-Aldrich) for 20 min. After five washes with 1 % acetic acid (Merck KGaA), the protein-bound dye was solubilized in 10 mM Tris base solution (Promega). Optical density values were determined at the wavelengths of 492 nm and 620 nm. The percentage of growth inhibition (Inh %) was calculated according to the formula: Inh % = (1 − [ODt/ODc] × 100) %, in which ODt and ODc are the optical density value of the test sample and the control sample, respectively. Data were represented as means ± standard error ($n \geq 3$). The IC_{50} value was determined by using Prism software with multivariate nonlinear regression and $R^2 > 0.9$. Camptothecin (Merck KGaA) was used as a positive control.

4. Conclusions

From the ethyl acetate extract of the stems of *Miliusa velutina*, six compounds (**1**–**6**) including two new cyclofarnesane sesquiterpenes (**1**, **2**) and four known phenol derivatives (**3**-**6**) were isolated and elucidated the chemical structures for the first time. The absolute configurations of two new compounds, miliutine A acid (**1**) and miliutine A methyl ester (**2**),

were identified based on ECD Cotton effects and NOESY spectrum. The six compounds (**1-6**) with cytotoxic percent values lower than 50%, showed none or weak on the cytotoxicity of cancer cell lines HepG2.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Quang Ton That ID: http://orcid.org/0000-0001-7485-3821

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