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Chemical constituents from *Neopestalotiopsis clavispora* culture medium with estrogenic effects in MCF-7 cells

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ABSTRACT

Estrogen is a vital hormone responsible for the development of the female reproductive system. Hence, estrogen deficiency in menopause increases various symptoms. This study aimed to discover biologically active compounds from an EtOAc-extract of *Neopestalotiopsis clavispora* culture medium which has previously shown estrogenic activity. A new α-pyrone (**1**) and a new tetramic acid derivative (**2**), together with hymenosetin (**3**) and pestalotiollide B (**4**), were isolated from the extract. The chemical structures of the new compounds were elucidated using MS and NMR spectroscopy, and the absolute configurations were established by quantum mechanical calculations of electronic circular dichroism and gauge-including atomic orbital NMR chemical shift, followed by DP4 + analysis. The isolated compounds were initially tested for their estrogenic activities using MCF-7 estrogen responsive human breast cancer cells. Hymenosetin (**3**) showed estrogenic activity by increasing the proliferation of MCF-7 cells at the concentration of 2.5 μM via the phosphorylation of estrogen receptor-α.

1. Introduction

Estrogen plays an important role in the development and regulation of the female reproductive system (Nilsson and Gustafsson, 2002). When the ovaries stop producing estrogen in most elderly women, various menopausal symptoms (Dalal and Agarwal, 2015), such as hormone-dependent cancers, cardiovascular diseases (Baker et al., 2003), mental disorders, and sexual problems occur. Hormone replacement therapy (HRT) has been used to treat these menopausal symptoms (Al-Safi and Santoro, 2014); however, it is often related to side effects such as breast and ovarian cancers (Mahavni and Sood, 2001). Therefore, developing alternatives to HRT with lower risks of side effects is considered as an attracting attention for treatment of the menopausal symptoms. Hence, several plant-derived compounds such as isoflavones, lignans, and coumestans, have been previously reported as possible alternatives to HRT (Cassidy et al., 2000; Kwon et al., 2021). In our ongoing project to discover biologically active compounds, an EtOAc-extract of *Neopestalotiopsis clavispora* culture medium was studied, showing estrogenic activity using MCF-7 estrogen responsive human breast cancer cells by increasing the proliferation of MCF-7 cells at the concentration of 5 μg/mL.

Neopestalotiopsis (Sporocadaceae) is an endophytic, plant pathogenic or saprobic found on living plants (Jeewon et al., 2003; Gerardo-Lugo et al., 2020), and is widely distributed throughout tropical and

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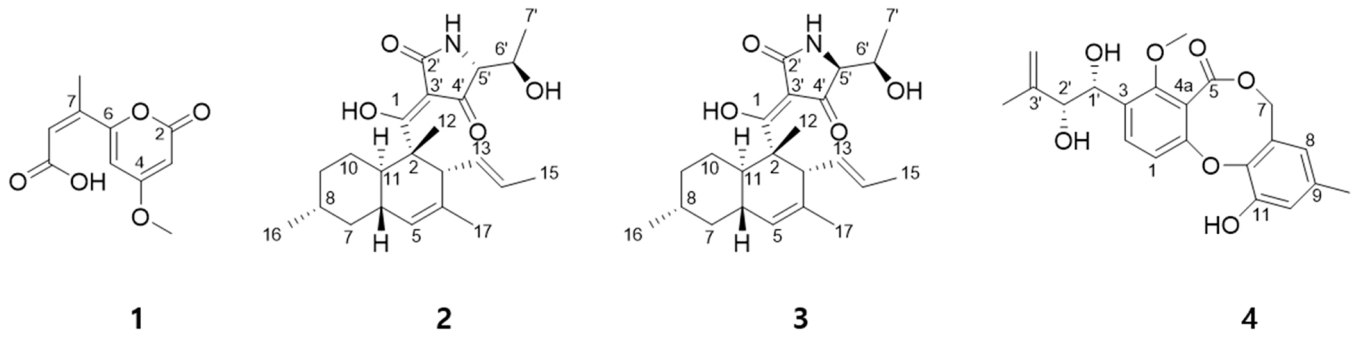

Fig. 1. The structures of compounds **1** – **4** isolated from *Neopestalotiopsis clavispora*.

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of compounds 1 and 2.

	1[a]		**2**[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1				200.6
2		162.5		49.5
3	5.77 (s)	90.8	3.10, br s	49.4
4		170.9		131.9
5	6.57 (s)	102.5	5.16, br s	125.7
6		158.9	1.81, m	39.1
7		140.6	0.88, m	42.6
			1.80, m	
8	6.48 (s)	122.1	1.53, d (6.1)	33.5
9		167.6	1.10, m	35.8
			1.77, m	
7-CH_3	2.26 (s)	13.5		
4-OCH_3	3.85 (s)	57.1		
10			1.06, m	28.3
			1.94, m	
11			1.67, m	39.8
12			1.43, br s	13.7
13			5.24, m	127.9
14			5.15, m	130.1
15			1.54, d (6.1)	17.8
16			0.92, d (6.6)	22.6
17			1.60, br s	22.2
2′				174.4
3′				100.0
4′				187.7
5′			3.78 (br s)	64.7
6′			4.06 (br s)	68.4
7′			1.19, d (5.6)	18.0
NH			6.30, br s	

[a]Recorded in DMSO-d_6.
[b]Recorded in $CDCl_3$

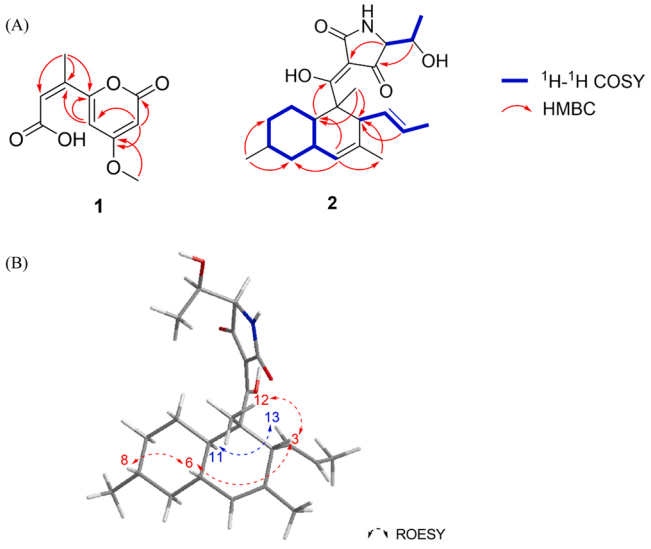

Fig. 2. (A) COSY (bold blue line) and key HMBC (red arrows) correlations for compound **1** and **2** and (B) key ROESY correlations of **2**.

temperate regions (Santos et al., 2020). The previous study of *Neopestalotiopsis* species reported the production of decalinoyltetramic acids (Zhao et al., 2015), which possess antibacterial (Zhao et al., 2015), antifungal (Halecker et al., 2014), and cytotoxic effects (Halecker et al., 2014). In our continuous search for new fungal-derived bioactive constituents (Kwon et al., 2021, 2020), *Neopestalotiopsis clavispora* with estrogenic activity was studied. A new α-pyrone (**1**) and a new tetramic acid derivative (**2**), together with two known compounds (**3** and **4**) [12, 14] were isolated from the EtOAc-extract of *N. clavispora* culture medium (Fig. 1). Herein, the isolation, structural determination and biological evaluation of all the isolated compounds are described.

2. Results and discussion

Compound **1**, a pale brown oil, was obtained and assigned the molecular formula $C_{10}H_{10}O_5$ via HRESIMS data, suggesting it had 6 degrees of unsaturation. The ^{1}H NMR spectral data showed the presence of three olefinic groups at δ_H 6.57 (1H, s, H-5), 6.48 (1H, s, H-8), and 5.77 (1H, s, H-3), a methyl group at δ_H 2.26 (3H, s, 7-CH_3) and a methoxy group at δ_H 3.85 (3H, s, 4-OCH_3). The ^{13}C NMR spectral data exhibited 10 resonances for six olefinic, two carbonyl, a methyl, and a methoxy carbons (Table 1). Consequently, the ^{1}H and ^{13}C NMR spectral data obtained for **1** were similar to those for acropyrone (Hammerschmidt et al., 2014), an α-pyrone derivative, except for the absence of a methyl signal, as evidenced by the HMBC correlation of H-3/C-2 and H-3/C-4. Moreover, the HMBC correlation of H-5/C-6 and C-7 indicated the connection of the 2-butanoic acid side chain to the α-pyrone moiety at C-6, and the correlation of CH_3-11/C-6, C-7 and C-8 confirmed the location of the methyl group at C-7 (Fig. 2A). The geometry of Δ (Kwon et al., 2021; Jeewon et al., 2003) was assigned to be *Z* by a gauge-including atomic orbital NMR chemical shift calculations, followed by DP4 + analysis (Grimblat et al., 2015), while the acropyrone was reported to be *E* (Fig. S14). Accordingly, the new compound **1** was elucidated as shown and named, neopestalopyrone.

Compound **2** was obtained as a yellow oil and assigned the molecular formula $C_{23}H_{33}NO_4$ via HRESIMS data, suggesting it had 8 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectra of **2** revealed the presence of three olefinic groups, three methylene groups, six methine groups including a hydroxy methine, five methyl groups, three carbonyl groups, and a nitrogen group, which were highly similar to those of hymenosetin (**3**) (Halecker et al., 2014) (Table 1). In the ^{1}H NMR spectrum of **2**, minor differences in the tetramic acid moiety at δ_H 4.06 (1H, br s, H-6′), 3.78 (1H, br s, H-5′), and 1.19 (3H, d, J = 5.6 Hz, H-7′) suggested that both compounds **2** and **3** have the same planar structure, as evidenced by the

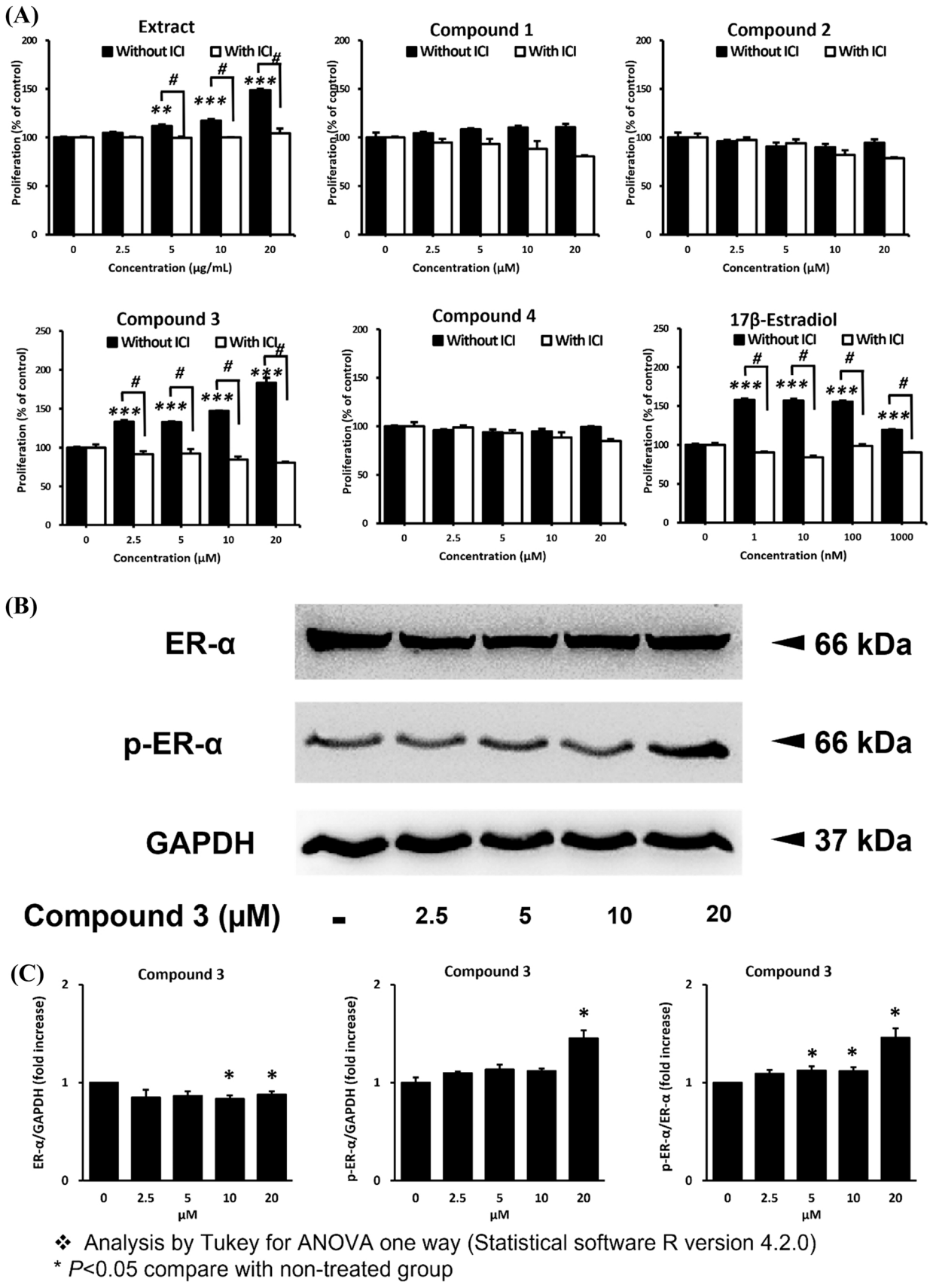

Fig. 3. Proliferation of MCF-7 cells after 96 h treatment. Cell viability is presented as a mean ± standard error of mean (SEM). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the non-treated group. $^{\#}P < 0.05$ compared with the ICI-treated group at the same concentration of samples (A). Western blotting of ER-α and phosphorylated ER-α (Ser118) proteins (B). Bar graph presents the densitometric quantification of western blot bands (C).

^{2}D NMR experiments, but different stereochemistry in the tetramic acid moiety (Table S1).

The relative configuration of **2** was determined by *J*-based configuration analysis (Matsumori et al., 1999) and ROESY correlations. $^{2}J_{C,H}$ and $^{3}J_{C,H}$ obtained from hetero half-filtered TOCSY NMR experiment afforded the relative configuration as shown. The small $^{2}J_{C\text{-}6',H\text{-}5'}$ (2.5 Hz) indicated an anti-orientation between H-5′ and hydroxyl group at C-6′. Both the large $^{2}J_{C\text{-}5',H\text{-}6'}$ (4.4 Hz) and small $^{3}J_{C\text{-}7',H\text{-}5'}$ (3.6 Hz) pointed to a gauche orientation between H-6′ and nitrogen group at C-5′, and H-5′ and C-7′, respectively. Thereby, the nitrogen group at C-5′ and the hydroxyl group at C-6′ was in gauche orientation. In addition, the relative configuration of the decalin moiety was determined by the comprehensive ROESY correlations from H-8/H-6, H-6/H-3, H-3/H-12, and H-11/H-13 (Fig. 2B). The absolute configuration was demonstrated by the quantum mechanical calculations, electronic circular dichroism (ECD) and a gauge-including atomic orbital NMR chemical shift. The computationally calculated ECD spectra of four possible stereoisomers, **2a** (2*S*,3*R*,6*S*,8*R*,5′*S*,6′*S*), **2b** (2*R*,3*S*,6*R*,8*S*,5′*S*,6′*S*), **2c** (2*R*,3*S*,6*R*,8*S*,5′*R*,6′*R*), and **2d** (2*S*,3*R*,6*S*,8*R*,5′*R*,6′*R*), were compared with the experimental ECD spectrum (Fig. S13); The calculated ECD spectra of **2a** and **2d** were good fit to the experimental ECD spectrum of **2**, indicating the absolute configuration in the decalin moiety as 2*S*,3*R*,6*S*,8*R*. To establish the absolute configuration of the tetramic acid moiety, the gauge-including atomic orbital NMR chemical shift calculation including DP4 + probability was applied and the calculated ^{1}H and ^{13}C NMR chemical shifts of the **2a** and **2d** were compared with the experimental values of **2**, which determined the structural equivalence of **2** and **2d** (2*S*,3*R*,6*S*,8*R*,5′*R*,6′*R*) with a 100% DP4 + probability (Fig. S15). Therefore, the new compound **2** was the 5′-*epimer* of hymenosetin (**3**) and named, 5′-*epi*-hymenosetin.

All the isolated compounds were evaluated for their estrogenic effects using MCF-7 estrogen responsive human breast cancer cells (Fig. 3). Compound **3** showed estrogenic activity from the concentration of 2.5 μM by increasing the proliferation of the MCF-7 cells. Estrogenic activity was evaluated after treatment with the compounds for 96 h and 17β-estradiol was used as a positive control (Fig. 3A). The cell proliferation of the compound-treated group continuously increased in a concentration-dependent manner to the highest concentration of 20 μM. However, the proliferation was suppressed in the presence of 500 nM ICI 187.780, a well-known estrogen receptor (ER) antagonist; therefore, the proliferation caused by compound **3** was regarded as estrogen-responsive proliferation.

The activation of ER-α protein, a major sub-type of ERs in MCF-7 cells was analyzed using western blotting analysis. After 96 h of treatment with compound **3**, ER-α and p-ER-α protein expression showed significant differences compared to the non-treated group with 5 μM; ER-α protein expression decreased, whereas the expression of p-ER-α increased (Figs. 3B and 3C). The results showed that compound **3** excited the phosphorylation of ER-α, leading to the induction of the cell proliferation in ER-positive breast cancer MCF-7 cells.

In conclusion, the present work demonstrated the isolation and structural elucidation of the new α-pyrone derivative, neopestalopyrone (**1**) and a new tetramic acid derivative, 5′-*epi*-hymenosetin (**2**), together with two known compounds (**3** and **4**) [12, 14], from an EtOAc-extract of *N. clavispora* culture medium. Hymenosetin (**3**) increased the proliferation of MCF-7 cell lines by phosphorylation ER-α, suggesting that compound **3** should be studied further.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phytol.2022.10.008.

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