Dendrofalconerol A Suppresses Migrating Cancer Cells *via* EMT and Integrin Proteins

PREMKAMOL PENGPAENG[1,4], BOONCHOO SRITULARAK[3,4] and PITHI CHANVORACHOTE[1,2,4]

Departments of [1]Pharmacology and Physiology and [3]Pharmacognosy and Pharmaceutical Botany, Chulalongkorn University, Bangkok, Thailand;
[2]Cell-Based Drug and Health Product Development Research Unit, Chulalongkorn University, Bangkok, Thailand;
[4]Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Abstract. *Background/Aim: Enhanced cell motility is a hallmark of highly metastatic cancer cells. The anti-migratory activity of Dendrofalconerol A (DF-A), a pure bis(bibenzyls) isolated from the stem of Dendrobium falconeri (Orchidaceae) is reported in the present study. Materials and Methods: Cytotoxicity effects of DF-A on H460 lung cancer cells was determined by the MTT assay. We also investigated the mechanism of DF-A-mediated EMT and integrin proteins level by western blotting. Results: DF-A at concentrations of 0.5-5 μM significantly reduced the protein level of migrating cells in a dose-dependent manner. The expression of migration-related integrins, including integrin β_1 and integrin α_4 was significantly reduced in response to DF-A treatment. Also, DF-A was shown to suppress epithelial to mesenchymal transition (EMT), as indicated by cadherin switch from N- to E-cadherin and decrease of Snail, Slug and vimentin. Conclusion: This study revealed the potential of DF-A, an anti-metastasis agent and the underlying mechanism in this in vitro assay with H460 cells, which leads to the development of a novel anti-metastatic agent.*

Recent studies in cancer have pointed-out the significant impact of an epithelial-mesenchymal transition (EMT) in the process of cancer cell dissemination. During cancer metastasis, the ability of cancer cells to alter their phenotype form epithelial to mesenchymal renders them resistant to anoikis with increased motility (1-3). In cancer biology, EMT has garnered significant attention since many researchers recognized EMT as a hallmark reflecting aggressiveness (4, 5). The molecular markers of the EMT process in cancer cells, such as down-regulation of E-cadherin together with up-regulation of N-cadherin was long shown to link with acquisition of anoikis resistance (6-8). Also, the increase of snail, slug and vimentin has been reported as an important molecular event of EMT (9).

Likewise, the alteration in the expression pattern of cellular integrins has been shown to associate with the aggressive potential of cancer cells (10-12). Indeed, cancer metastasis is a multistep process beginning with cancer cell detachment from the extracellular matrix (ECM) (13, 14). Integrins are proteins playing a key role in interacting with ECM and provide survival signals for the attached cells (15, 16). In general, integrins consist of alpha and beta subunits that provide initiating triggers for activation of intracellular signals (16). Studies have indicated that the expression of integrin α_4, α_5, α_v, β_1 and β_5 is tightly associated with increased migratory and invasive behavior of metastatic cancer cells (10-12). An increase of integrin β1 was found to promote resistance to chemotherapy (17, 18). In addition, the decrease of β_3, β_4 and β_5 integrins was shown to suppress tumor growth and angiogenesis (19, 20).

In line with the anti-metastasis drug discovery research, the present study investigated the potential of dendrofalconerol A (DF-A; (4,6-Dimethoxy-9-(4-methoxybenzyl)-8-[2-(4-methoxyphenyl)ethyl]-9H-xanthene-2,3,5-triol) (Figure 1), a methanol extract from the aerial parts of *Dendrobium falconeri* Hook (*Orchidaceae*) (21), on EMT, as well as integrin expressions. Based on the aforementioned properties that this agent possesses we also present the ability of this agent to inhibit EMT, as well as suppress aggressive related integrins, DF-A appears to be a promising target for further development for anti-metastasis approaches.

Abbreviations: MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; TBST, tris-buffered saline with 0.1% Tween; epithelial-mesenchymal transition, EMT; E-cadherin, E-cad; N-cadherin, N-cad.

Correspondence to: Dr. Pithi Chanvorachote, Associated Professor, Department of Pharmacology and Physiology, Cell-Based Drug and Health Product Development Research Unit and Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. Tel: +66 22188344, Fax: +66 22188340, e-mail: pithi.c@chula.ac.th and pithi_chan@yahoo.com

Key Words: Dendrobium falconeri, dendrofalconerol A, EMT, integrin, suppression, migration, lung cancer.

Materials and Methods

DF-A preparation. Dendrofalconerol A (>98%) was isolated from the aerial parts of *Dendrobium falconeri*. Briefly, the dried stems of this plant (800 g) were powdered and extracted with MeOH at room temperature. The MeOH extract was filtered and evaporated under reduced pressure to give a viscous mass (73 g). This material was subjected to vacuum-liquid chromatography on silica gel (EtOAc-hexane gradient) to give fractions A-K. Fraction I (1.51 g) was separated by CC (silica gel; EtOAc-haxane, gradient and EtOAc-hexane, 1:4) to give 35 fractions. Fraction 25 (216 mg) was further purified by Sephadex LH20 (MeOH) to give dendrofalconerol A (29 mg). The structure of DF-A was determined through analysis of its spectroscopic data.

Cell culture and DF-A treatment. The non-small cell lung cancer H460 cell line used in this study was obtained from American Type Culture Collection (Manassas, VA, USA). H460 cells were cultured in RPMI medium. Media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and 100 units/ml penicillin/streptomycin. Cells were maintained in a humidified incubator containing 5% CO_2 at 37˚C. DF-A was dissolved in DMSO and diluted with sterile PBS to achieve the working concentrations. The percentage of DMSO in the working solution was less than 0.1, which showed no toxicity in H460 cell.

Chemicals. Trypsin, Hoechst 33342, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PBS, dimethysulfoxide (DMSO) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Integrin β_1, integrin α_4, vimentin, Snail, Slug, E-cadherin, N-cadherin and β-actin rabbit antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell viability assay. To determine DF-A-mediated cytotoxicity, cell viability was determined by the MTT assay, which measures cellular capacity to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to purple formazan crystals by the mitochondria dehydrogenase enzyme. After treatment, the medium was replaced by 100 μl/well of MTT solution (0.4 mg/ml) and cells were incubated for 4 h at 37˚C. Subsequently, the MTT solution was removed and a 100 μl/well of DMSO were added to dissolve the formazan crystals. At the end of the assay period, the intensity was measured at 570 nm using a microplate reader (SpectraMax® M5, Molecular Devices, City, Ca, USA). All analyses were performed in at least three independent replicate cultures. The cell viability was calculated from the optical density (OD) ratio of treated to non-treated control cells and presented as a percentage of the non-treated controls.

Nuclear staining assay. Cells were treated with or without DF-A in RPMI serum medium under various concentrations for 24 h at 37˚C. Apoptotic and necrotic cell death was determined by Hoechst 33342/propidium iodide (PI) co-staining. Then, cells were incubated with 10 μM of Hoechst 33342 dye and 5 μg/ml of PI for 30 min at 37˚C in the dark. Finally, cells were visualized under a fluorescence microscope (Olympus IX51 with DP70, Olympus, Center Valley, PA, USA). The blue fluorescent Hoechst dye detected apoptotic cells, while the red fluorescent propidium iodide detected necrotic cells.

Western blot analysis. Cells were harvested and lysed on ice for 60 min. After the sample had been prepared, the protein content of cell lysate was determined using the BSA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein of each sample was separated by size using SDS-PAGE and then transferred into nitrocellulose membranes. The membrane was blocked in 5% skim milk in TBST (25 mmol/l Tris-HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature and then probed with appropriate primary antibodies at 4˚C overnight and washed three times with TBST for 8 min. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies according to the primary antibodies for 2 h at room temperature. Lastly, the signal of immunoreactive proteins was detected by enhanced chemiluminescence (Supersignal West Pico; Pierce). Protein expression (E-cadherin (E-cad), N-cadherin (N-cad), Snail, Slug, vimentin, integrin β_1, Integrin α_4) was investigated and β-actin was used as a loading of control in each treatment.

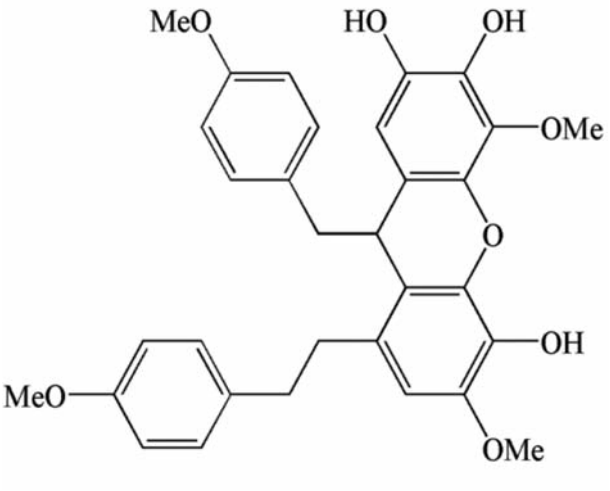

Figure 1. *Structure of DF-A ($C_{32}H_{32}O_8$) MW. 544.*

Statistical analysis. Mean data from at least three independent experiments were normalized to result in the non-treated control. All data were presented as the means±standard error of the mean (S.E.M.). Statistical differences between means were determined using an analysis of variance (ANOVA) and *post-hoc* test at a significance level of $p<0.05$.

Results

Evaluation of the cytotoxic effect of DF-A on H460 cells. First, the non-toxic concentrations of DF-A was characterized, by the H460 cells were treated with various concentrations of DF-A (0-100 μM) for 24 h, then cell viability was measured by the MTT assay. Figure 2A shows that a concentration of 10 μM significantly induced the death of H460 cells when compared to untreated control cells. Approximately 20% of the cells died when treated with DF-A (10 μM), while 85% of the cells died at the highest concentration of DF-A. Thus, we used the non-toxic concentrations of less than 10 μM DF-A on H460 cells

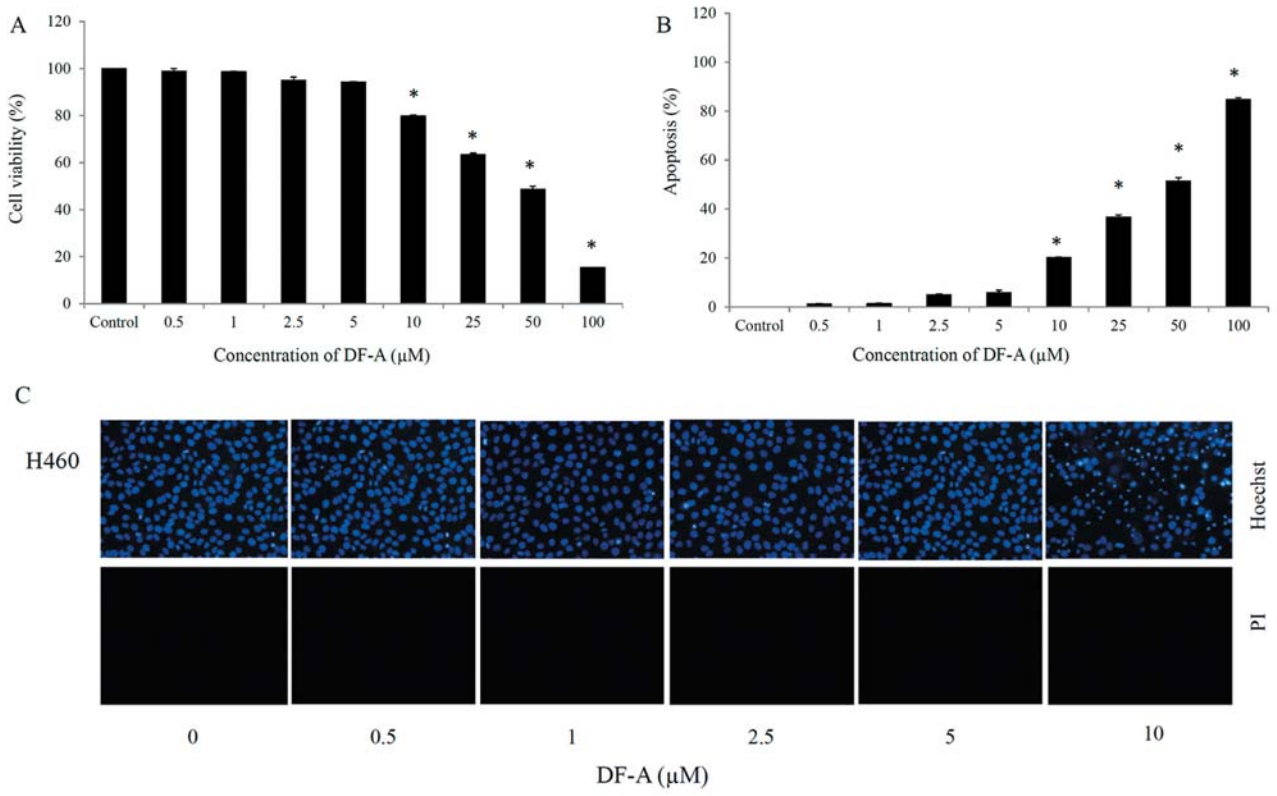

Figure 2. *The cytotoxic effects of DF-A on lung cancer H460 cells. Cells were treated under various concentrations with DF-A (0-100 μM) for 24 h. (A, B) Percentage of cell viability was determined by the MTT assay and percentage of apoptotic cells, respectively. Values are means of independent triplicate samples±SE. *p<0.05 versus untreated control. (C) The mode of cell death of H460 cells was determined by Hoechst 33342/PI co-staining. Apoptotic cells were detected by blue fluorescent Hoechst dye and visualized under a fluorescence microscope.*

for further experiments. To confirm the above results, we also tested the mode of cell death mechanism after cells were treated with DF-A, by Hoechst33342/PI co-staining assay. The nuclear staining assay indicated that 0-5 μM of DF-A caused no condensed and/or fragmented nuclei compared to non-treated control cells, as presented in hoechst staining. Additionally, PI staining showed no detectable change in nuclear red fluorescence observed during that period (Figure 2C).

DF-A decreases cell migration via suppression of epithelial to mesenchymal transition and integrins. Recently, studies have indicated the critical role of EMT, as well as integrins in enhancing cancer cell motility. Having shown that DF-A possesses anti-migration effects, we further investigated the underlying mechanism of its action. Cells were incubated with DF-A, allowed to migrate for 24 h and then the levels of proteins involved in cell migration, namely E-cad, N-cad, Snail, Slug, vimentin, integrin β_1, integrin α_4, were determined by western blotting. We found that treatment of cells with DF-A significantly suppressed the EMT process. The expression levels of EMT markers including vimentin, Snail and Slug were found to be significantly reduced in response to DF-A treatment (Figure 3A). Also, the cadherin switch form N- to E-cadherin further confirmed these results suggesting the EMT suppressing effect by DF-A. Furthermore, the level of integrins which have been shown to function in cell migration such as integrin β_1 and integrin α_4 was significantly decreased in the DF-A-treated cells (Figure 3B). Taken together, these results indicate that DF-A inhibits the migration of H460 cells by suppressing the activation of EMT and integrins.

Discussion and Conclusion

EMT is a complicated process of cancer cell dissemination and essential to the developmental of cancer metastasis (1, 2). After cancer cells detach from their primary site and spread to other sites, they can alter their phenotype from epithelial to mesenchymal (1-3). This process allows cancer cells to resist anoikis and promote cell motility that promotes to metastasis and leads to extremely aggressive cancerous states and, thus, rapid death of patients. In the present study,

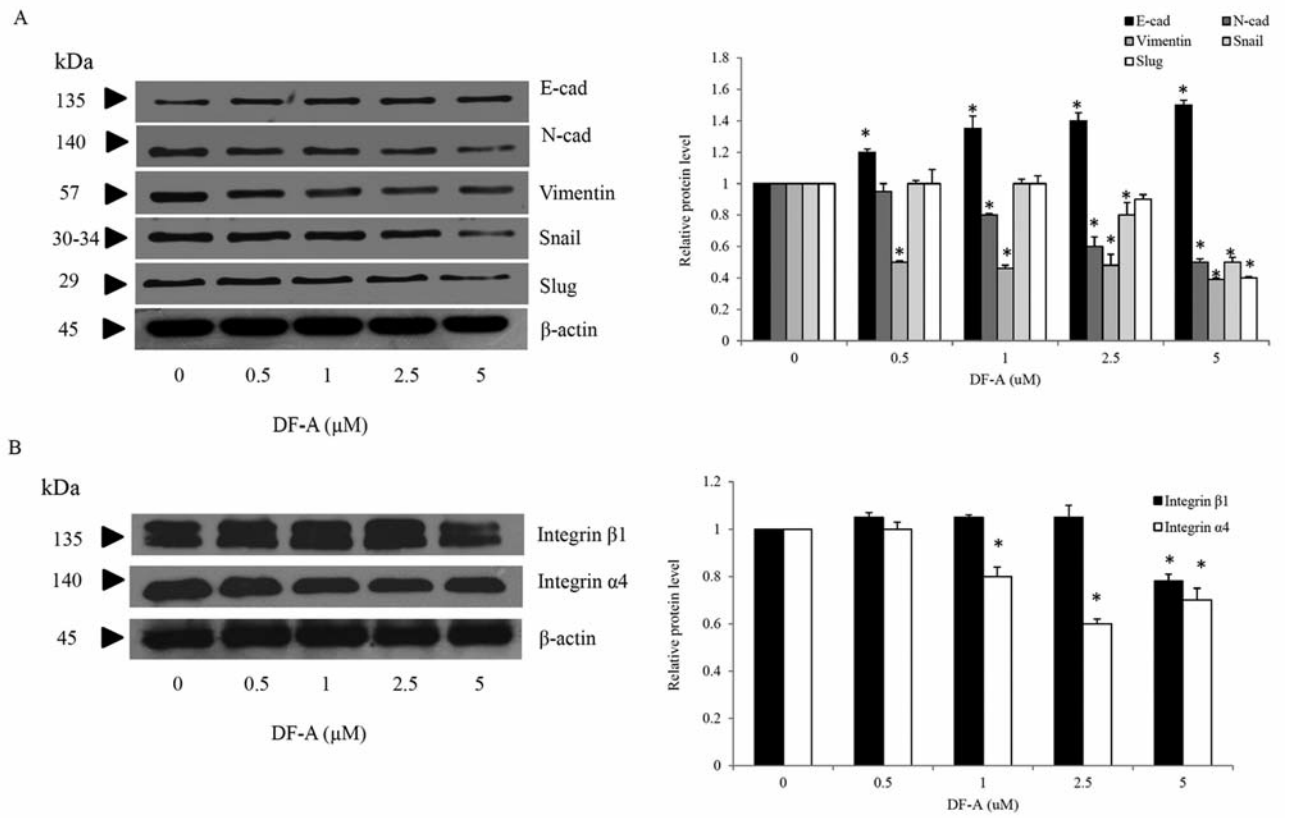

Figure 3. *Alteration of migratory-related proteins was determined by western blotting; β-actin was used as loading control to confirm equal sample loading. Cells were treated with or without DF-A (0-5 μM) for 24 h. (A) Expression of E-cadherin (E-cad), N-cadherin (N-cad), vimentin, Snail and Slug (B. The levels of integrin β_1 and integrin α_4 in response to DF-A treatment.*

we investigated the potential of DF-A on EMT and focused on the E-caherin, N-cadherin and EMT markers (Snail, Slug, vimentin). The non-toxic concentrations of DF-A could decrease cell migration by up-regulation of E-cadherin and down-regulation of N-cadherin, vimentin, Snail and Slug. Also, we investigated the role of integrins regarding their ability to mediate cell migration. The previous studies have reported that the expression level of integrin, especially, integrin β_1 and α_4 is associated with the migratory behavior of cancer cells (11, 12). Our investigation demonstrated that DF-A resulted in a significant down-regulation of integrins, including integrin β_1 and integrin α_4 (Figures 3A and 3B).

In summary, we reported a novel finding about DF-A activity on the inhibition of lung cancer cell migration through regulation of EMT as well as integrin proteins involved in cell motility. Based on these results DF-A seems a promising compound for cancer therapy treatment.

Conflicts of Interest

The Authors declare that there is no conflict of interest in this research.

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References

1 Hogstrand C, Kille P, Ackland ML, Hiscox S and Taylor KM: A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and STAT3 (signal transducer and activator of transcription 3). Biochem J *445*: 229-237, 2013.

2 Nurwidya F, Takahashi F, Murakami A and Takahashi K: Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. Cancer Res Treat *44*: 151-156, 2012.

3 Hernandez-Caballero M: Molecular Mechanisms of Metastasis: Epithelial-Mesenchymal Transition, Anoikis and Loss of Adhesion. Carcinogenesis, book edited by Kathryn Tonissen, DOI: 10.5772/55399, 2013.

4 Hanahan D and Weinberg Robert A: Hallmarks of Cancer: The Next Generation. Cell *144*: 646-674, 2011.

5 Wang Y and Zhou BP: Epithelial-mesenchymal Transition – A Hallmark of Breast Cancer Metastasis. Cancer Hallm *1*: 38-49, 2013.
6 Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. The J Clin Invest *119*: 1420-1428, 2009.
7 Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y and Johnson KR: Cadherin switching. J Cell Sci *121*: 727-735, 2008;121.
8 Chunhacha P, Sriuranpong V and Chanvorachote P: Epithelial-mesenchymal transition mediates anoikis resistance and enhances invasion in pleural effusion-derived human lung cancer cells. Oncology Lett *5*: 1043-1047, 2013.
9 Klymkowsky MW and Savagner P: Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am J Pathol *174*: 1588-1593, 2009.
10 Felding-Habermann B: Integrin adhesion receptors in tumor metastasis. Clin Exp Metastasis *20*: 201-213, 2003.
11 Ganguly KK, Pal S, Moulik S and Chatterjee A: Integrins and metastasis. Cell Adh Migr *7*: 251-261, 2013.
12 Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A and Mueller BM: Integrin activation controls metastasis in human breast cancer. Proc Natl Acad of Sci USA *98*: 1553-1358, 2001.
13 Geiger TR and Peeper DS: Metastasis mechanisms. Biochim Biophys Acta *1796*: 293-308, 2009.
14 Hunter KW, Crawford NP and Alsarraj J: Mechanisms of metastasis. Breast cancer Res *10(Suppl 1)*: S2, 2008.
15 Huttenlocher A and Horwitz AR: Integrins in cell migration. Cold Spring Harb Perspect Biol *3*: a005074, 2011.
16 Boudreau NJ and Jones PL: Extracellular matrix and integrin signalling: the shape of things to come. Biochem J *339*: 481-488, 1999.
17 Luan W and Zhao L: Role of integrin β1 in sensitivity to chemotherapy of pulmonary adenocarcinoma A549. Chin-Ger J Clin Oncol *11*: 80-82, 2012.
18 Zhang H, Ozaki I, Mizuta T, Matsuhashi S, Yoshimura T, Hisatomi A, Tadano J, Sakai T and Yamamoto K: β1-integrin protects hepatoma cells from chemotherapy induced apoptosis *via* a mitogen-activated protein kinase dependent pathway. Cancer *95*: 896-906, 2002.
19 Xiong J, Balcioglu HE and Danen EHJ: Integrin signaling in control of tumor growth and progression. Int J Biochem Cell Biol *45*: 1012-1015, 2013.
20 Stupack DG and Cheresh DA: Integrins and angiogenesis. Curr Top Dev Biol *64*: 207-228, 2004.
21 Sritularak B and Likhitwitayawuid K: New Bisbibenzyls from *Dendrobium falconeri*. Hel Chim Acta *92*: 740-744, 2009.

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