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5-Demethylovalicin, as a Methionine Aminopeptidase-2 Inhibitor Produced by *Chrysosporium*

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Abstract—5-Demethylovalicin was isolated from the fermentation broth *Chrysosporium lucknowense* and the structure was identified by spectroscopic methods. 5-Demethylovalicin inhibited the recombinant human MetAP-2 ($IC_{50}=17.7$ nM) and the growth of human umbilical vein endothelial cells (HUVEC; $IC_{50}=100$ nM) in cell proliferation assay without cytotoxicity on the transformed and cancer cell lines.

Introduction

Angiogenesis, the formation of new blood vessels from an existing vascular bed, is known to be essential for the solid tumor formation as well as wound healing and embryonic development. Angiogenesis is regulated by the balance between angiogenic and angiostatic factors. In local tumor environment this balance is altered and highly favors angiogenesis. The inhibition of angiogenesis is therefore emerging as a promising therapy for cancer.[1] However, targeting tumor angiogenesis is complicated by the factors that multiple angiogenic factors are implicated, and raising the question whether or not inhibition of a single factor will be efficacious.

Because the anti-angiogenesis agent fumagillin bound covalently and modified the enzyme methionine aminopeptidase (MetAP),[2,3] the methionine aminopeptidase (type 2) was suggested as a common target for angiogenesis inhibitors.[4] The structure and function of the enzyme MetAP2 were identified at molecular level. Biochemical and structural evidence has shown that histidine 231 was the site of covalent attachement.[5,6]

During a process to find MetAP2 inhibitors from fungal sources, 5-demethylovalicin (**1**) and ovalicin (**2**) were isolated from the mycelial extracts of the fungus *Chrysosporium lucknowense* F80642.[7,8] In this paper, we described the isolation, structure determination, and anti-angiogenic activity of the compound **1**.

Results and Discussion

Isolation and Structure determination of 1

The culture broth (8 L) was extracted with acetone (8 L) and filtered on a filter paper to collect filtrates. The filtrate was concentrated to give a 3.8 g of yellow residue and the crude residue was loaded on a silica gel column (Merck, Kieselgel 60, 230–400 mesh, 6×25 cm) and eluted with increasing concentration of EtOAc in *n*-hexane (up to 60% EtOAc). The resulting active fractions were then separated on a C_{18} gel chromatography (Merck, Lichroprep RP-18, 40–63 μm; $CH_3CN/H_2O=20:80$ equilibrated; 4×25 cm) by increasing of CH_3CN concentration. The active constituents were loaded on a Sephadex LH-20 column and eluted with MeOH. Finally 35 mg of **1** was separated on two sets of preparative TLC.

Compound **1** was isolated as a gummy solid and the FABMS of **1** exhibited the molecular ion at m/z 283.1554 $(M+H)^+$ corresponding to the molecular formula $C_{15}H_{23}O_5$ (calculated 283.1545). The HMQC experiment and DEPT spectral data reveal three methyl,

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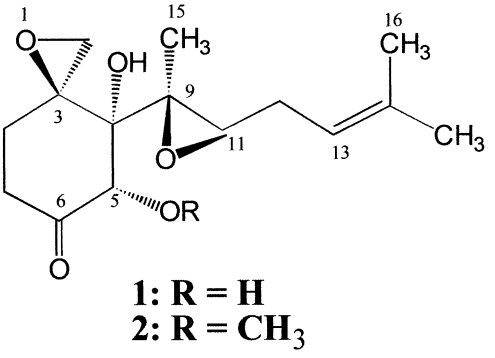
four methylene, three methine, and five quaternary carbons including a carbonyl carbon. ^{1}H–^{1}H correlations in COSY, H-8–H-10, indicated the presence of a vinyl group and an epoxide methine. Also, a long range coupling between H-11 (dt, $J=6.8$, 1.6 Hz) and H-13 (dt, $J=7.2$, 1.6 Hz) was observed in TOCSY experiment. The two hydroxy peaks of 5-OH and 4-OH were assigned at δ 3.70 and 3.21 based on the HMBC spectrum, respectively. Stereochemistry of H-5 and H-14 was confirmed by ROESY spectrum. The vinyl and epoxide group are typical groups in ovalicin analogues,[9] which are well known angiogenesis inhibitors. The cyclohexanone skeleton was confirmed by the strong two- and three-bond HMBC correlations from C-3 (δ 59.95) to H-2b (δ 2.73) and H-7 (δ 2.75, 2.60), and by the correlations of carbonyl carbon at C-6 (δ 208.20) with the protons on C-8 (δ 1.50, 2.67) and C-5 (δ 4.67). The exocyclic epoxide ring on cyclohexanone was confirmed by the chemical shifts and coupling constant (δ 2.73, 3.09, d, $J=4.4$ Hz), which are the typical epoxide proton peaks, and a strong correlation between C-3 (δ 59.95) and H-2 in HMBC spectrum. The structure was finally confirmed in comparison with the spectral data of ovalicin (**2**),[9,10] and reported spectral data.[11]



Biological activities of 1

The 5-demethylovalicin (**1**) strongly inhibited the enzyme hMetAP2 (recombinant human MetAP, type 2) with IC_{50} value of 17.7 nM. But **1** showed 2000-fold less potent activity to another type of the enzyme eMetAP1 (*Escherichia coli* MetAP, type 1; $IC_{50}=35$ μM). Therefore, **1** showed specific inhibitory activity on the (type 2)-MetAP. Compound **1** and **2** exhibited slightly stronger activity than fumagillin, which also well known angiogenesis inhibitor and have a long aliphatic group at C-4, on agar diffusion assay on *S. cerevisiae* (map1::HIS3) as in Figure 1.[12]

Compound **1** was also exhibited specific inhibitory activity for the growth of HUVEC that is an in vitro tool to determine angiogenesis process, in dose-dependent mode ($IC_{50}=25.99$ pg/μL, 100 nM). However, compound (**1**) did not inhibit the growth of the normal fibroblast cell (NIH3T3), transformed cells (K-*ras*-transformed), and cancer cell lines (see Fig. 2).

5-Demethylovalicin (**1**) has similar activities in comparison with other reported ovalicins with methyl group on C-5. Therefore, **1** may be a useful lead compound for the development and structure design of MetAP2 inhibitors, and the study of structure–activity relationship.

In 1998, the crystal structure of MetAP2 was determined by Liu et al., and they found that a covalent bond formed between an epoxide group of on the cyclohexane ring and a histidine residue (His-231) in the active site of the enzyme.[13] Liu et al. also found that only the epoxide rather than side chain epoxide of fumagillin was involved in the covalent bonding to MetAP2.[13] This result raises the possibility for a more potent analogue with a various group on C-4 of fumagillin and C-5 of ovalicin, respectively. Therefore, the preparation of analogues of **1** at C-5 might shed a light to the development of new MetAP2 inhibitors for antitumor drug.

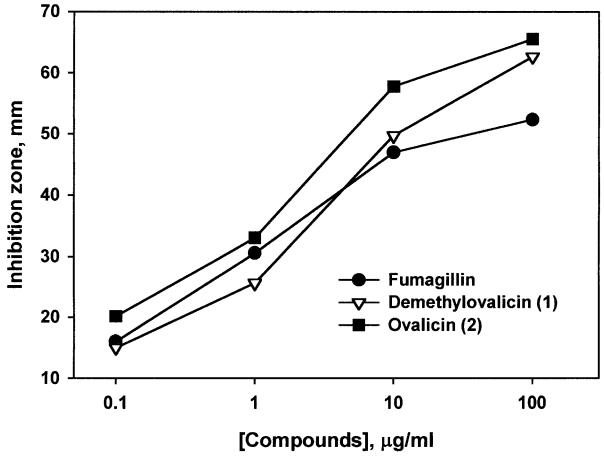


Figure 1. Determination of MetAP2 inhibitory activities of the compounds using agar diffusion assay on the recombinant yeast *Saccharomyces cerevisiae* (map1::HIS3).

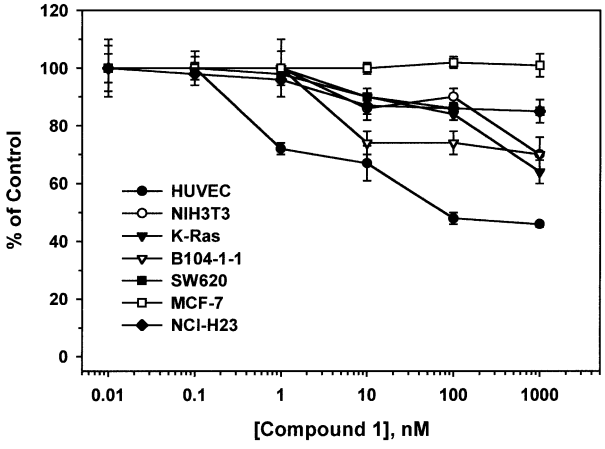


Figure 2. Growth-inhibition assay using the colorimetric method with WST-1.

Experimental

General experimental procedures

The UV spectra were recorded in MeOH on a Shimadzu UV-265 instrument. The ^{1}H NMR spectra were recorder in $CDCl_3$ on a Bruker AMX-400 NMR spectrometer at 400 MHz, while the ^{13}C NMR data were recorded on the same instrument at 100 MHz. Mass spectra were supplied by the Mass Spectrometer Facility, Department of Chemistry, University of California at Riverside.

Organism and fermentation

The fungi sp. F80642 was isolated from a soil sample collected in Mt. Keryong, Chungnam. The soil suspension was activated on PDA (Potato dextrose broth 20 g, agar 18 g, chloramphenicol 50 ppm, nalidixic acid 20 ppm, D.W 1 L) and Littman oxgall agar (glucose 10 g, oxgall 15 g, pepton 1 g, agar 18 g, chloramphenicol 50 ppm, nalidixic acid 20 ppm, D.W 1 L) to isolate fungi. Strain F80642 was selected among the colonies by its MetAP2 inhibitory activity on the selective assay plate containing the MetAP1-deleted *S. cerevisiae*. The strain was identified as *C. lucknowense* by Korean Collection for Type Culture (KCTC, http://kctc.kribb.re.kr) and deposited as KCTC0640BP. The fungus activated on potato dextrose agar plate and inoculated into 500 mL shake flasks containing 100 mL of YM broth (yeast extract 0.3 g, malt extract 0.3 g, peptone 0.5 g, and dextrose 1.0 g) and cultivated on a rotary shaker at 170 rpm and at 28 °C for 3 days. The seed culture of 240 mL was then transferred into 14 L Fermentor (Marubishi MJ-N type) containing 8 L of production medium (glucose 160 g, yeast extract 16 g, polypeptone 40 g, $MgSO_47H_2O$ 4 g, KH_2PO_4 8 g, initial pH = 5.6–5.8). The fermentation was carried out at 28°C for 5 days with agitation of 180 rpm and with aeration of 4 L air/min.

Isolation

The culture broth (8 L) was extracted with acetone (8 L) and filtered on a Whatman No.1 filter to collect filtrate. The filtrates were concentrated in vacuo and re-extracted into EtOAc. The EtOAc fractions were then concentrated to give a 3.8 g of yellow residue. The crude residue was loaded on a silica gel column (Merck, Kieselgel 60, 230–400 mesh, 6×25 cm) and eluted with increasing concentration of EtOAc in *n*-hexane (up to 60% EtOAc). The active constituent was detected on TLC (*n*-hexane/EtOAc = 60:40 for normal phase, $R_f = 0.59$; $CH_3CN/H_2O = 55:45$ for reverse phase, $R_f = 0.47$) and on *map1* assay plates for MeOH-soluble parts. The resulting active fractions were then separated on a C_{18} gel chromatography (Merck, Lichroprep RP-18, 40–63 μm; $CH_3CN/H_2O = 20:80$ equilibrated; 4×25 cm) by increasing of CH_3CN concentration. The active constituents were loaded on a Sephadex LH-20 column and eluted with MeOH. Finally 23 mg of **1** was separated on two sets of prep-TLC ($CHCl_3$/MeOH = 20:1 for first normal phase; $CH_3CN/H_2O = 55:45$ for second reverse phase).

5-Demethylovalicin (1). UV (MeOH) λ_{max} 272 (218); $[\alpha]_D^{25}$ −25.6 (*c* 0.003, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.19 (1H, dt, *J* = 7.2, 1.6 Hz, H-13), 4.67 (1H, d, *J* = 2.8 Hz, H-5), 3,70 (1H, d, *J* = 3.6 Hz, 5-OH), 3.21 (1H, s, 4-OH), 3.09 (1H, d, *J* = 4.4 Hz, H-2a), 2.91 (1H, dt, *J* = 6.8, 1.6 Hz, H-11), 2.75 (1H, m, 7-Ha), 2.73 (1H, d, *J* = 4.4 Hz, H-2b), 2.67 (1H, m, H-8a), 2.60 (1H, m, H-7b), 2.43 (1H, m, H-12a), 2.16 (1H, m, H-12b), 1.76 ((3H, s, H-16), 1.68 (3H, s, H-17), 1.50 (1H, m, H-8); 1,48 (3H, s, H-15). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.20 (s, C-6), 135.51 (s, C-14), 117.90 (d, C-13), 77.68 (s, C-4), 77.41 (d, C-5), 60.33 (s, C-9), 59.95 (s, C-3), 56.70 (d, C-11), 50.74 (t, C-2), 35.11 (t, C-7), 30.20 (t, C-8), 26.96 (t, C-12), 25.71 (q, C-16), 17.98 (q, C-17), 14.47 (q, C-15).

Bioassay

Inhibition of the MetAP2 activity was determined on as agar plate containing the MetAP1 deleted yeast strain *map1*, *S. cerevisiae* (map1::HIS3) that was gift from Yie-Hwa Chang (St Louis Univ.). Sterile filter disks impregnated with 10 μL of culture extracts were placed on the assay plate and incubated at 30 °C for 3 days.[3] Control plates were prepared by wild strain *S. cerevisiae* sp. *w303* and recombinant strain null MetAP2 (map2::URA3).

MetAP enzymatic assay

Recombinant human MetAP2 was expressed and purified from insect cells in a method described by Zuo et al.[12] Various concentrations of 5-demethylovalicin (**1**) in methanol and methanol control were each incubated with 15 nM recombinant human MetAP2 or yeast MetAP1 in buffer A (20 mM HEPES, pH 7.5, 150 mM KCl and 1.5 mM $CoCl_2$) for 10 min at 37 °C. To begin the enzymatic reaction, Met-Gly-Met-Met was added to a final concentration of 2 mM and incubated at 37 °C. After 15 min, EDTA was added to a concentration of 10 mM in order to quench the reaction. Released methionine was quantified as reported previously.[4]

Endothelial cell proliferation assay

Human umbilical vein endothelial cell (HUVEC) was cultivated by endothelial growth medium (EGM®, Clonetech) at 37 °C under 5% CO_2 in humidified incubator. NIH3T3 (normal mouse fibroblast), K-Ras (*K-ras*-transformed NIH3T3) and B104-1-1 (neu*-transformed NIH3T3; ErbB-2 activated) cell lines were cultivated in DMEM (Dulbecco's modified eagle medium) containing 10% fetal bovine serum in 5% CO_2 incubator at 37 °C. SW620 (colon cancer), MCF-7 (breast cancer) and NCI-H23 (lung cancer) cell lines were cultivated in RPMI 1640 media at the same condition. Cell proliferation assays were carried out by the WST-1 (Roche Molecular Biochemicals) protocol. Confluently growing (80%) cells were trypsinized, transferred into microplate (3000–5000 cells per well) and incubated at 37 °C under 5% CO_2 for 1 day. After removal of the culture media from the microplate wells, compound **1** and **2** (in 0.1% DMSO in fresh culture media) were

treated for 48 h (NIH3T3, K-Ras and B104-1-1) and 96 h (HUVEC) respectively. Resulting metabolically active cells were detected by ELISA reader (Bio-Rad; 450 nm) after 1–4 h of incubation with WST-1.[12]

Acknowledgements

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