5-Demethoxyfumagillol, a Potent Angiogenesis Inhibitor Isolated from *Aspergillus fumigatus*

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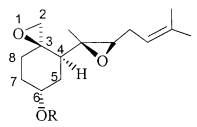
A novel angiogenesis inhibitor, 5-demethoxyfumagillol (1), was obtained by isolation, purification and saponification of cultured broth of *Aspergillus fumigatus*. The structure was assigned as (3*R*,4*R*,6*R*)-4-[(2*R*,3*R*)-2-methyl-3-(3-methyl-but-2-enyl)-oxiranyl]-1-oxa-spiro[2,5]octan-6-ol (1) by spectroscopic analysis and confirmed by independent synthesis from fumagillol (3). In addition, 6-*O*-(chloroacetylcarbamoyl)-5-demethoxyfumagillol (7) showed a potential anti-angiogenic activity in CAPE cells *in vitro*.

Key words 5-demethoxyfumagillol; angiogenesis inhibitor; *Aspergillus fumigatus*; fermentation; semi-synthetic fumagillol derivative

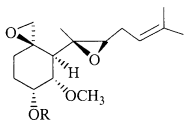
Angiogenesis, the phenomenon of generating a new capillary vessels, has a close connection with various diseases[1] such as the growth and transfer of solid cancer, rheumatoid arthritis, diabetic retinopathy, and psoriasis. In 1971, Judah Folkman of the Medical College of Harvard University, U.S.A., suggested the novel concept of treating solid tumors by inhibiting angiogenesis.[2] Recent clinical studies on anti-angiogenic agents seem to indicate that the problem of tolerance associated with conventional anti-cancer agents could be alleviated, probably by acting directly on the endothelial cells rather than on tumor cells of a living organism.

Fifty years ago, Hanson and Eble reported that cultures of a fungus *Aspergillus fumigatus* (IMI-069714) inhibited *Staphylococcus aureus* 209 bacteriophage.[3] The structure and stereochemistry of the active constituent fumagillin was determined by X-ray crystallographic studies.[4] Also, in 1990, Ingber *et al.*[5] reported that fumagillin (**2**) showed potent anti-angiogenic activity and inhibited endothelial cell proliferation *in vitro* and tumor-induced angiogenesis *in vivo*.[6,7] However, the effectiveness of **2** as an inhibitor of tumor growth was limited because it produced severe weight loss as a side effect. Ingber *et al.* started chemical modification of **2** in an effort to obtain analogues having the potent anti-angiogenic activity of **2** with less toxicity. As a result, one of the semi-synthetic derivatives, TNP-470 (**4**), has been found to have more potent anti-angiogenic activity and be less toxic compared to **2**.[8—10] TNP-470 (**4**) is currently one of the most promising small molecule inhibitors of angiogenesis and is being evaluated in phase III clinical trials as a potential anti-cancer drug.[11—14]



5-Demethoxyfumagillol (**1**) : R=H

6-*O*-(chloroacetylcarbamoyl)-5-demethoxyfumagillol (**7**) : R= $CONHCOCH_2Cl$



Fumagillin (**2**) : R=$CO(CH^{I}{=}CH)_4CO_2H$

Fumagillol (**3**) : R=H

TNP- 470 (**4**) : R= $CONHCOCH_2Cl$

Chart 1

In the course of our search for a new type of anti-angiogenesis inhibitors from cultured broths of soil microorganisms, 5-demethoxyfumagillol (**1**), a novel anti-angiogenic substance, was obtained by basic hydrolysis of 5-demethoxyfumagillin.[15]

In the present paper, we describe the fermentation, isolation, and saponification of 5-demethoxyfumagillin, and structural assignment of **1** by spectroscopic analysis and independent synthesis (Chart 2). In addition, the preparation of a 5-demethoxyfumagillol analogue with a carbamoyl side chain and its anti-angiogenic activity are reported.

Chemistry To confirm the structure of 5-demethoxyfumagillol (**1**), semi-synthetic **1** was synthesized from fumagillol (**3**) as a starting material. First, oxidation of **3** by the Collins reagent,[16] followed by reductive elimination of the resulting 6-ketofumagillol (**5**) by treatment with samarium(II) diiodide (SmI_2),[17] produced the desired demethoxylated 6-ketofumagillol **6** in 50% yield. Then, stereoselective reduction of ketone **6** with L-selectride (lithium tri-*sec*-butylborohydride, 1.0 M solution in tetrahydrofuran) furnished 83% yield of the desired **1**, along with 13% of epimeric 6-*epi*-5-demethoxyfumagillol (**1′**). Finally, in order to examine the anti-angiogenic activities of demethoxyfumagillol derivatives, we attempted chemical modification of **1**. 6-*O*-(Chloroacetylcarbamoyl)-5-demethoxyfumagillol (**7**) was obtained by treatment of **1** with chloroacetyl isocyanate in the presence of a stoichiometric amount of DMAP in dichloromethane in 70% yield (Chart 2).

Results and Discussion

Several types of angiogenesis inhibitors such as angiostatic steroids,[18] retinoid[19] and cartilage factors[20] have been discovered, although they are not clinically useful due to toxicity and potency. Therefore, it is of interest to search for new types of angiogenesis inhibitors.

In the course of our extensive screening program for novel angiogenesis inhibitors from soil microorganisms, we found that a novel 5-demethoxyfumagillol (**1**), produced by the saponification of 5-demethoxyfumagillin isolated from the cultured broth of *Aspergillus fumigatus* (IMI-069714), showed potent anti-proliferation activity against calf pul-

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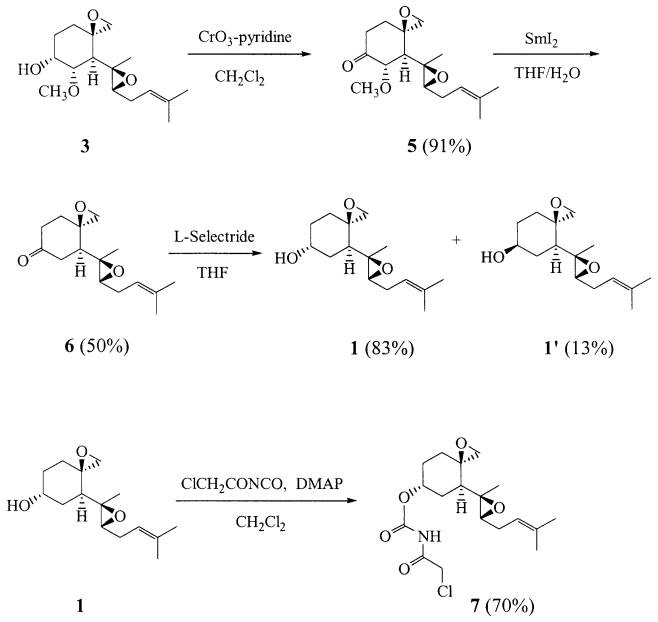


Chart 2

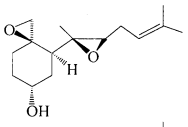
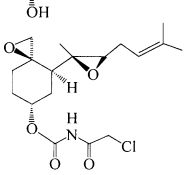
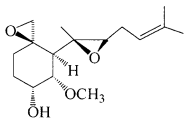
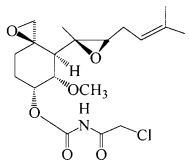
monary artery endothelial (CPAE) cells. Spectral data for 5-demethoxy fumagillin were very similar to those of known fumagillin (**2**) except for the 5-methoxy moiety. Saponification of 5-demethoxyfumagillin to remove the polyene moiety gave the corresponding 5-demethoxyfumagillol (**1**) in 48% yield. The structure of **1**, a basic hydrolysate, was established by an extensive spectroscopic analysis of NMR (^{1}H, ^{13}C), FT-IR, and HR-Mass (fast atom bombardment, FAB).

In order to definitively confirm the structure of 5-demethoxyfumagillol (**1**), semi-synthetic compound **1** was independently prepared from fumagillol (**3**) as previously described in Chart 2. Thus, the structure of 5-demethoxyfumagillol (**1**) was elucidated to be (3*R*,4*R*,6*R*)-4-[(2*R*,3*R*)-2-methyl-3-(3-methyl-but-2-enyl)-oxiranyl]-1-oxa-spiro[2,5]-octan-6-ol.

In order to examine the *in vitro* inhibitory activity of **1** and its derivative against endothelial cell proliferation, chemical modification of **1** was carried out. Chemical modification of **1** gave 6-*O*-(chloroacetylcarbamoyl)-5-demethoxyfumagillol (**7**) using chloroacetyl isocyanate in the presence of DMAP as a representative compound. The anti-proliferation activity and cytotoxicity of a novel **1** and **7** were examined in CPAE cells and lymphoma (L5178Y, mice) cells, respectively. The anti-proliferation activity of **1** was very similar to that of **3**, whereas **7** showed at least eight times more potent anti-proliferation activity compared to **7**, as described in Table 1. However, both **1** and its carbamoyl derivative **7**, exhibited less potent activity compared to TNP-470. Taking into consideration these results, the methoxy moiety on the 5-position of fumagillol compounds seems to be necessary and important for the anti-proliferation activity.

Currently, efforts are under way to elucidate the structure–activity relationship of these fumagillin analogues as potential anti-cancer agents.

Table 1. Anti-proliferation Activity of Fumagillol and Demethoxyfumagillol Analogues

Compound	Structure	CPAE[b]	L5178Y[b]
		IC_{50} (μM)[b]	IC_{50} (μM)
1		7.06	>39.6
7		1.147	>2.67
3		8.09	>35.4
4[a]		0.0011	>24.5

a) The preparation of reference compound (**4**) was carried out following reported procedure.[21] *b*) Anti-proliferation activities were colorimetrically measured by SBR (CPAE cell) and MTT (L5178Y) method and IC_{50} values were estimated by Probits method (Pharm/PCS^R).

Experimental

All reactions were conducted under anhydrous conditions in solvents dried over molecular sieves type 4 Å under a nitrogen atmosphere and performed using oven dried glassware. Melting points were determined on a Buchi 510 capillary apparatus and are uncorrected. IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer. NMR spectra were recorded on a Bruker DPX 400 MHz instrument and performed in $CDCl_3$ using tetramethylsilane as the internal reference except where indicated otherwise. The coupling constants (J) are reported in Hz. Mass spectra were recorded on a HP 5989B mass spectrometer. Flash chromatography was performed using Merck silica gel 60 (230—400 mesh) according to the procedure reported previously.[22]

Preparation of 5-Demethoxyfumagillol by Fermentation. Fermentation A suspension of spores [1.0×10^3 spores/ml, 10% glycerol, 1% Tween 80, 5% lactose (w/v)] of *Aspergillus fumigatus* (IMI-069714) was inoculated in pre-culture medium (CSL 40 g/l, glucose 30 g/l, $CaCO_3$ 10 g/l) in a concentration of 1% (v/v), and cultured with rotary shaking at 28 °C. After 30 h the pre-culture was inoculated in a 250 l jar fermentor containing 18 l of culture medium (CSL 50 g/l, glucose 30 g/l, $CaCO_3$ 10 g/l) in a concentration of 1%, and cultured at 28 °C for 60—70 h.

Isolation of 5-Demethoxyfumagillin The culture was filtered through a filter paper, and the filtrate (about 1.1 l) was applied to a HP-20 column to adsorb the active material. The HP-20 column was washed with 50 ml of 50% aqueous methanol and eluted with 50 ml of methanol/ethanol (1 : 1, v/v) solution. The active portions were combined and concentrated under reduced pressure. A proper amount of chloroform was added to dissolve the residue. The same amount of distilled water was added to the chloroform solution and the mixture was centrifuged to separate the chloroform layer and the layer was concentrated under reduced pressure. About 9 g of the concentrate was adsorbed on a silica gel column of 9 g volume and the column was eluted with a mixed solvent of chloroform/methanol/acetic acid (100 : 5 : 0.1, v/v). The active portions were combined and concentrated under reduced pressure (about 150 mg) and the residue was dissolved in a small amount of ethyl acetate. The solution was left overnight at −20 °C to induce precipitation. The precipitated material (120 mg) containing fumagillin was washed with cold ethyl acetate 2—3 times to obtain a mixed sample having high purity. The mixture was separated by medium pressure liquid chromatography (MPLC) with dichloromethane/methanol (98 : 2, v/v) as elution solvent to give the crude title compound 5-demethoxyfumagillin as an off-white foam product (6.5 mg, 35.5% yield). IR (neat) ν_{max} cm^{-1}: 3855, 3647, 3510, 2920, 1695, 1680, 1650, 1628, 1537, 1520, 1362, 1080. ^{1}H-NMR (400 MHz,

$CDCl_3$) δ: 7.33—7.21 (m, 2H), 6.64—6.59 (m, 2H), 6.51—6.46 (m, 2H), 5.95 (dd, J=4.04, 15.3 Hz, 2H), 5.36 (br s, 1H), 5.19 (t, J=7.54 Hz, 1H), 2.91 (d, J=4.36 Hz, 1H), 2.45—2.35 (m, 1H), 2.17—1.79 (m, 7H), 1.75 (s, 3H), 1.66 (s, 3H), 1.15 (s, 3H), 0.93—0.88 (m, 1H).

Preparation of 5-Demethoxyfumagillol (1) To 5-demethoxyfumagillin (6 mg), 0.1 N NaOH solution (0.6 ml) was added and the mixture was stirred at room temperature for 6 h. The reaction mixture was diluted with ethyl ether (2.5 ml), and the organic layer was separated and washed with water (1 ml) and brine (1 ml), and dried over anhydrous magnesium sulfate. After filtering, the filtrate was concentrated by evaporation under reduced pressure. The obtained residue was purified by silica gel column chromatography with ethyl acetate/*n*-hexane (1 : 1, v/v) to obtain the title compound, 5-demethoxyfumagillol (**1**) as a colorless oil (2.5 mg, 48% yield). IR (neat) ν_{max} cm^{-1}: 3850, 3732, 3647, 3440, 2920, 1698, 1680, 1652, 1558, 1507, 1460, 1382, 980. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.19—5.15 (m, 1H), 4.31—4.29 (m, 1H), 2.86 (d, J=4.4 Hz, 1H), 2.39 (m, 1H), 2.42—2.33 (m, 1H), 2.27—1.78 (m, 1H), 1.74 (s, 3H), 1.65 (s, 3H), 1.14 (s, 3H), 1.12—1.03 (m, 1H).

Semi-synthetic Procedure for 5-Demethoxyfumagillol (1) and 6-*O*-(Chloroacethylcarbamoyl)-5-demethoxyfumagillol (7). (3*R*,4*R*,5*S*)-5-Methoxy-4-[(2*R*,3*R*)-2-methyl-3-(3-methyl-but-2-enyl)-oxiranyl]-1-oxa-spiro[2.5]octan-6-one (5) To a solution of pyridine (1.0 ml) in dry dichloromethane (14 ml) at room temperature was added chromium(VI) oxide (420 mg). After stirring for 30 min at the same temperature, a solution of fumagillol **3** (200 mg, 0.71 mmol) in dry dichloromethane (7 ml) was added dropwise and the resulting mixture was stirred at room temperature for 6 h under N_2 atmosphere. The reaction mixture was filtered through celite to remove residual chromium. The filtrate was concentrated *in vacuo* to leave a pale yellowish oil which was purified by column chromatography on SiO_2 with ethyl acetate/*n*-hexane (1 : 4, v/v) to give the title oxidized ketofumagillol compound **5** as a colorless oil (180 mg, 91% yield). IR (neat) ν_{max} cm^{-1}: 2917, 1730, 1558, 1455, 1114, 457, 418 cm^{-1}. ^{1}H-NMR (400 MHz , $CDCl_3$) δ: 5.19 (t, J=7.5 Hz, 1H), 4.08 (d, J=10.3 Hz, 1H), 3.51 (s, 3H), 3.06 (d, J=4.3 Hz, 1H), 2.73 (d, J=4.3 Hz, 1H), 2.72—2.65 (m, 1H), 2.60 (t, J=6.4 Hz, 1H), 2.52 (dt, J=14.0, 4.3 Hz, 1H), 2.42—2.37 (m, 1H), 2.18—2.12 (m, 1H), 2.06 (td, J=13.6, 5.4 Hz, 1H), 1.88 (d, J=10.5 Hz, 1H), 1.75 (s, 3H), 1.73—1.69 (m, 1H), 1.65 (s, 3H), 1.29 (s, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 207.14, 135.11, 118.28, 83.25, 60.52, 58.67, 58.60, 58.49, 53.66, 51.86, 36.87, 33.27, 27.42, 25.76, 18.02, 13.92. HR-MS *m*/*z*: Calcd for $C_{16}H_{24}O_4$ (M^+), 280.1675. Found: 280.1666.

(3*R*,4*R*)-4-[(2*R*,3*R*)-2-Methyl-3-(3-methyl-but-2-enyl)-oxiranyl]-1-oxa spiro[2.5]octan-6-one (6) To a pre-cooled (−78 °C) and stirred solution of 6-ketofumagillol compound **5** (135 mg, 0.48 mmol) in THF/H_2O (10 : 1, v/v, 15 ml) was added 0.1 M SmI_2 solution in tetrahydrofuran (9.6 ml, 0.96 mmol) with a syringe pump under N_2 atmosphere. The resultant dark blue mixture was stirred at the same temperature for 30 min, and then allowed to warm to room temperature for 4 h. The reaction mixture was poured into saturated aqueous K_2CO_3 (50 ml) and extracted with ethyl acetate (3×25 ml). The combined organic layer was washed with water (50 ml), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* and purified by column chromatography on silica gel with ethyl acetate/*n*-hexane (1 : 4, v/v) to give the title 5-demethoxy-6-ketofumagillol **6** as a colorless oil (60 mg, 50% yield). IR (neat) ν_{max} cm^{-1}: 3851, 3733, 3647, 2917, 1716, 1558, 1455, 1386, 1253, 1139, 909, 836, 457 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.15 (t, J=7.3 Hz, 1H), 2.94 (d, J=4.3 Hz, 1H), 2.75 (d, J=4.3 Hz, 1H), 2.74—2.67 (m, 3H), 2.58 (ddd, J=14.4, 4.7, 1.7 Hz, 1H), 2.44—2.40 (m, 2H), 2.12 (m, 1H), 2.02—1.98 (m, 1H), 1.93—1.89 (m, 1H), 1.83 (dd, J=10.7, 4.7 Hz, 1H), 1.75 (s, 3H), 1.65 (s, 3H), 1.26 (s, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 208.36, 135.14, 118.06, 62.21, 60.07, 58.45, 51.66, 47.61, 40.93, 38.63, 32.26, 27.50, 25.76, 17.98, 13.84. HR-MS *m*/*z*: Calcd for $C_{15}H_{22}O_3$ (M^+), 250.1569. Found: 250.1560.

(3*R*,4*R*,6*R*)-4-[(2*R*,3*R*)-2-Methyl-3-(3-methyl-but-2-enyl)-oxiranyl]-1-oxa-spiro[2.5]octan-6-ol (1) To a pre-cooled (−78 °C), stirred solution of 5-demethoxy-6-ketofumagillol **6** (24 mg, 0.096 mmol) in dry tetrahydrofuran (2 ml) a 1.0 M L-selectride solution in tetrahydrofuran (0.144 ml, 0.144 mmol) was added dropwise by syringe pump under N_2 atmosphere. The reaction mixture was stirred at −78 °C for 1 h, and quenched by addition of methanol (5 ml) and saturated NH_4Cl solution (30 ml). The mixture was extracted with ethyl acetate (100 ml), washed with water (60 ml), dried over anhydrous Na_2SO_4, and filtered and concentrated *in vacuo* to leave an oil residue which was purified by column chromatography on silica gel with ethyl acetate/*n*-hexane (1 : 2, v/v) as elution solvent to afford the first major fraction, the title compound 5-demethoxyfumagillol **1**, as a colorless oil (20 mg, 83%). IR (neat) ν_{max} cm^{-1}: 3851, 3733, 3647, 3444, 2922, 1698, 1683, 1652, 1558, 1540, 1507, 1456, 1385, 986, 445. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.18 (t, J=7.5 Hz, 1H), 4.31 (t, J=2.5 Hz, 1H), 2.87 (d, J=4.3 Hz, 1H), 2.71 (dd, J=7.0, 6.1 Hz, 1H), 2.53 (d, J=4.3 Hz, 1H), 2.39 (m, 1H), 2.24 (td, J=13.4, 4.8 Hz, 1H), 2.12 (m, 1H), 2.01—1.94 (m, 2H), 1.91—1.84 (m, 2H), 1.81—1.78 (m, 1H), 1.75 (s, 3H), 1.65 (s, 3H), 1.15 (s, 3H), 1.09 (dt, J=13.8, 3.1 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 134.90, 118.35, 65.66, 64.19, 60.39, 59.64, 51.10, 42.14, 33.16, 30.20, 29.17, 27.60, 25.75, 18.00, 13.67. HR-MS *m*/*z*: Calcd for $C_{15}H_{24}O_3$ (M^+), 252.1725. Found: 252.1739.

6-*epi*-5-Demethoxyfumagillol (1′) Further purification of the minor fraction gave the title stereoisomer product **1′** as a colorless oil (3 mg, 13%). IR (neat) ν_{max} cm^{-1}: 3851, 3733, 3647, 3445, 2918, 1698, 1683, 1652, 1558, 1540, 1507, 1456, 1071, 491, 418. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 5.16 (t, J=7.5 Hz, 1H), 3.76 (m, 1H), 2.88 (d, J=4.3 Hz, 1H), 2.67 (dd, J=7.2, 5.9 Hz, 1H), 2.58 (d, J=4.3 Hz, 1H), 2.41 (m, 1H), 2.22—2.18 (m, 1H), 2.14—2.08 (m, 1H), 2.20 (m, 1H), 1.89—1.83 (m, 2H), 1.80—1.77 (m, 1H), 1.74 (s, 3H), 1.70—1.67 (m, 1H), 1.65 (s, 3H), 1.42 (dd, J=12.8, 3.4 Hz, 1H), 1.29 (dt, J=14.0, 3.4 Hz, 1H), 1.18 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 135.48, 118.62, 70.07, 64.60, 60.69, 58.82, 50.92, 47.19, 35.57, 33.27, 33.01, 27.96, 26.15, 18.40, 13.99. HR-MS *m*/*z* Calcd for $C_{15}H_{24}O_3$ (M^+), 252.1725. Found: 252.1773.

5-Demethoxy-6-*O*-(chloroacetylcarbamoyl)fumagillol (7) To a solution of 5-demethoxyfumagillol **1** (100 mg, 0.39 mmol) in dry dichloromethane (10 ml) was added dimethylaminopyridine (50 mg, 0.40 mmol) and chloroacetyl isocyanate (95 mg, 0.79 mmol). After stirring for 1 h, the reaction mixture was quenched with saturated NH_4Cl (5 ml) and extracted with dichloromethane (30 ml). The organic layer was washed with water (10 ml), and brine (10 ml), respectively, dried with anhydrous Na_2SO_4, and concentrated by evaporation under reduced pressure to leave an oil which was purified by column chromatography on SiO_2 with ethyl acetate/*n*-hexane (1 : 4, v/v) to give the title compound as an off-white foam (102 mg, 70.3% yield). IR ν_{max} cm^{-1}: 3280, 2960, 1790, 1755, 1725, 1490, 1380, 1230, 1190. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.30 (m, 1H), 1.16 (s, 3H), 1.65 (s, 3H), 1.75 (s, 3H), 2.41—1.81 (m, 8H), 2.60 (d, J=4.2 Hz, 1H), 2.66 (m, 1H), 2.92 (d, J=4.2 Hz, 1H), 4.49 (m, 1H), 5.16 (t, J=7.3 Hz, 1H), 7.83 (br s, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 19.3, 22.6, 24.8, 25.3, 25.8, 39.6, 49.6, 57.5, 59.6, 67.3, 125.8, 133.2, 136.0, 157.3. HR-MS *m*/*z* Calcd for $C_{18}H_{26}ClNO_5$ (M^+), 371.1573. Found: 371.1525.

Anti-proliferation Activities The anti-proliferation activities of fumagillol (**3**) and 5-demethoxyfumagillol derivatives (**1**) and **7** were evaluated as follows. Endothelial cells from calf pulmonary artery (CPAE, ATCC CRL-209) were plated on 96-well microtiter plates (2×10^4 cells/ml) and incubated with MEM medium supplemented with 10% fetal bovine serum and 100 μg/ml endothelial cell growth supplement (ECGS). Murine leukemia L5178Y cells were plated on 96-well microtiter plates (2×10^4 cell/ml) and incubated with RPMI 1640 medium supplemented with 10% fetal bovine serum. CPAE cells and the other cells were incubated for 4 and 3 d at 37 °C in a 5% CO_2 incubator, respectively. The anti-proliferation activities were colorimetrically measured by SRB (CPAE cells) or MTT (L5178Y cells) method and the IC_{50} values were estimated by Probits method (Pharm/PCS^R). The biological data for fumagillol and 5-demethoxyfumagillol derivatives are summarized in Table 1.

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