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Synthesis and antiproliferative activity of ligerin and new fumagillin analogs against osteosarcoma

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ABSTRACT

Ligerin (**1**) is a natural chlorinated merosesquiterpenoid related to fumagillin (**2**) exhibiting a selective antiproliferative activity against osteosarcoma cell lines and an *in vivo* antitumor activity in a murine model. Semisynthesis of ligerin analogs was performed in order to study the effects of the C3-spiroepoxide substitution by a halogenated moiety together with the modulation of the C6 chain. Results showed that all derivatives exhibited an *in vitro* antiproliferative activity against osteosarcoma cell lines and that chlorohydrin compounds were equally or more active than their spiroepoxy analogs. Among semisynthetic analogs, the parent compound **1** was the best candidate for further studies since it exhibited higher or equivalent activity compared to TNP470 (**3**) against SaOS2 and MG63 human osteosarcoma cells with a four times weaker toxicity against HFF2 human fibroblasts. Quantitative video-microscopy analysis was conducted and allowed a better understanding of the mechanism of its antiproliferative activity.

1. Introduction

Despite its low incidence (around 300 new cases/year in Europe), osteosarcoma is the most frequent malignant primary bone tumor. It affects predominantly children, teenagers and young adults and accounts for 8.9% of cancer-related deaths in children [1,2]. No satisfactory treatment is currently available and no significant improvement in prognosis has been noticed since the advent of combined chemotherapy in the 90's. The 5-year survival rate for patients diagnosed with osteosarcoma is still remaining between 60% and 70% without metastasis [2]. Current therapy consists in combining surgery and multiagents chemotherapy (neoadjuvant and post-surgery) based principally on methotrexate, ifosfamide, doxorubicin and cisplatin treatments. All these drugs induce significant side effects, highlighting the need to improve current treatment strategies.

Fumagillin (**2**) is a merosesquiterpene isolated for the first time in 1949 from a crude extract of a strain of *Aspergillus fumigatus* [3,4]. First studied and used in clinical medicine for its antimicrobial activity in human and veterinary health [5], this molecule has known a renewed interest since 1990 because of its anti-angiogenic properties [6]. Given the high toxicity of this compound, many syntheses of derivatives were done and led to the synthesis of the first clinically developed analog: the 6-O-chloroacetylcarbamoyl-fumagillol named TNP470 (**3**). This compound presented a strong activity against adenocarcinoma but its clinical development was stopped in phase II trial due to its neurotoxicity [7–10]. The instability and the toxicity of TNP-470 are likely due, at least in part, to the presence of three functional groups chemically labile or metabolically unstable, the two epoxides (the spiroepoxide and the 1′-2′ epoxide on the C4 side chain) and the chloroacetyl moiety at C6.

The mechanism of action of this class of compounds remains not fully resolved, but the methionine aminopeptidase-2 (MetAP2) has been identified as their main molecular target. This metalloprotease is responsible for the removal of the methionine residue from newly synthetized polypeptides, allowing their further myristoylation and functionalization. Inhibition of this enzyme would cause cell-cycle arrest. Most of these sesquiterpenes inhibit selectively and irreversibly the MetAP2 through insertion of the C4 chain in the active pocket, mimicking the terminal part of native proteins, and via the formation of a covalent bond between the C-7 of the

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spiroepoxide group and a nitrogen of the 231-histidine residue of the enzyme [11].

In 2005, Rodeschini [12] defined the role of the two epoxides for the binding of fumagillin analogs to the active site of MetAP2 which involves two water molecules, a binuclear metal center and the 231-histidin residue [13]. As the removal of the 1′-2′ epoxide doesn't impact the activity of the molecule, it would most likely be involved in the orientation of the side chain for the recognition by the enzyme [14]. Griffith et al. defined the importance of the second epoxide, demonstrating that the removal of the ring epoxide dramatically lowered the activity of fumagillol against MetAP2 [15]. Since its first discovery and use in medicine, structural modifications on the sesquiterpene backbone concerned mainly the C4-branched chain in order to improve the affinity for the hydrophobic channel surrounding the catalytic pocket [16]. Among dozens of synthetic derivatives of fumagillin, two analogs have recently been undergoing development in phase I clinical trials. CKD-732 is studied for the treatment of refractory solid cancer [17] and in combination with capecitabine and oxaliplatine for the treatment of metastatic colorectal cancer in patients who progressed on chemotherapy based on irinotecan [18]. PPI-2458 is evaluated for the treatment of non-Hodgkin lymphomas and several solid tumors [19]. These two compounds corroborate the interest of this chemical family in the search for new anticancer drugs.

In the course of our search for new drugs against osteosarcoma, ligerin (**1**), a new natural compound related to fumagillin (**2**), has previously been isolated from a marine-derived strain of *Penicillium* sp. (Fig. 1). Ligerin, the 3-hydroxy, 3-chloromethylene, 6-(3-carboxy-1-oxopropyl)-fumagillol was the second natural product exhibiting a halogenated moiety in place of the common spiroepoxide. Assayed against various murine cell lines, it exhibited a selective antiproliferative activity against osteosarcoma compared to non-tumor cells [20].

This work was focused on the semisynthesis of new halogenohydrin analogs related to ligerin, in order to investigate the impact of halogen atoms such as chlorine or bromine on the antiproliferative activity compared to their spiroepoxide analogs. Structural modulations of C6-side chain were also explored, maintaining the terminal carboxylic acid moiety. Bioactivities as well as selectivity of all compounds were evaluated using *in vitro* assays against both murine and human osteosarcoma and non-tumor cell lines and were compared to TNP470 (**3**) and reference anticancer compounds. Further studies on the ligerin bioactivity were also conducted, in order to get a better understanding of its antiproliferative mechanism.

2. Results and discussions

2.1. Chemistry

In order to evaluate the effect of the spiroepoxide opening and its substitution by a halogenomethylene moiety on the cytotoxicity of this chemical series, 6-O-succinylfumagillol (**1a**) and fumagillol (**4a**) were synthesized together with their respective chlorohydrin analogs, ligerin (**1**) and 7-chloro-fumagillol (**4**) (Scheme 1). Given that only two brominated compounds have been described in the literature in the fumagillin series [21], the synthesis of a bromohydrin analog of ligerin was also performed (**5**). In a second time, some ligerin analogs with different C6 moieties were synthesized, by introducing a heteroatom or a benzene ring in the side chain or by extending the length of the carboxylic acid side chain.

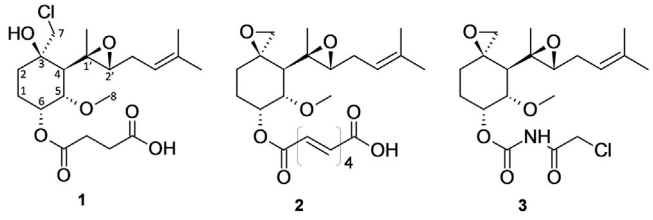


Fig. 1. Structures of ligerin (**1**), fumagillin (**2**) and TNP470 (**3**).

For that purpose, a first step consisted in preparing **4a**, the saponification product of (+)-fumagillin. Instead of the classical two steps process consisting in a first purification of the fumagillin dicyclohexylamine salt contained in a commercial product (Fumidil B®) followed by the hydrolysis of the C-6 ester, a one-step reaction was developed. In this way, the entire commercial preparation was directly submitted to alkaline hydrolysis using 0.5 N NaOH, allowing to purify **4a** from the reaction mix by liquid/liquid partition after acidification. From **4a**, each structural modulation was obtained with the same semisynthetic approach. Compound **4a** was esterified using different anhydrides to afford compounds **1a**, **6a**, **7a**, **8a**. To obtain the chlorohydrin or bromohydrin analogs (compounds **1**, **5**–**8**), a halogenation step was further performed using the corresponding halogenous salt, i.e. LiCl or LiBr. The structures of all compounds were confirmed by ^{1}H and ^{13}C NMR analyses.

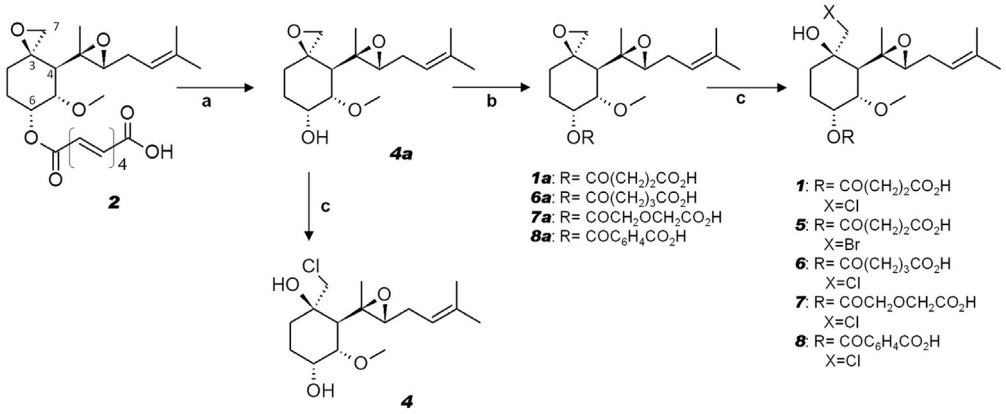
2.2. Pharmacology

2.2.1. Antiproliferative activity

The antiproliferative activity of halogenated compounds (**1**, **4** and **5**) and their respective spiroepoxide analogs (**1a**, **4a**) was tested *in vitro* against two osteosarcoma cell lines: one murine (POS-1) and one human (SaOS2).

As shown in Fig. 2, compounds **1a** and **1** were more active than **4a** and **4** as no IC_{50} could be measured in the range of 0.40–2100 nM for these last compounds. This first result confirms that a C6 side-chain is required for a high activity against these cancer cells [22]. The C7 halogen substitution was shown to have a weaker influence on the cytotoxicity. The activity of the chlorohydrins and their respective spiroepoxide analogs was found to be equivalent except for **1** against SaOS2 cell line which was more active than its spiroepoxide analog **1a** and in the same manner for **4** and **4a** against POS-1 cells. Similar activity was also obtained for **3** and its halogenohydrin derivative against the two osteosarcoma cell lines. Nonetheless, substitution of the chlorine atom by a bromine atom decreased the activity as **5** exhibited an intermediate activity compared to **1** and other analogs. IC_{50} of **5** against POS-1 and SaOS2 cells were respectively 272 nM and 801 nM whereas IC_{50} of **1** were 78 nM and 137 nM.

In literature, the two epoxides have often been described as essential for the binding to MetAP2, the target enzyme of this class of compounds [12], but the impact of the C3 epoxide opening on the activity remains unclear. In this way, it has been reported that compounds for which the spiroepoxide was opened were less active than their intact analog. For instance, the C3-methylthiomethyl derivative of **3** was over 100 times less active than the parent compound on both MetAP2 inhibition and HUVEC assays [23], and halogenation of the spiroepoxide of fumagillin derivatives led to a 10 fold decrease of their activity on the enzyme [24]. On the contrary, Hayashi et al. have shown that chlovalicin, a natural chlorinated analog in the fumagillol series, was 3 times more active than its epoxidized form ovalicin [25]. More recently, in a *in vitro* metabolization study, Arico-Muendel et al. have shown that, after exposure of the fumagillin analog PPI-2458 to an acidic treatment mimicking the stomach milieu, some metabolites formed were chlorohydrin analogs. These compounds were found as effective as their epoxide precursor on a MetAP2 inhibition assay



Scheme 1. Synthetic routes for compounds **1**, **5**–**8**. Reagents and conditions: a) 0.5 N NaoH, rt, 18 h; b) anhydride, DMAP/dry pyridine, rt, 24 h; c) LiCl or LiBr, THF, acetic acid, rt, 24 h.

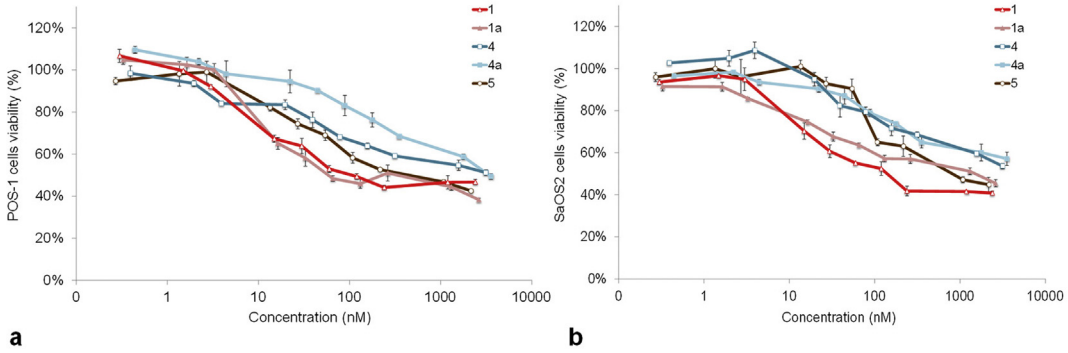


Fig. 2. Effects of compounds **1**, **1a**, **4**, **4a** and **5** against POS-1 (a) or SaOS2 (b) cell proliferation after 72 h exposure. Cell viability was measured by XTT assay. Data are mean ± SD of triplicate experiments.

and against HUVEC proliferation [19], as we observed for **1a** and **1** against osteosarcoma cells. Because of a rapid re-cyclization observed in rat plasma to afford the initial epoxide form they hypothesized that chlorohydrins lack intrinsic activity [19]. In the present study, the observation of a lower cytotoxicity for the bromohydrin analog **5** should tend to envisage that the C7 methylene substitution has an effective involvement in the activity of these compounds. Nevertheless, a molecular docking study performed with **1** and **5** using the 3-dimensional structure of the human MetAP2/fumagillin complex (PDB code: 1Boa) [11] revealed that nor a chlorine nor a bromine atom can prevent the interaction of the C7 methylene with the His231 residue of MetAP2, as it has been described for **2** and **3** [11] (data not shown).

In a second time, the modulation of the C6 moiety was explored. Cytotoxicity of compounds **6**–**8** was evaluated against a panel of three osteosarcoma cell lines, one murine (POS-1) and two human (SaOS2 and MG63) and two non-tumor fibroblasts L929 (murine) and HFF2 (human) (Table 1). All compounds displayed an antiproliferative activity associated with a dose effect against osteosarcoma cells, even if IC_{50} could not be reached for compounds **5**–**8** against MG63 and for **8** against SaOS2 (maximum 42–48% inhibition). For all tested compound except **7**, activity was higher against murine than human cell lines.

Results showed that all derivatives exhibited a lower activity compared to the parent compound **1**, whatever the cell line. For example, against POS-1 compounds **6**–**8** were found to be 3 to 17 times less active than **1**. Thus, the introduction of a heteroatom or a benzene ring in the succinyl moiety or the lengthening of the carbon chain could not improve the antiproliferative activity of **1**. As the different IC_{50} observed for the three synthetized compounds were cell line-dependent, no clear structure–activity relationship could be deduced.

For determination of selectivity against cancer cells and toxicity assessment, activities against osteosarcoma cells were compared with cytotoxicity against non-tumor fibroblastic cell lines. All

Table 1
Antiproliferative activity of compounds **1**, **3**, **5**–**8** and reference anticancer molecules against three tumor cell lines (one murine POS1, and two human SaOS2 and MG63) and two non-tumor cell lines (one murine L929 and one human HFF2), expressed as IC_{50} [nM] and SI (selectivity index).

Compound	Murine cell lines			Human cell lines			
	IC_{50} (nM)		SI[b]	IC_{50} (nM)[a]			SI[b]
	POS-1	L929		SaOS2	MG63	HFF2	
1	78	>2300	>29	137	1459	>2300	>17
3	2	>2300	>961	508	1521	1979	4
5	272	>2100	>8	801	>2100	>2100	>3
6	234	>2300	>10	863	>2300	>2300	>3
7	1394	>2300	>2	629	>2300	>2300	>4
8	230	>2100	>9	>2100	>2100	>2100	
Paclitaxel	95	521	5	52	NT	NT	
Vinscristine	75	419	6	11	NT	NT	
Doxorubicin	43	161	4	48	NT	NT	
Irinotecan	6300	6500	1	NT	NT	NT	
Fludarabine	5700	17,500	3	NT	NT	NT	

[a] NT: Non Tested.
[b] The selectivity index (SI) was calculated as the ratio between the IC_{50} values on normal cells *versus* cancer cells from the same origin (L929/POS-1; HFF2/SaOS2).

compounds induced a higher inhibition of cancer cell proliferation *versus* the non-tumor one from the same origin (murine or human). In this way, none of the compounds showed a sufficient activity allowing to establish an IC_{50} value against L929, and a maximum of 40% of inhibition of cell viability was attained in the range of concentrations tested. Furthermore, all compounds were at least 3 times more potent against human cancer cells than against HFF2. The selectivity index of compounds **6**–**8** was compared to **1** by calculating the ratio between the IC_{50} values against normal cells *versus* cancer cells from the same origin, highlighting that ligerin seems to be the most selective compound against osteosarcoma cells, with SI found over 29 and 17 against murine and human cell lines respectively.

As among all compounds tested, **1** appeared to be the more potent analog, its antiproliferative activity and selectivity for osteosarcoma were compared to the phase II engaged drug candidate, TNP-470 (**3**) the 6-O-chloroacetylcarbamoyl semisynthetic derivative of fumagillol. Against murine osteosarcoma, **3** was found to be more potent than **1** with IC_{50} values of 2 and 78 nM, respectively. On the contrary, **1** exhibited a higher activity against human SaOS2 (IC_{50} of 137 and 801 nM for **1** and **3** respectively), whereas the two compounds exhibited a similar activity against MG63 with a maximal diminution of cell viability of 55% within the range of concentrations tested. Against the non-tumor L929 and HFF2 cell lines, the cytotoxicity of **1** was weaker than **3** in the range 0.30–230 nM. Although **3** showed a high selectivity against murine cells ($SI > 961$), **1** exhibited a better ratio against human cell lines, with a selectivity index 4 times higher than the one of **3**. For instance, at a concentration of 30 nM, the decrease of MG63 and SaOS2 viability was 30–35% for both compounds, whereas the proliferation of HFF2 fibroblasts was inhibited of 15% and 43% for **1** and **3** respectively.

Furthermore, **1** was found to be at least 70 times more active than irinotecan and fludarabine against POS-1 and showed equipotent efficacy with vincristine and paclitaxel. Compared to the five anticancer drugs tested, the selectivity index of **1** was higher against murine cell lines. For instance, **1** presented a selectivity index at least 7 times higher than the one of the most active drug doxorubicin.

2.2.2. Effect of ligerin on cell replication/viability

Further studies were performed to determine whether **1** exhibited a cytotoxic or a cytostatic activity. After 72 h exposure to **1**, viable POS-1 and L929 cells were counted using a trypan blue dye exclusion assay. Two positive controls were used, apomine as reference compound for cytostatic activity and staurosporin for cytotoxicity. Contrary to the staurosporin treatment which induced immediate cell death against POS-1, 81% of cells were found to be living cells after a contact with 0.7 μM of **1**, a concentration which induced 54% inhibition of POS-1 proliferation. This result was similar to the proportion of living cells in the assay performed with the solvent negative control (82% living cells) and slightly higher than with apomine (67%). On L929, equivalent percentages were obtained for **1**, apomine and negative control.

The effect of ligerin on the cell division duration and on the morphology of L929 and POS-1 cells was evaluated thanks to a time lapse analysis using quantitative videomicroscopy performed with a reversed microscope and a camera taking a picture of each well of a 24-well plate every 10 min. Each experiment, i.e. negative control, staurosporine, apomine and **1** was performed in triplicate. Cells were then counted and growth curves were constructed as shown in Fig. 3. With a treatment consisting of 0.7 μM of ligerin, POS-1 proliferation was slowed down and the doubling time of cell population was twice longer, 30 h instead of 15 h for negative control. This effect was weaker against L929, as the cell cycle duration increased from 17 h for negative control to 22 h for the ligerin-treated cells. Videomicroscopy also allowed the observation of the effects induced by the tested compounds on cell shape and movements. In normal conditions, POS-1 cells are continuously exhibiting unilateral movements, and they require an initial contact between them to engage their division. In the presence of **1**, cell movements were dramatically perturbed and cell divisions were observed even when cells were isolated, without any previous contact with other cells. However, no morphological modifications were noticed on osteosarcoma cells, contrary to what occurred on L929 which exhibited a rounded form in place of their characteristic fibroblastic shape (Fig. 4). Then, at the tested concentration, **1** exhibited a selective activity against osteosarcoma. As it was observed for apomine, this activity was characterized as cytostatic.

3. Conclusion

In conclusion, new analogs of the fumagillin-related natural compound ligerin **1** were semisynthesized using a simplified three-step process and were investigated for their antiproliferative activity against osteosarcoma and normal cell lines. Results showed that both halogenohydrin and spiroepoxy compounds exhibited an antiproliferative activity against murine or human cancer cell lines higher than against normal fibroblastic cells. Chlorohydrins **1** and **4** were found to be at least as active as their epoxy derivatives against SaOS2 and POS1 osteosarcoma cell lines, whereas the bromohydrin **5** showed a reduced activity, emphasizing the role of the C7 substituents. Compared to synthetized compounds and the reference drug candidate **3**, **1** exhibited a higher activity against human SaOS2 and MG63 cancer cells together with a lower cytotoxicity against normal cells. Furthermore, **1** was evaluated as more than 70 times more active than irinotecan and fludarabine and as potent as vincristine and paclitaxel against POS-1 cells. Pharmacological investigations highlighted that **1** exerted its antiproliferative activity through a cytostatic mechanism. Further studies will be performed in order to better understand the mechanism of action of **1** at the molecular level.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

Reagents were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Solvents from Carlo Erba SDS (Val de Reuil, France) were distilled before use. Fumidil B® was obtained from Ceva Santé Animale (Libourne, France). Progress of reactions was monitored by thin layer chromatography on Silica Gel plates Alugram® Xtra SIL G/UV_{254} (Macherey–Nagel, Hoerdt, France). 1D and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker 500 MHz spectrometer fitted with a TCI cryoprobe. Chemical shift are expressed in δ (ppm) with TMS as internal standard and coupling constants in Hertz. HRMS analyses were performed with an IT-TOF mass spectrometer composed of an ESI ion source and a hybrid Ion Trap-Time-Of-Flight mass analyzer (Shimadzu, Kyoto, Japan).

4.1.2. Semisynthesis

Each semisynthetic approach started from **2**. The first step consisted of an alkaline hydrolysis of **2** contained in the commercial product Fumidil B®. For that purpose, 50 g of Fumidil B® were dissolved in 1 L of NaOH (0.5 N) and the reaction was stirred at room temperature during 18 h. 5% citric acid was added before extraction with diethyl ether. Then, organic phase was dried with $NaHCO_3$/anhydrous Na_2SO_4 and concentrated under vacuum, to give **4a** (yellow oil, 609 mg). The second step consisted of the

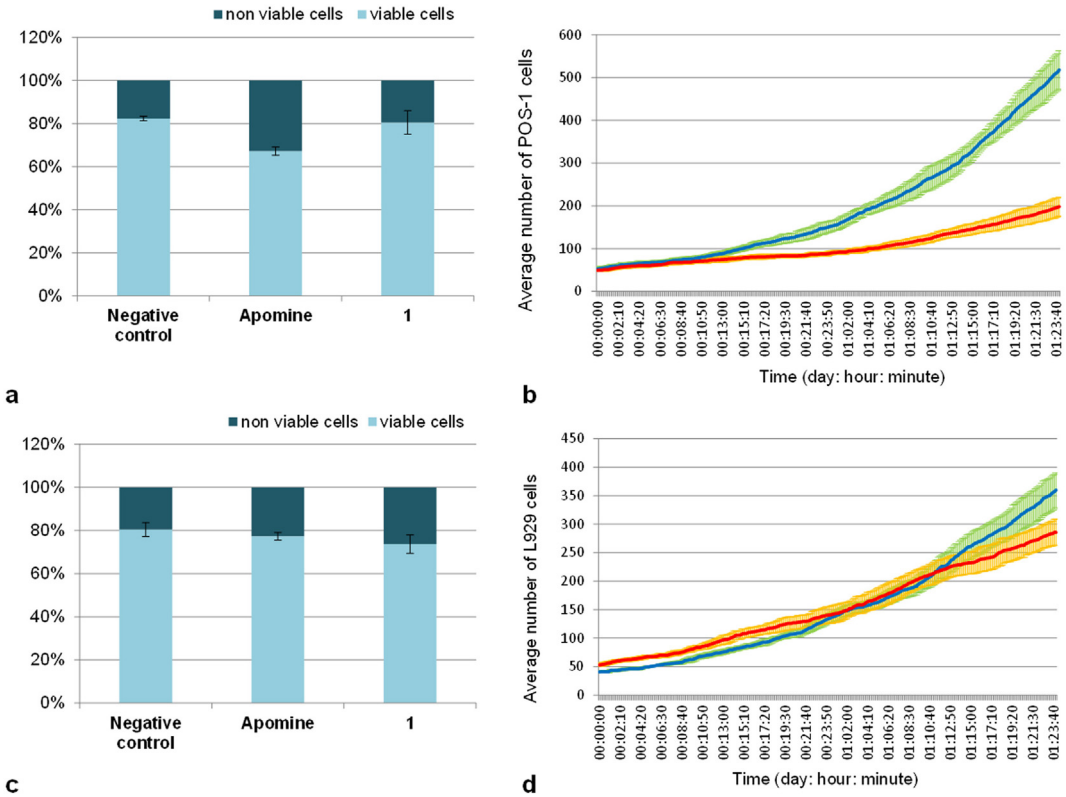


Fig. 3. Effect of 0.7 μM **1** on cell viability and cell proliferation of POS-1 (a, b) and L929 (c, d) cells. a, c Trypan blue exclusion assay after 72 h incubation (values ±SD of four independent experiments). b, d Growth curves of cells observed every 10 min during 2 days by quantitative videomicroscopy. Red line average number of cells exposed to **1**, (orange area: ±SD of triplicates); blue line average number of cells exposed to negative control, (green area: ±SD of triplicates). Apomine concentration 10 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

esterification of **4a**. This compound (600 mg, 2.12 mmol, 1 eq) was dissolved with DMAP (dimethylaminopyridine, 330 mg, 2.75 mmol, 1.30 eq), succinic anhydride (827 mg, 8.28 mmol, 3.9 eq) in 4 mL of anhydrous pyridine. The reaction was stirred at room temperature during 24 h. The reaction mix was extracted with AcOEt and washed with water. The organic phase was then extracted with a saturated solution of $NaHCO_3$ before the acidification until pH4 with NaH_2PO_4. The mixture was finally extracted with AcOEt and dried using Na_2SO_4anh before concentration under vacuum to obtain compound **1a** (yellow oil, 620 mg). This compound (600 mg,

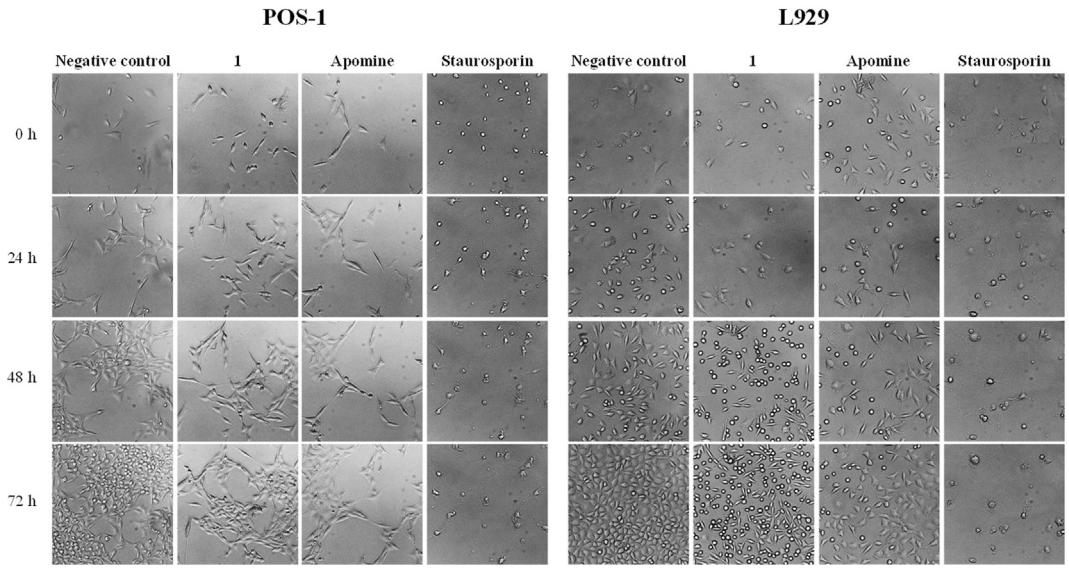


Fig. 4. Morphological changes of POS-1 (left) and L929 (right) cells after 0, 24, 48, 72 h exposure to negative control, 0.7 μM **1**, 10 μM apomine, 1 μM staurosporin (G×10 magnification).

1.56 mmol, 1 eq) was dissolved with LiCl (285 mg, 6.80 mmol, 4.35 eq) in 5 mL of THF at 0 °C and acetic acid (450 μL, 486 mg, 8.1 mmol, 5.2 eq) was then added. Mixture was warmed to room temperature and the reaction was stirred during 24 h. Ligerin **(1)** (colorless oil, 401 mg) was purified by liquid chromatography on a silica gel column (elution by cyclohexane and CH_2Cl_2/MeOH 10:1 (v/v)). Compounds **5**–**8** were synthesized with the same semisynthetic approach; only the anhydride used for the esterification step and the halogenous salt for the last step changed (succinic anhydride and LiBr for **5**, glutaric anhydride and LiCl for **6**, diglycolic anhydride and LiCl for **7**, phthalic anhydride and LiCl for **8**). For compound **4**, the halogenation step with LiCl was performed with **4a**. Compound **3** was synthesized according to the method described by Marui et al. [26].

*4.1.2.1. 6-(3-Carboxy-1-oxopropyl)-fumagillol (**1a**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 405.1896 (calcd for $C_{20}H_{30}O_7Na$, 405.1884, Δ 3.0 ppm); ^1H NMR ($CDCl_3$) δ: 1.10 & 2.05 (m; 2H); 1.2 (s; 3H); 1.68 (s; 3H); 1.76 (s; 3H); 1.82–1.86 (m; 2H); 1.98 (m; 1H); 2.17 & 2.39 (m; 2H); 2.56 & 2.96 (d; $J = 10$ Hz; 2H); 2.63 (t; $J = 5.5$ Hz; 1H); 2.71 (s; 4H); 3.40 (s; 3H); 3.63 (m; 1H); 5.19 (t; $J = 7.0$ Hz; 1H); 5.68 (s; 1H); ^{13}C NMR ($CDCl_3$) δ: 18.3 (CH_3); 26.0 (CH_2); 26.1 (CH_3); 27.7 (CH_2); 29.7 (CH_2); 29.7 (CH_2); 29.7 (CH_2); 48.1 (CH); 51.2 (CH_2); 57.1 (CH_3); 59.6(C); 59.7 (C); 61.5 (CH); 67.2 (CH); 79.2 (CH); 118.7 (CH); 135.4 (C); 14.0 (CH_3); 171.9 (C); 175.7 (C).

*4.1.2.2. Ligerin (**1**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 441.1656 (calcd for $C_{20}H_{31}ClO_7Na$, 441.1651, Δ 1.3 ppm); ^1H NMR ($CDCl_3$) δ: 1.40 & 1.96 (m; 2H); 1.51 (s; 3H); 1.68 (s; 3H); 1.76 (s; 3H); 1.82–1.86 (m; 2H); 2.19 & 2.47 (m; 2H); 2.39 (m; 1H); 2.73 (s; 4H); 2.98 (t; $J = 5.5$ Hz; 1H); 3.28 (s; 3H); 3.3 (m; 1H); 3.52 & 3.85 (d; $J = 10$ Hz; 2H); 5.21 (t; $J = 7.0$ Hz; 1H); 5.52 (s; 1H); ^{13}C NMR ($CDCl_3$) δ: 17.9 (CH_3); 22.2 (CH_3); 23.4 (CH_2); 25.8 (CH_3); 27.4 (CH_2); 29.3 ($2CH_2$); 43.2 (CH); 50.3 (CH_2); 56.6 (CH_3); 62.3 (CH); 64.0 (C); 66.4 (CH); 76.7 (C); 78.4 (CH); 118.2 (CH); 134.8 (C); 171.5 (C); 176.9 (C).

*4.1.2.3. 7-Chloro-fumagillol (**4**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 341.1502 (calcd for $C_{16}H_{27}ClO_4Na$, 341.1490, Δ 3.74 ppm); ^1H NMR ($CDCl_3$) δ: 1.36 & 2.10 (m; 2H); 1.48 (s; 3H); 1.67 (s; 3H); 1.74 (s; 3H); 1.82–1.86 (m; 2H); 2.18 & 2.45 (m; 2H); 2.40 (m; 1H); 2.98 (t; $J = 5$ Hz; 1H); 3.33 (m; 1H); 3.35 (s; 3H); 3.51 & 3.81 (d; $J = 10.8$ Hz; 2H); 4.22 (s; 1H); 5.20 (t; $J = 6.8$ Hz; 1H); ^{13}C NMR ($CDCl_3$) δ: 18.2 (CH_3); 22.5 (CH_3); 23.6 (CH_2); 26.1 (CH_3); 27.8 (CH_2); 30.0 (CH_2); 42.6 (CH); 51.0 (CH_2); 56.6 (CH_3); 62.5 (CH); 63.6 (C); 64.2 (CH); 76.5 (C); 80.8 (CH); 118.6 (CH); 135.1 (C).

*4.1.2.4. 7-Bromo-6-(3-carboxy-1-oxopropyl)-fumagillol (**5**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 485.1133 (calcd for $C_{20}H_{31}BrO_7Na$, 485.1145, Δ 2.47 ppm); ^1H NMR ($CDCl_3$) δ: 1.48 & 1.99 (m; 2H); 1.513 (s; 3H); 1.68 (s; 3H); 1.76 (s; 3H); 1.81 (m; 2H); 2.19 & 2.48 (m; 2H); 2.48 (m; 1H); 2.74 (s; 4H); 2.99 (t; $J = 5.5$ Hz; 1H); 3.29 (m; 1H); 3.29 (s; 3H); 3.50 & 3.75 (d; $J = 10$ Hz; 2H); 5.20 (t; $J = 7.0$ Hz; 1H); 5.51 (s; 1H); ^{13}C NMR ($CDCl_3$) δ: 17.6 (CH_3); 22.0 (CH_3); 23.3 (CH_2); 25.5 (CH_3); 27.0 (CH_2); 28.8 (CH_2); 28.8 (CH_2); 29.9 (CH_2); 39.9 (CH_2); 43.7 (CH); 56.7 (CH_3); 62.3 (CH); 64.0 (C); 66.1 (CH); 78.7 (C); 78.7 (CH); 118.2 (CH); 135.4 (C); 171.4 (C); 177.5 (C).

*4.1.2.5. 7-Chloro-6-(4-carboxy-1-oxobutoxy)-fumagillol (**6**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 455.1811 (calcd for $C_{21}H_{33}ClO_7Na$, 455.1807, Δ 0.88 ppm); ^1H NMR ($CDCl_3$) δ: 1.40 & 2.03 (m; 2H); 1.51 (s; 3H); 1.68 (s; 3H); 1.80 (s; 3H); 1.83 (m; 2H); 2.03 & 2.20 (m; 2H); 2.20 (m; 1H); 2.42–2.56 (m; 6H); 2.98 (t; $J = 5.5$ Hz; 1H); 3.31 (s; 3H); 3.32 (m; 1H); 3.51 & 3.7 (d; $J = 10$ Hz; 2H); 5.20 (t; $J = 7.3$ Hz; 1H); 5.52 (s; 1H); ^{13}C NMR ($CDCl_3$) δ: 17.9 (CH_3); 20.4 (CH_2); 22.7 (CH_3); 23.5 (CH_2); 25.9 (CH_3); 27.4 (CH_2); 29.0 (CH_2); 33.6 ($2CH_2$); 43.1 (CH); 50.0 (CH_2); 56.6 (CH_3); 62.3 (CH); 64.0 (C); 65.8 (CH); 76.7 (C); 78.6 (CH); 118.1 (CH); 134.8 (C); 172.6 (C); 176.1 (C).

*4.1.2.6. 7-Chloro-6-[[(2-carboxymethoxy)acetyl]oxy]-fumagillol (**7**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 457.1596 (calcd for $C_{20}H_{31}ClO_8Na$, 457.1600, Δ 0.85 ppm); ^1H NMR ($CDCl_3$) δ: 1.35 & 1.98 (m; 2H); 1.48 (s; 3H); 1.68 (s; 3H); 1.75 (s; 3H); 1.82–1.86 (m; 2H); 2.18 & 2.46 (m; 2H); 2.39 (m; 1H); 2.96 (t; $J = 5.5$ Hz; 1H); 3.29 (s; 3H); 3.34 & 3.85 (d; $J = 10$ Hz; 2H); 3.48 (s; 1H); 4.12–4.45 (m; 4H); 5.18 (t; $J = 7.5$ Hz; 1H); 5.58 (s; 1H); ^{13}C NMR ($CDCl_3$) δ: 17.8 (CH_3); 22.7 (CH_3); 23.4 (CH_2); 25.9 (CH_3); 27.4 (CH_2); 29.0 (CH_2); 43.1 (CH); 50.3 (CH_2); 56.9 (CH_3); 62.2 (CH); 63.3 (C); 66.8 (CH); 67.5 (CH_2); 68.3 (CH_2); 76.0 (C); 78.4 (CH); 118.0 (CH); 134.8 (C); 169.4 (C); 176.2 (C).

*4.1.2.7. 7-Chloro-6-[(2-carboxybenzoyl)oxy]-fumagillol (**8**).* Colorless oil; HRESIMS *m/z* $[M+Na]^+$ 489.1655 (calcd for $C_{24}H_{31}ClO_7Na$, 489.1651, Δ 0.82 ppm); ^1H NMR ($CDCl_3$) δ: 1.48 & 1.93 (m; 2H); 1.51 (s; 3H); 1.68 (s; 3H); 1.76 (s; 3H); 1.82–1.93 (m; 2H); 2.19 & 2.47 (m; 2H); 2.23 (m; 1H); 2.98 (t; $J = 5.5$ Hz; 1H); 3.39 (m; 1H); 3.39 (s; 3H); 3.57 & 3.90 (d; $J = 10$ Hz; 2H); 5.21 (t; $J = 7.0$ Hz; 1H); 5.77 (s; 1H); 7.58 (d; $J = 8$ Hz; 4H); 7.84 (d; $J = 8$ Hz; 4H); ^{13}C NMR ($CDCl_3$) δ: 17.8 (CH_3); 22.7 (CH_3); 23.2 (CH_2); 25.8 (CH_3); 27.3 (CH_2); 29.1 (CH_2); 43.4 (CH); 50.4 (CH_2); 56.8 (CH_3); 62.4 (CH); 64.1 (C); 67.6 (CH); 76.0 (C); 78.7 (CH); 118.4 (CH); 128.8 (CH); 129.0 (CH); 129.6 (C); 130.9 (CH); 131.2 (CH); 134.8 (C); 134.9 (C); 167.4 (C); 171.8 (C).

4.2. Biological activities

4.2.1. Cell lines and cell culture

Three cancer cell lines POS-1 (murine osteosarcoma), MG63 and SaOS2 (human osteosarcomas ATCC number CRL-1427 and HTB-85), and two non-tumor cell lines, L929 (murine fibroblasts, ATCC number CCL-1) and HFF2 (human fibroblasts) were cultured. L929 and POS-1 cell lines were grown in RPMI 1640 medium (Gibco®, Grand Island, New York) supplemented with 5% (v/v) of fetal bovine serum. MG63, SaOS2 and HFF2 cell lines were grown in DMEM medium (Gibco® Grand Island, New York) supplemented with fetal bovine serum 10% (v/v). All the cell lines were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

4.2.2. XTT assay

In order to evaluate the antiproliferative activity of the semi-synthetic compounds, cells were sown in 96-well plates (100 μL/well at a density of $1 \cdot 10^4$ cells/mL for POS-1, HFF2 and L929, $1.5 \cdot 10^4$ for SaOS2 and MG63). After 24 h incubation at 37 °C with 5% CO_2 to allow cell attachment, and renewal of culture medium, cells were exposed to various concentrations of tested compounds for 72 h. Cytotoxic activity was determined by colorimetric method using tetrazolium salts. An XTT solution (sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate, Roche Applied Science) was added in each well (50 μL/well) and plates were incubated for 4 h at 37 °C, 5% CO_2. Absorption at 450 nm was measured with a plate reader (Wallace Victor 2™ 1420 MultiLabel Counter Perkin Elmer®). IC_{50} were determined as the concentration that inhibited cell viability by 50%.

4.2.3. Quantitative videomicroscopy

The cell growth was evaluated thanks to a time lapse experiment using quantitative videomicroscopy. Cells were grown in 24-well plates. A negative control and two positive controls (apomine and staurosporin treatment) were used. Assays were performed in

triplicate with 0.7 μM ligerin, 10 μM apomine and 1 μM staurosporin. The plates were incubated at 37 °C in a 5% CO_2 atmosphere. Pictures of each well were taken in a unique optical field every 10 min for 72 h with a reversed microscope (Shutter Leica) at a G×10 magnification. Data acquisition was performed using the software MetaMorph™, multi-dimensional acquis 31. The 433 pictures obtained were analyzed with the software ImageJ (NIH, USA) and assembled to create movies allowing observation of the cell phenotypes throughout the treatment. Cells were counted on each picture for each well and cell viability was assessed by a trypan blue dye exclusion assay.

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Appendix A. Supplementary data

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References

[1] G. Ottaviani, N. Jaffe, Cancer Treatment and Research 152 (2009) 15–32.
[2] M.L. Broadhead, J.C.M. Clark, D.E. Myers, C.R. Dass, P.F.M. Choong, Sarcoma (2011).
[3] F.R. Hanson, T.E. Elbe, Journal of Bacteriology 58 (1949) 527–529.
[4] T.E. Elbe, F.R. Hanson, Antibiotica et Chemotherapia 1 (1951) 54–58.
[5] L. Bailey, Nature 171 (1953) 212–213.
[6] J. Folkman, D. Ingber, T. Fujita, T. Kanamaru, Patent EP325199A2 Takeda Chemical Industries, Ltd., Japan, 1989.
[7] V. Castronovo, D. Belotti, European Journal of Cancer 32A (1996) 2520–2527.
[8] P. Bhargava, J.L. Marshall, N. Rizvi, W. Dahut, J. Yoe, M. Figuera, K. Phipps, V.S. Ong, A. Kato, M.J. Hawkins, Clinical Cancer Research 5 (1999) 1989–1995.
[9] R.S. Herbst, T.L. Madden, H.T. Tran, G.R. Blumenschein Jr., C.A. Meyers, L.F. Seabrooke, F.R. Khuri, V.K. Puduvalli, V. Allgood, H.A. Fritsche Jr., L. Hinton, R.A. Newman, E.A. Crane, F.V. Fossella, M. Dordal, T. Goodin, W.K. Hong, Journal of Clinical Oncology 20 (2002) 4440–4447.
[10] B. Datta, Biochimica et Biophysica Acta 1796 (2009) 281–292.
[11] S. Liu, J. Widom, C.W. Kemp, C.M. Crews, J. Clardy, Science 282 (1998) 1324–1327.
[12] V. Rodeschini, P. Van de Weghe, C. Tarnus, J. Eustache, Tetrahedron Letters 46 (2005) 6691–6695.
[13] C. Drahl, B.F. Cravatt, E.J. Sorensen, Angewandte Chemie International Edition 44 (2005) 5788–5809.
[14] V. Rodeschini, J.-G. Boiteau, P. Van de Weghe, C. Tarnus, J. Eustache, Journal of Organic Chemistry 69 (2004) 357–373.
[15] E.C. Griffith, Z. Su, S. Niwayama, C.A. Ramsay, Y.-H. Chang, J.O. Liu, Proceedings of the National Academy of Sciences of the United States of America 95 (1998) 15183–15188.
[16] E.C. Griffith, Z. Su, B.E. Turk, S. Chen, Y.-H. Chang, Z. Wu, K. Biemann, J.O. Liu, Chemistry & Biology 4 (1997) 461–471.
[17] S.J. Shin, H.C. Jeung, J.B. Ahn, S.Y. Rha, J.K. Roh, K.S. Park, D.H. Kim, C. Kim, H.C. Chung, Investigational New Drugs 28 (2010) 650–658.
[18] S.J. Shin, J.B. Ahn, K.S. Park, Y.J. Lee, Y.S. Hong, T.W. Kim, H.R. Kim, S.Y. Rha, J.K. Roh, D.-H. Kim, C. Kim, H.C. Chung, Investigational New Drugs 30 (2012) 672–680.
[19] C.C. Arico-Muendel, B. Belanger, D. Benjamin, H.S. Blanchette, T.M. Caiazzo, P.A. Centrella, J. DeLorey, E.G. Doyle, U. Gradhand, S.T. Griffin, S. Hill, M.T. Labenski, B.A. Morgan, G. O'Donovan, K. Prasad, S. Skinner, N. Taghizadeh, C.D. Thompson, J. Wakefield, W. Westlin, K.F. White, Drug Metabolism and Disposition 41 (2013) 814–826.
[20] M. Vansteelandt, E. Blanchet, M. Egorov, F. Petit, L. Toupet, A. Bondon, F. Monteau, B. Le Bizec, Y.F. Pouchus, R. Le Bot, O. Grovel, Journal of Nature Products 76 (2013) 297–301.
[21] S. Kishimoto, S. Marui, T. Fujita, Patent EP415294A2 Takeda Chemical Industries, Ltd., Japan, 1991.
[22] C.K. Han, S.K. Ahn, N.S. Choi, R.K. Hong, S.K. Moon, H.S. Chun, S.J. Lee, J.W. Kim, C.I. Hong, D. Kim, J.H. Yoon, K.T. No, Bioorganic & Medicinal Chemistry Letters 10 (2000) 39–43.
[23] B.E. Turk, Z. Su, J.O. Liu, Bioorganic & Medicinal Chemistry 6 (1998) 1163–1169.
[24] M. Fardis, H.-J. Pyun, J. Tario, H. Jin, C.U. Kim, J. Ruckman, Y. Lin, L. Green, B. Hicke, Bioorganic & Medicinal Chemistry 11 (2003) 5051–5058.
[25] M. Hayashi, Y.-P. Kim, S. Takamatsu, S. Preeprame, T. Komiya, R. Masuma, H. Tanaka, K. Komiyama, S. Omura, Journal of Antibiotics 49 (1996) 631–634.
[26] S. Marui, F. Itoh, Y. Kozai, K. Sudo, S. Kishimoto, Chemical & Pharmaceutical Bulletin 40 (1992) 96–101.