Design and Synthesis of Fluoroacylshikonin as an Anticancer Agent

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ABSTRACT A series of shikonin derivatives, selectively acylated by various fluorinated carboxylic acids at the side chain of shikonin, were synthesized and their anticancer activity evaluated, in which eight compounds are reported for the first time. Among all the compounds tested, compound **S7** showed the most potent anticancer activity against B16-F10 (malignant melanoma cells), MG63 (human osteosarcoma cells), and A549 (lung cancer cells) with IC_{50} 0.39 ± 0.01, 0.72 ± 0.04 and 0.58 ± 0.02 μmol/L. Docking simulation of compound **S7** was carried out to position **S7** into a tubulin active site to determine the probable binding conformation. All the results suggested that compound **S7** may be a potential anticancer agent. *Chirality 25:757–762, 2013.*

KEY WORDS: fluoroacylshikonin; tubulin inhibitor; anticancer activity

INTRODUCTION

Shikonin (Fig. 3, **S12**) is an active naphthoquinone compound, which is mainly isolated from the roots of the traditional oriental medicinal herb *Lithospermium erythrorhizon*. It is used as a natural medicine treating for burns, inflammations, wounds, and ulcers in the Far East and Europe.[1] Over the past few decades, there has been much development into the research of shikonin and its derivatives, which are used as potential drugs for cancers.[2–4]

Several researchers synthesized and evaluated numerous new shikonin derivatives, aiming at finding novel anticancer drugs that are effective and have fewer side effects. For many shikonin derivatives, most modifications were focused on the hydroxyl group of the side-chain. Xuan and Hu[5] reported on α-methyl -n-butylshikonin (Fig. 1, **a1**) and β, β-dimethylacrylshikonin (Fig. 1, **a2**); their acyl groups contained respectively a methyl and double bond, having a strong anticancer activity against HL60 and K562 cell lines. Rao et al.[6] reported that β-HIVS (Fig. 1, **β-HIVS**) displayed the most potent anticancer activity and selective cytotoxicity towards DU-145. Moreover, researchers also found that isovalerylshikonin (Fig. 1, **a3**) and SH-7 (Fig. 1, **SH-7**) displayed prominent inhibitory actions on leukemia HL-60 cells and A549 lines to induce apoptosis, and both compounds were stronger than their mother compound shikonin.[7–9] It is well known that shikonin and its derivatives contain a naphthazarin skeleton. According to previous studies, the mechanism for the anticancer action of naphthazarin derivatives was the inhibition of DNA topoisomerase-I that could be one of the mechanisms for its anticancer action.[10] However, recently Acharya et al.[11] demonstrated that naphthazarin is a microtubule inhibitor in a cell-free system and in A549 cells. Naphthazarin induced cell death by activating apoptosis and autophagy pathways. Naphthazarin treatment could depolymerize interphase microtubules and disorganized spindle microtubules. These latest research results have helped us in evaluating anticancer activity.

In last 5 decades, there has been much development in the use of fluorine in medicinal chemistry, when it was introduced into some complex structures to improve pharmacological activity. In 1957, Heidelberger and co-workers[12,13] synthesized 5-fluorouracil (5-FU) (Fig. 1, **5-FU**) and evaluated its anticancer activity, which was found to have significant tumor-inhibiting activity. It is a landmark contribution to medicinal chemistry. Since then, a new era of fluorine introduced into anticancer drugs was heralded. Kar et al.[14] found that F-Cpd5 (Fig. 1, **F-Cpd5**) was a more potent inhibitor for the hepatocellular carcinoma line Hep3B than its parent Cpd5 (Fig. 1, **Cpd5**). F-Cpd5 also displayed fewer side effects for normal cells than Cpd5 and has a considerable selective effect on tumor cells compared with normal cells and incurs less reactive oxygen species (ROS). Fulvestrant (Faslodex) (Fig. 2) is a typical fluorine-containing drug, which was approved by the U.S. Food and Drug Administration (FDA) for the treatment of hormone receptor-positive metastatic breast cancer.

The above result reveals that fluorine displays a special effect in the anticancer fluoro-drug molecule. Several reasons can be used to explain how fluorine improves the effects of drugs.[15] First, the fluorine introduced into drugs usually leads to increased lipid solubility. So the rates of absorption and transport of drugs in vivo are enhanced. Second, the presence of the high electronegativity of fluorine often leads to altering the drugs' physicochemical property and bioactivity. Third, in special cases, fluorine replacing hydrogen in the specific location of drugs can block some essential biochemical reactions, such as 5-FU. Combining these findings mentioned, we synthesized a series of shikonin derivatives (**S1–S8**) which were selectively acylated by various fluorinated carboxylic acids at the side chain of shikonin.

MATERIALS AND METHODS

All chemicals (reagent grade) used were purchased from Alfa Aesar (UK) and Nanjing Chemical Reagent Co. (Nanjing, China). Separation of the compounds by column chromatography was carried out with Silica Gel 60 (200–300 mesh ASTM; E. Merck, Darmstadt, Germany). All the

Additional Supporting Information may be found in the online version of this article.

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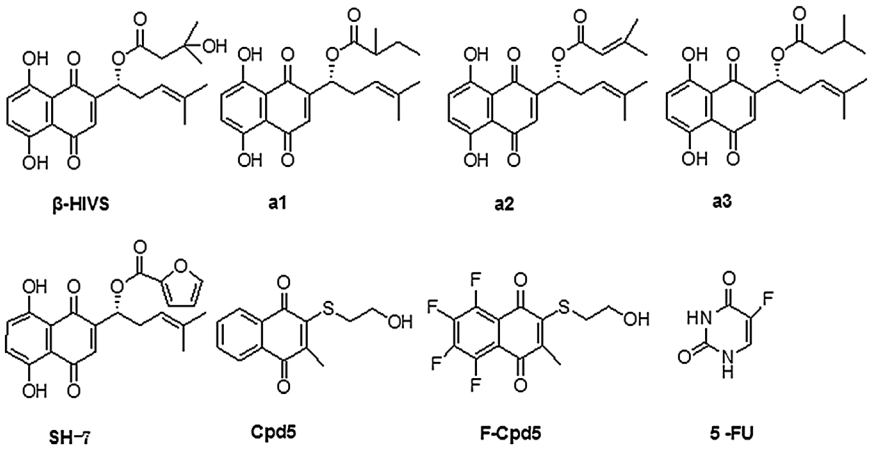


Fig. 1. Structures of acylshikonins, Cpd5, F-Cpd5, and 5-FU.

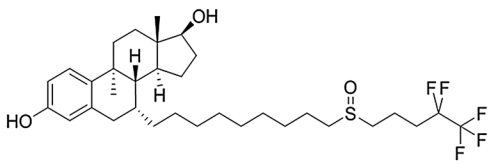


Fig. 2. Structure of fulvestrant.

^{1}H NMR spectra were recorded on a Bruker DRX 500 spectrometer in $CDCl_3$. All the ^{19}F NMR spectra were recorded on a Bruker Avance III 500 spectrometer in $CDCl_3$. Chemical shifts (δ) for ^{1}H NMR and ^{19}F NMR spectra are reported in ppm (δ). Melting points (uncorrected) were measured on an XT4 MP Apparatus (Taike, Beijing, China). The electrospray ionization, mass spectrometry {ESI-MS} spectra were recorded on a Mariner System 5304 mass spectrometer. Thin-layer chromatography (TLC) was carried out on the glass-backed silica gel sheets (silica gel 60 Å GF254) and visualized in UV light (254 nm). Elemental analyses were performed on a CHN–O–Rapid instrument and were within 0.4% of the theoretical values.

General Procedure for Preparation of Compounds

Shikonin 0.288 g (1 mmol) was dissolved in 10 mL of anhydrous dichloromethane, and 0.416 g (2.02 mmol) of dicyclohexylcarbodiimide (DCC) was added to the reaction system. The reaction mixture was stirred under nitrogen atmosphere in an ice bath for 20 min. 0.049 g (0.4 mmol) of 4-(dimethylamino) pyridine (DMAP) was added and stirring in the ice bath for a further 10 min. Then 1 mmol of carboxylic acid was added to the reaction mixture and stirred in the ice bath for 40 min. While stirring, the product was checked with TLC continually. After 40 min stirring, the reaction was stopped. After removal of solvent by evaporation, the product of the reaction was dissolved by normal hexane. Then the impurity was filtered and the solvent was collected. After the removal of solvent again, the residue was purified by column chromatography on silica gel with EtOAc – petroleum ether (1:8) to afford the target compounds. After the target compound was purified by column chromatograph, the compounds were dissolved by normal hexane and filtered three times, aiming at better removal of dicyclohexylurea (DCU) and obtaining higher purity compounds.

2-[1-(2, 2-difluoroacetyl)-4-methyl-3-pentenyl] naphthazarin (S1). Red powder, 73% yield. Mp: 65.4-66.6°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.58 (s, 1H); 12.39 (s, 1H); 7.19 (s, 2H); 7.08 (s, 1H); 6.23 (dd, J_1 = 4.5 Hz, J_2 = 6.5 Hz, 1H); 5.99 (t, J = 53.5 Hz, 1H); 5.13 (t, J = 7.5 Hz, 1H); 2.75-2.70 (m, 1H); 2.62-2.57 (m, 1H); 1.72 (s, 3H); 1.61 (s, 3H). ^{19}F NMR (500MHz, $CDCl_3$) δ: -126.45 (s, 2F). ESI-MS: calcd. for $C_{18}H_{16}F_2O_6$ ([M-H]$^-$), 365.09 found 365.42. Anal. Calcd. for $C_{18}H_{16}F_2O_6$: C, 59.02; H, 4.40; O, 26.21. Found: C, 58.94; H, 4.65; O, 26.14.

2-[1-(2-fluoropropionyl)-4-methyl-3-pentenyl] naphthazarin (S2). Red powder, 53% yield. Mp: 109.3-110.8°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.59 (s, 1H); 12.41 (s, 1H); 7.19 (s, 2H); 7.04 (s, 1H); 6.16 (dd, J_1 = 4.5 Hz, J_2 = 7.5Hz, 1H); 5.16-5.01 (m, 2H); 2.71-2.66 (m, 1H); 2.58-2.51 (m, 1H), 1.71 (s, 3H); 1.61 (dt, J_1 = 6 Hz, J_2 = 24 Hz, 6H). ^{19}F NMR (500 MHz, $CDCl_3$) δ: -184.41 (s, 1F). ESI-MS: calcd. for $C_{19}H_{19}FO_6$ ([M-H]$^-$), 361.12 found 361.33. Anal. Calcd. for $C_{19}H_{19}FO_6$: C, 62.98; H, 5.29; O, 26.41. Found: C, 62.78; H, 5.35; O, 26.29.

2-[1-(2, 2-difluoropropionyl)-4-methyl-3-pentenyl] naphthazarin (S3). Red powder, 66% yield. Mp: 67.2-69.0°C. ^{1}H NMR (500MHz, $CDCl_3$) δ: 12.58 (s, 1H); 12.40 (s, 1H); 7.19 (s, 2H); 7.07 (s, 1H); 6.18 (dd, J_1 = 4.5 Hz, J_2 = 7.5 Hz 1H); 5.13 (t, J = 7.5 Hz, 1H); 2.74-2.69 (m, 1H); 2.61-2.55 (m, 1H); 1.85 (t, J = 18.5 Hz, 3H); 1.71 (s, 3H); 1.61 (s, 3H). ^{19}F NMR (500MHz, $CDCl_3$) δ: -98.91 (s, 2F). ESI-MS: calcd. for $C_{19}H_{18}F_2O_6$ ([M-H]$^-$), 379.11 found 379.50. Anal. Calcd. for $C_{19}H_{18}F_2O_6$: C, 60.00; H, 4.77; O, 25.24. Found: C, 59.91; H, 4.93; O, 25.19.

2-[1-(3, 3, 3-trifluoropropionyl)-4-methyl-3-pentenyl] naphthazarin (S4). Red powder, 57% yield. Mp: 96.6-99.1°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.58 (s, 1H); 12.40 (s, 1H); 7.19 (s, 2H); 7.02 (s, 1H); 6.16 (dd, J_1 = 5 Hz, J_2 = 7 Hz 1H); 5.12 (t, J = 7 Hz, 1H); 3.27 (q, J = 10 Hz, 2H), 2.70-2.65 (m, 1H); 2.56-2.50 (m, 1H); 1.71 (s, 3H); 1.60 (s, 3H). ^{19}F NMR (500 MHz, $CDCl_3$) δ: -63.15 (s, 3F). ESI-MS: calcd. for $C_{19}H_{17}F_3O_6$ ([M-H]$^-$) 397.10, found 397.42. Anal. Calcd. for $C_{19}H_{17}F_3O_6$: C, 57.29; H, 4.30; O, 24.10. Found: C, 57.20; H, 4.49; O, 24.01.

2-[1-(2, 2, 3, 3-tetrafluoropropionyl)-4-methyl-3pentenyl] naphthazarin (S5). Red powder, 39% yield. Mp: 76.4-77.5°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.59 (s, 1H); 12.40 (s, 1H); 7.20 (s, 2H); 7.10 (s, 1H); 6.29 (dd, J_1 = 3.5 Hz, J_2 = 5.5 Hz, 1H); 5.99 (t, J = 53 Hz, 1H); 5.13 (t, J = 5 Hz, 1H); 2.78-2.72 (m, 1H); 2.65-2.59 (m, 1H); 1.73 (s, 3H); 1.63 (s, 3H). ^{19}F NMR (500MHz, $CDCl_3$) δ: -120.35 (s, 2F); -139.23 (s, 2F) ESI-MS: calcd. for $C_{19}H_{16}F_4O_6$ ([M+H]$^+$), 417.09, found 417.48. Anal. Calcd. for $C_{19}H_{16}F_4O_6$: C, 54.81; H, 3.87; O, 23.06. Found: C, 54.68; H, 3.98; O, 22.96.

2-[1-(2, 2, 3, 3, 4, 4, 4-heptafluorobutyryl)-4-methyl-3-pentenyl] naphthazarin (S6). Red powder, 42% yield. Mp: 67.1-68.4°C. ^{1}H NMR (500MHz, $CDCl_3$) δ: 12.60 (s, 1H); 12.40 (s, 1H); 7.20 (s, 2H); 7.10 (s, 1H); 6.32 (dd, J_1 = 4.5Hz, J_2 = 7.5 Hz, 1H); 5.12 (t, J = 7 Hz, 1H); 2.77-2.72 (m, 1H); 2.66-2.60 (m, 1H); 1.72 (s, 3H); 1.62 (s, 3H). ^{19}F NMR (500 MHz, $CDCl_3$) δ: -79.95 (s, 3F); -113.09 (s, 2F); -124.80 (s, 2F). ESI-MS: calcd. for $C_{20}H_{15}F_7O_6$ ([M-H]$^-$) 483.08, found 483.25. Anal. Calcd. for $C_{20}H_{15}F_7O_6$: C, 49.60; H, 3.12; O, 19.82. Found: C, 49.51; H, 3.23; O, 19.78.

2-[1-(2-methyl-4, 4, 4-trifluorobutyryl)-4-methyl-3-pentenyl] naphthazarin (S7). Red powder, 68% yield. Mp: 35.1-36.4°C ^{1}H NMR (500MHz, $CDCl_3$) δ: 12.58 (s, 1H); 12.40 (s, 1H); 7.18 (s, 2H); 6.99 (s, 1H); 6.09-6.05 (m, 1H); 5.13 (t, J = 5.5 Hz, 1H); 2.93-2.87 (m, 1H); 2.74-2.61 (m, 2H); 2.55-2.49 (m, 1H); 2.25-2.15 (m, 1H); 1.71 (s, 3H); 1.61 (d, J = 4Hz, 3H); 1.37-1.33 (m, 3H). ^{19}F NMR (500 MHz, $CDCl_3$) δ: -64.82 (s, 3F). ESI-MS: calcd. for $C_{21}H_{21}F_3O_6$ ([M-H]$^-$), 426.13 found 425.50. Anal. Calcd. for $C_{21}H_{21}F_3O_6$: C, 59.15; H, 4.96; O, 22.51. Found: C, 59.06; H, 5.10; O, 22.42.

2-[1-(4, 4, 4-trifluorobutyryl)-4-methyl-3-pentenyl] naphthazarin (S8). Red powder, 74% yield. Mp: 91.3-92.9°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.60 (s, 1H); 12.43 (s, 1H); 7.20 (s, 2H); 7.01 (s, 1H); 6.09 (dd, J_1 = 5.5 Hz, J_2 = 6.5 Hz, 1H); 5.13 (t, J = 7 Hz, 1H); 2.71-2.63 (m, 3H) ; 2.55-2.47 (m,3H) ; 1.72 (s, 3H); 1.61 (s, 3H). ^{19}F NMR (500 MHz, $CDCl_3$) δ: -66.98 (s, 3F). ESI-MS: calcd. for $C_{20}H_{19}F_3O_6$ ([M-H]$^-$), 411.11 found 411.17. Anal. Calcd. for $C_{20}H_{19}F_3O_6$: C, 58.25; H, 4.64; O, 23.28. Found: C, 58.08; H, 4.81; O, 23.20.

2-[1-(acetyl)-4-methyl-3-pentenyl] naphthazarin (S9). Red powder, 83% yield. Mp: 85.0-86.0°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.50 (s, 1H); 12.44 (s, 1H); 7.20 (s, 2H); 7.01 (s, 1H); 6.05 (dd, J_1 = 4.5 Hz, J_2 = 7 Hz, 1H); 5.14 (t, J = 7 Hz, 1H); 2.67-2.61 (m, 1H); 2.53-2.47 (m, 1H); 2.16 (s, 3H); 1.71 (s, 3H); 1.60 (s 3H). ESI-MS: calcd. for $C_{18}H_{18}O_6$ ([M-H]$^-$) 329.11, found 329.33.

2-[1-(propionyl)-4-methyl-3-pentenyl] naphthazarin (S10). Red powder, 81% yield. Mp: 64.0-66.0°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.61 (s, 1H); 12.45 (s, 1H); 7.21 (s, 2H); 7.00 (s, 1H); 6.06 (dd, J_1 = 4.5 Hz, J_2 = 7 Hz, 1H); 5.14 (t, J = 7 Hz, 1H); 2.67-2.62 (m, 1H); 2.53-2.42 (m, 3H); 1.71 (s, 3H); 1.60 (s, 3H); 1.20 (t, J = 7.5 Hz, 3H). ESI-MS: calcd. for $C_{19}H_{20}O_6$ ([M-H]$^-$) 343.14, found 343.37.

2-[1-(butyryl)-4-methyl-3-pentenyl] naphthazarin (S11). Red powder, 81% yield. Mp: 84.7-85.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.59 (s, 1H); 12.43 (s, 1H); 7.19 (s, 2H); 7.00 (s, 1H); 6.05 (dd, J_1 = 5 Hz, J_2 = 7 Hz, 1H); 5.14 (t, J = 6.5Hz, 1H); 2.66-2.61 (m, 1H); 2.52-2.46 (m, 1H); 2.42-2.36 (m, 2H); 1.73-1.68 (m, 5H); 1.60 (s, 3H); 0.99 (t, J = 7.5 Hz, 3H). ESI-MS: calcd. for $C_{20}H_{22}O_6$ ([M -H]$^-$) 357.14, found 357.17.

Antiproliferative Activities Assay

The antiproliferative activity of the prepared compounds **S1–S12** against B16-F10, MG63, and A549 cell lines were determined using a standard (MTT)-based colorimetric assay (BioTek, Highland Park, Winooski, VT).[19] Target tumor cell lines were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. After diluting to 2×10^4 cells mL-1 with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The subsequent incubation was permitted at 37 °C, 5% CO_2 atmosphere for 24 h before the cytotoxicity assessments. Tested samples at preset concentrations were added to six wells with colchicines as positive reference. After 48 h exposure, 40 μL of phosphate-buffered saline (PBS) containing 2.5 mg/mL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)) was added to each well. Four hours later, 100 μL extraction solutions (10% SDS–5% isobutyl alcohol–0.01 ML HCl) was added. After an overnight incubation at 37 °C, the optical density was measured at a wavelength of 570 nm on an enzyme-linked immunosorbent assay (ELISA) microplate reader. The results are summarized in Table 1, and the analysis of standard deviation and statistical significance were carried out using DPS v. 7.5. In the concentration range from 0.0 μmol/L to 10.0 μmol/L, the interference of each compound was at the same level, which would not affect our results.

Effects on Tubulin Polymerization

Bovine brain tubulin was purified as described previously.[20] To evaluate the effect of the compounds on tubulin assembly in vitro,[21] varying concentrations were preincubated with 10 μM tubulin in glutamate buffer at 30 °C and then cooled to 0°C. After addition of GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30 °C and the assembly of tubulin was observed turbid metrically. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after 20 min incubation. The optical density was measured at a wavelength of 350 nm on an ELISA microplate reader. The results are summarized in Table 1, and the analysis of standard deviation and statistical significance were carried out using DPS v. 7.5.

Cell Cycle Analysis

Hela cells were plated in 6-well plates (5.0×10^3 cells/well) and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated with the compound **S7** at its respective IC_{50} concentrations. Untreated cells (control) or cells treated with the compound's solvent (DMSO) were included. DMSO was used at the highest concentration in the experiments. Following 24-h treatment, cells were centrifuged and fixed in 70% ethanol at 4 °C for at least 12 h and subsequently resuspended in PBS containing 0.1 mg/mL RNase A and 5 μg/mL propidium iodide (PI). Cellular DNA content, for cell cycle distribution analysis, was measured by flow cytometry using FACNcan cytofluorometer (PT. Madagasi Brosa, JI. Batang Hari No. 73, Propinsi Sumatera Utara, Indonesia) plotting at least 20,000 events per sample, as previously described.[22,23]

TABLE 1. Inhibition (IC_{50}) of B16-F10, MG63 and A549 cells proliferation and inhibition of tubulin polymerization by compounds S_1–S_{12}

		IC_{50} (μM)			
Compounds	Starting material	B16-F10	MG63	A549	Tubulin
S1	CF_2H-COOH	1.43±0.31**	1.76±0.54P**	1.68±0.46**	9.98±0.96**
S2	CH_3CFH-COOH	1.19±0.09**	1.44±0.36**	1.26±0.14**	8.31±0.62**
S3	CH_3CF_2_COOH	0.86±0.06**	1.16±0.21**	0.98±0.05**	4.92±0.35**
S4	CF_3CH_2-COOH	0.97±0.04**	1.35±0.34**	1.05±0.08**	6.10±0.59**
S5	CF_2HCF_2-COOH	0.60±0.03**	0.97±0.26**	0.66±0.04**	3.23±0.12**
S6	$CF_3(CF_2)_2$-COOH	1.05±0.21**	1.26±0.36**	1.18±0.34**	8.01±0.63**
S7	$CF_3CH_2CH(CH_3)$-COOH	0.39±0.01**	0.72±0.04**	0.58±0.02**	2.59±0.13**
S8	$CF_3(CH_2)_2$-COOH	0.75±0.03**	1.05±0.24**	0.85±0.04**	5.26±0.47**
S9	CH_3-COOH	2.02±0.56**	2.28±0.37**	2.55±0.22**	11.94±0.93**
S10	CH_3CH_2-COOH	1.94±0.48**	2.05±0.63**	1.65±0.34**	12.39±0.97**
S11	$CH_3(CH_2)_2$-COOH	2.11±0.62**	2.93±0.66**	2.78±0.25**	15.76±1.14**
S12	—	3.72±0.45	5.67±0.82	5.06±0.77	30.18±1.91**
Colchicine	—	—	—	—	1.50±0.06

IC_{50} for B16-F10, MG63 and A549:
compared to **S12 (shikonin) $P < 0.01$. IC_{50} for tubulin: **compared to colchicine $P < 0.01$.

The percentage of cells in the G1, S, and G2/M phases of the cell cycle and the percentage of cells in the sub-G1 peak were determined using WINMDI (Scripps Research Institute, San Diego, CA) after cell debris exclusion.

Docking Simulations

Molecular docking of compounds **S7** into tubulin (PDB code: 1SA0) was carried out using the Lamarckian genetic algorithm of Auto-Dock 4.0.3.

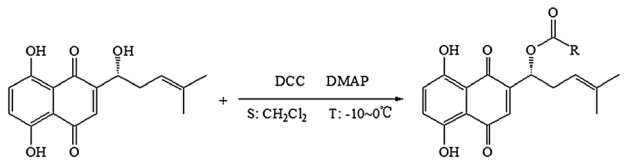
RESULTS AND DISCUSSION

Chemistry

We synthesized a series of novel shikonin derivatives (Fig. 3) possessing acyl group at the side-chain hydroxyl of shikonin in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylaminol) pyridine (DMAP). The synthesis of shikonin derivatives **S1—S11** followed the general reaction pathway is outlined in Scheme 1. Eight compounds (**S1–S8**) are reported for the first time. The reaction in molar ratio of shikonin/DMAP (1:0.4) resulted in good yields: 39%–83%. All of the synthetic compounds gave satisfactory analytical and spectroscopic data. ^{1}H NMR, ^{19}F NMR and ESI-MS spectra were consistent with the assigned structures.

Biological Activities

All derivatives of shikonin (**S1–S11**) were evaluated for their ability to produce anticancer activity against three human cancer cell lines, B16-F10 (malignant melanoma cells), MG63 (human osteosarcoma cells), and A549 (lung cancer cells). The results were summarized in Table 1. For the compounds (**S1–S11**) in Table 1, we can see that per acylshikonin (**S1–S11**) displays higher inhibitory effects against B16-F10, MG63, and A549 than shikonin (**S12**). Thus, we conclude that the acyl group of acylshikonin is very important for the enhancement of potency. Moreover, all fluoroacylshikonin (**S1–S8**) that contained fluorine in their structure clearly exhibited higher inhibitory effects against B16-F10, MG63, and A549 than the other three acylshikonin (**S9–S11**). In Figure 3, we see that the compound **S9** associated with compound **S1** and both contained the same number of carbon atoms in their acyl groups (**S10** for **S2**, **S3**, **S4**, and **S5**; **S11** for **S6** and **S8**). However, we also find in Table 1 that **S9** is a less strong inhibitor against B16-F10, MG63, and A549 than compound **S1**. It is the same situation for **S10** and **S11** that both were less strong, respectively, than **S2–S5** and **S6–S8**.Therefore, for compounds (**S1_S8**), we infer that fluorine atoms improved the anticancer activity. Among all the compounds (**S1–S12**), **S7** displayed the most potent inhibitory activity (IC_{50} = 0.39 ± 0.01 μmol/L for B16-F10, IC_{50} = 0.72 ± 0.04 μmol/L for MG63, and IC_{50} = 0.58 ± 0.02 μmol/L for A549). Compound **S7** bearing a methyl group showed more potent activity than compound **S8** without a methyl group. the structure–activity relationships in this compound (**S7**) demonstrated that the methyl group may contribute to the enhancement of anticancer activity. Previously, Walle et al.[16,17] reported that methylated flavones were more inhibited for the proliferation of cancer cells than the unmethylated flavones. Therefore, we infer that methylation on the acyl group of acylshikonin enhanced the antican-



Scheme 1. General synthesis of shikonin derivatives (S1–S11). Reagents and conditions: (a) piperidine, pyridine, 80-90°C, 4–5 h. (b) CH2Cl2, DCC, DMAP, -10-0°C, overnight.

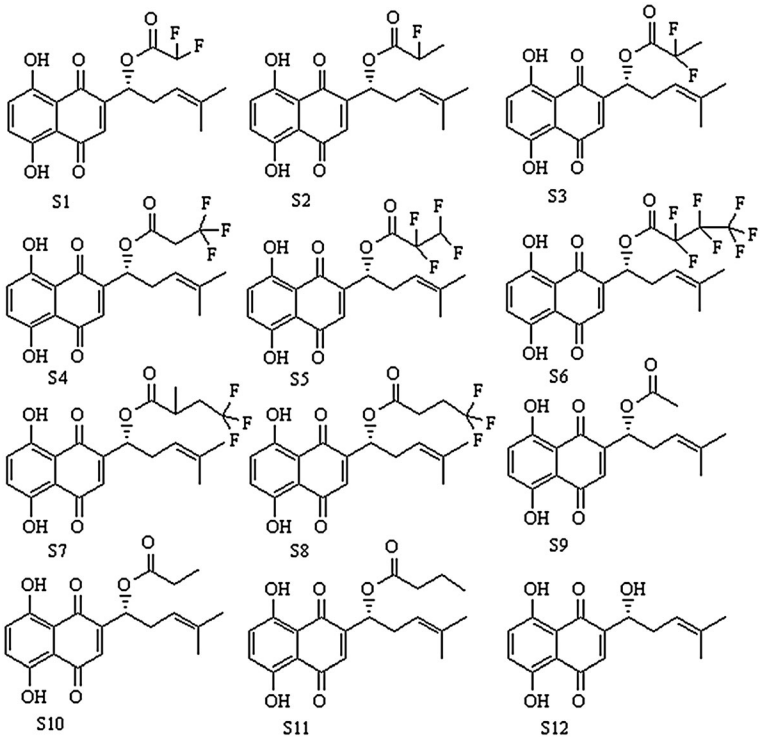


Fig. 3. Shikonin and acylshikonins.

cer activity. But there could be another possibility. The addition of the methyl group on the alkyl chain would affect the solubility of the compounds, and the increased activity might be the effect of solubility. Whether the methylation could increase the activity or not still requires more experiments for proof.

Recently, Acharya et al.[11] also demonstrated that naphthazarin is a microtubule inhibitor in cell-free systems and in A549 cells. Encouraged by these new findings, we also designed an assay in which compounds (**S1–S12**) interact with tubulin. As shown in Table 1, compounds **S7** and **S5** showed strong inhibitory effect (**S7**, IC_{50} = 2.59 ± 0.13 μmol/L; **S5**, IC_{50} = 3.23 ± 0.12 μmol/L, and colchicines, IC_{50} = 1.50 ± 0.06 μmol/L). Combining the above results, we inferred that **S7** and **S5** directly interacted with tubulin. Furthermore, cell cycle analysis of compound **S7** was performed using flow cytometry. Cell cycle analysis (Fig. 4) indicated that compound **S7** strongly induced G2/M arrest in B16-F10 cells. The effect was observed after treatment with **S7** (1 μmol/L) for 24 h. About 47% of the cells were arrested in the G2/M phase in the presence of 1 μmol/L. According to these findings, we inferred that compound **S7** may be a potent antitubulin agent.

To obtain a better understanding of the potency of compound **S7** and guide further SAR studies, we proceeded to examine the interaction of compound **S7** with tubulin (PDB code: 1SA0). All docking runs were carried out with the LigandFit Dock protocol of Discovery Studio 3.1. The binding modes of compound **S7** and tubulin are depicted in Figure 5 (left). All the amino acid residues which had interactions with tubulin are exhibited. In the binding model, compound **S7** is nicely bound to the active site of tubulin by

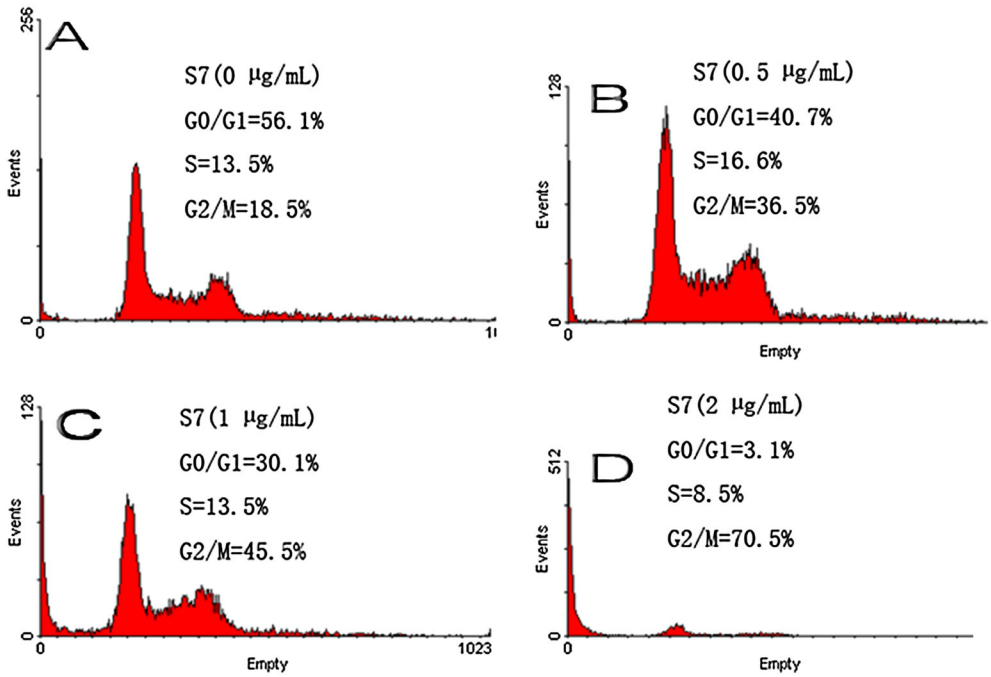


Fig. 4. Cell cycle of B16-F10 cells treated with compound S7 using flow cytometry. Cells were harvested after treatment with S7 for 24 h and subjected to cell cycle analysis. The percentage of each cycle phase is indicated.

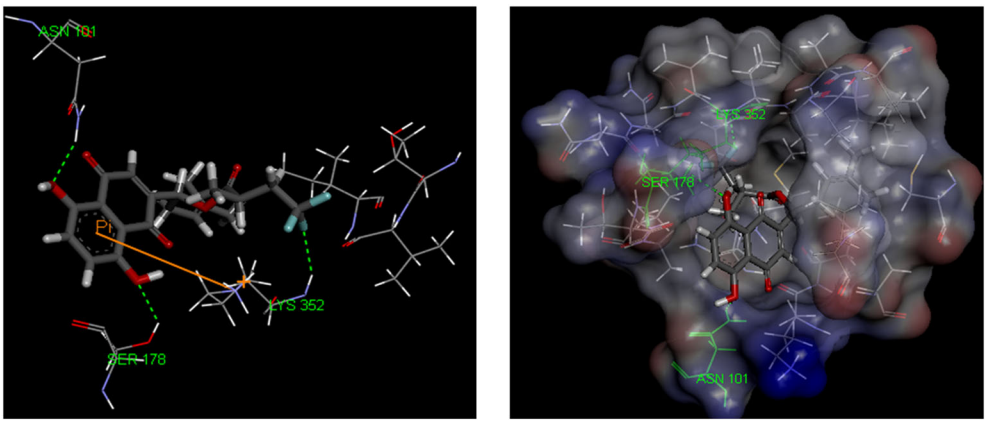


Fig. 5. Left: Binding mode of compound S7 with tubulin (PDB code: 1SA0). The dotted lines show the hydrogen bonds. Right: 3D model of the interaction between compound S7 and the colchicines binding site. The dotted lines show the hydrogen bonds.

forming three hydrogen bonds with LYS 352 (angle F···H–N = 133.654°, distance= 2.223 Å) , ASN 101 (angle O···H–N = 152.506°, distance = 2.228 Å), and SER 178 (angle O···H–O = 102.179°, distance = 2.447 Å). Moreover, the benzene ring of ligand **S7** formed a cation–π interaction with LYS 352 (distance = 6.793 Å). Cation–π interactions can play an important role in stabilizing the 3D structure of a protein.[18] Therefore, compound **S7** was closely bound to the active site of tubulin by two types interactions, mentioned above. In Figure 5 (right), the 3D model of the interaction between compound **S7** and the active site of tubulin are depicted. The molecular docking results showed that compound **S7** may be a potential antitubulin inhibitor.

CONCLUSIONS

To summarize, we synthesized 11 novel shikonin derivatives possessing an acyl group at the side-chain hydroxyl of shikonin and assessed their anticancer activity against three cancer cell lines in vitro (B16-F10, MG63 and A549). Most compounds exhibited significant inhibitory activity on three cell lines. Among the compounds, **S7** showed the most potent anticancer activity, with IC_{50} = 0.39 ± 0.01 μmol/L for B16-F10, 0.72 ± 0.04 μmol/L for MG63, and 0.58 ± 0.02 μmol/L for A549., The introduction of fluoroacyl substituents to shikonin was confirmed to increase the cytotoxicities of shikonin. Compound **S7** demonstrated the most potent antitubulin activity that inhibited the polymerization of tubulin, with IC_{50} of 2.56 μmol/L, which was compared with the positive control colchicine (IC_{50} of 1.50 μmol/L). Docking simulation was performed to study the probable binding model. We then proceeded to examine the interaction of compound **S7** with tubulin structure (PDB code: 1SA0). Antitubulin and anticancer assay results adequately confirmed that compound **S7** is a potential anticancer agent.

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LITERATURE CITED

1. Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. Angew Chem Int Ed 1999;38:270–300.
2. Hashimoto S, Xu M, Masuda Y, Aiuchi T, Nakajo S, Cao J, Miyakoshi M, Ida Y, Nakaya K. -Hydroxyisovalerylshikonin inhibits the cell growth of various cancer cell lines and induces apoptosis in leukemia HL-60 cells through a mechanism different from those of fas and etoposide. J Biochem 1999;125:17–23.
3. Zhou W, Peng Y, Li SS. Semi-synthesis and anti-tumor activity of 5, 8-O-dimethyl acylshikonin derivatives. Eur J Med Chem 2010;45:6005–6011.
4. Lu L, Qin A, Huang H, Zhou P, Zhang CY, Liu JB. Shikonin extracted from medicinal chinese herbs exerts anti-inflammatory effect via proteasome inhibition. Eur J Med Chem 2009;658:242–247.
5. Xuan YY, Hu H. Naturally-occurring shikonin analogues — A class of necroptotic inducers that circumvent cancer drug resistance. Cancer Lett 2009;274:233–242.
6. Rao Z, Liu X, Zhou W, Yi J, Li SS. Synthesis and antitumour activity of b-hydroxyisovalerylshikonin analogues. Eur J Med Chem 2011;46:3934–3941.
7. Ahn BZ, Baik KU, Kweon GR, Lim K, Hwangl BD. Acylshikonin analogues: synthesis and inhibition of DNA Topoisomerase-I. J Med Chem 1995;38:1044–1047.
8. Yang F, Chen Y, Duan WH, Zhang C, Zhu H, Ding J. SH-7, a new synthesized shikonin derivative, exerting its potent antitumor activities as a topoisomerase inhibitor. Int J Cancer 2006;119:1184–1193.
9. Yoon Y, Kim YO, Lim NY, Jeon WK, Sung HJ. Shikonin, an ingredient of lithospermum erythrorhizon induced apoptosis in HL 60 human premyelocytic leukemia cell line. Planta Med 1999;65:532–535.
10. Verma RP. Understanding topoisomerase I and II in terms of QSAR. Bioorg Med Chem 2005;13:1059–1067.
11. Acharya BR, Bhattacharyya S, Choudhury D, Chakrabarti G. The microtubule depolymerizing agent naphthazarin induces both apoptosis and autophagy in A549 lung cancer cells. Apoptosis 2011;16:924–939.
12. Duschinsky R, Pleven E, Heidelberger C. The synthesis of 5-fluoropyrimidines. J Am Chem Soc 1957;79:4559–4560.
13. Heidelberger C, Chaudhuri N, Danneberg P. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature 1957;179:663–666.
14. Kar S, Wang MF, Ham SW, Carr BI. Fluorinated Cpd 5, a pure arylating K-vitamin derivative, inhibits human hepatoma cell growth by inhibiting Cdc25 and activating MAPK. Biochem Pharmacol 2006;72:1217–1227.
15. Filler R, Saha R. Fluorine in medicinal chemistry: a century of progress and a 60-year retrospective of selected highlights. Future Med Chem 2009;1:777–791.
16. Walle T, Walle UK. Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells. J Pharm Pharmacol 2007;59:857–862.
17. Walle T. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? Cancer Biol 2007;17:354–362.
18. Jannifer CM, Dougherty DA. The cation–B interaction. Chem Rev 1997;97:1303–1324.
19. Boumendjel A, Boccard J, Carrupt PA, Nicolle E, Blanc M, Geze A, Choisnard L, Wouessidjewe D, Matera EL, Dumontet C. Antimitotic and antiproliferative activities of chalcones: forward structure±activity relationship. J Med Chem 2008;51:2307–2310.
20. Hamel E, Lin CM. Separation of active tubulin and microtubule-associated proteins by ultracentrifugation and isolation of a component causing the formation of microtubule bundles. Biochemistry 1984;23:4173–4184.
21. Hamel E. Evaluation of antimitotic agents by quantitative comparisons of their effects on the polymerization of purified tutulin. Cell Biochem Biophys 2003;38:1–22.
22. Vasconcelos MH, Beleza SS, Quirk C, Maia LF, Sambade C, Guimarães JE. Limited synergistic effect of antisense oligonucleotides against bcr-abl and transferrin receptor mRNA in leukemic cells in culture. Cancer Lett 2000;152:135–143.
23. Neves MP, Cidade H, Pinto M, Silva AM, Gales L, Damas AM, Lima RT, Vasconcelos H, Nascimento MSJ. Prenylated derivatives of baicalein and 3,7-dihydroxyflavone: synthesis and study of their effects on tumor cell lines growth, cell cycle and apoptosis. Eur J Med Chem 2011;46:2562–2574.