Brief Articles

Antitumor Agents. 239. Isolation, Structure Elucidation, Total Synthesis, and Anti-Breast Cancer Activity of Neo-tanshinlactone from *Salvia miltiorrhiza*

Xihong Wang,[†] Kenneth F. Bastow,[†] Chang-Ming Sun,[§] Yun-Lian Lin,[§] Hsi-Jung Yu,[⊥] Ming-Jaw Don,[§] Tian-Shung Wu,[‡] Seikou Nakamura,[†] and Kuo-Hsiung Lee[†,*]

Natural Products Laboratory, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan, National Research Institute of Chinese Medicine, Taipei, Taiwan, and Department of Chemistry, Chinese Culture University, Taipei, Taiwan

Received June 8, 2004

Neo-tanshinlactone (**1**) was isolated and synthesized for the first time and evaluated in vitro against several human cancer cell lines. Compound **1** showed significant inhibition against two ER+ human breast cancer cell lines and was 10-fold more potent and 20-fold more selective as compared to tamoxifen citrate. Compound **1** also potently inhibited an ER−, HER-2 overexpressing breast cancer cell line. Therefore, this novel compound merits further development as an anti-breast cancer drug candidate.

Introduction

Breast cancer, the most frequent cancer in women, is the second leading cause of cancer-related death, and the present breast cancer therapies achieve meaningful clinical results in only 30−40% of patients.[1] Estrogens are well recognized to play the predominant role in breast cancer development and growth, and much effort has been devoted to the blockade of estrogen formation and action.[1,2] However, expression of the HER-2 receptor is also a significant factor associated with breast cancer morbidity.[3] It is also becoming clearer that crosstalk between estrogen and growth factor receptor pathways occurs and likely is a factor in the pathology and treatment of breast cancer.[4]

The most widely used therapy for breast cancer, which has shown benefits at all stages of the disease, is the use of an antiestrogen such as tamoxifen.[1] Over 30 years of clinical trials and use have established the clinical merit of tamoxifen but have also highlighted the critical need for the next generation of drugs that are more broadly efficacious and have fewer potential side-effects, especially those from estrogen receptor agonism.[5]

"Tanshen", the rhizome of *Salvia miltiorrhiza* Bunge, has been used in traditional Chinese medicine (TCM) for the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction. The herb has also been applied for hemorrhage, dismenorrhea, miscarriage, swelling, and insomnia.[6,7] Numerous tanshinones were isolated from *S. miltiorrhiza*, and diverse medicinal actions were reported, including antitumor,[6] antioxidant,[8] antimicrobial,[9] antiplatelet aggregation,[10,11] and antiallergic activities.[12] The characteristic structural feature of the major constituents from Tanshen is the presence of *ortho*- or *para*-diketones.

As a part of our interest in plant-derived antitumor drug discovery, a minor component, neo-tanshinlactone (**1**), was isolated from the EtOH extract of *S. miltiorrhiza*. The structure of **1**, as elucidated by extensive NMR spectroscopy and mass spectrometry, is unique. For the first time, compound **1** was synthesized, and the novel structure was unambiguously determined. Herein we report the isolation, structure elucidation, and the total synthesis of compound **1** as well as its selective activity against human breast cancer cell lines of clinical significance.

Results and Discussion

The concentrated ethanolic extract of the root of *S. miltiorrhiza* was suspended in water and then extracted with EtOAc. The EtOAc extract was subjected to repeated silica gel chromatography and preparative TLC to obtain compound **1** (Figure 1).

Compound **1** was obtained as white needles with a molecular formula of $C_{17}H_{12}O_3$ determined by HREIMS ($[M]^+$, *m/z* 264.0786). The IR spectrum showed absorption for a carbonyl group (1726 cm^{-1}). The ^1H and ^{13}C NMR spectra of **1** were similar to those of tanshinlactone (Figure 1), which was isolated from the same species by Luo et al.[13] The ^1H NMR spectrum ($CDCl_3$) of **1** revealed an ABX pattern for 1,2,3-aromatic protons at δ 8.42 (d, J = 8.0 Hz), 7.49 (t, J = 8.0 Hz), and 7.41 (d, J = 8.0 Hz), and AB pattern for *ortho*-aromatic protons at δ 7.84 (d, J = 8.5 Hz), and 7.79 (d, J = 8.5 Hz), one vinyl proton at δ 7.39 (q, J = 1.5 Hz), and two methyls at 2.68 (s) and 2.37 (d, J = 1.5 Hz). One signal at δ_C 158.7 in the ^{13}C NMR spectrum of **1** indicated the presence of a lactone ring. This lactone ring is the major difference between neotanshinlactone and the tanshi-

* To whom correspondence should be addressed. Phone: 919-962-0066. Fax: 919-966-3893. E-mail: khlee@unc.edu.
† University of North Carolina.
‡ National Cheng Kung University.
§ National Research Institute of Chinese Medicine.
⊥ Chinese Culture University.

10.1021/jm040112r CCC: $27.50
Published on Web 10/12/2004

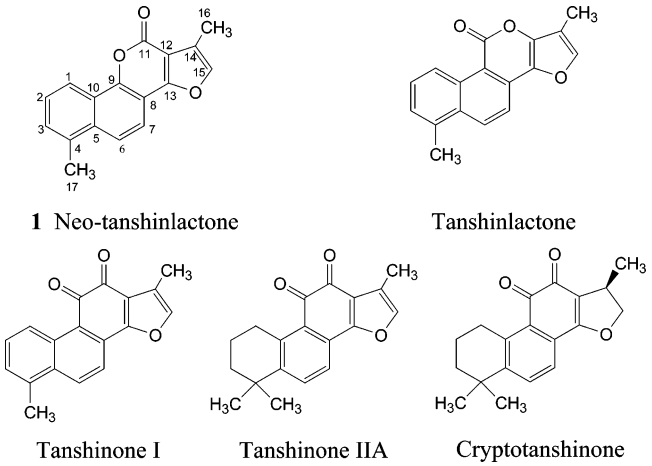


Figure 1. Structures of neo-tanshinlactone (**1**), tanshinlactone, and tanshinones.

nones. On the basis of the above information, the carbonyl group could be connected to either C-9 or C-12, and the structure was ambiguous on the basis of previous work.[13] We have now determined the location of the carbonyl group by HMBC. The observation of three-bond couplings of the vinyl methyl (H-16) to quaternary carbon C-12 (δ_C 110.3) and H-1 to quaternary carbon C-9 (δ_C 149.6) indicated that the carbonyl group should be connected to C-12. Because C-9 is connected to the oxygen atom, C-9 was shifted downfield. The complete assignment of the ^{1}H and ^{13}C NMR signals of **1** was based on extensive COSY, HMQC, and HMBC data. Thus, the compound was established to have structure **1** (Figure 1) and was named as neo-tanshinlactone.

To confirm neo-tanshinlactone (**1**)'s unique structure and to obtain this minor phytochemical constituent in larger quantity, we decided that total chemical synthesis was necessary. Our synthetic strategy (Scheme 1) was to first prepare 5-methyl-1-naphthol (**6**).[14,15] Treatment of **6** with malonic acid in the presence of PPA at 75 °C yielded **7**.[16] Reaction of **7** with chloroacetone in the presence of HOAc/NH_4OAc in toluene/ethanol[17] gave the target product **1**. Spectroscopic data of the synthesized product were identical with those of the natural product **1**.

Compound **1** was tested for in vitro anticancer activity against a variety of human cancer cell lines and showed unique specific activity. Compound **1** was active against the MCF-7 cancer cell line at 0.6∼1.2 μg/mL but showed insignificant activity against other cell lines in the panel at concentrations up to 10 μg/mL (Table 1). Extended bioassay studies showed that **1** was active against estrogen receptor positive (ER+) human breast cancer cell lines (MCF-7 and ZR-75-1) with ED_{50} values of 0.6 μg/mL and 0.3 μg/mL, respectively, but was inactive against two ER− cell lines (MDA MB-231, and HS 587-T) with ED_{50} > 10 μg/mL (Table 2, Figure 2). Interestingly, **1** was also active against a HER-2-overexpressing breast cancer cell line (SK-BR-3, HER-2++), but was essentially inactive against epidermal growth factor receptor (EGFR) overexpressing skin cancer or androgen receptor (AR)-dependent prostate cancer cell lines (A431 and LN−CaP, respectively). The three major tanshinones from *Salvia miltiorrhiza*, tanshinone I, tanshinone IIA, and cryptotanshinone (Figure 1), were tested against MCF-7 and MDA MB-231 cell lines. The results are shown in Table 3. The selected tanshinones showed significant activity against the two cell lines with ED_{50} values of <1 μg/mL but without selectivity. We also compared the activity and selectivity of neo-tanshinlactone (**1**) and tamoxifen citrate (TAM), which is widely used as an estrogen receptor modulator, against four human breast cancer cell lines MCF-7, ZR-75-1, MDA MB-231, and HS 587-T. Tamoxifen citrate was active against ER+ human breast cancers (MCF-7 and ZR-75-1) with ED_{50} values of 5.0 μg/mL and 3.6 μg/mL, respectively, but was less active against ER− cell lines (MDA MB-231 and HS 587-T) with ED_{50} values of 7.0 μg/mL and 8.5 μg/mL, respectively (Table 3, Figure 2). On the basis of these direct comparisons, compound **1** is 10-fold more potent and 20-fold more selective than tamoxifen citrate against ER+ and HER-2++ breast cancer in vitro.

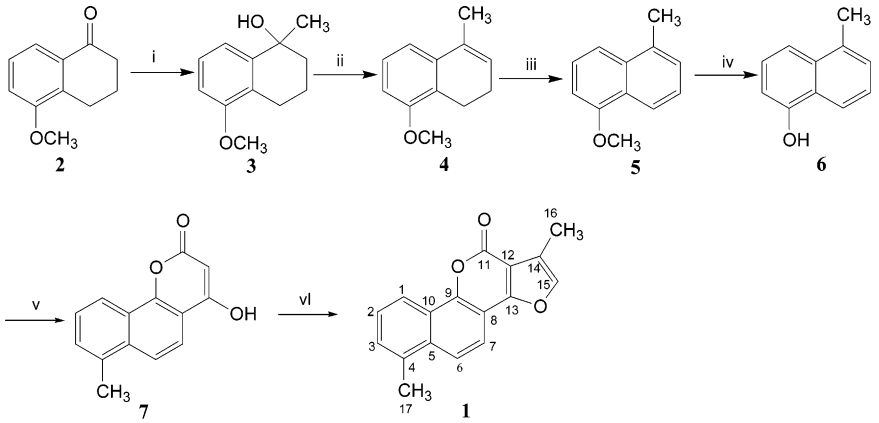
In conclusion, we isolated and synthesized a new compound, neo-tanshinlactone (**1**), with strong and selective anti-breast cancer activity. This compound might be a useful lead for developing novel and promis-

Table 1. Cytotoxicity of Neo-tanshinlactone (**1**)

compound	MCF-7[a]	MDA-MB-231	A549
neo-tanshinlactone (**1**)	<2.5 (57)	>20 (17)	>20 (10)

[a] Cell line: ED_{50} μg/mL. % inhibition at test concentration is the bracketed value.

Scheme 1. Synthesis of Neo-tanshinlactone (**1**)[a]



[a] (i) CH_3MgBr, Et_2O, reflux, 5 h; (ii) $ZnCl_2$/concd HCl, benzene, 5 h; (iii) 10% Pd/C, triglyme, reflux 3 days; (iv) BBr_3, CH_2Cl_2, reflux, 3 h; (v) malonic acid, PPA, 75 °C, 3 h; (vi) chloroacetone, HOAc/NH_4OAc, toluene/EtOH, reflux, 24 h.

Table 2. In Vitro Anticancer Profiles of Neo-tanshinlactone (**1**) and Tamoxifen Citrate (TAM)

	mean ED_{50} values (μg/mL)/cell line										
compd	MCF-7 (ER+)	MDA-MB-231 (ER−)	ZR-75-1 (ER+)	HS 587-T (ER−)	SK-BR-3 (ER-)	A431	LN-CaP	SW620	PC-3	KB	KB-VIN
1	0.6	10	0.3	16	0.2	>10 (35)	>10 (28−36)	>10 (22)	10	>10 (18)	>10 (9)
TAM	5	8	5	9	5	7	6	4	10	9	9

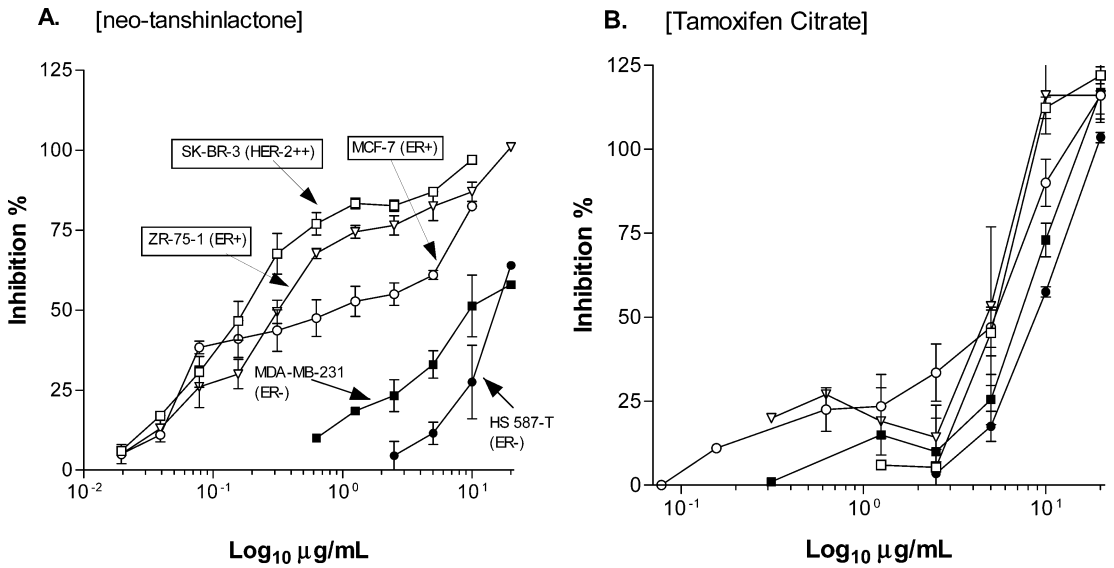


Figure 2. In vitro anticancer profiles of neo-tanshinlactone and tamoxifen citrate (TAM). Data are mean and standard error from several independent experiments ($n = 2-4$). Neo-tanshinlactone is shown in panel A and tamoxifen citrate is shown in panel B. The specific tumor cells used are: SK-BR-3 (open squares), HS 587-T (closed circles), ZR-75-1 (open triangles), MCF-7 (open circles), MDA-MB-231 (closed squares). Labeled arrows are also used to identify cell lines in panel A to facilitate review.

Table 3. Cytotoxicity of Three Major Tanshinones

compound	MCF-7[a]	MDA-MB-231
tanshinone I	0.82	0.75
tanshinone IIA	0.38	0.70
cryptotanshinone	0.41	0.16

[a] Cell line: ED_{50} in μg/mL.

ing anti-breast cancer drug candidates. Current data suggest that neo-tanshinlactone (**1**)'s mechanism of action is complex. It is unlikely to be estrogen-receptor-mediated since SK-BR-3 is a receptor-negative cell line.[4] Effects on estrogen formation were not tested, but MCF-7 has 20-fold lower aromatase activity than SK-BR-3,[4] yet the latter cell line is 3-fold more sensitive (Table 2, Figure 2). Selective target-interaction downstream of the estrogen and HER-2 receptor could account for the activity profile of compound **1** since cross-talk between the signaling pathways is known[4] but not fully understood. Synthesis of analogues of compound **1** is in progress with an aim to further improve the pharmacological profiles for preclinical testing.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanaco micro-melting point apparatus and are uncorrected. Infrared spectra were obtained on a Nicolet Avatar 320 FTIR spectrophotometer. Nuclear magnetic resonance spectra were recorded on a Varian UnityInova-500 spectrometer. Chemical shifts are reported in parts per million (δ) units relative to internal tetramethylsilane. The EIMS spectrum was measured on a Finnigan GCQ GC/MS spectrometer at 30 eV. HREIMS was recorded on a Finnigan MAT 95S mass spectrometer. Column chromatography was performed with E. Merck 230−400 mesh silica gel.

Plant Material. The dried roots of *Salvia miltiorrhiza* were purchased from a local herbal drug store in Taipei, and identified by Mr. Jun-Chih Ou, a research fellow of National Research Institute of Chinese Medicine (NRICM). A voucher specimen was deposited in the herbarium of NRICM.

Extraction and Isolation. Slices of the dried roots of *S. miltiorrhiza* (5 kg) were extracted with EtOH (3 × 10 L) at room temperature. The combined EtOH extracts were concentrated in vacuo. The residue was then partitioned between EtOAc and H_2O. The concentrated EtOAc extract (2.2 kg) was subjected to column chromatography over silica gel and eluted with *n*-hexane/EtOAc (4:1), *n*-hexane/EtOAc (1:1), and EtOAc successively. The first fraction was rechromatographed on silica gel using a gradient of *n*-hexane/EtOAc (10:1 to 2:1). The subfraction was further chromatographed on silica gel eluted with *n*-hexane/CH_2Cl_2 (4:1) to give **1** [160 mg, $R_f = 0.52$ (*n*-hexane/EtOAc = 5:1)].

Neo-tanshinlactone (1). White solid (*n*-hexane/EtOAc); mp 173−175 °C; IR (KBr) ν_{max} 1726, 1619, 1578, 1379, 1172, 1074, 1010, 770 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.37 (d, $J = 1.5$ Hz, CH_3-16), 2.68 (s, CH_3-17), 7.39 (d, $J = 1.5$ Hz, H-15), 7.41 (d, $J = 8.0$ Hz, H-3), 7.49 (t, $J = 8.0$ Hz, H-2), 7.79 (d, $J = 8.5$ Hz, H-7), 7.84 (d, $J = 8.5$ Hz, H-6), 8.42 (d, $J = 8.0$ Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 8.6 (CH_3-16), 19.6 (CH_3-17), 108.0 (C-8), 110.3 (C-12), 116.6 (C-7), 120.4 (C-14), 120.7 (C-1), 120.8 (C-6), 123.5 (C-10), 126.9 (C-2), 128.9 (C-3), 133.2 (C-5), 134.6 (C-4), 141.1 (C-15), 149.6 (C-9), 158.7 (C-11, C-13); EIMS *m/z* (%): 264 (100, M^+), 184 (13), 165 (9), 128 (9); HREIMS *m/z* 264.0788 (M^+) (Calcd for $C_{17}H_{12}O_3$: 264.0786).

Synthesis of 5-Methyl-1-naphthol (6). 5-Methoxy-1-tetralone (**2**) (2 g, 11.3 mmol) was dissolved in 50 mL of anhydrous diethyl ether, and methylmagnesium bromide (9.5 mL, 28.4 mmol) was slowly added to the solution at 0 °C. The mixture was then heated to reflux and stirred for 3 h. After cooling, the reaction mixture was extracted with diethyl ether. Without purification, the resulting crude product (**3**) was dehydrated using concentrated HCl and zinc chloride. After purification, the product **4** (1.9 g) was obtained. 10% Pd/C (2.3

g) was added to a solution of **4** in triglyme (15 mL), and the mixture was heated to reflux for 3 days to furnish compound **5** (1.4 g). To a solution of **5** in anhydrous dichloromethane (20 mL) was added a solution of boron tribromide in dichloromethane (1.0 M, 24 mL) dropwise at 0 °C. The mixture was heated to reflux and stirred for 3 h to give the desired naphthol intermediate **6** (1.2 g) after silica gel chromatography. The total yield over four steps (**2** → **6**) was 70%. mp 93−94 °C; ^{1}H NMR ($CDCl_3$) δ 2.67 (s, 3H), 5.30 (s, 1H), 6.83 (d, J = 7.5 Hz, 1H), 7.31−7.41 (m, 3H), 7.58 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H).

Synthesis of 4-Hydroxy-7-methyl-benzo[*h*]chromen-2-one (7). The mixture of 5-methyl-1-naphthol (**6**) (1.0 g, 6.33 mmol), malonic acid (658 mg, 6.33 mmol), and PPA (10 g) was heated at 75 °C for 3 h. After the reaction, ice−water was added to the black residue. The solid was filtered, dissolved in 10% Na_2CO_3 solution, and stirred overnight. The basic solution was filtered, and the filtrate was acidified with 2 N HCl solution until the pH was about 4. The precipitate was then filtered and purified by silica gel chromatography to yield **7** (620 mg, 43%) as a yellow solid. mp 223−225 °C; ^{1}H NMR (DMSO-d_6) δ 2.69 (s, 3H), 5.70 (s, 1H), 7.57−7.61 (m, 2H), 7.86 (d, J = 9.0 Hz, 1H), 7.92 (d, J = 9.0 Hz, 1H), 8.23 (d, J = 7.5 Hz, 1H), 12.8 (br s, 1H).

Synthesis of Neo-tanshinlactone (1). To a solution of **7** (50 mg, 0.22 mmol) in toluene (8 mL) was added a mixture of HOAc (66 mg, 1.1 mmol) and NH_4OAc (80 mg, 1.1 mmol) in EtOH (2 mL) and chloroacetone (103 mg, 1.1 mmol). The mixture was refluxed 24 h. After cooling, the mixture was diluted with H_2O and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by column chromatography to give **1** (35 mg, 60%) as a white solid. The spectroscopic data of synthetic compound **1** were identical with those of the natural product **1**.

In Vitro Anticancer Assay.[18] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 1500−7500 cells per well with compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were fixed with cold 50% trichloroacetic acid and then stained with 0.4% sulforhodamine B (SRB). The absorbency at 562 nm was measured using a microplate reader after solubilizing the bound dye. The mean ED_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant (Figure 2). The following human tumor cell lines were used in the assay: A549 (non small cell lung cancer), MCF-7 (estrogen receptor positive breast cancer), ZR-75-1 (estrogen receptor positive breast cancer), MDA-MB-231 (estrogen receptor negative breast cancer), HS 587-T (estrogen receptor negative breast cancer), SK-BR-3 (HER-2-overexpressing breast cancer), A431 (EGFR-overexpressing skin cancer), LN-CaP (AR-dependent prostate cancer), SW620 (colon cancer), PC-3 (prostate cancer), KB (nasopharyngeal carcinoma), KB-VIN (vincristine-resistant KB subline). All cell lines were obtained from the Lineberger Comprehensive Cancer Center (UNC−CH) or from ATCC (Rockville, MD) and were cultured in RPMI-1640 medium supplemented with 25 mM HEPES, 0.25% sodium bicarbonate, 10% fetal bovine serum, and 100 μg/mL kanamycin.

Acknowledgment. This investigation was supported by grant CA 17625 from National Cancer Institute awarded to K. H. Lee, and by the National Science Council of Republic of China and National Research Institute of Chinese Medicine.

References

(1) Labrie, F.; Labrie, C.; Bélanger, A.; Simard, J.; Gauthier, S.; Luu-The, V.; et al. EM-652 (SCH 57068), a third generation SERM acting as pure antiestrogen in the mammary gland and endometrium. *J. Steroid Biochem. Mol. Biol.* **1999**, *69*, 51−84.
(2) Jordan, V. C.; Gradishar, W. J. Molecular mechanisms and future uses of antiestrogens. *Mol. Aspects Med.* **1997**, *18*, 187−247.
(3) Slamon, D. J.; Godolphin, W.; Jones, L. A., et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **1989**, *244*, 707−712.
(4) Kinoshita, Y.; Chen, S. Induction of aromatase (CYP19) expression in breast cancer cells through a nongenomic action of estrogen receptor α. *Cancer Res.* **2003**, *63*, 3546−3555.
(5) Dhingra, K. Antiestrogens-tamoxifen, SERMs and beyond. *Invest. New Drugs* **1999**, *17*, 285−311.
(6) Ryu, S. Y.; Lee, C. O.; Choi, S. U. In vitro cytotoxicity of tanshinones from *Salvia miltiorrhiza*. *Planta Med.* **1997**, *63*, 339−342.
(7) Wu, W.-L.; Chang, W.-L.; Chen, C.-F. Cytotoxic activities of tanshinones against human carcinoma cell lines. *Am. J. Chin. Med.* **1991**, *19*, 207−216.
(8) Cao, E.-H.; Liu, X.-Q.; Wang, J.-J.; Xu, N.-F. Effect of natural antioxidant tanshinone II-A on DNA damage by lipid peroxidation in liver cells. *Free Radic. Biol. Med.* **1996**, *20*, 801−806.
(9) Honda, G.; Koezuka, Y.; Tabata, M. Isolation of an antidermatophytic substance from the root of *Salvia miltiorrhiza*. *Chem. Pharm. Bull.* **1988**, *36*, 408−411.
(10) Onitsuka, M.; Fujiu, M.; Shinma, N.; Maruyama, H. B. New platelet aggregation inhibitors from Tan-Shen; radix of *Salvia miltiorrhiza* Bunge. *Chem. Pharm. Bull.* **1983**, *31*, 1670−1675.
(11) Lin, H. C.; Ding, H. Y.; Chang, W. L. Two new fatty diterpenoids from *Salvia miltiorrhiz. J. Nat. Prod.* **2001**, *64*, 648−650.
(12) Ryu, S. Y.; Lee, C. O.; Choi, S. U. Inhibition of mast cell degranulation by tanshinones from the roots of *Salvia miltiorrhiza*. *Planta Med.* **1999**, *65*, 654−655.
(13) Luo, H.-W.; Ji, J.; Wu, M.-Y.; Yong, Z.-G.; Niwa, M.; and Hirata, Y. Tanshinlactone, a novel seco-abietanoid from *Salvia miltiorrhiza*. *Chem. Pharm. Bull.* **1986**, *34*, 3166−3168.
(14) Suh, Y.-G.; Shin, D.-Y.; Min, K. H.; Hyun, S.-S.; Jung, J.-K.; Seo, S.-Y. Facile construction of the oxaphenalene skeleton by *peri* ring closure, formal synthesis of Mansonone F. *Chem. Commun.* **2000**, 1203−1204.
(15) Mayer, P.; Alibert, S.; Brunel, P.; Imbert, T. Synthesis of benzo-[de]chromene analogue of Efaroxan. *Heterocycles* **2000**, *55*, 387−392.
(16) Kamijo, H.; Takido, T.; Nakazawa, T.; Itabashi, K.; Seno, M. Synthesis of 3-hydroxy-1H-phenalen-1-ones from methylthionaphthalenes with malonic acids. *Nippon Kagaku Kaishi* **1993**, *11*, 1257−1262.
(17) Risitano, F.; Grassi, G.; Foti, F.; Bilardo, C. A convenient synthesis of furo[3,2-c]coumarins by a tandem alkylation/intramolecular aldolization reaction. *Tetrahedron Lett.* **2001**, *42*, 3503−3505.
(18) Nakanishi, Y.; Chang, F.-R.; Liaw, C.-C.; Wu, Y.-C.; Bastow, K. F.; Lee, K.-H. Acetogenins as selective inhibitors of the human ovarian 1A9 tumor cell line. *J. Med. Chem.* **2003**, *46*, 3185.

JM040112R