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ISOLATION, STRUCTURE ELUCIDATION, AND SYNTHESES OF ISONEOCRYPTOTANSHINONE II AND TANSHINLACTONE A FROM *SALVIA MILTIORRHIZA*

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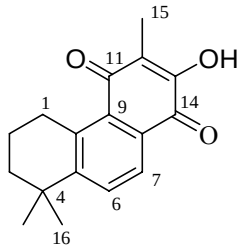
Abstract – Two new components, isoneocryptotanshinone II (**1**) and tanshinlactone A (**2**), were isolated from an EtOH extract of *Salvia miltiorrhiza*. The structures of **1** and **2** were established by spectroscopic methods and total syntheses. Compound (**2**) exhibited moderate cytotoxic activities with a CD_{50} range of 6.87–8.85 μg/mL against the HeLa (cervical epitheloid carcinoma), HepG2 (hepatocellular carcinoma) and OVCAR-3 (ovarian adenocarcinoma) cell lines.

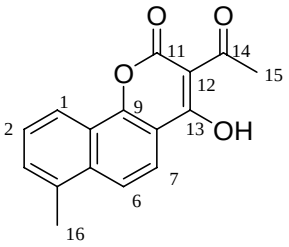
INTRODUCTION

The dried roots of *Salvia miltiorrhiza* under the name Tanshen has been used in traditional Chinese medicine (TCM) for the treatment of hemorrhage, menstrual disorder, miscarriage, and swelling.[1] According to literature reports, a lot of diterpenoid tanshinones were isolated and identified from *S. miltiorrhiza* and many of them were also reported to exhibit diverse biological activities such as antitumor,[2] antioxidant,[3] antimicrobial,[4] antiplatelet aggregation,[5,6] and antiallergic activities.[7] As a part of our continuing interest in bioactive constituents,[8-10] two new components, isoneocryptotanshinone II (**1**) and tanshinlactone A (**2**), were further isolated from the root of *S. miltiorrhiza*. Herein we report the isolation and structure elucidation of **1** and **2** on the basis of spectroscopic data; in addition, compounds

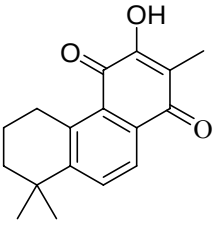
(**1**) and (**2**) were synthesized for spectroscopic data comparison. Compounds (**1**) and (**2**) were also evaluated for their cytotoxic activities against selected cancer cell lines.



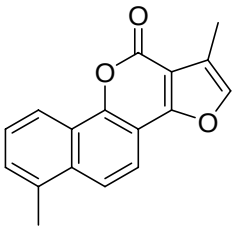
Isoneocryptotanshinone II (**1**)



Tanshinlactone A (**2**)



Neocryptotanshinone II (**3**)



Neotanshinlactone (**4**)

RESULTS AND DISCUSSION

The concentrated EtOH extract of *S. miltiorrhiza* was suspended in H_2O and partitioned successively with EtOAc. The concentrated EtOAc extract was chromatographed on silica gel. Repeated chromatography of the fractions resulted in the isolation of two new components (**1**) and (**2**).

Compound (**1**) was obtained as a yellow solid with a molecular formula of $C_{17}H_{18}O_3$ determined by HR-EIMS spectrum ($[M]^+$, *m*/*z* 270.1257). The ^1H and ^{13}C NMR spectra of **1** were similar to those of neocryptotanshinone II (**3**) which was previously isolated from the same species.[11] The ^1H NMR spectrum ($CDCl_3$) of **1** indicated an AB pattern for two *ortho*-aromatic protons at δ 7.64 (d, *J* = 8.5 Hz) and 7.93 (d, *J* = 8.5 Hz), three methylenes at δ 3.25 (t), 1.76–1.78 (m), and 1.64–1.66 (m), two methyl signals at δ 2.04 (s, 3H) and 1.30 (s, 6H), and a hydroxyl group at δ 7.08 (s). The ^{13}C NMR spectrum of **1** showed two signals at δ 181.5 and 187.9, which revealed the presence of 1,4-quinone moiety. On the basis of the HMBC spectrum, the correlations of H-7 and hydroxyl group to carbonyl group C-14 (δ_C 181.5) as well as methyl (H-15) to carbonyl group C-11 (δ_C 187.9) suggested that the methyl group connected to C-12 and, on the other hand, the hydroxyl group connected to C-13. The complete assignments of the ^1H and ^{13}C NMR spectra of **1** were shown in Table 1, based on the extensive COSY, HMQC, and HMBC spectral data. Thus, the compound was established to have structure (**1**) and was named as isoneocryptotanshinone II.

Compound (**2**) was obtained as a yellow solid with a molecular formula of $C_{16}H_{12}O_4$ determined by HR-EIMS spectrum ($[M]^+$, *m*/*z* 268.0737). The IR spectrum showed absorption for two carbonyl groups at 1728 and 1628 cm^{-1}. The ^1H and ^{13}C NMR spectra of **2** were similar to those of neotanshinlactone (**4**) which was previously isolated from the same species and the structure of **4** was confirmed by total synthesis.[8] The ^1H NMR spectrum ($CDCl_3$) of **2** revealed five aromatic protons at δ 8.41–8.43 (m), 7.95 (d, *J* = 9.0 Hz), 7.84 (d, *J* = 9.0 Hz), and 7.52–7.54 (m, 2H), and two methyls at δ 2.80 (s) and 2.71 (s). In

the EIMS spectrum, a fragmentation peak at *m*/*z* 226 indicated the presence of an acetyl group. One signal at δ_C 160.1 in ^{13}C NMR spectrum of **2** indicated the presence of a lactone ring. The location of carbonyl group of lactone ring connected to either C-9 or C-12 was determined by HMBC spectrum. The observation of the three-bond couplings of acetyl methyl (H-15) to quaternary carbon C-12 (δ_C 101.1) and H-1 to quaternary carbon C-9 (δ_C 153.6) indicated that the location of carbonyl group of lactone ring should be connected to C-12. Because C-9 is connected to the oxygen atom, C-9 was shifted down-field. The complete assignments of the ^1H and ^{13}C NMR spectra of **2** were shown in Table 1, based on the extensive COSY, HMQC, and HMBC spectral data. Thus, the compound was established to have structure (**2**) and was named as tanshinlactone A. The ^1H and ^{13}C NMR spectra of **2** and **4** were very similar to each other except the chemical shifts of acetyl group in **2** and the furan ring in **4**.[8]

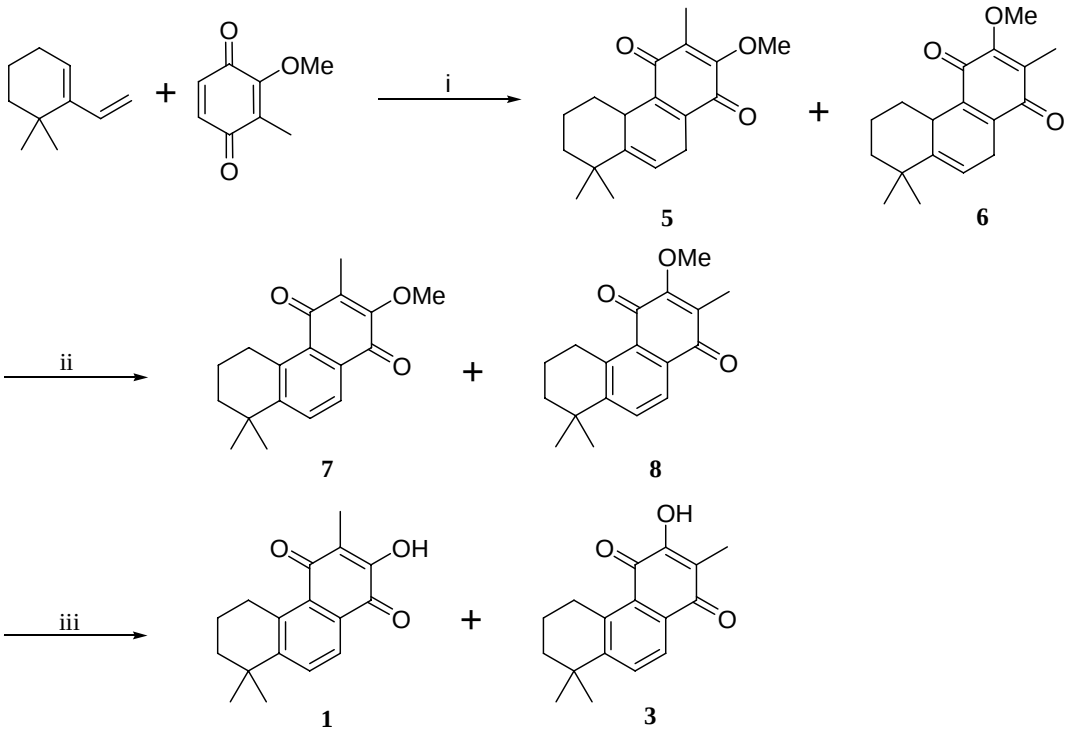
Table 1. NMR spectral Data for **1**, **2** and **3** in $CDCl_3$

	1		**2**		**3**	
position	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)	δ_C
1	3.25 (t, 6.5)	30.1	8.41–8.43 (m)	121.6	3.25 (t, 6.5)	29.9
2	1.76–1.78 (m)	19.4	7.52–7.54 (m)	127.1	1.80–1.85 (m)	19.1
3	1.64–1.66 (m)	37.9	7.52–7.54 (m)	131.3	1.64–1.67 (m)	37.7
4		35.3		134.8		34.8
5		155.6		136.2		152.8
6	7.64 (d, 8.5)	131.2	7.84 (d, 9.0)	120.7	7.72 (d, 8.5)	133.3
7	7.93 (d, 8.5)	124.2	7.95 (d, 9.0)	119.1	7.99 (d, 8.5)	124.9
8		128.6		110.1		132.5
9		130.3		153.6		126.5
10		140.6		122.8		140.9
11		187.9		160.1		182.8
12		121.7		101.1		153.6
13		151.4		178.9		117.8
14		181.5		205.9		185.3
15 (CH_3)	2.04 (s)	9.02	2.80 (s)	30.2	2.04 (s)	8.42
16 (CH_3)	1.30 (s)	31.9	2.71 (s)	19.6	1.31 (s)	31.8
17 (CH_3)	1.30 (s)	31.9			1.31 (s)	31.8
OH	7.08 (s)				7.59 (s)	

The location of methyl and hydroxyl groups is the major difference between **1** and **3.** To confirm the structures of **1** and **3**, total chemical syntheses of **1** and **3** were carried out and the synthetic strategy was shown in Scheme 1.

The starting materials of 6,6-dimethyl-1-vinylcyclohexene and 2-methoxy-3-methyl-1,4-benzoquinone were prepared by known literature procedures.[12,13] Two common literature methods were used for cycloaddition reaction.[14,15] Treatment of 2-methoxy-3-methyl-1,4-benzoquinone and 6,6-dimethyl-1-vinylcyclohexene with boron trifluoride in CH_2Cl_2 at -40 ºC yielded a mixture of cycloadducts (**5**) and (**6**),[14] which were then converted to a mixture of aromatized products (**7**) and (**8**) in 41% yield by reaction

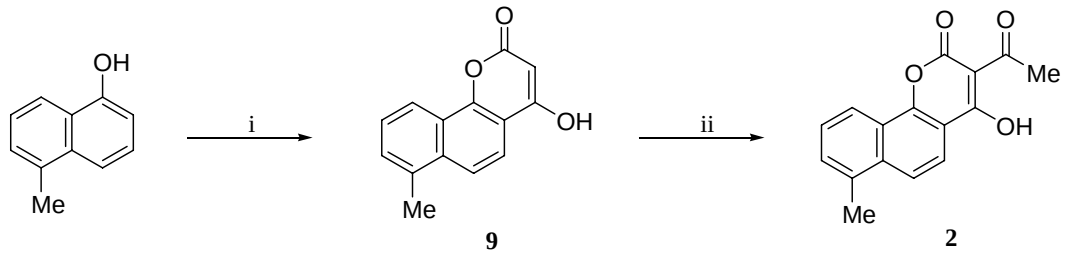
with DDQ in benzene under reflux. The ratio of **7** and **8** was 3.6 : 1 on the basis of methoxy peak in ^{1}H NMR spectrum. When the ultrasound-promoted cycloaddition was carried out with subsequent aromatization with DDQ, a 63% yield of **7** and **8** with poor regioselectivity (1 : 2) was obtained.[15] The regioisomers of **7** and **8** were separated by a reverse-phase C-18 column using 70% acetonitrile in water as eluent. Demethylation with boron trichloride, compounds (**7**) and (**8**) were converted to the target compounds (**1**) and (**3**), respectively.[16] Spectroscopic data of synthesized compound (**1**) were identical with those of natural product (**1**). On the basis of the HMBC spectrum of compound (**3**), the observation of three-bond couplings of aromatic proton H-7 and methyl (H-15) to carbonyl group C-14 (δ_C 185.3) indicated that the methyl group should be connected to C-13 and hydroxyl group should be connected to C-12. The complete assignments of the ^{1}H and ^{13}C NMR spectra of **3** were shown in Table 1, based on the extensive COSY, HMQC, and HMBC spectral data. Thus, compound (**3**) was established to have structure neocryptotanshinone II. The chemical shifts of H-6 and H-7 and carbonyl carbon C-11 and C-14 were the major difference in the ^{1}H and ^{13}C NMR spectral data between **1** and **3** (Table 1).



Scheme 1. Reagents and conditions: (i) $BF_3{\cdot}Et_2O$, CH_2Cl_2 or ultrasound, neat; (ii) DDQ, benzene, reflux; (iii) BCl_3, CH_2Cl_2.

To confirm the unique structure of **2**, a simple synthesis of **2** was carried out and shown in Scheme 2. The starting 5-methyl-1-naphthol was prepared from 2-amino-3-methylbenzoic acid and furan by a known literature procedure.[17] Treatment of 5-methyl-1-naphthol and malonic acid with phosphoryl chloride and zinc chloride yielded 4-hydroxy-7-methylbenzo[*h*]chromen-2-one (**9**) as a yellow solid in 43% yield.[18]

Reaction of **9** with acetic anhydride and DMAP in dichloromethane gave the target product (**2**) in 48% yield.[19] Spectroscopic data of synthesized compound (**2**) were identical with those of natural product (**2**).



Scheme 2. Reagents and conditions: (i) malonic acid, $POCl_3$, $ZnCl_2$, 75 ºC; (ii) Ac_2O, DMAP, CH_2Cl_2.

On the basis of literature reports, many tanshinones represented the anticancer activity.[2,9] Therefore, compounds (**1**, **2**, **3, 7** and **8**) were evaluated for cytotoxic activity against the HeLa (cervical epitheloid carcinoma), HepG2 (hepatocellular carcinoma) and OVCAR-3 (ovarian adenocarcinoma) cell lines by using MTT method and cisplatin as positive control. Unfortunately, compounds (**3**, **7**, and **8**) did not show any cytotoxicity, and **1** showed weak activity. However, compound (**2**) exhibited moderate activities with a CD_{50} range of 6.87–8.85 μg/mL against three cancer cell lines (Table 2).

Table 2. Cytotoxicity (CD_{50}, μg/mL) of **1**, **2**, **3**, **7**, and **8** against human cancer cell lines.

Compound	HeLa	HepG2	OVCAR-3
1	32.2	35.2	32.7
2	7.75	6.87	8.85
3	> 40	> 40	> 40
7	> 40	> 40	> 40
8	> 40	> 40	> 40
Cisplatin	2.41	4.31	7.35

In conclusion, we reported here the isolation and structure elucidation of two new components (**1**) and (**2**) from *S. miltiorrhiza*, and provided a convenient synthetic method for preparation of **1** and **2** in only a few steps from starting materials. Among the tested compounds, **2** showed the best potency against three cancer cell lines.

EXPERIMENTAL

General Experimental Procedures

Melting points were determined with a Yanaco micro-melting point apparatus and are uncorrected. IR spectra were obtained on a Nicolet Avatar 320 FTIR spectrophotometer. NMR spectra were recorded on a Varian Unity Inova-500 spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C). Chemical shifts are

reported in parts per million (δ) units relative to internal tetramethylsilane. The EIMS spectra were measured with direct insertion probe on a Finnigan GCQ spectrometer at 30 eV. HR-EIMS spectral data were recorded on a Finnigan MAT 95S mass spectrometer. An ultrasound cleaner (Branson 5210, 47 kHz, 140 W) was used for ultrasonication. Column chromatography was performed with E. Merck 230–400 mesh silica gel and a Lobar RP-18 (40–63 μm) column. Preparative TLC was carried out on precoated silica gel plates (Merck Art. 113895).

Plant Material

The dried roots of *S. miltiorrhiza* were purchased from a local herbal drug store in Taipei. The plant materials were identified by Mr. Jun-Chih Ou, a former 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 (10 L x 3) at rt for a week. The combined EtOH extracts were concentrated in *vacuo*. The residue was then partitioned between EtOAc and H_2O. The concentrated EtOAc extract (285 g) was subjected to 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 mixtures of *n*-hexane/EtOAc under gradient condition (10 : 1 to 2 : 1) to give tanshinone IIA (1.25 g), cryptotanshinone (520 mg), and tanshinone I (180 mg). The subfraction in front of tanshinone IIA was further purified by preparative TLC using *n*-hexane/EtOAc (6 : 1) as the mobile phase to give **1** [8 mg, R_f = 0.57 (*n*-hexane/EtOAc; 5 : 1)]. The subfraction behind tanshinone I was further purified by preparative TLC using *n*-hexane/EtOAc (3 : 1) as the mobile phase to give **2** [10 mg, R_f = 0.31 (*n*-hexane/EtOAc; 5 : 1)].

Isoneocryptotanshinone II (1)

Yellow solid ($CHCl_3$/*n*-hexane); mp 144–145 ºC; IR (KBr) ν_{max} 3361, 2932, 1649, 1390, 1318, 1281, 1269, 1137, 1084, 1069, 754 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; EIMS *m/z* (%) 270 (100) [M^+], 255 (78), 241 (26), 227 (43), 171 (27); HR-EIMS *m/z* 270.1257 (calcd for $C_{17}H_{18}O_3$, 270.1250).

Tanshinlactone A (2)

Yellow solid ($CHCl_3$/*n*-hexane); mp 177–179 ºC; IR (KBr) ν_{max} 1728, 1628, 1598, 1564, 1484, 1419, 1379, 1193, 1014, 776 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; EIMS *m/z* (%) 268 (100) [M^+], 226 (44), 202 (34), 184 (57), 128 (23); HR-EIMS *m/z* 268.0737 (calcd for $C_{16}H_{12}O_4$, 268.0730).

Syntheses of 5,6,7,8-tetrahydro-2-methoxy-3,8,8-trimethylphenanthrene-1,4-dione (7) and 5,6,7,8-tetrahydro-3-methoxy-2,8,8-trimethylphenanthrene-1,4-dione (8):

Method A. To a solution of 2-methoxy-3-methyl-1,4-benzoquinone (760 mg, 5.0 mmol) in CH_2Cl_2 (50 mL) at -40 ºC was added $BF_3{\cdot}Et_2O$ (2.13 g, 15.0 mmol). After stirring for 20 min, 6,6-dimethyl-1-vinylcyclohex-1-ene (748 mg, 5.5 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was continuously stirred at -40 ºC for 3 h. Water was added and the reaction mixture was then warmed to rt. The resulting mixture was extracted with CH_2Cl_2 (100 mL) three times. The combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered, and evaporated. The residue was purified by silica gel chromatography eluting with 5% EtOAc in hexane to give the mixture of **5** and **6** as a yellow oil (1.02 g). Without separation, DDQ (545 mg, 2.4 mmol) was added to the mixture of **5** and **6** in benzene (20 mL) under nitrogen. The reaction mixture was then refluxed for 12 h. After cooling, the reaction mixture was filter through celite. The filtrate was then concentrated and the resulting residue was subjected to a silica gel chromatographic column and then eluted with 5% EtOAc in hexane to give the mixture of **7** and **8** (583 mg, 41%, **7** : **8**, 3.6 : 1). Pure **7** and **8** was obtained by chromatography on a RP-18 column eluting with 70 % acetonitrile in water. Compound (**7**) is a yellow solid (EtOAc/*n*-hexane); mp 66–67 ºC; IR (KBr) ν_{max} 2928, 2859, 1660, 1638, 1579, 1457, 1312, 1263, 1239, 1141, 1095, 998 cm^{-1}; ^1H NMR ($CDCl_3$), δ 1.30 (s, 6H), 1.64–1.66 (m, 2H), 1.75–1.80 (m, 2H), 2.03 (s, 3H), 3.22 (t, *J* = 6.5 Hz, 2H), 4.03 (s, 3H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.90 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 9.66, 19.4, 30.0, 31.9, 35.1, 37.9, 60.7, 124.2, 129.7, 130.8, 131.5, 133.2, 139.7, 154.0, 156.1, 181.6, 188.4; EIMS *m/z* (%) 284 (100) [M^+], 269 (65), 255 (50), 241 (60). Compound (**8**) is a yellow oil; IR (KBr) ν_{max} 2930, 2866, 1663, 1650, 1566, 1455, 1311, 1267, 1201, 1162, 1138, 1098, 991 cm^{-1}; ^1H NMR ($CDCl_3$), δ 1.29 (s, 6H), 1.65–1.67 (m, 2H), 1.76–1.79 (m, 2H), 2.01 (s, 3H), 3.20 (t, *J* = 6.5 Hz, 2H), 4.00 (s, 3H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.91 (d, *J* = 8.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 8.82, 19.3, 29.9, 31.9, 34.9, 37.9, 60.7, 124.2, 129.0, 129.2, 131.3, 132.0, 139.7, 153.3, 159.1, 183.5, 186.2; EIMS *m/z* (%) 284 (100) [M^+], 269 (60), 255 (30), 241 (45).

Method B. A mixture of 2-methoxy-3-methyl-1,4-benzoquinone (152 mg, 1.0 mmol) and 6,6-dimethyl-1-vinylcyclohex-1-ene (408 mg, 3.0 mmol) was placed in a test tube and subjected to ultrasonication at 45 ºC for 6 h. The mixture was purified by silica gel chromatography eluting with 5% EtOAc in hexane to give the mixture of **5** and **6** as yellow oil (230 mg). Without separation, DDQ (545 mg, 2.4 mmol) was added to the mixture of **5** and **6** in benzene (20 mL) under nitrogen. The reaction mixture was then refluxed for 12 h. After cooling, the reaction mixture was filter through celite. The filtrate was then concentrated and the resulting residue was subjected to a silica gel chromatographic column and then eluted with 5% EtOAc in hexane to give a mixture of **7** and **8** (178 mg, 63%, **7** : **8**, 1 : 2).

Synthesis of isoneocryptotanshinone II (1)

To a solution of **7** (375mg, 1.32 mmol) in CH_2Cl_2 (20 mL) at -78 ºC was added BCl_3 (1.0 M in CH_2Cl_2,

1.6 mL) and the mixture was stirred for 2 h. Water was added and the reaction mixture was extracted with CH_2Cl_2 three times. The combined organic layers were washed with water, dried over Na_2SO_4, filtered, and evaporated. The residue was purified by silica gel chromatography eluting with 10% EtOAc in hexane to give **1** (293 mg, 82%) as a yellow solid. The ^1H and ^{13}C NMR, mp, and EIMS spectral data of synthetic compound (**1**) were identical with those of the natural product isoneocryptotanshinone II.

Synthesis of neocryptotanshinone II (3)

Compound (**3**) was prepared using the same procedure as for **1** starting from compound (**8**), and was obtained in 85% yield as a yellow solid: mp 153–154 ºC ($CHCl_3$/*n*-hexane); IR (KBr) ν_{max} 3337, 2925, 1654, 1630, 1565, 1375, 1349, 1325, 1204, 1081, 754 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; EIMS *m/z* (%) 270 (100) [M^+], 255 (56), 241 (39), 227 (41), 171 (20).

4-Hydroxy-7-methyl-benzo[*h*]chromen-2-one (**9**)

To a mixture of 5-methyl-1-naphthol (1.0 g, 6.33 mmol), malonic acid (659 mg, 6.33 mmol), and $ZnCl_2$ (2.15 mg, 15.8 mmol) was added $POCl_3$ (1.7 mL, 18.3 mmol). The mixture was heated at 75 ºC for 24 h. The black residue was shaken with ice water. The solid was filtered, washed with water and dissolved in hot saturated aqueous $NaHCO_3$ solution. The basic solution was filtered and the filtrate was carefully acidified with 1% sulfuric acid. The precipitate was then filtered and air dried. The crude residue was recrystallized from methanol to yield **9** (620 mg, 43%) as a yellow solid, which was used in the next step without further purification: ^1H 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); ^{13}C NMR (DMSO-d_6) δ 19.2, 90.7, 110.9, 118.8, 119.9, 120.1, 122.4, 127.1, 129.5, 133.9, 134.7, 151.0, 162.0, 166.7.

Synthesis of tanshinlactone A (2)

To a solution of **9** (50 mg, 0.22 mmol) in dry CH_2Cl_2 (15 mL) was added DMAP (35 mg, 0.29 mmol) and Ac_2O (224 mg, 2.2 mmol). After the mixture was stirred at rt for 48 h, the mixture was then concentrated and the residue was subjected to column chromatography eluting with 10% EtOAc in hexane to give **2** (28 mg, 48%) as a yellow solid. The ^1H and ^{13}C NMR, mp, and EIMS spectral data were identical with those of the natural product tanshinlactone A.

Cytotoxicity assay. The cell line culture conditions[20] and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay for CD_{50} were carried out according to the procedures previously described.[21]

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