Determination of tanshinones in Danshen (*Salvia miltiorrhiza*) by high-performance liquid chromatography with fluorescence detection after pre-column derivatization

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Abstract

Introduction - Tanshinones are a major class of bioactive ingredients in the traditional herbal medicines, Danshen (*Salvia miltiorrhiza*). A sensitive and reliable determination method for tanshinones is useful to ensure the quality of Danshen.

Objective – To develop a sensitive and selective analytical method for tanshinones by high-performance liquid chromatography (HPLC) with fluorescence detection after pre-column derivatization.

Methodology - The proposed method depends on derivatization reaction of tanshinones with 4-carbomethoxybenzaldehyde and ammonium acetate forming intensely fluorescent imidazole derivative.

Results - The proposed method provided excellent sensitivity with the detection limits of 3.3 nM (66 fmol/injection), 3.2 nM (64 fmol/injection) and 2.0 nM (40 fmol/injection) for cryptotanshinone, tanshinone I and tanshinone IIA, respectively, without necessity of complicated instrumentations. The developed method is successfully applied to quantify the contents of tanshinones in Danshen.

Conclusion - The developed method is the first analytical method for tanshinones by fluorescence detection. Since the derivatization reaction is selective for the *o*-quinone structure of tanshinone, the developed method will become a suitable mean for the discovering of tanshinone type diterpenoids from herbal samples.

Keywords: tanshinone; *Salvia miltiorrhiza*; high-performance liquid chromatography; fluorescence detection; fluorogenic derivatization.

Short abstract

We present a sensitive and selective method for the quantification of tanshinones by HPLC with fluorescence detection after the pre-column derivatization. The proposed method depends on derivatization reaction of tanshinones to form highly fluorescent imidazole derivative with 4-carbomethoxybenzaldehyde and ammonium acetate as reagents. The contents of cryptotanshinone, tanshinone I and tanshinone IIA were successfully determined in Danshen by the developed method.

Introduction

Danshen (*Salvia miltiorrhiza*) is among the most widely used traditional herbal medicines. It has been usually used for the cure of cardio- and cerebrovascular diseases and hyperlipidemia (Zhou *et al.*, 2005; Deng *et al.*, 2014; Su et al., 2015). Tanshinones, including cryptotanshinone, tanshinone I and tanshinone IIA (Fig. 1), are a major class of bioactive ingredients in Danshen (Cao *et al.*, 2016a). It has been reported that tanshinones exhibit several beneficial effects on human health such as antioxidant activity, anti-inflammatory, improvement of blood circulation and inhibition of amyloid aggregation (Niu *et al.*, 2000; Fan *et al.*, 2009; Li *et al.* 2008; Wang *et al.*, 2013). Since tanshinones contribute to therapeutic effects of Danshen, a sensitive and reliable simultaneous determination method for tanshinones is essential to ensure the quality of Danshen (Zhou *et al.*, 2006). In addition, selective detection method for tanshinones should be useful to discover new bioactive components having tanshinone structure existing in Danshen.

Many chromatographic methods have been described for simultaneous assay of tanshinones: micellar electrokinetic chromatography with ultraviolet (UV) detection (Cao *et al.*, 2016b), high-performance liquid chromatography (HPLC) with UV detection (Chang *et al.*, 2008; Wang *et al.*, 2014; Wang *et al.*, 2015), electrochemical detection (ECD) (Chen *et al.*, 2012b; Chen *et al.*, 2013) and LC with mass spectrometry either with electrospray ionizaion mass spectrometry (LC-ESI-MS) (Chen *et al.*, 2012a), LC with tandem mass spectrometry (LC-MS/MS) (Xu *et al.*, 2016; Zhang *et al.*, 2016) or LC with quadrupole time-of flight tandem mass spectrometry (LC-Q-TOF-MS/MS) (Zhou *et al.*, 2009). However, HPLC-UV methods does not have adequate selectivity

and sensitivity to detect trace levels of tanshinones. While HPLC-ECD methods exhibit greater sensitivity than HPLC-UV methods, they require moderately complicated instruments such as multi electrode arrays. Additionally, the electrode of ECD can be easily deactivated by repeated sample injections. LC-MS based methods can provide good sensitivity and excellent selectivity, but these involve complex and costly instrumentations which are not probably available in all laboratories. While, fluorescence detection can provide high sensitivity with simple instrumentation. However, since tanshinones do not have intrinsic fluorescence themselves, direct fluorescence detection of tanshinones is difficult.

Previously, we designed an HPLC method with high sensitivity and selectivity for the assay of quinones such as 9,10-phenanthrenquinone after pre-column fluorogenic derivatization (Kishikawa *et al*., 2004; Kishikawa *et al*., 2011). This method depends on conversion of quinone to intensely fluorescent imidazole derivative via the reaction with aromatic aldehyde and ammonium acetate. Since tanshinones are diterpenoid quinones, we considered that they could be converted to fluorescent derivatives by this reaction in a similar way to 9,10-phenanthrenequinone. In this study, we developed an HPLC method for simultaneous determination of tanshinones using 4-carbomethoxybenzaldehyde and ammonium acetate as derivatization reagents, and the content of tanshinones was determined in Danshen via the developed method.

Experimental

Material and reagents

Tanshinone IIA, 4-carbomethoxybenzaldehyde, acetic acid and 9,10-phenanthrenequinone (an internal standard, IS) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Cryptotanshinone and tanshinone I were from Funakoshi Co. (Tokyo, Japan). Benzaldehyde and 4-methoxybenzldehyde were from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Methanol (HPLC grade) was from Kanto Chemical (Tokyo). Distilled water was obtained by using Auto Still WG 203 water device (Yamato Scientific Co. Ltd., Tokyo). Other chemicals were of extra pure grade. Danshen was purchased from Uchida Wakanyaku Co. Ltd. (Tokyo), and the tested Danshen (lot no. DOK0324) was harvested in Sichuan (China) in November 2014. 4-Carbomethoybenzaldehyde and ammonium acetate were prepared in methanol and acetic acid, respectively, just before the derivatization reaction. Also, acetic acid was used to catalyze the formation reaction of arylimidazole derivative (El-Maghrabey *et al*., 2015).

Fluorescence derivatization and measurement procedures

To 100 μL of methanol solution of tanshinones, 100 μL each of 0.3 M 4-carbomethoxybenzaldehyde in methanol and 1.6 M ammonium acetate in acetic acid were successively added. This solution was heated at 100 ºC for 30 min. Fluorescence spectra of the reaction product were measured by a Shimadzu (Kyoto) RF-1500 spectrofluorometer after 20 times dilution with methanol. For HPLC analysis, 20 μL of the reaction mixture was injected into the HPLC system following filtration with 0.45 μm membrane filter.

HPLC system and conditions

The chromatographic system comprised of a Shimadzu LC-10AT pump, a Rheodyne (Cotati, CA, USA) 7125 injector with loop size of 20-μL, a Shimadzu RF-10A fluorescence detector, and an EZChrom Elite chromatography data acquisition system (Scientific Software, Pleasanton, CA, USA). The separation was achieved on a DAISO-PAK SP-120-5-ODS-AP (250 mm x 4.6 mm, i.d., DAISO Co., Ltd., Osaka) using isocratic elution with methanol:water mixture (90:10, v/v%) at a flow rate of 1.0 mL/min at ambient temperature. The fluorescence detector wavelengths were set at 375 nm for excitation and at 515 nm for emission.

Extraction procedure for tanshinones from Danshen

Fifty mg of Danshen was weighed into a test tube after sieving. To the test tube, 10 mL of methanol was added as an extraction solvent. Tanshinones were extracted by an ultrasonication for 20 min at ambient temperature. After centrifugation, 50 μL of supernatant was collected followed by 20 times dilution with methanol. Then, the derivatization procedure was applied to the diluted solution.

Results and discussion

Fluorescence characteristics of tanshinone derivative

At first, the fluorescence spectra of the tanshinone derivatives prepared by reaction with three types of aromatic aldehydes including benzaldehyde, 4-carbomethoxybenzaldehyde and 4-methoxybenzaldehyde were scanned. Figure S1 shows the fluorescence spectra obtained from cryptotanshinone after derivatization with

4-carbometoxybenzaldehyde. Although cryptotanshinone does not have fluorescence (Fig. S1A), the formed derivative emits ingtense fluorescence. Table 1 lists the optimum excitation and emission wavelengths and the relative fluorescence intensity obtained from the reaction mixture of tanshinones. Among the tested aromatic aldehydes, 4-carbomethoxybenzaldehyde derivatives provided highest fluorescence intensity at longest wavelength regions. This result is good accordance with our previous results for the fluorescence derivatization of 9,10-phenanthrenequinone (Kishikawa *et al.*, 2011). Therefore, we selected 4-carbomethoxybenzaldehyde as the derivatization reagent for further studies.

Identification of fluorescent tanshinone derivative.

Figure 2 shows typical chromatograms of a standard solution of tanshinones after the derivatization and the reagent blank. The peaks of the derivatives of cryptotanshinone, tanshinone I, tanshinone IIA and IS were detected at a retention time of 23, 24, 29 and 8 min, respectively (Fig. 2A). While the reagent blank does not have significant fluorescence since both 4-carbometxoybenzaldehyde and ammonium acetate are non-fluorescent (Fig. 2B). So as to explain the structures of the fluorescent derivative, the peak fraction of each derivative was analyzed by a Quattro Micro mass spectrometer (Waters, Milford, MA, USA) with positive electrospray ionization (ESI). As shown in the ESI-MS spectra (Fig. S2), the abundant ion peaks for cryptotanshinone, tanshinone I, tanshinone IIA derivatives were found at *m/z* 441, 421 and 439, respectively. These peaks are corresponding to the fluorescent imidazole derivatives. In addition, we examined ^{1}H-NMR study for synthesized cryptotanshinone derivative. The obtained data (Supplementary information, Figure S3) also suggested the formation

of imidazole derivative. The formation of imidazole derivative was also confirmed in the reaction between 9,10-phenanthrenequinone and aromatic aldehyde (Kishikawa *et al.*, 2011). Therefore, the derivatization reaction was proposed to occur as shown in Fig. 3.

Optimization of derivatization reaction conditions

To achieve higher sensitivity, optimization study of the derivatization conditions was conducted using standard solution of tanshinones. The concentration of 4-carbometoxybenzaldehyde was optimized in the range of 0.05-0.5 M. The highest and constant peak areas were attained using more than 0.2 M (Fig. 4A); 0.3 M was selected as optimal reagent concentration. The concentration of ammonium acetate was investigated in the range of 0.5-2.0 M. The peak area reached the maximum using 1.6 M ammonium acetate then slightly declined (Fig. 4B); accordingly, 1.6 M ammonium acetate was used for further studies. Increasing the reaction temperature caused substantial increase in the peak area, thus, 100 °C was selected to be the best reaction temperature. Heating for more than 20 min yielded the highest and constant peak areas (Fig. 4C); 30 min was selected as optimal reaction time.

Calibration curve, detection limit and repeatability

Calibration curves were constructed as the peak area ratio of tanshinone to IS against tanshinones concentration. The, regression equations, linear ranges, correlation coefficients (r^2) and limits of detection (LODs) were abridged in Table 2. Good linearity between concentrations and peak area ratios was confirmed in the range of 0.005 to 2.5 μM with r^2 more than 0.999. The LODs (S/N = 3) for cryptotanshinone,

tanshinone I and Tanshinone IIA were 3.3 nM (66 fmol/injection), 3.2 nM (64 fmol/injection) and 2.0 nM (40 fmol/injection), respectively. As summarized in Table 3, the sensitivity of the developed method was 22-124 times greater than those of HPLC-UV methods, 5-37 times greater than those of HPLC-ECD methods, 15-17 times greater than those of the LC-MS method, 5.5-26 times higher than those of the LC-Q-TOF-MS method and 2-3.4 times higher than those of the LC-MS/MS method reported by Xu *et al*. (2016). Although the proposed method was less sensitive when compared to the LC-MS/MS method reported by Zhang *et al*. (2016), excellent sensitivity could be achieved without requiring complex and expensive instrumentations in contrast to the LC-MS/MS analysis.

Examination of the method reproducibility was done using three different concentrations of tanshinones (0.025, 0.25 and 2.5 μM). The values of relative standard deviation (%RSD) express the precision. %RSD values for intra-day (n = 5) and inter-day (n = 5) precision were 2.0-8.5% and 1.7-9.4%, respectively (Table S1). It was confirmed that the proposed method had sufficient reproducibility.

Determination of tanshinones in Danshen

Determination of tanshinones in the extract from Danshen was accomplished using the developed method. Tanshinones were extracted ultrasonically with methanol according to the previous report (Zhang *et al*., 2016). A chromatogram of the extract after the derivatization is shown in Fig. 5A. The peaks of cryptotanshinone, tanshinone I and tanshinone IIA derivative were clearly detected in the chromatogram. The recoveries of the developed method were assessed using Danshen spiked with 0.84, 4.2 and 21 μmol/g of tanshinones. As shown in Table 4, the proposed method

provided high recoveries between 96.4-108% with RSD less than 6.6% (n=4). The concentrations of cryptotanshinone, tanshinone I and tanshinone IIA found in the tested sample were 3.98, 2.15 and 10.60 μmol/g, respectively. These values were in accordance with those stated in the earlier studies (Chen *et al*., 2012b; Zhang *et al*., 2016). Furthermore, we determined the concentrations of these tanshinones in the same Danshen sample by the reported HPLC-UV method (Chang *et al*., 2008). The concentrations of cryptotanshinone, tanshinone I and tanshinone IIA obtained by the reference method were 3.90, 2.17 and 10.36 μmol/g, respectively. Since the determined values obtained by the proposed method were not significantly different with those attained by the reference method, the reliability of the developed method was proven. On the other hand, in addition to cryptotanshinone, tanshinone I and tanshinone IIA, two significant peaks were detected at 17 min (peak 4 in Fig. 5A) and 27 min (peak 5 in Fig. 5A). Since these peaks were disappeared without the derivatization reaction in the same manner as the three tanshinones (Fig. 5B), it could be considered that these peaks were also derived from tanshinones in Danshen. When the fractions of peak 4 and 5 were subjected to ESI-MS analysis, these fractions gave the same abundant ion peak at m/z at 423.1. Therefore, it could be suggested that these peaks were derived from dihydrotanshinones such as 15,16-dihydrotanshinone I and 1,2-dihydrotanshinone. Considering higher hydrophilicity of 15,16-dihydrotanshinone I (log*P* = 3.677) compared to 1,2-dihydrotanshinone (log*P* = 4.351), the peak 4 detected at 17 min and peak 5 detected at 27 min could be attributed to 15,16-dihydrotanshinone I and 1,2-dihydrotanshinone, respectively. In this way, the dveloped method would be beneficial to discover the presence of unknown tanshinones in natural samples, in addition to the determination of tanshinone contents for the

quality assessment of traditional herbal medicines.

Conclusion

Tanshinone could form highly fluorescent imidazole derivative after reaction with 4-carbomethoxybenzaldehyde and ammonium acetate. Depending on this finding, a sensitive and selective method for the determination of tanshinones by HPLC-fluorescence detection after the pre-column derivatization was developed. Application of the developed method to determine contents of three tanshinones in Danshen was successfully achieved. The present study is the first effort to determine tanshinones by fluorescence detection. The developed method would be suitable to determine tanshinone contents in traditional medicines because of its high sensitivity with simple instrumentations. Additionally, since the derivatization reaction is selective for the *o*-quinone structure of tanshinones, the developed method will become a promising tool for the discovery of tanshinone type diterpenoids from herbal samples.

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Table 1. Fluorescence characteristics of the reaction mixture of tanshinones with aromatic aldehydes in the presence of ammonium acetate

Aromatic aldehyde	Tanshinone	λex, nm	λem, nm	RFI[a]
Benzaldehyde	Cryptotanshinone	295	395	17
	Tanshinone I	290	375	40
	Tanshinone IIA	290	370	27
4-Carbomethoxybenzaldehyde	Cryptotanshinone	375	515	25
	Tanshinone I	320	455	100
	Tanshinone IIA	355	475	94
4-Methoxybenzaldehyde	Cryptotanshinone	350	395	38
	Tanshinone I	315	375	91
	Tanshinone IIA	320	370	60

[a]Fluorescence intensity of the reaction mixture of cryptotanshinone with 4-carbomethoxybenzaldehyde was taken as 100

Table 2. Calibration curves and limits of detection for tanshinones

Tanshinone	Calibration curve[a]				LOD[b], nM (fmol/injection)
	Range, μM	Slope[c]	Intercept[c]	*r*	
Cryptotanshinone	0.005-2.5	2.77 ± 0.07	-0.02 ± 0.01	0.999	3.3 (66)
Tanshinone I	0.005-2.5	4.18 ± 0.13	-0.08 ± 0.02	0.999	3.2 (64)
Tanshinone IIA	0.005-2.5	5.12 ± 0.19	-0.02 ± 0.03	0.999	2.0 (40)

[a]Peak area ratio versus concentration.

[b]LOD at a S/N ratio of 3.

[c]Data presented as mean ± standard error of three experiments.

Table 3. Comparison of sensitivity of the proposed method with previous methods

Method	LOD, nM			References
	Cryptotanshinone	Tanshinone I	Tanshinone IIA	
HPLC-UV	169	182	85.0	Chang *et al*., 2008
HPLC-UV	250	69.3	255	Wang *et al*., 2014
HPLC-UV	314	188	248	Wang *et al*., 2015
HPLC-ECD	26.4	23.8	74.0	Chen *et al*., 2012b
HPLC-ECD	17.2	42.4	25.1	Chen *et al*., 2013
LC-MS	55.1	48.9	34.7	Chen *et al*., 2012a
LC-Q-TOF-MS	18.2	43.5	51.0	Zhou *et al*., 2009
LC-MS/MS	6.8	-[a]	6.8	Xu *et al*., 2016
LC-MS/MS	0.007	0.007	0.001	Zhang *et al*., 2016
HPLC-FL	3.3	3.2	2.0	Proposed method

[a] not analyzed

Table 4. Recovery of the proposed method for tanshinones in Danshen

Tanshinone	Added, μmol/g	Found, μmol/g	Recovery%[a]	RSD% (n = 4)
Cryptotanshinone	0	3.98	-	2.1
	0.84	4.82	100	6.6
	4.20	8.07	97.3	4.5
	21.0	26.5	107	1.5
Tanshinone I	0	2.15	-	3.0
	0.84	2.99	99.3	3.8
	4.20	6.20	96.4	2.8
	21.0	22.6	97.4	3.7
Tanshinone IIA	0	10.6	-	2.5
	0.84	11.4	102	6.5
	4.20	14.8	102	6.2
	21.0	33.3	108	1.8

[a]Recovery was calculated as [(found amount)-(original amount)]/(added amount)×100.

Figure captions

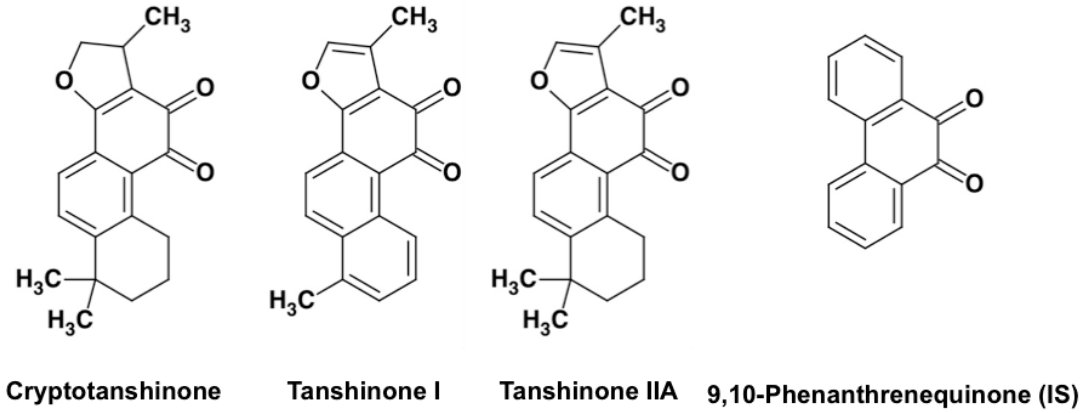
Figure 1. Chemical structures of tanshinone and 9,10-phenanthrenequinone (IS).

Figure 2. Chromatograms of (A) 0.25 μM standard solution of tanshinones after derivatization and (B) reagent blank. Peaks: 1, cryptotanshinone; 2, tanshinone IIA; 3, tanshinone I; IS, 9,10-phenanthrenequinone.

Figure 3. Fluorescence derivatization reaction of cryptotanshinone with 4-carbomethoxybenzaldehyde in the presence of ammonium acetate.

Figure 4. Effects of (A) 4-carbomethoxybenzaldehyde concentration, (B) ammonium acetate concentration and (C) reaction time on the peak area of fluorescent derivative.

Figure 5. Chromatograms of the methanol extract from Danshen (A) with and (B) without of the derivatization reaction. Peaks: 1, cryptotanshinone; 2, tanshinone IIA; 3, tanshinone I; IS, 9,10-phenanthrenequinone; 4 and 5, unknown fluorescent peaks.



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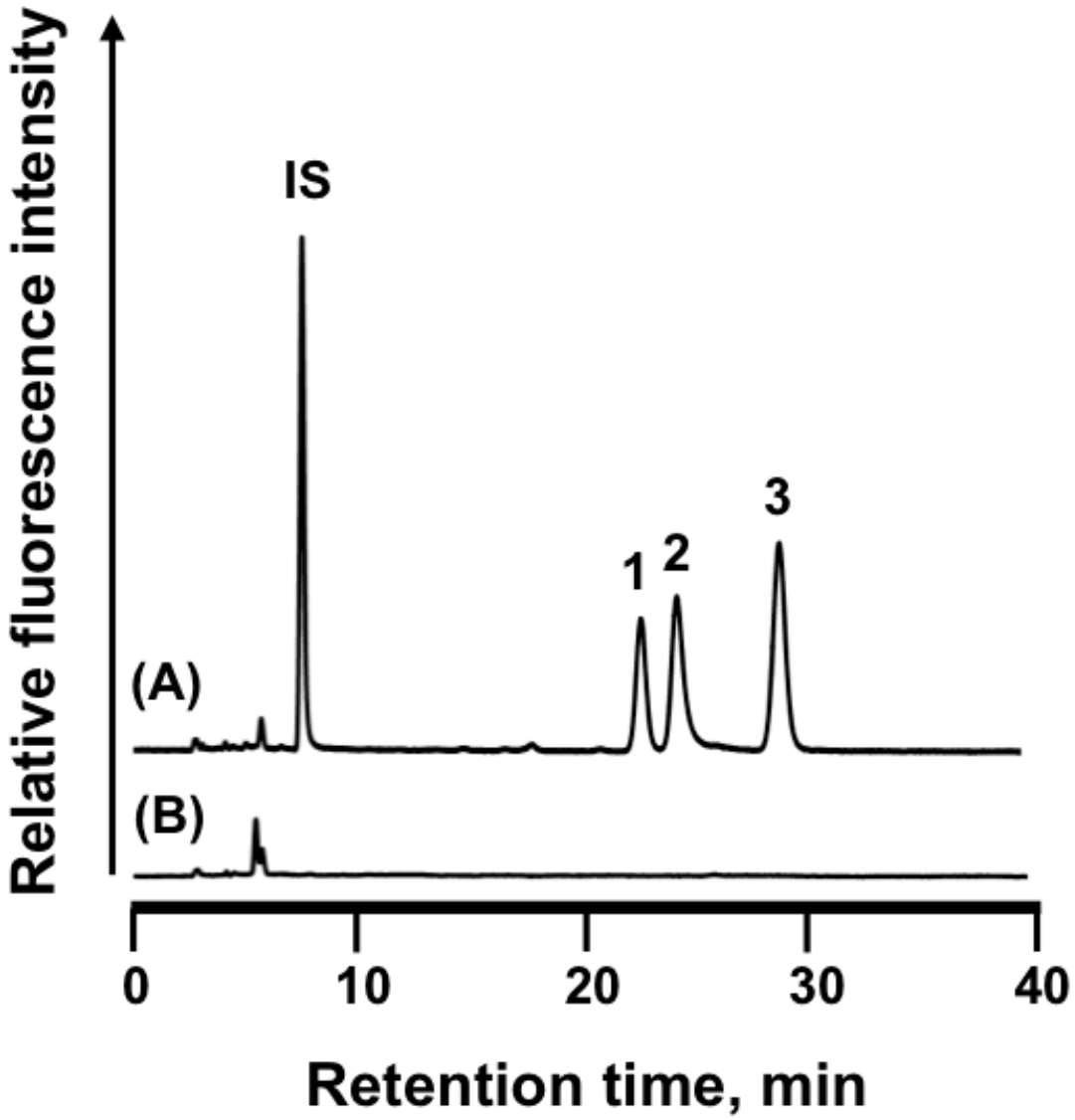

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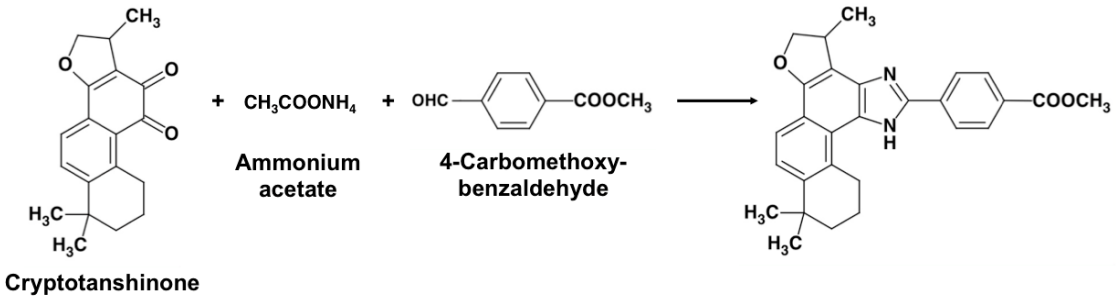

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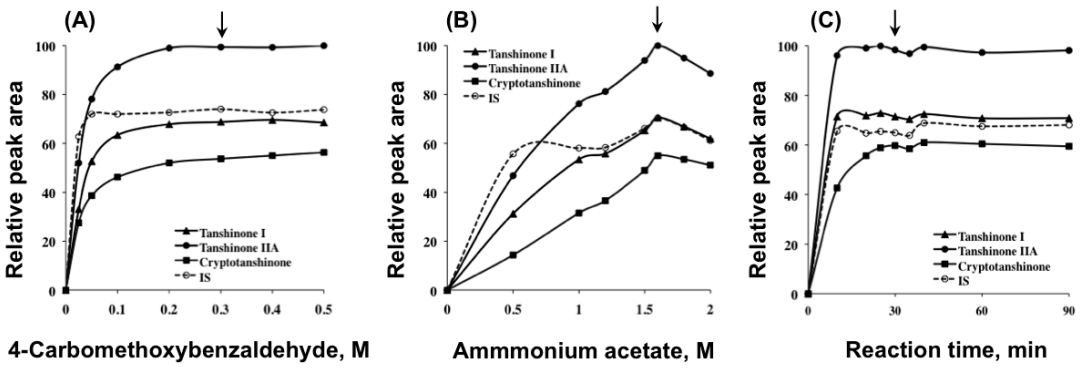

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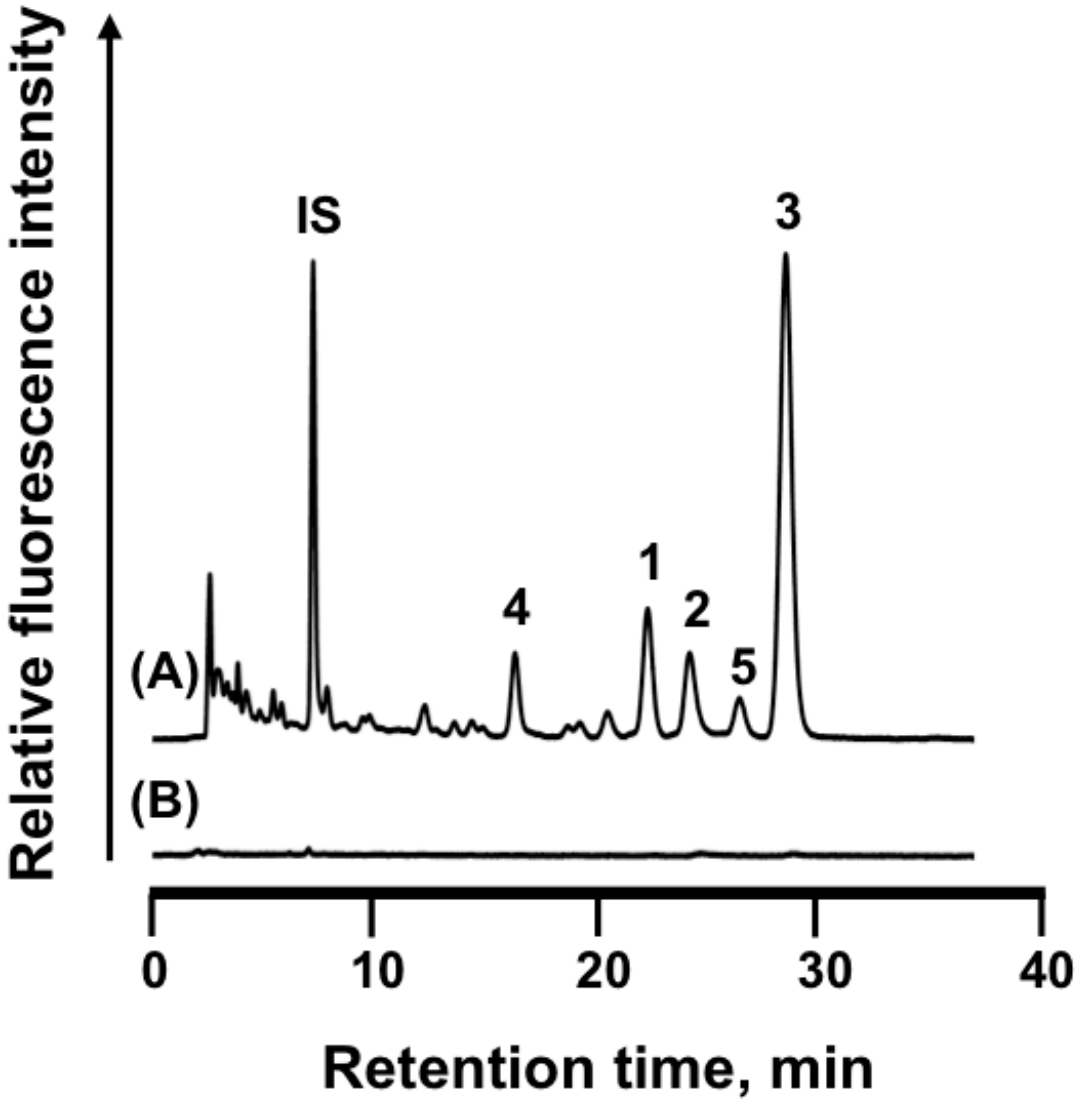

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Supporting Information

Table S1. Intra-day and inter-day precision of the proposed method for analysis of tanshinones

Tanshinone	Concentration, μM	Precision (RSD%)	
		Intra-day (n = 5)	Inter-day (n = 5)
Cryptotanshinone	0.025	8.5	8.6
	0.25	2.1	3.4
	2.5	2.3	3.9
Tanshinone I	0.025	6.6	9.4
	0.25	2.0	2.8
	2.5	2.4	2.5
Tanshinone IIA	0.025	4.3	6.5
	0.25	2.3	1.7
	2.5	2.3	2.6

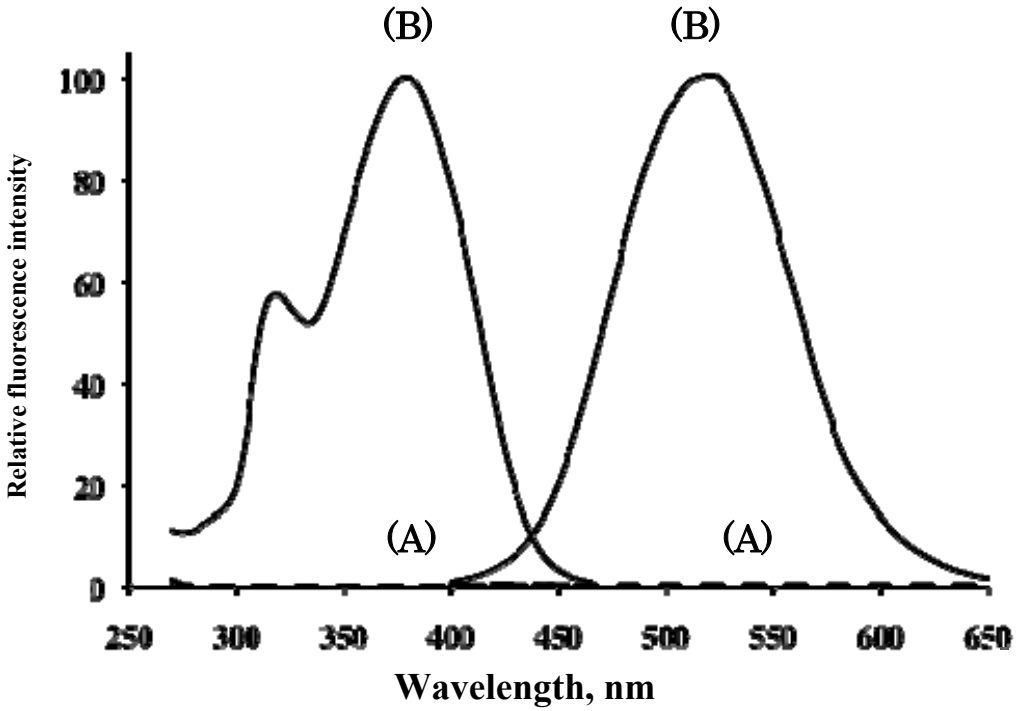
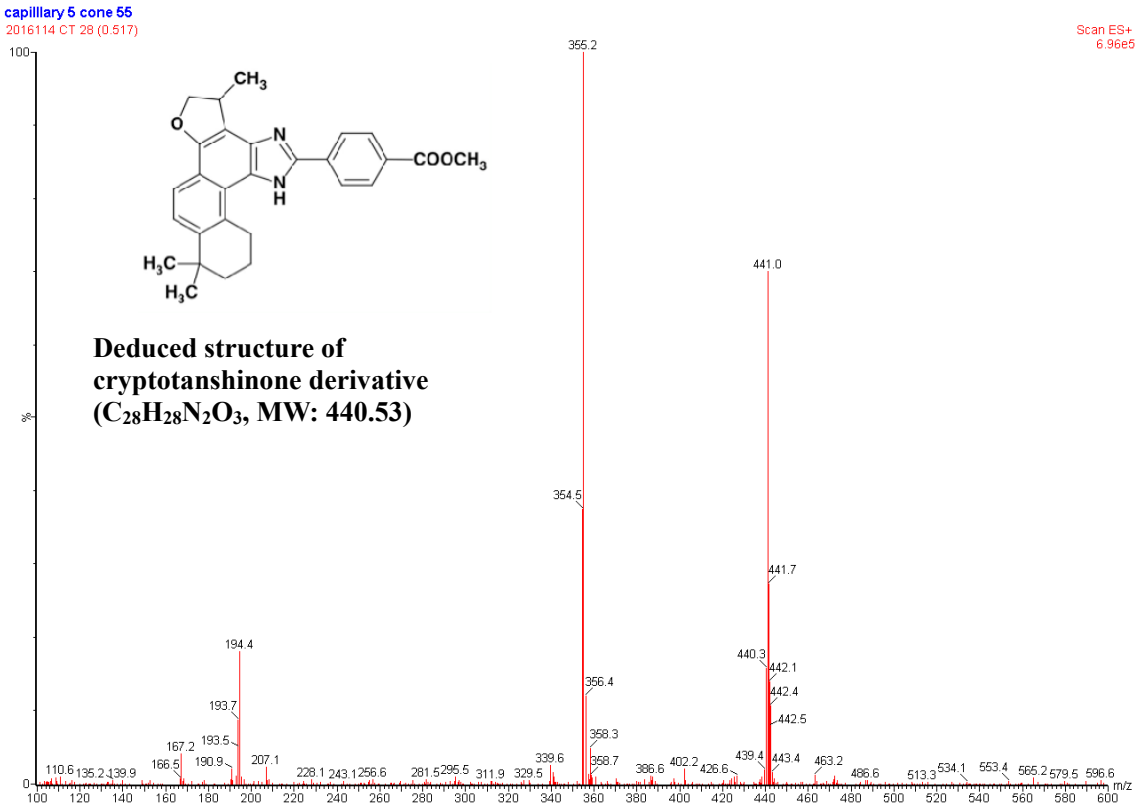


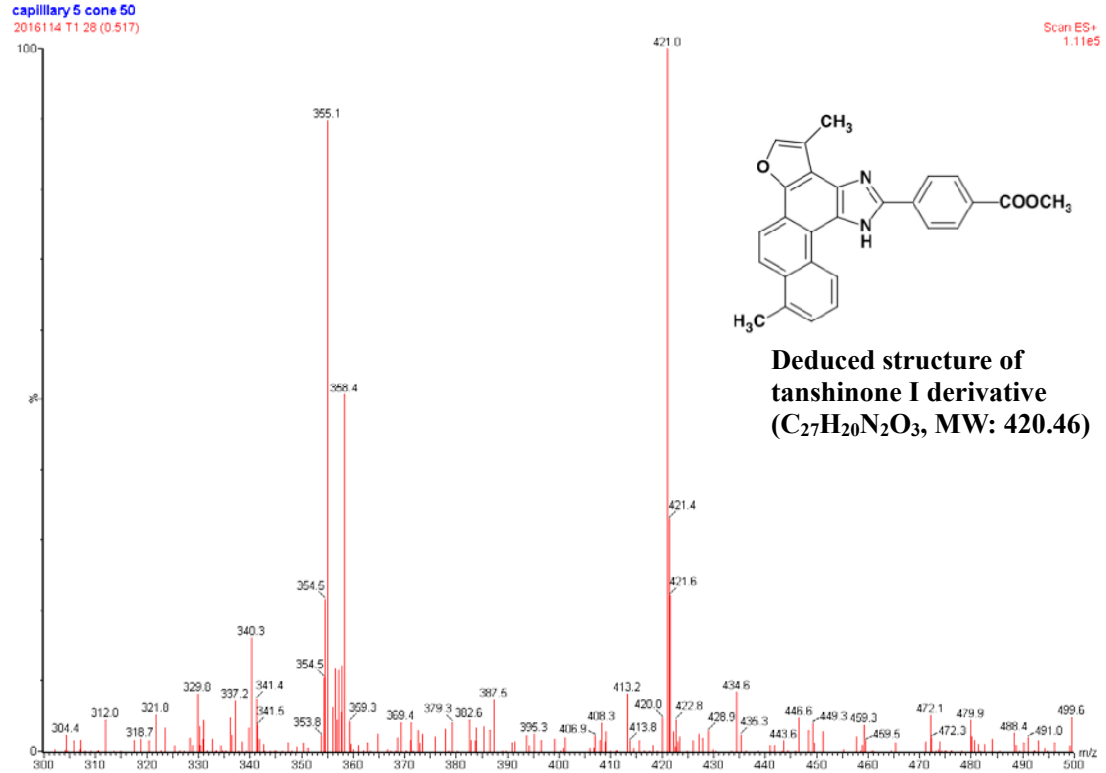
Figure S1. Excitation and emission spectra of 5 μM cryptotanshinone (A, dashed line) before and (B, solid line) after reaction with 4-carbomethoxybenzaldehyde in the presence of ammonium acetate. Excitation and emission wavelengths were 375 nm and 515 nm, respectively.

(A)



Deduced structure of cryptotanshinone derivative ($C_{28}H_{28}N_2O_3$, MW: 440.53)

(B)



Deduced structure of tanshinone I derivative ($C_{27}H_{20}N_2O_3$, MW: 420.46)

(C)

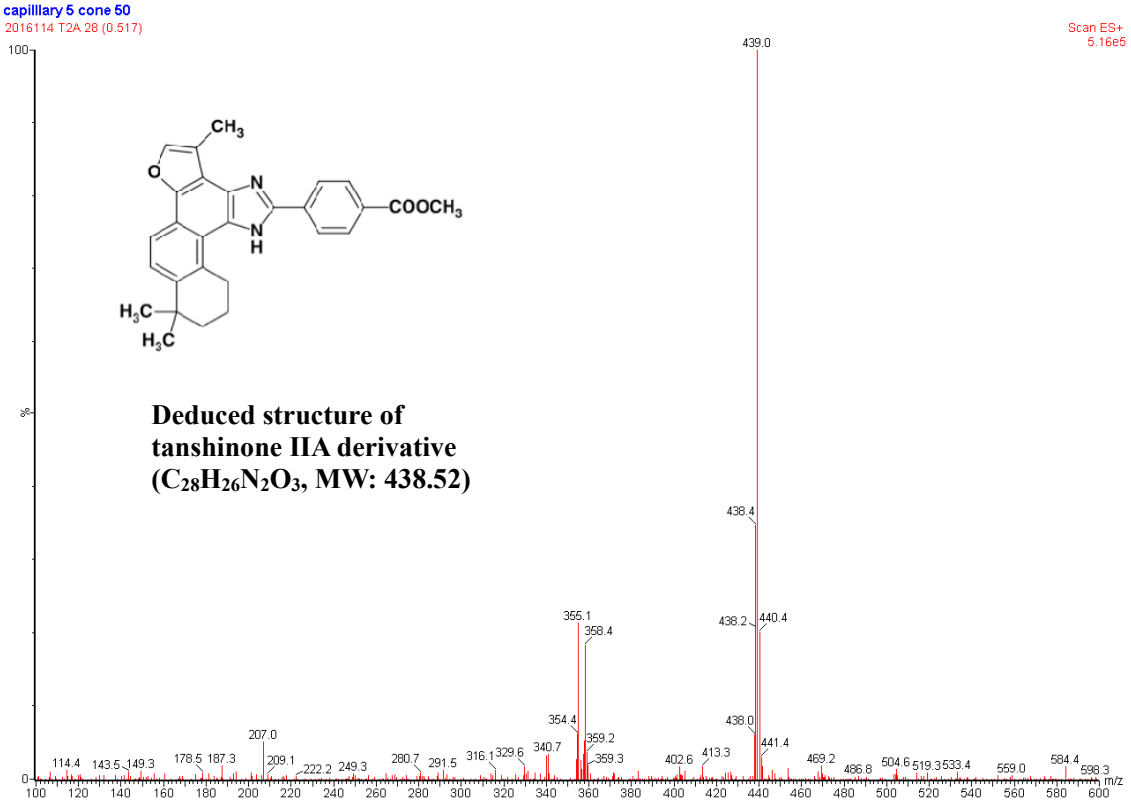


Figure S2. Electrospray Ionization mass spectra of (A) cryptotanshinone, (B) tanshinone I and (C) tanshinone IIA derivative.

Synthesis of cryptotanshinone derivative and ^{1}H NMR spectral data

One hundred mg of ammonium acetate (1.3 mmol), 8.2 mg of 4-carbomethoxybenzaldehyde (0.05 mmol), and 14.8 mg of cryptotanshinone (0.05 mmol) were dissolved in 0.5 mL of acetic acid. The mixture was heated with stirring at 100 ◦C for 10 h. The reaction mixture was cooled down then poured into ice-cold water. A dark orange precipitate is formed which is filtered, dried then recrystallized in a small volume of ethyl acetate to give orange brown crystals; yield: 12 mg, 54%, mp: >300 ºC (MP-53 melting point apparatus, Yanagimoto, Kyoto). The obtained orange-brown compound was subjected to ^{1}H-NMR studies using Varian Inova500 spectrometer (Varian, CA, USA). Figure S3 shows the ^{1}H-NMR spectrum of cryptotanshinone derivative. The ^{1}H-NMR (500 MHz, DMSO-d6) d (ppm) data were as follows: d 1.35 (s, 6H, 2CH_3), d 1.45 (d, J = 6.5 Hz, 3H, CH_3), d 1.74 (t, J = 4 Hz, 2H, CH_2), d 1.94 (m, 2H, CH_2), d 3.29 (m, 2H, CH_2), d 3.78 (m, 1H, CH), d 3.90 (s, 3H, CH_3), d 4.43 (q, J = 4.5 Hz, 1H, CH), d 4.88 (t, J = 4.5 Hz, 1H, CH), d 7.51 (d, J = 8.5 Hz, 1H, Ar-H), d 7.76 (d, J = 8.5 Hz, 1H, Ar-H), d 8.13 (d, J = 8.5 Hz, 2H, 2Ar-H), d 8.35 (d, J = 8.5 Hz, 2H, 2Ar-H), d 13.04 (s, 1H, NH). The peak at 13.04, which is characteristic for the imidazole N-H, prove the condensation of cryptotanshinone and 4-carbomethoxybenzaldehyde and ammonium acetate to give the imidazole derivative. Also, the absence of aldehyde proton peak that could appear at d 9.5-10.5 suggests the same assumption.

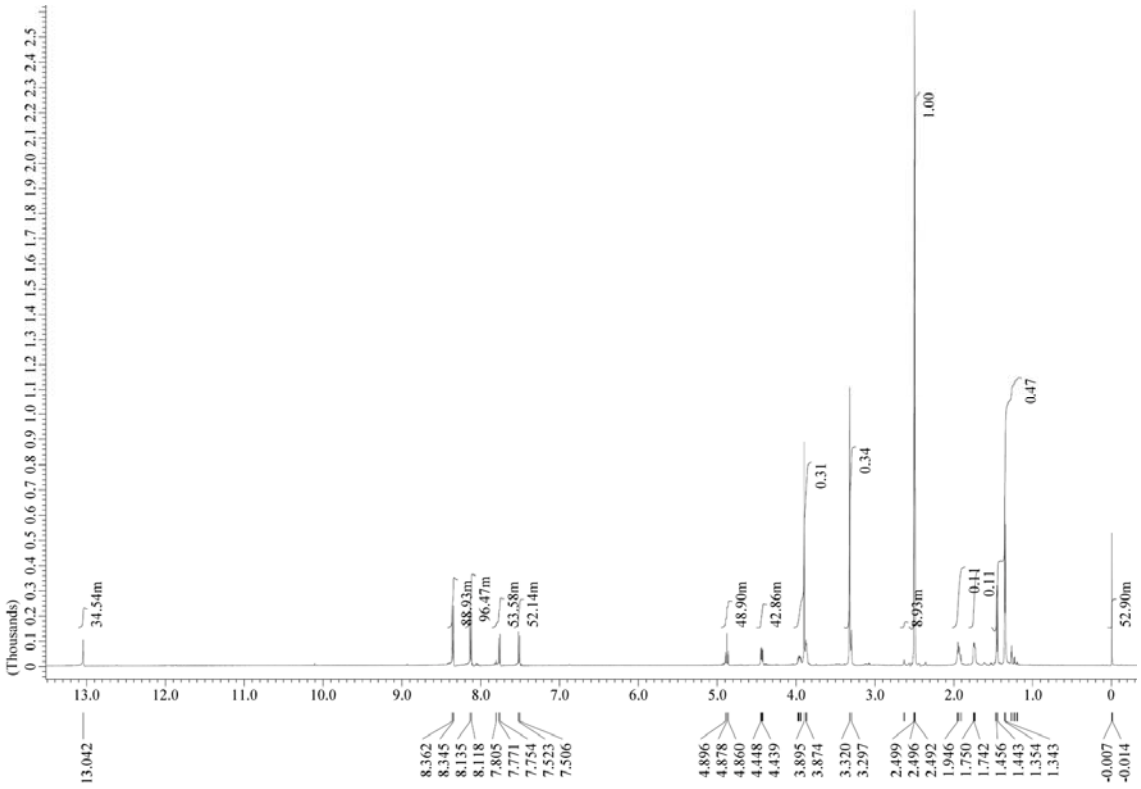


Figure S3. ^{1}H-NMR spectrum of cryptotanshinone derivative.