Bioorganic Chemistry 85 (2019) 357–363

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Orobanone analogues from acid-promoted aromatization rearrangement of curcumol inhibit hypoxia-inducible factor-1 (HIF-1) in cell-based reporter assays

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ARTICLE INFO

Keywords:
Orobanone
Curcumol
Aromatization
Acid catalysis
HIF-1 transcription

ABSTRACT

In this paper, the mechanism of orobanone analogues formation via aromatization rearrangement of curcumol was minutely explored. Aromatization of curcumol with acetone under acidic condition was selected as the model reaction. The formation of a stable aromatic system was the driving force for this reaction. Based on the model reaction, other four new orobanone analogues were prepared through curcumol reacting with different carbonyl compounds. The results showed that the stability of carbocation, which was generated from the carbonyl compounds, and the steric hindrance were main factors affecting the aromatization. We also synthesized the analogue of aromaticane B using compound **2**. *In vitro* anti-proliferative activity of some derivatives were tested by MTT assay. Two derivatives showed weak anti-tumor effect on two cancer cell lines (HepG2 and MCF7) under normoxia. Four orobanone analogue **2**, **5**, **6** and **9** significantly inhibited hypoxia-induced HIF-1 luciferase reporter activity in HeLa cells with the IC_{50} values of 13.6, 6.6, 2.4 and 18.2 μM, respectively.

1. Introduction

Curcumae rhizome is a kind of Chinese traditional medicine, which is the dry rhizome of Curcuma phaeocaulis Val., Curcuma kwangsiensis or Curcuma wenyujin [1,2]. It has a high content of essential oil. Curcumol (1) is one of the major active ingredients of the essential oil, nominated *(3S-(3α, 3aα, 5α, 6α, 8a.β))-Octahydro-3-methyl-8-methylene-5-(1-methylethyl)-6H-3a,6-epoxy azulen-6-ol* (Fig. 1). Curcumol has been reported with many bioactivities, such as antifungal activity [3], anti-proliferative activity [4–6], anti-inflammation [7], and anti-virus, etc. Recently, orobanone isolated from Curcuma zedoaria showed antibiotic effect on Bacillus cereus (Fig. 1) [8]. Similarly, as the orobanone analogues, Phaeocaulisins D and phaeocaulisins L, isolated from rhizomes of Curcuma phaeocaulis, exhibited inhibitory activity against lipopolysaccharide-induced NO production in RAW 264.7 macrophages. Phaeocaulisins L showed moderate activity (IC_{50} 54.27 ± 4.23 μM) compared to phaeocaulisins D (IC_{50} 5.9 μM) (Fig. 1) [9,10]. Aromaticane B isolated from Curcuma aromatica showed notable antioxidant effects on oxidative injury induced by H_2O_2 [11].

So far, all the tested orobanone and its analogues are natural products except compound **2** (Fig. 1). Almost thirty years ago, compound **2**, an analogue of orobanone with antitumor activity, was prepared by the reaction of curcumol with HBr in acetone [12,13]. As we know, there is none report of the synthesis of orobanone. Meanwhile, the main skeleton of orobanone is 2, 3-dihydroazulen-6(1H)-one, of which the available synthetic methods are very limited, including intramolecular cyclization [14–18], intermolecular addition [19,20], local oxidation [21] and aromatization after the elimination of epoxy bridge [22]. It was interesting that curcumol can be converted into another kind of natural product analogues by the aromatization rearrangement catalyzed by proton acid. Due to the similar antifungal [3,6] and anti-inflammatory activities [7,9,10] between orobanone analogues and curcumol, it was assumed that orobanone and/or its analogues have anti-tumor effect, which had not been reported. Thus the reaction of curcumol with different carbonyl compounds might provide us the opportunity to synthesize more orobanones analogues and be meaningful to find more anti-proliferative candidates.

The model reaction in our study was that curcumol reacted with acetone catalyzed by hydrobromic acid. Based on two important intermediates, we proposed the mechanism of the model reaction. Successionally, the reaction of curcumol with different carbonyl compounds was studied and other four new orobanone analogues were

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https://doi.org/10.1016/j.bioorg.2019.01.013
Received 28 September 2018; Received in revised form 29 December 2018; Accepted 6 January 2019
Available online 10 January 2019
0045-2068/

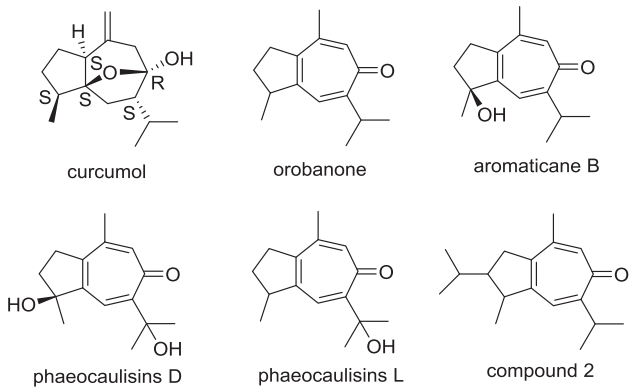
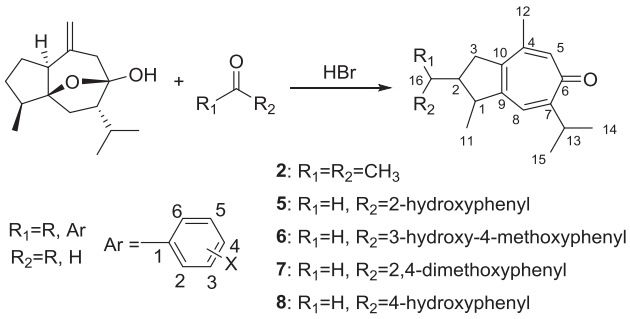


Fig. 1. The chemical structure of curcumol, orobanone, aromaticane B, phaeocaulisins D, phaeocaulisins L and compound **2**.



Scheme 1. Synthesis route of orobanone analogues.

successful synthesized (Scheme 1).

Hypoxia inducible factor-1 (HIF-1) plays pivotal roles by regulating angiogenesis, invasion, metastasis, glucose uptake, and cell survival during hypoxic tumor microenvironment [23]. Natural products have been widely explored as HIF-1 inhibitors with particular effectiveness in cancerous diseases [24,25]. Recent researches about the anti-tumor activity of curcumol have demonstrated that curcumol can inhibit cancer cell proliferation [4–6,26–30], induce cell cycle arrest [31,32], induce apoptosis [5,30,32–34] and suppress cancer cell metastasis [35,36] via different pathways and targets. It was reported that MAPK [5] and PI3K-Akt [27,31,35] were involved in the signaling pathways suppressed by curcumol. Most importantly, both signaling pathways are related to the downregulation of HIF-1 transcription and expression [37]. Herein, we hypothesized that curcumol and the orobanone analogues may have anti-tumor effect via inhibiting HIF-1 expression.

2. Results and discussion

2.1. Chemistry

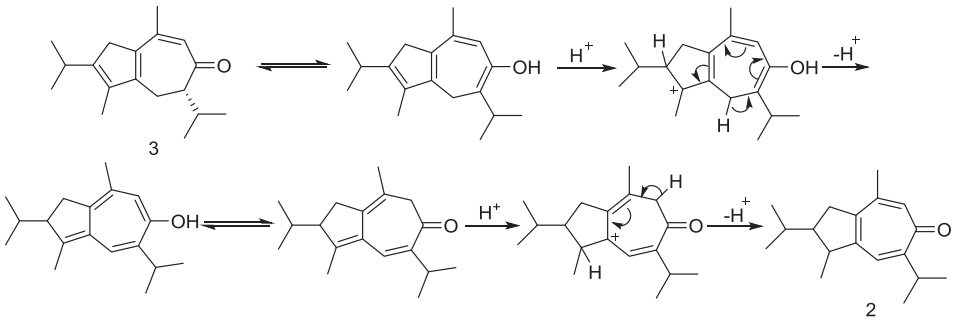
Aromatization of curcumol in acetone under the catalysis of hydrogen bromide has been reported [12,13]. However, the mechanism of this reaction has never been reported. In our study, since the difficulty in preparing acetone-hydrogen bromide solution, hydrobromic acid was used. Unexpectedly, this change caused lower yield and more complicated post-processing compared to acetone-hydrogen bromide solution. When the mixture of acetone and hydrobromic acid was dehydrated by phosphorus pentoxide in succession to sodium sulfate, the final product was crystallized from the brick-red oily solution at room temperature in a reasonable yield (53–75%).

The aromatization rearrangement process monitored by TLC indicated that several intermediate compounds were involved. First, compound **3**, a yellow crystalline, was isolated by column chromatography. Compound **3**, with two double bonds in the five-membered ring, was an isomeric compound of compound **2**. Therefore, we can confirm that double bond shift occured during the process (Scheme 2). Interestingly, the increased unsaturation made the seven-membered rings possible to form a stable aromatic system. The result indicated that unsaturated bonds of the molecule had a further shift under the influence of proton. All the unsaturated bonds moved to seven-membered rings leading to the generation of compound **2**. It was believed that the formation of a stable aromatic system was the driving force for this reaction. In other words, the process of aromatization promoted the occurrence of the reaction.

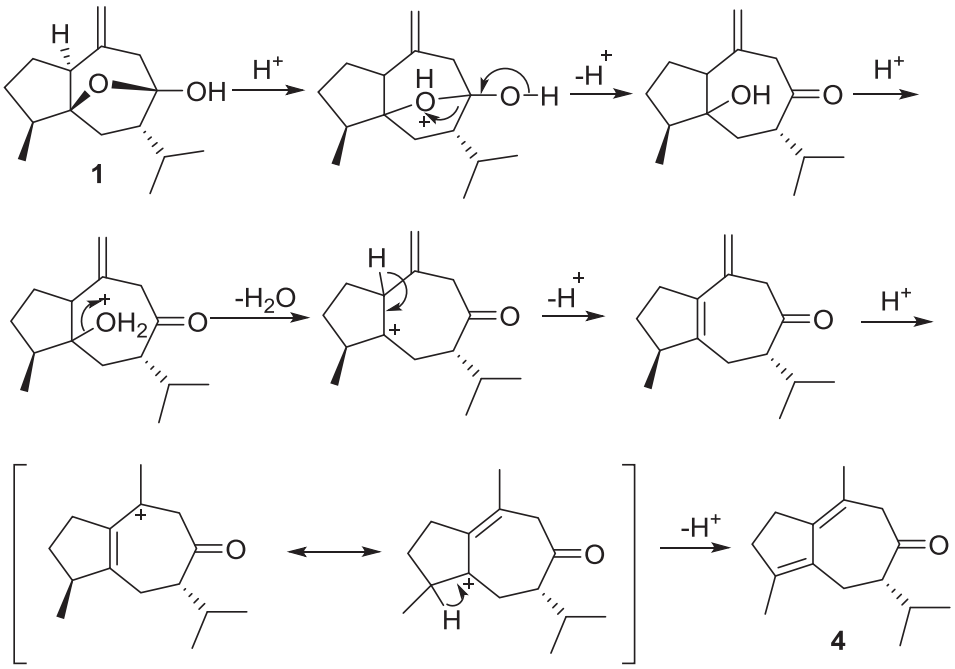
From structures of compound **2** and **3**, we could figure out that curcumol underwent ring-opening of oxygen-bridge first. However whether acetone participated in the ring-opening was not clear. The reaction of curcumol catalyzed by hydrobromic acid in THF without actone was studied as well. Compound **4** is the main product. The probable ring-opening route was shown below (Scheme 3).

Since there are two exocyclic double-bonds, compound **4** is prone to aromatization by rearrangement. This means that the reaction tends to produce a product with the largest number of alkyl groups. Comparing compound **3** with compound **4**, we speculated a very complex process (Scheme 4). First, the double bond migration occured under the catalysis of protonic acid. Second, carbocation was formed by a union of a carbonyl compound and a proton, which attacked л electrons of double bond. This electrophilic addition reaction was a key step in the process, which generated new carbon-carbon bond and carbocation simultaneously. The carbocation further underwent E_1 elimination and a double bond was formed subsequently. These two steps resembled the electrophilic substitution reaction on benzene. After that, a new hydroxyl group was formed through addition-elimination reaction. Under the catalysis of a proton, the hydroxyl group left in the form of a water molecule. This process formed another carbocation. Then a new carbon-carbon double bond was formed through E_1 elimination. At last, compound **3** was generated by double bond migration.

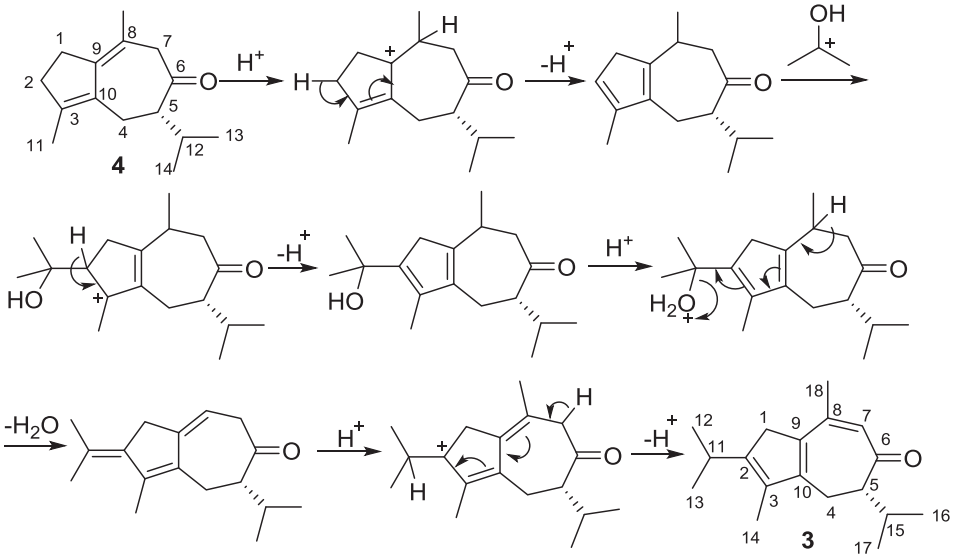
Based on the model reaction, a series of different carbonyl compounds were selected to react with curcumol. Several new trienone compounds **5–8** were prepared (Table 1). In the process, we noticed that the reaction of other carbonyl compounds needed longer time and produced more by-products with strong acid under reflux. We also found that the reaction did not take place between curcumol and aliphatic carbonyl compounds except for acetone. Alicyclic ketones did



Scheme 2. A possible pathway of the tropone formation.



Scheme 3. A supposed mechanism for ring-opening and dehydration of Curcumol.



Scheme 4. A supposed pathway of the addition-elimination reaction of Curcumol.

not carry out the reaction either. Aromatic aldehydes with electron-donating group can carry out the reaction smoothly. But aromatic ketones did not undergo this reaction.

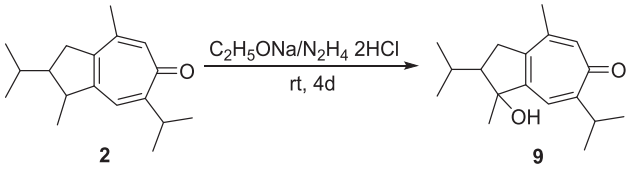
Interestingly, the analogue of aromaticane B was synthesized based on the aromatization rearrangement of curcumol accidentally (Scheme 5). The mechanism of this reaction needs further study.

Table 1
Aromatization of curcumol with several carbonyl compounds.

Carbonyl compounds	Reaction time (h)	Product	Yield
Acetone	2	**2**	75%
O-hydroxybenzaldehyde	8	**5**	59%
Vanillin	8	**6**	59%
2,4-dimethoxybenzaldehyde	10	**7**	53%
p-hydroxybenzaldehyde	10	**8**	53%

According to the assumption, carbonyl compounds are supposed to be transformed into carbocations during the reaction. The stability of carbocation is the key factor in this reaction. Therefore, compounds such as benzaldehyde can carry out the reaction especially when there are electron-donating groups on the benzene ring. Carbocation formed from alicyclic ketones is less stable and unable to participate in the aromatization of curcumol. In addition, although they can form a stable carbocation, benzophenone and acetophenone cannot undergo this kind of reaction due to the bigger steric hindrance (Table S1).

The structures of **2** and **6** have been confirmed by X-ray analysis (Fig. 2). Suitable crystal of compound **2** grew at room temperature from a solution of normal hexane, and suitable crystal of compound **6** grew at room temperature from a solution of ethyl acetate. Since there are two chiral carbon atoms in compound **2** or compound **6**, the single crystal is composed of their enantiomers. This could be additional powerful proof of the supposed double bond migration process.



Scheme 5. Synthesis route of the analogue of aromaticane B.

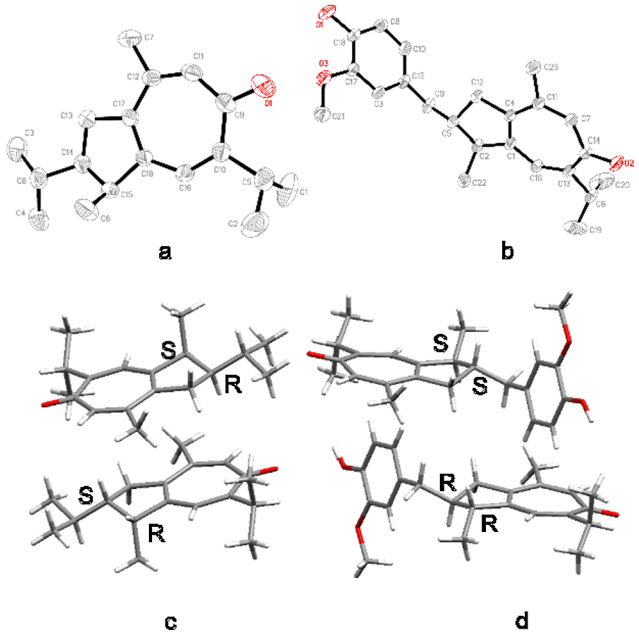


Fig. 2. (a) Molecular structure of compound **2** with the thermal ellipsoids shown at the 30% probability; (b) Molecular structure of compound **6** with the thermal ellipsoids shown at the 30% probability; (c) Enantiomers of compound **2** in the crystal; (d) Enantiomers of compound **6** in the crystal.

2.2. Effect of compounds on cell proliferation

The anti-inflammatory activity of natural orobanone analogues is similar to curcumol [9,10]. Curcumol is an antiproliferative agent [4–6,21–26]. It was reported that compound **2** had anti-tumor activity [12]. Therefore, we tested the *in vitro* anti-proliferative activity of the products to figure out whether the orobanone analogues reserved the anti-tumor activity under normoxia. After the first primary screening, three derivatives were selected and tested for their cytotoxic activity against two cancer cell lines (HepG2 and MCF7) by the MTT assay (Fig. S1). Notably, both compound **5** and **6** showed a better result than curcumol in the assay (Table 2). However, there was no selective inhibition between HepG2 and L-O2 by the orobanone analogues we tested. Surprisingly, the survival rates of the cancer cells were high at the low concentrations (< 50.0 μM) of compound **5** and **6**. When it was at 10.0 μM, compound **5** induced hormesis. However, the survival rates of cancer cells declined quickly at the concentrations from 50.0 μM to 100.0 μM of compound **5** and **6** (Fig. S1). The dose-response curves of compound **5** and **6** were abnormal, possibly due to as yet undetermined feedback, off-target, or non-specific mechanisms. There is no self-aggregation phenomenon when the analogues are under the high concentrations during the experiment and predicted by Aggregator Advisor [38].

Table 2
The results of the activity test.

Compounds	IC_{50}(μM)		
	MCF7	HepG2	L-O2
2	> 100.0	> 100.0	49.7 ± 0.7
5	35.4 ± 0.7	55.1 ± 1.1	48.5 ± 0.4
6	51.3 ± 13.3	89.6 ± 1.0	80.3 ± 0.9
Belinostat	0.1 ± 0	1.9 ± 0.1	3.2 ± 0.2
Curcumol	> 100.0	> 100.0	> 100.0

Table 3
Inhibition of HIF-1 transcriptional activity in HeLa cell-based HRE and CMV dual luciferase assay and cell growth inhibition.

Compounds	HRE IC_{50} (μM)[a]	GI_{50} (μM)[b]
10	7.8 ± 0.7	10.8 ± 1.9
Curcumol	68.5 ± 6.2	> 100.0
2	13.6 ± 1.1	40.1 ± 6.9
5	6.6 ± 0.8	15.8 ± 6.3
6	2.4 ± 0.6	0.7 ± 0.1
9	18.2 ± 9.4	24.0 ± 2.3

[a] HeLa cells were incubated for 12h with or without drugs under the normoxic or hypoxic condition.

[b] HeLa cells were incubated for 72h with various concentrations of compounds under the normoxic condition.

2.3. Inhibition of HIF-1 transcription

We tested curcumol and the orobanone analogues about their inhibition of HIF-1 transcriptional activity (Table 3), reported phenoxyacetanilide **10** [39] by Nakamura's groups as reference substance (see Fig. 3). For the first time, we found curcumol could inhibit HIF-1 transcription with IC_{50} of 68.5 ± 6.19 μM, which was identical with our speculation. The orobanone analogues showed better inhibition than curcumol, especially for compound **6** that was synthesized by curcumol and vanillin [40]. The inhibitory activity of compound **6** is nearly three times of that of reference compound **10**. The result indicated that the aromatization of curcumol was beneficial to improve the inhibition of HIF-1 transcription. The aromatic substituents on C2 also elevated the compounds' inhibition on HIF-1 transcription. Nevertheless, the hydroxylation of C1 did not show significant effect. Therefor, 2-aromatic substituted orobanone analogues may become a novel kind of HIF-1 inhibitors.

3. Conclusions

We herein synthesized six orobanone analogues. Five of the products were synthesized through the mechanism of aromatization

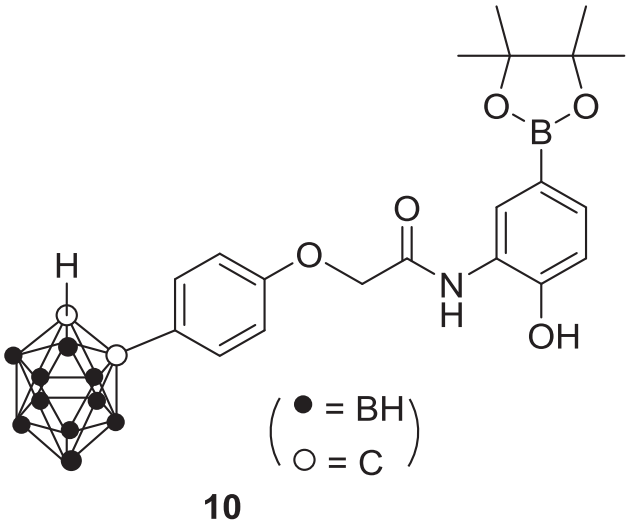


Fig. 3. The structure of reference substance **10.**

rearrangement of curcumol we proposed in this paper. We supposed the stability of carbocation generated from the carbonyl compounds and the steric hindrance of carbonyl compound were main factors that affect the aromatization. The activity tests revealed the orobanone analogues products had lower IC_{50} concentration than curcumol, which demonstrated the analogue preserved the antitumor activity. Notably, curcumol and orobanone analogues inhibited HIF-1 transcription under hypoxia. Orobanone's aromaticity and 2-aromatic substituents contribute to the orobanone analogues' improved activity against HIF-1transcrition. Interestingly, the synthesis of compound **9** was based on compound **2** accidently, which may be used as a novel method of hydroxylation and needs further exploring. Further detailed mechanistic studies of HIF-1 inhibition by compound **6** is currently under investigation in our research group.

4. Experimental section

4.1. Chemistry

4.1.1. General

Solvents and chemicals were reagent grade or better and obtained from commercial sources. NMR spectra were recorded on Bruker Advance II spectrometer. Analytical thin layer chromatography (TLC) was performed on silica gel plates produced by Qingdao Haiyang Chemical Co. Ltd (200 × 200 mm, 0.2–0.25 mm) with F254 indicator. Detection: under 254 nm UV light or color development by spraying with vanillin-concentrated sulfuric acid reagent. Compound 2 has been reported with limited spectroscopic data.

4.1.2. Typical procedure for the aromatization arrangement of curcumol

Method A: 8 ml 40% of hydrobromic acid solution was added to 80 ml acetone and dehydrated by anhydrous sodium sulfate for 3 h. Phosphorus pentoxide was used to further dehydrate for another 15 min. After filtering the desiccant, 5.0 g curcumol was dissolved in the filtrate and heated to reflux for 3 h. The reaction solution was neutralized by saturated solution of sodium bicarbonate and then extracted by ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was removed under vacuum. The residue was brown oil. Hereafter this is designated as method A.

Method B: The solution of 0.50 g curcumol and carbonyl compounds (molar ratio 1:1) in 30 ml THF was mixed with 5 ml 40% of hydrobromic acid. The mixture was heated to reflux for 8 h. The reaction solution was neutralized by saturated solution of sodium bicarbonate and then extracted by ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was removed under vacuum. The residue is oil-like substance and purified by silica gel chromatograph. Hereafter this is designated as method B.

4.1.2.1. 2,3-dihydro-1,4-dimethyl-2,7-diisopropyl-azulen-6(1H)-on (2). Compound **2** was prepared according to method A. The crude product was crystallized directly and washed with a small amount of petroleum ether to afford compound 2 (75%). White crystal; mp 81–83 °C; ^{1}H NMR(400 MHz, $CDCl_3$): δ 7.08(1H, s, H-8), 6.99(1H, s, H-5), 3.46 (^{1}H, hept, J = 6.8 Hz, H-13), 3.07(1H, p, J = 7.2 Hz, H-1), 2.82(1H, dd, J = 16.8, 7.6 Hz, H-3a), 2.54(1H, dd, J = 16.8, 11.4 Hz, H-3b), 2.22(3H, s, H-12), 1.92(1H, m, H-2), 1.71(1H, dhept, J = 10.8, 6.4 Hz, H-16), 1.17(3H, d, J = 6.4 Hz, H-11), 1.16(3H, d, J = 6.8 Hz, H-14 or H-15), 1.04(3H, d, J = 6.8 Hz, H-14 or H-15), 0.98(6H, d, J = 6.4 Hz, H-17, H-18); ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.4(C-6), 158.6(C-7), 151.8(C-9), 144.9, 144.8(C-4, C-10), 138.9(C-5), 130.7(C-8), 48.7(C-2), 46.7(C-1), 38.6(C-3), 29.7(C-13), 28.3(C-16), 25.0(C-12), 22.4, 22.5, (C-14, C-15), 21.6, 21.7(C-17, C-18), 14.2(C-11); HRMS (EI) (m/z): $[M]^+$ calcd for $C_{18}H_{26}O$: 258.1995, found: 258.1984.

4.1.2.2. 4,5-dihydro-3,8-dimethyl-2,5-diisopropyl-azulen-6(1H)-one (3). Compound **3** was prepared according to method A. The reaction time was shortened to 1hr. The crude product was purified by silica gel chromatograph to afford compound 3 (20%). Yellow crystal; mp 81–82 °C ^{1}H NMR(400 MHz, $CDCl_3$): δ 5.88(1H, s, H-7), 3.07(2H, s, H-1), 2.96(1H, hept, J = 6.8 Hz, H-11), 2.70(1H, dd, J = 16.8, 7.2 Hz, H-4a), 2.59(1H, dd, J = 16.8, 2.4 Hz, H-4b), 2.17(1H, ddd, J = 8.8, 7.2, 2.4 Hz, H-5), 2.08(3H, s, H-18), 2.02(1H, dhept, J = 8.8, 6.8 Hz, H-15), 1.91(3H, s, H-14), 1.14(3H, d, J = 6.8 Hz, H-12 or H-13), 1.13(3H, d, J = 6.8 Hz, H-13 or H-12), 0.88(3H, d, J = 6.8 Hz, H-16 or H-17), 0.83(3H, d, J = 6.8 Hz, H-16 or H-17); ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.3(C-6), 151.9(C-2) 151.4(C-3), 146.0(C-8), 136.1(C-9), 135.6(C-10), 124.9(C-7), 54.8(C-5), 39.9(C-1), 27.7(C-11), 25.7(C-15), 25.0(C-18), 23.5(C-4), 23.0, 22.9(C-12, C-13), 21.2, 20.1(C-16, C-17), 10.8(C-14); HRMS (EI) (m/z): $[M]^+$ calcd for $C_{18}H_{26}O$: 258.1995, found: 258.1984.

4.1.2.3. (S)-3,8-dimethyl-5-isopropyl-2,4,5,7-tetrahydroazulen-6(1H)-one (4). 1.00 g curcumol was completely dissolved in 30 ml THF and mixed with 5 ml 40% of hydrobromic acid. The mixture was heated to reflux for 4hr. The reaction solution was neutralized by saturated solution of sodium bicarbonate and extracted with ethyl acetate. The combined extract was dried by anhydrous sodium sulfate. Ethyl acetate was evaporated under vacuum. The residue was a yellow oil-like substance. The residue was purified by silica gel chromatograph to afford compound **4** in a 60% yield. Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.79(1H, d, J = 13.6 Hz, H-7a), 2.75(1H, d, J = 13.6 Hz, H-7b), 2.57(1H, dd, J = 14.2, 3.2 Hz, H-4), 2.34–2.41(5H, m, H-1, H-2, H-4), 2.12–2.15(2H, m, H-5, H-12), 1.76(6H, s, H-11, H-15), 0.98(3H, d, J = 6.4 Hz, H-13 or H-14), 0.91(3H, d, J = 6.4 Hz, H-14 or H-13). ^{13}C NMR (100 MHz, $CDCl_3$): δ 210.5(C-6), 143.8(C-9), 141.7(C-3), 135.4(C-10), 113.8(C-8), 57.2(C-5), 48.1(C-7), 35.5(C-2), 30.9(C-12), 28.0(C-1), 23.7(C-4), 22.8(C-15), 18.8, 20.8(C-13, C-14), 14.8(C-11); HRMS (EI) (m/z): $[M]^+$ calcd for $C_{15}H_{22}O$: 218.1676, found: 218.1671.

4.1.2.4. 2,3-dihydro-1,4-dimethyl-2-(2-hydroxybenzyl)-7-isopropyl-azulen-6(1H)-one(5). The substrate was salicylaldehyde. Compound **5** was prepared according to method B in 59% yield(0.40 g). White solid; mp 163–164 °C; α_D^{20} + 2.5 (c 0.57, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.07–8.18(3H, m, Ar-H, H-8), 7.01–7.06(1H, d, Ar-H), 6.83–6.91(2H, m, Ar-H, H-5), 3.50(1H, hept, J = 6.8 Hz, H-13), 2.96(1H, dd, J = 13.6, 6.8 Hz, H-16a), 2.852(1H, m, H-1), 2.85(1H, dd, J = 13.6, 6.0 Hz, H-3a), 2.61(1H, dd, J = 13.6, 8.4 Hz, H-3b), 2.61(1H, dd, J = 17.2, 6.0 Hz, H-16b), 2.32(1H, m, H-2), 2.16(3H, s, H-12), 1.24(3H, d, J = 7.2 Hz, H-11), 1.18(3H, d, J = 6.8 Hz, H-14 or H-15), 1.17(3H, d, J = 6.8 Hz, H-14 or H-15); ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.5, 158.6, 154.6, 151.7, 146.0, 145.7, 138.7, 131.3, 130.3, 127.2, 126.9, 120.1, 115.5, 50.5, 44.3, 40.5, 39.7, 30.0, 25.2, 22.6, 22.5, 19.7; HRMS (EI) (m/z): $[M]^+$ calcd for $C_{22}H_{26}O_2$: 322.1942, found: 322.1933.

4.1.2.5. 2,3-dihydro-1,4-dimethyl-2-(4-hydroxy-3-methoxybenzyl)-7-isopropyl-azulen-6(1H)-one(6). The substrate was vanillin. Compound **6** was prepared according to method B in 54% yield (0.40 g). White solid; mp 140–142 °C; α_D^{20} + 6.0 (c 0.42, $CHCl_3$);^{1}H NMR (400 MHz, $CDCl_3$): δ 7.07(1H, s, H-8), 7.00(1H, s, H-2′), 6.87(1H, d, J = 8.4 Hz, H-6′), 6.69(1H, dd, J = 8.4, 2.0 Hz, H-5′), 6.68(1H, s, H-5), 3.89(3H, s, -OCH3), 3.47(1H, hept, J = 6.8 Hz, H-13), 2.92(1H, dd, J = 17.6, 8.0 Hz, H-16a), 2.87(1H, m, H-1), 2.80(1H, dd, J = 14.0, 6.4 Hz, H-3a), 2.57(1H, dd, J = 14.0, 8.8 Hz, H-3b), 2.51(1H, dd, J = 17.6, 6.0 Hz, H-16b), 2.20–2.35(1H, m, H-2), 2.18(3H, s, H-12), 1.23(3H, d, J = 6.8 Hz, H-11), 1.17(3H, d, J = 6.8 Hz, H-14 or H-15), 1.16(3H, d, J = 6.8 Hz, H-14 or H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.4, 158.5, 148.9, 146.5, 144.8, 144.4, 144.0, 139.0, 132.3, 129.9, 121.5, 114.4, 111.4, 55.9, 50.2, 45.7, 40.5, 40.3, 29.9, 25.1, 22.6, 22.5, 19.8. HRMS (EI) (m/z): $[M]^+$ calcd for $C_{23}H_{28}O_3$: 352.2037, found: 352.2038.

4.1.2.6. 2,3-dihydro-1,4-dimethyl-2-(2,4-dimethoxybenzyl)-7-isopropyl-azulen-6(1H)-one(7). Compound **7** was prepared according to method

B in 53% yield(0.41 g). The reaction time was 10 h and the substrate was 2, 4-methoxybenzaldehyde. Yellow oil; α_D^{20} + 5.4(c0.31, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.09(1H, s, H-8), 7.024(1H, d, *J* = 2.4 Hz, H-3′), 7.018(1H, dd, *J* = 8.4, *J* = 2.4 Hz, H-5′), 6.46(1H, s, H-5), 6.34(1H, d, *J* = 8.4 Hz, H-6′), 3.80(3H, s, -OCH_3), 3.79(3H, s, -OCH_3), 3.48(1H, hept, *J* = 6.4 Hz, H-13), 2.90(1H, dd, *J* = 17.6, 8.0 Hz, H-16a), 2.77(1H, dd, *J* = 13.6, 6.0 Hz, H-3a), 2.75–2.90(1H, m, H-1), 2.57(1H, dd, *J* = 13.6, 6.0 Hz, H-3b), 2.55(1H, dd, *J* = 17.6, 8.0 Hz, H-16b), 2.18–2.28(1H, m, H-2), 2.17(3H, s, H-12), 1.21(3H, d, *J* = 6.8 Hz, H-11), 1.18(3H, d, *J* = 6.8 Hz, H-14 or H-15), 1.17(3H, d, *J* = 6.8 Hz, H-14 or H-15). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.3, 159.4, 158.5, 158.2, 149.5, 145.1, 145.0, 138.9, 130.6, 130.2, 121.2, 103.9, 98.5, 55.3, 55.2, 50.4, 44.3, 40.4, 34.4, 29.9, 25.1, 22.6, 22.5, 19.6. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{24}H_{30}O_3$: 366.2198, found: 366.2195.

4.1.2.7. 2,3-dihydro-1,4-dimethyl–2-(4-hydroxybenzyl)-7-isopropyl azulen-6(1H)-one(8). Compound **8** was prepared according to method B in 53% yield(0.39 g). The reaction time was 10 h and the substrate was p-hydroxybenzaldehyde. Light brown solid; mp 180–181 °C; α_D^{20} + 2.0 (c 0.50, CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.24(1H, s, H-8), 8.00(1H, s, H-5), 7.02(2H, d, *J* = 8.0 Hz, H-3′, H-5′), 6.81(2H, d, *J* = 8.0 Hz, H-2′, H-6′), 3.72(1H, hept, *J* = 6.8 Hz, H-13), 3.49(1H, s, -OH), 3.18–3.27(2H, m, H-16), 2.88(1H, dd, *J* = 5.6 Hz, *J* = 13.6 Hz, H-3a), 2.80–2.86(1H, m, H-1), 2.66(1H, dd, *J* = 8.0 Hz, *J* = 13.6 Hz, H-3b), 2.60(3H, s, H-12), 2.40–2.44(1H, m, H-2), 1.41 (3H, d, *J* = 6.8 Hz, H-11), 1.32(6H, d, *J* = 6.8 Hz, H-14, H-15). ^{13}C NMR (100 MHz, DMSO-D_6): δ 184.2, 157.6, 155.9, 149.5, 145.8, 145.6, 138.3, 130.9, 130.5, 130.2, 115.6, 49.9, 45.8, 40.6, 39.3, 30.0, 25.1, 22.7, 22.6, 20.1. HRMS (EI) (*m/z*): $[M]^+$ calcd for $C_{22}H_{26}O_2$: 322.1941, found: 322.1933.

4.1.2.8. 1-hydroxy-2,3-dihydro-1,4-dimethyl-2,7-diisopropyl-azulen-6(1H)-one(9) was prepared in this way. Dissolve the 1 g metal sodium in 20 ml ethyl alcohol. After the sodium was completely reacted, 1.9 g hydrazine hydrochloride was added, then 0.50 g compound 1 was added, React at the room temperature for 4 days, and the yellow reaction mixture was obtained. Added 40 ml water to the reaction system, then the yellow reaction mixture was extracted with ethyl acetate, dried with anhydrous sodium sulfate then filter. The organic solvent was removed by reducing pressure distillation, Separate and purify the crude product by column chromatography, and the mobile phase is petroleum ether- ethyl acetate (5:1). The product is white powdery solid, with the yield of 41%. ^{1}H NMR ($CDCI_3$) δ7.31(1H, s, H-8) δ7.01(1H, s, H-5) δ3.44(1H, m, *J* = 6.8 Hz, H-13) δ2.86(1H, dd, *J* = 7.6 Hz, *J* = 16.8 Hz, H-3a) δ2.28(1H, dd, *J* = 10.4 Hz, *J* = 16.8 Hz, H-3b) δ2.22(3H, s, H-12) δ1.90(1H, s, -OH) δ1.81–1.86(1H, m, H-16) δ1.69–1.77(1H, m, H-3) δ1.27 (3H, s, H-11) δ1.18(6H, d, *J* = 6.0 Hz, H-14, H-15) δ1.16(3H, d, *J* = 6.4 Hz, H-18 or H-17) δ1.02(3H, d, *J* = 6.4 Hz, H-17 or H-18). ^{13}C NMR ($CDCI_3$) δ21.55, 21.90(C-17, C-18), 22.35, 22.44(C-14, C-15), 22.50(C-11), 24.41(C-12), 28.69(C-2), 30.03(C-13), 36.99(C-3), 58.20(C-16), 84.64(C-1), 128.20(C-8), 139.67(C-5), 142.45(C-10), 144.36(C-4), 151.34(C-9), 159.43(C-7), 185.54(C-6). HRMS (EI) (*m/z*): $[M]^+$ calcd for C18H26O2: 274.1933, found: 2274.1957.

4.1.3. X-ray data

General information: Data set was collected with a Smart APEX2 CCD diffractometer. Programs used: data collection APEX2 (Bruker, 2005),[16] data reduction SAINT-Plus,[17] structure solution SHELXS97 (Sheldrick, 2008),[18] structure refinement SHELXL97 (Sheldrick, 2008),[18] graphics SHELXTL. Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication. CCDC-767167(**2**) and CCDC-767168(**6**) contain the supplementary crystallographic data for this paper. This data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(1223)336–033; E-mail:deposit@ccdc.cam.ac.uk].

4.1.3.1. X-ray crystal structure analysis for compound 2. Formula C18H26O, M = 258.39, colorless crystal 0.36 × 0.28 × 0.24 mm, a = 9.618 (5) Å, b = 12.634 (7) Å, c = 13.196 (7) Å, α = 90.00°, β = 93.834 (8)°, γ = 90.00°, V = 1599.9 (15) Å3, ρcalcd = 1.073 g cm − 3, μ = 0.06 mm − 1, Z = 4, monoclinic, space group *P*2(1)/n, λ = 0.71073 Å, T = 293 (2) K, 6888 measured reflections, 2813 independent reflections (Rint = 0.018), and 2207 observed reflections [I > 2σ(I)], 172 refined parameters, R = 0.052, w*R* (F2) = 0.158, max(min) residual electron density 0.21(−0.25) e Å − 3, hydrogen atoms calculated and refined as riding atoms.

4.1.3.2. X-ray crystal structure analysis for compound 6. Formula C23H28O3, M = 352.45, colorless crystal 0.35 × 0.29 × 0.24 mm, a = 8.441 (2) Å, b = 10.674 (3) Å, c = 11.047 (3) Å, α = 80.023 (4)°, β = 85.520 (4)°, γ = 85.735 (4)°, V = 975.4 (5) Å3, ρcalcd = 1.200 g cm^{-3}, μ = 0.08 mm − 1, Z = 2, triclinic, space group *P*-1, λ = 0.71073 Å, T = 293 (2) K, 4761 measured reflections, 3385 independent reflections (Rint = 0.015), and 2986 observed reflections [I > 2σ(I)], 236 refined parameters, R = 0.049, w*R* (F2) = 0.153, max(min) residual electron density 0.30(−0.30) e Å − 3, hydrogen atoms calculated and refined as riding atoms.

4.2. Biology

4.2.1. MTT assay for cell growth inhibition

The inhibition of the title compounds against HepG2 human liver cancer cells and L-O2 human liver cells were evaluated using a standard MTT-based colorimetric assay. Five thousand corresponding cells per well were seeded into 96-well plates and incubated at 37 °C, 5%CO2 for 24 h. And then 100 μL a series of concentration of drug-containing medium were dispensed into wells to maintain the final concentration as 100, 90, 80, 70, 60 and 50 μmol/L. Each concentration was in triplicate. After 48 h incubation, cell survival was determined by the addition of 20 μL MTT (Sigma Aldrich, St. Louis, USA) work solution (5 mg/mL MTT dissolved in Phosphate Buffer Solution (PBS)). After post-incubation at 37 °C for 4 h, the medium was discarded following by adding 100 μL DMSO (SigmaeAldrich, St. Louis, USA). The plates were then vortexed for 10 min for complete dissolution. The optical absorbance was measured at 570 nm. The data represented the mean of three independent experiments in triplicate and were expressed as mean ± SD. The IC_{50} value was defined as the concentration at which 50% of the cells could survive. HeLa cells were incubated for 60 h with various concentrations (400 nM to 100 μM) of compounds under the normoxic condition, and viable cells were tested by the MTT assay. The drug concentration required to inhibit cell growth by 50% (IC_{50}) was determined from semi-logarithmic dose–response plots, and results represent means ± SD of triplicate samples.

4.2.2. Cell-based reporter assays for hypoxia-inducible factor-1 (HIF-1) inhibition

HeLa cells expressing HRE-dependent firefly luciferase reporter construct (HRE-Luc) and constitutively expressing CMV-driven Renilla luciferase reporter with SureFECT Transfection Reagent were established with Cignal™ Lenti Reporter (SABiosciences, Frederick, MD) according to the manufacturer's instructions. The consensus sequence of HRE was 5′-TACGTGCT-3′ from the erythropoietin gene. Cells stably expressing the HRE-reporter gene were selected with puromycin. The cells were incubated for 12 h with or without drugs under the normoxic or hypoxic condition (1% O_2). After incubation, the luciferase assay was performed using a Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. The drug concentration

required to inhibit the relative light units by 50% (IC_{50}) was determined from semi-logarithmic dose–response plots, and the results represent means ± SD of triplicate samples.

Acknowledgements

We gratefully acknowledge the financial support of the Young Teachers Program of Dalian University of Technology. The bioactivity evaluation part of this work was supported by the National Science Foundation for Young Scientists of China (No. 21606036).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.013.

References

[1] Committee for the Pharmacopoeia of PR China, Pharmacopoeia of PR China, part I, China Medical Science and Technology Press, Beijing, China, 2015.
[2] Y. Zhou, M. Xie, Y. Song, W.P. Wang, H.R. Zhao, Y.X. Tian, Y. Wang, S.J. Bai, Y.C. Zhao, X.Y. Chen, Evid-based Compl. Alt. Med. 3 (2016) 1–30.
[3] C.Q. Chen, L. Li, F.S. Zhang, Q. Chen, C. Chen, X.R. Yu, Q.Y. Liu, J.K. Bao, Z.F. Long, PLoS One 13 (2018) e0194284, https://doi.org/10.1371/journal.pone.0194284.
[4] P. Guo, Y.W. Wang, B.X. Weng, X.K. Li, S.L. Ye, F.Q. Yang, J. Asian Nat. Prod. Res. 16 (2014) 53–58, https://doi.org/10.1080/10286020.2013.857660.
[5] J. Wang, F. Huang, Z. Bai, B.X. Chi, J.C. Wu, X. Chen, Int. J. Mol. Sci. 16 (2015) 19851–19867, https://doi.org/10.3390/ijms160819851.
[6] L. Zhou, E.D. Wei, B.T. Zhou, G.W. Bi, L. Gao, T.Y. Zhang, J.F. Huang, Y. Wei, B. Ge, Biomed. Pharmacother. 104 (2018) 798–805, https://doi.org/10.1016/j.biopha.2018.05.101.
[7] X. Chen, C.J. Zong, Y. Gao, R.L. Cai, L. Feng, J. Lu, F. Liu, Y. Qi, Eur. J. Pharmcol. 723 (2014) 339–345, https://doi.org/10.1016/j.ejphar.2013.11.007.
[8] C.S. Jeong, S.H. Shim, Nat. Prod. Sci. 21 (2015) 147–149.
[9] Y. Liu, J.H. Ma, Q. Zhao, C.R. Liao, L.Q. Ding, L.X. Chen, F. Zhao, F. Qiu, J. Nat. Prod. 76 (2013) 1150–1156, https://doi.org/10.1021/np400202f.
[10] J.H. Ma, Y. Wang, Y. Liu, S.Y. Gao, L.Q. Ding, F. Zhao, L.X. Chen, F. Qiu, J. Asian Nat. Prod. Res. 17 (2015) 532–540, https://doi.org/10.1080/10286020.2015.1046449.
[11] S.J. Dong, B.C. Li, W.F. Dai, D. Wang, Y. Qin, M. Zhang, J. Nat. Prod. 80 (2017) 3093–3102, https://doi.org/10.1021/acs.jnatprod.6b01100.
[12] J.H. Hu, X.W. Han, Z.Y. Yang, Bopuxue Zazhi 3 (1986) 241–248.
[13] Y.T. Guo, X. Du, Yaoxue Xuebao 24 (1989) 707–710.
[14] A.S. Kende, K. Koch, Tetrahedron Lett. 27 (1986) 6051–6054, https://doi.org/10.1016/S0040-4039(00)85396-4.
[15] S.K. Hong, H. Kim, Y. Seo, S.H. Lee, J.K. Cha, Y.G. Kim, Org. Lett. 12 (2010) 3954–3956 https://pubs.acs.org/doi/abs/10.1021/ol1017849.
[16] Y.S. Zheng, E.H. Ghazvini Zadeh, Y. Yuan, Eur. J. Org. Chem. 2016 (2016) 2115–2119, https://doi.org/10.1002/ejoc.201600321.
[17] L.T. Scott, P. Grutter, R. Chamberlain, J. Am. Chem. Soc. 106 (1984) 4852–4856 https://pubs.acs.org/doi/abs/10.1021/ja00329a036.
[18] M. Zhang, N. Liu, W.P. Tang, J. Am. Chem. Soc. 135 (2013) 12434–12438, https://doi.org/10.1021/ja406255j.
[19] T. Nakazawa, Y. Niimoto, I. Murata, Tetrahedron Lett. 6 (1978) 569–572, https://doi.org/10.1002/chin.197823140.
[20] H.K. Chang, Y.C. Liao, R.S. Liu, J. Org. Chem. 21 (2007) 8139–8141 https://pubs.acs.org/doi/10.1021/jo701458b.
[21] K. Yamamoto, Y. Matsue, I. Murata, Chem. Lett. 8 (1981) 1071–1074.
[22] H.J. Zhang, L. Hu, Z.Q. Ma, R.N. Li, Z. Zhang, C. Tao, B. Cheng, Y. Li, H.F. Wang, H.B. Zhai, Angew. Chem. Int. Ed. 55 (2016) 11638–11641, https://doi.org/10.1002/anie.201605879.
[23] V. Petrova, M. Annicchiaricopetruzzelli, G. Melino, I. Amelio, Oncogenesis 7 (2018) 10–23, https://doi.org/10.1038/s41389-017-0011-9.
[24] D.G. Nagle, Y.D. Zhou, Curr. Drug Targets 7 (2006) 355–369, https://doi.org/10.2174/138945006776054979.
[25] Y. Xia, H.K. Choi, K. Lee, Eur. J. Med. Chem. 49 (2012) 24–40, https://doi.org/10.1016/j.ejmech.2012.01.033.
[26] H. Zhang, L.R. Zhang, H.J. Jiang, J.G. Gao, J.Q. Du, Z. Yang, Zhonghua Shiyan Waike Zazhi 32 (2015) 2444–2447 https://10.3760/cma.j.issn.1001-9030.2015.10.038.
[27] G. Chen, Y.H. Wang, M.Q. Li, T.J. Xu, X.L. Wang, B. Hong, Y.C. Niu, Eur. J. Pharm. Sci. 65 (2014) 21–28, https://doi.org/10.1016/j.ejps.2014.09.001.
[28] J. Wang, K. Diao, Y.F. Hou, Y.H. Xiong, X. Chen, Zhonghua Zhongyiyao Zazhi 28 (2013) 3024–3027.
[29] L.Z. Huang, J. Wang, F.T. Lu, F.C. Yang, X. Chen, X. Hong, X.S. Jiang, Zhongguo Zhongyao Zazhi 38 (2013) 1812–1815, https://doi.org/10.4268/cjcmm20131132.
[30] X. Chen, J. Wang, X.S. Jiang, J.H. Zeng, F.X. Huang, Zhongguo Shiyan Fangjixue Zazhi 17 (2011) 157–159.
[31] J. Wang, X.M. Li, Z. Bai, B.X. Chi, Y. Wei, X. Chen, J. Ethnopharmacol. 210 (2018) 1–9, https://doi.org/10.1016/j.jep.2017.06.037.
[32] W.W. Zhang, Z.P. Wang, T.S. Chen, Med. Oncol. 28 (2011) 307–314, https://doi.org/10.1007/s12032-010-9431-5.
[33] F. Li, Q. An, Y.B. Zhou, D.L. Li, C. Han, X.J. Zhang, Q.C. Kan, Int. J. Clin. Exp. Med. 9 (2016) 9999–10005 http://www.ijcem.com/files/ijcem0021655.pdf/.
[34] H. Wang, Y. Wang, X.J. Jiang, Z.Z. Wang, B. Zhong, Y.F. Fang, Int. Immunopharmacol. 21 (2014) 375–382, https://doi.org/10.1016/j.intimp.2014.05.021.
[35] L. Ning, H. Ma, Z.Y. Jiang, L. Chen, L. Li, Q.F. Chen, H.Y. Qi, Integ. Cancer. Ther. 15 (2016) 216–225, https://doi.org/10.1177/1534735416642865.
[36] H.Y. Qi, L. Ning, Z.Y. Yu, G.J. Dou, L. Li, J. Agric. Food Chem. 65 (2017) 3074–3082, https://doi.org/10.1021/acs.jafc.7b00573.
[37] D. Singh, R. Arora, P. Kaur, B. Singh, R. Mannan, S. Arora, Cell Biosci. 7 (2017) 62–71, https://doi.org/10.1186/s13578-017-0190-2.
[38] J.J. Irwin, D. Duan, H. Torosyan, A.K. Doak, K.T. Ziebart, T. Sterling, G. Tumanian, B.K. Shoichet, J. Med. Chem. 58 (2015) 7076–7087 https://10.1021/acs.jmedchem.5b01105.
[39] G.Z. Li, S. Azuma, H. Minegishi, H. Nakamura, J. Organomet. Chem. 798 (2015) 189–195, https://doi.org/10.1016/j.jorganchem.2015.05.029.
[40] E.J. Park, Y.M. Lee, T.I. Oh, B.M. Kim, B.O. Lim, J.H. Lim, Int. J. Mol. Sci. 18 (2017) E532, https://doi.org/10.3390/ijms18030532.