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Synthesis, Anticancer Activity, Structure-Activity Relationship and Mechanism Investigation of Falcarindiol Analogues

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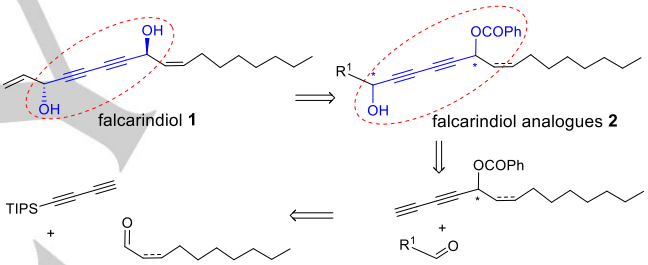
‡These authors contributed equally to this work.

Abstract: Forty samples of optically active falcarindiol analogues are synthesized by using the easily available *C*2 symmetric (*R*)- and (*S*)-1,1'-binaphth-2-ol (BINOL) in combination with $Ti(O^iPr)_4$, Zn powder and EtI. Their anticancer activities on Hccc-9810, HepG2, MDA-MB-231, Hela, MG-63 and H460 cells are assayed to elucidate their structure-activity relationships. These results showed that the falcarindiol analogue (3*R*,8*S*)-**2i** with the terminal double bond has the most potent anti-proliferation effect on Hccc-9810 cells with IC_{50} value of 0.46 μM. The falcarindiol analogue (3*R*,8*S*)-**2i** can induce obvious Hccc-9810 cell apoptosis in a concentration-dependent manner by Hoechst staining and flow cytometry analysis. The proposed mechanism suggests that the falcarindiol analogue (3*R*,8*S*)-**2i** increases LDH release and MDA content, and reduces the levels of SOD activity, which lead to the accumulation of oxidative stress and induce apoptosis in Hccc-9810 cells.

Introduction

Cholangiocarcinoma (CCA) is one of the most common primary hepatic malignancy in the world, and it could be considered as a type of gastro-intestinal cancers[1]. In spite of the advances in diagnosis and treatment, CCA-related deaths have increased markedly in the past decades. Most CCA patients are in the advanced incurable stage, and not amenable to surgical extirpation or liver transplantation[2]. Current chemotherapy is the main treatment for metastatic CCA, however, high drug resistance usually lowers the efficacy of chemotherapy drugs[3]. Therefore, novel therapeutic strategies and/or effective anticancer agents against this malignancy are urgently needed. Falcarindiol (3*R*,8*S*)-**1** is a polyacetylene found in many medicinal and dietary plants belonging to the families *Araliaceae*, *Panax ginseng, Apiaceae* and celery, and it has been shown to contribute to the cytotoxic, anti-inflammatory and potential anticancer properties[4-7]. In particular, the anti-proliferation effect of falcarindiol (3*R*,8*S*)-**1** on colon cancer and breast cancer cell lines were confirmed repeatedly[8-10]. However, sensitivity of human CCA cells such as Hccc-9810 to falcarindiol and its analogues has not been reported.



Scheme 1. A shorter pathway for asymmtric addition of 1,3-diyne to aldehydes to generate chiral falcarindiol analogues **2**

Falcarindiol **1** and its analogues **2** are characterized by a conjugated diyne with two chiral carbinol motif and a long hydrocarbon chain. The low content of falcarindiol analogues **2** in natural resources limits their further study and application[11]. Total synthesis of the falcarindiol analogues **2** has become an important practical choice in solving this problem, in which the construction of the chiral diyndiol is the key step. In 1999, Cai *et al* synthesized falcarindiol **1** for the first time by using L-tartaric and D-xylose as chiral sources to construct two chiral acetylenic alcohol intermediates followed by cross-coupling[12]. Thomas *et al* also prepared falcarindiol by asymmetric ynone reductions to form chiral propargyl alcohols followed by copper-catalyzed Cadiot-Chodkiewicz cross-coupling[13]. A shorter pathway requires asymmetric addition of 1,3-diyne to aldehydes to generate chiral falcarindiol analogues **2** (Scheme 1). Some chiral amino alcohols-based reaction systems have been developed for the asymmetric addition of 1,3-diyne to an aldehyde[14-16]. Trost *et al* demonstrated an enantioselective 1,3-diyne addition to an aldehyde by using the ProPhenol catalyst system in the presence of $ZnMe_2$ and triphenylphosphine oxide[17]. Pu *et al* found that 1,1'-binaphth-2-ol (BINOL) in combination with $ZnEt_2$ and $Ti(O^iPr)_4$ can catalyze the highly enantioselective addition of various 1,3-diynes to an aldehyde[18]. In this catalytic

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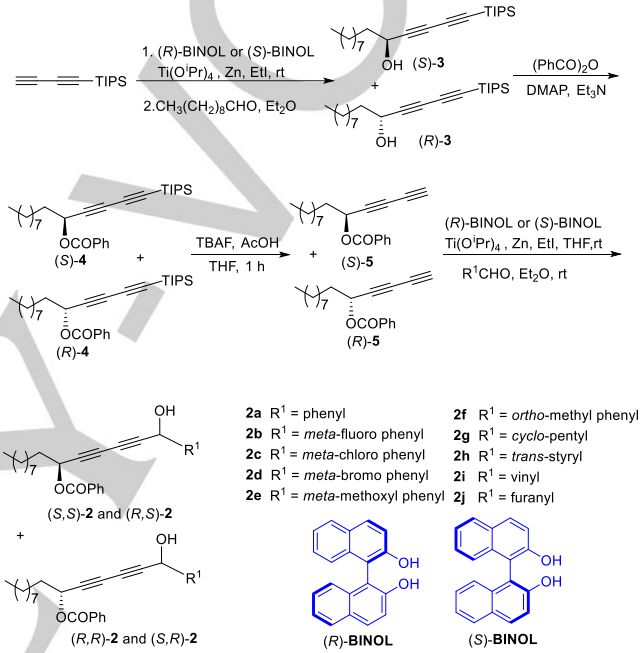
system, biscyclohexylamine (Cy_2NH) was added to facilitate the deprotonation of a terminal 1,3-diyne to form a nucleophilic diynylzinc, and chiral falcarindiol analogues can be efficiently synthesized from buta-1,3-diynyl-triisopropylsilane (TIPS) in short steps[19]. Although the two chiral centers of the falcarindiol analogues can be constructed by using the BINOL-Ti(O^iPr)$_4$-$ZnEt_2$-Cy_2NH catalytic system[19], the direct use of $ZnEt_2$ present an important safety issue for application in large scale industrial production due to its moist and air-sensitivity[20]. Recently, we have developed highly enantioselective syntheses of chiral conjugated diynols by using the chiral amino alcohol as well as the BINOL systems to promote the asymmetric 1,3-diyne addition to an aldehyde[16]. In this BINOL system, both enantiomers of the chiral conjugated diynol were prepared with high enantioselectivity by using the BINOL-Ti(O^iPr)$_4$ complex to activate the reaction of Zn with EtI under very mild reaction conditions,[16,20] which avoid the spontaneously combustible $ZnEt_2$. Moreover, these optically active conjugated diynols have demonstrated potential anticancer activities with significant differences against HepG2 and HeLa cancer cells[16]. However, the synthesis of chiral diyndiols as falcarindiol analogues were not examined by using the simple and practical BINOL-based catalytic system.

Herein, we report the synthesis of a number of chiral falcarindiol analogues by the BINOL-Ti(O^iPr)$_4$-Zn-EtI catalytic system and the screening assay of its anticancer activity on Hccc-9810, HepG2, MDA-MB-231, Hela, MG-63 and H460 cells. The falcarindiol analogue (3*R*,8*S*)-**2i** was found to have the most potent anticancer activity with IC_{50} values of 0.46 μM on Hccc-9810 cell lines in a dose-dependent manner. The possible mechanisms and structure-function relationships in anti-proliferation activity of the falcarindiol analogue (3*R*,8*S*)-**2i** on Hccc-9810 cell lines were also studied.

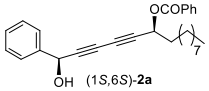
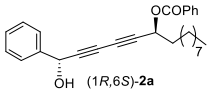
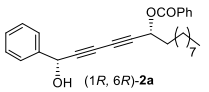
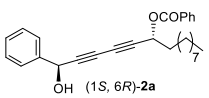
Results and Discussion

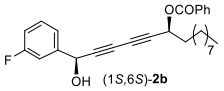
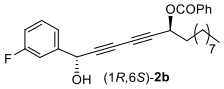
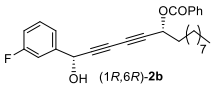
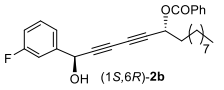
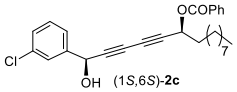
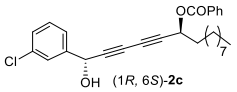
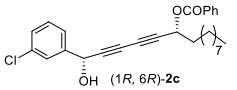
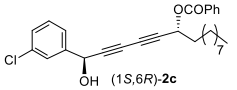
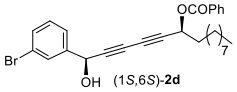
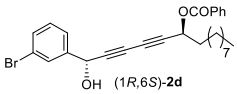
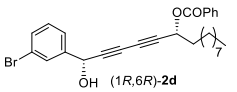
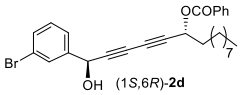
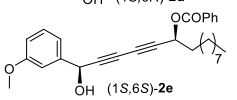
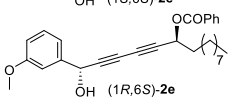
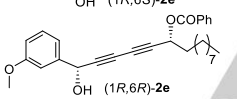
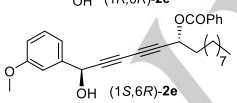
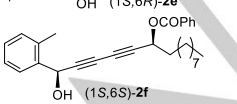
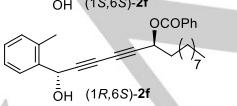
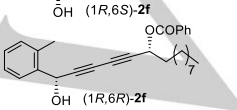
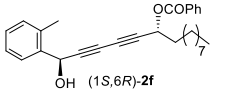
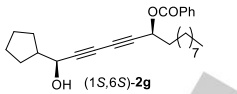
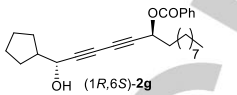
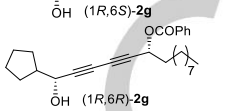
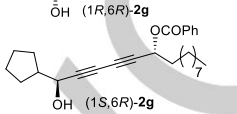
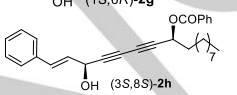
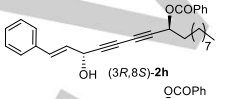
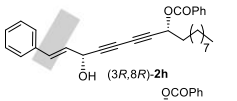
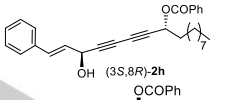
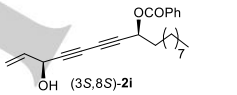
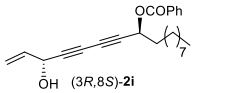
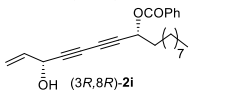
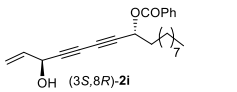
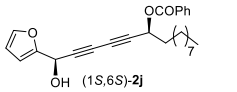
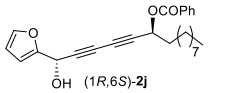
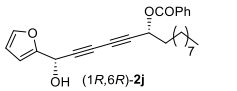
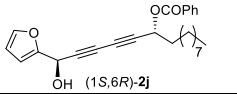
We first synthesized the conjugated diynols (*S*)-**3** and (*R*)-**3** by asymmetric addition of buta-1,3-diynyl-TIPS to 1-decanal catalyzed by the BINOL-Ti(O^iPr)$_4$-Zn-EtI system (Scheme 2)[21]. In the construction of the first chiral center, (*R*)-BINOL gave the diynol (*S*)-**3** with an enantiomeric excess (ee) of 91% and yield of 79%, while (*S*)-BINOL produced the diynol (*R*)-**3** with 93% ee and 82% yield (see Supporting Information, SI). After protection of the hydroxyl group of **3** with benzoic anhydride and removal of the TIPS group of **4** with tetrabutylammonium fluoride (TBAF), the resulting benzoylated diynols (*S*)-**5** and (*R*)-**5** were subjected to the second asymmetric addition to a variety of aromatic, aliphatic and α,β-unsaturated aldehydes by using the same catalyst system (Scheme 2). The second chiral center was constructed with either (*R*)- or (*S*)-configuration induced by the corresponding (*S*)- or (*R*)-BINOL-based catalyst system. This is the first time that the falcarindiol analogues **2** are synthesized by using the BINOL-based catalyst system. The results for the synthesis of the falcarindiol analogues **2** are summarized in Table 1. As shown in Table 1, 40 samples of falcarindiol analogues **2** were obtained with 18-57% yield. The reactions of (*S*)-**5** and (*R*)-**5** with *meta*- or *ortho*-substituted benzaldehydes containing electron-withdrawing or donating substituents gave the corresponding falcarindiol analogues **2** with excellent diastereomeric ratio (dr) and ee value (Entries 1-24). High diastereoselectivity and enantioselectivity were also observed for the benzoylated diynols (*S*)-**5** and (*R*)-**5** addition to cyclopentyl formaldehyde, α,β-unsaturated aldehydes and α-furan formaldehyde (Entries 25-40). The configuration of falcarindiol analogues **2** was determined by comparing the optical rotation with the literature.[22]



Scheme 2. Total synthesis of the falcarindiol analogues **2** by using the BINOL-Ti(O^iPr)$_4$-Zn-EtI system.

Table 1. Results for the synthesis of the falcarindiol analogues **2**

Entry	Falcarindiol analogues **2**	Yield (%)	dr [a]	ee [a] (%)	$[\alpha]_D^{25}$ [b]
1	 (1*S*,6*S*)-**2a**	24	14:1	86	-5.7
2	 (1*R*,6*S*)-**2a**	18	>20:1	99	+8.5
3	 (1*R*, 6*R*)-**2a**	37	>20:1	99	+2.7
4	 (1*S*, 6*R*)-**2a**	31	12.7:1	85	-14.7

5	 (1*S*,6*S*)-**2b**	40	7.3:1	75	-4.4
6	 (1*R*,6*S*)-**2b**	30	13.5:1	86	+3.2
7	 (1*R*,6*R*)-**2b**	39	9.4:1	80	+4.1
8	 (1*S*,6*R*)-**2b**	30	12:1	84	-2.9
9	 (1*S*,6*S*)-**2c**	57	20.5:1	91	-1.6
10	 (1*R*, 6*S*)-**2c**	40	14.2:1	87	+4.1
11	 (1*R*, 6*R*)-**2c**	52	7.6:1	77	+6.9
12	 (1*S*,6*R*)-**2c**	44	11.4:1	84	-14.6
13	 (1*S*,6*S*)-**2d**	21	15.1:1	89	-13.5
14	 (1*R*,6*S*)-**2d**	33	14.4:1	87	+3.4
15	 (1*R*,6*R*)-**2d**	24	11.5:1	84	+5.5
16	 (1*S*,6*R*)-**2d**	20	11.4:1	83	-19.8
17	 (1*S*,6*S*)-**2e**	22	11.4:1	84	-6.6
18	 (1*R*,6*S*)-**2e**	25	4.1:1	64	+6.1
19	 (1*R*,6*R*)-**2e**	19	12.4:1	84	+11.1
20	 (1*S*,6*R*)-**2e**	20	12.4:1	85	-68.8
21	 (1*S*,6*S*)-**2f**	37	11.1:1	83	+6.9
22	 (1*R*,6*S*)-**2f**	37	11.1:1	83	-17.4
23	 (1*R*,6*R*)-**2f**	42	26.6	93	-2.6
24	 (1*S*,6*R*)-**2f**	38	13:1	86	+4.9
25	 (1*S*,6*S*)-**2g**	29	12.7:1	85	+13.5
26	 (1*R*,6*S*)-**2g**	31	12:1	85	-3.8
27	 (1*R*,6*R*)-**2g**	36	11.7:1	84	-3.7
28	 (1*S*,6*R*)-**2g**	27	11.7:1	84	+3.3
29	 (3*S*,8*S*)-**2h**	26	13:1	86	-5.8
30	 (3*R*,8*S*)-**2h**	38	13:1	86	+46.5
31	 (3*R*,8*R*)-**2h**	32	12:1	85	+13.7
32	 (3*S*,8*R*)-**2h**	40	13:1	86	-7.2
33	 (3*S*,8*S*)-**2i**	39	15.3:1	88	+6.9
34	 (3*R*,8*S*)-**2i**	44	12:1	85	+10.2[b]
35	 (3*R*,8*R*)-**2i**	35	10:1	82	-19.1
36	 (3*S*,8*R*)-**2i**	45	8.5:1	79	-19.8
37	 (1*S*,6*S*)-**2j**	24	15.5:1	88	-14.0
38	 (1*R*,6*S*)-**2j**	24	4:1	60	+3.7
39	 (1*R*,6*R*)-**2j**	29	93:1	97	+9.8
40	 (1*S*,6*R*)-**2j**	37	7:1	75	-17.5

[a] Determined by chiral HPLC analysis. [b] The product configuration was determined by comparing the optical rotation reported in the references 22.

It was reported that certain polyacetylenes such as falcarindiol **1** have shown potential anti-proliferation activity against certain cancer cell lines[8-10]. These polyacetylenes can alkylate the

cysteine residue of the active site in certain targeted proteins of cancer cells *via* direct thiol addition to the conjugated triple bond.[23-24] With 40 samples of falcarindiol analogues in hand, we screened their anticancer activities on Hccc-9810, HepG2, MDA-MB-231, Hela, MG-63 and H460 cell lines by CCK-8 assay, and meanwhile the natural falcarindiol **1** was selected as a positive control (Table 1S in SI). As shown in Table 1S, the 40 samples exhibited different anti-proliferation effects on these cell lines at the adopted concentrations (1-300 μM). It was found that the falcarindiol analogues **2a-h** containing phenyl and cyclo-pentyl group almost did not inhibit the cancer cells growth in these selected cell lines. The falcarindiol analogues **2i** and **2j** with terminal ethylenic bond and furanyl group showed obvious anti-proliferation effects on human cholangiocarcinoma Hccc-9810, hepatocellular carcinoma HepG2, breast cancer MDA-MB-231, cervical cancer HeLa and osteosarcoma MG-63 cell lines. The effects of various concentrations the falcarindiol analogues **2i** and **2j** on Hccc-9810, HepG2, MDA-MB-231, HeLa and MG-63 cells were evaluated (Table 2S in SI). These results showed that the falcarindiol analogue (3*R*,8*S*)-**2i** can significantly inhibit the growth of Hccc-9810, HepG2, MDA-MB-231, Hela and MG-63 cell lines in a dose-dependent manner with the half maximal inhibitory concentration (IC_{50}) of 0.46 μM, 10.56 μM, 2.63 μM, 5.27 μM and 15.81 μM at 24 h respectively as shown in Figure 1. In particular, the falcarindiol analogue (3*R*,8*S*)-**2i** showed the greatest sensitivity on Hccc-9810 cell lines with IC_{50} of 0.46 μM.

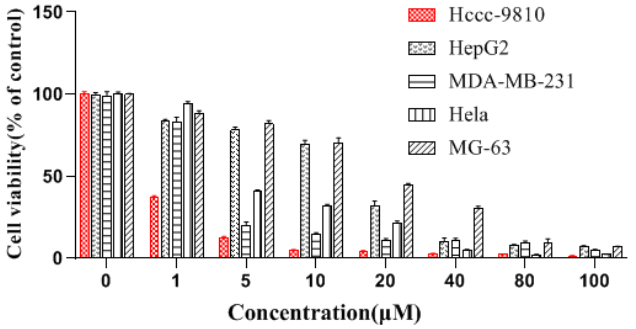
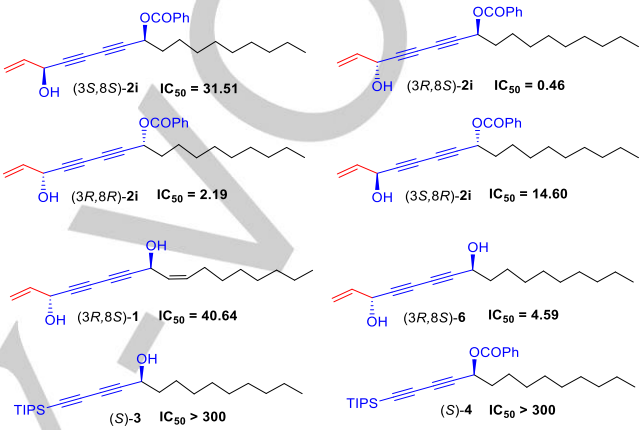


Figure 1. Effects of various concentrations of the falcarindiol analogue (3*R*,8*S*)-**2i** on five different human cancer cells at 24 h. x ± s, n = 3. **P < 0.01 vs control group at the indicated time.

We further analyzed the structure-activity relationships of the falcarindiol analogues **2i** on growth inhibition of Hccc-9810 cells. The stereoisomers (3*R*,8*S*)-**2i** and (3*R*,8*R*)-**2i** with a *R* configuration at the 3-position showed IC_{50} of 0.46 μM and 2.19 μM and they contributed greater inhibition activity than the isomers (3*S*,8*R*)-**2i** and (3*S*,8*S*)-**2i** with a *S* configuration at the 3-position for which gave IC_{50} of 14.66 μM and 31.51 μM (Scheme 3). The (*R*)- or (*S*)-configuration at the 8-position showed smaller effect on the anti-proliferation effects on Hccc-9810 cells. When the benzoyl group at the 8-position in (3*R*,8*S*)-**2i** was removed to give (3*R*,8*S*)-**6**, a IC_{50} of 4.59 μM was observed (Scheme 3). The conjugated diynols (*S*)-**3** and (*S*)-**4** with the TIPS group exhibited no cytotoxicity at concentration above 300 μM, which may be due to the more protective effects of its lipophilic structure than the anticancer activity on Hccc-9810 cells[21]. The anti-proliferation data of the falcarindiol (3*R*,8*S*)-**1** (IC_{50} = 40.64 μM) showed that the double bond in the middle of the carbon chain contributed less cytotoxicity compared to the single bond in the falcarindiol analogue (3*R*,8*S*)-**6** (Scheme 3). Thus, the falcarindiol analogue (3*R*,8*S*)-**2i** has potential as a therapeutic agent with cytostatic activity against Hccc-9810 cells.



Scheme 3. Structure-activity relationships of the falcarindiol analogues **2i** on growth inhibition of Hccc-9810 cells.

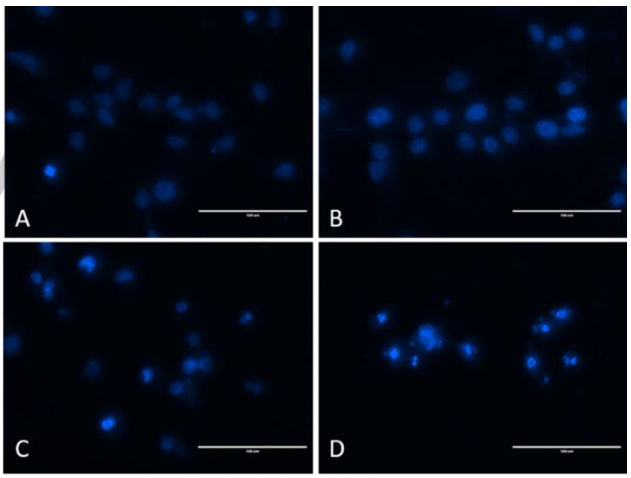


Figure 2 Effect of (3*R*,8*S*)-**2i** on apoptosis morphology of Hccc-9810 cells determined by Hoechst33258 staining (200×). A: Control group; B, C, D: Treated group with 0.5, 1 and 2 μM (3*R*,8*S*)-**2i**.

Apoptosis morphology of Hccc-9810 cells induced by the falcarindiol analogue (3*R*,8*S*)-**2i** was determined by Hoechst33258 staining under a fluorescent microscope. As shown in Figure 2, After Hccc-9810 cells were treated with various concentrations of (3*R*,8*S*)-**2i** for 24 hours, the classic apoptosis characteristics in cells with morphology change, chromatin condensation and apoptotic bodies were found with

Hoechst staining compared to the control group (A in Figure 2). With the increase of concentration from 0.5 μM, 1 μM to 2 μM, (3*R*,8*S*)-**2i** could induce obvious apoptosis in Hccc-9810 cells (B, C and D in Figure 2).

In order to confirm the falcarindiol analogue (3*R*,8*S*)-**2i** induced apoptosis, Hccc-9810 cells in suspension culture were subjected to double staining based on annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) by flow cytometry analysis and comparison with the deprotected (3*R*,8*S*)-**6**. As shown in Figure 3, the population of cells indicated a shift from viable cells to early stage and late stage apoptosis after the treatment with (3*R*,8*S*)-**2i** at different concentrations (0.5 μM, 1 μM and 2 μM) for 24 h by comparison with the control group (A in Figure 3). The percentage of apoptotic cells (annexin-FITC+/PI- and FITC+/PI+ cells) induced by (3*R*,8*S*)-**2i** were increased in a concentration-dependent manner with 8.01%, 45.98% and 51.68% respectively (B, C and D in Figure 3), while the treatment of Hccc-9810 cells with (3*R*,8*S*)-**6** at concentration of 0.5 μM, 1.0 μM and 2.0 μM induced a less increase of apoptosis with 10.19%, 12.29% and 27.08% (Figure 1S in SI). In addition, most of the apoptotic cells were in the lower right quadrant indicating that the apoptotic cells were in the early stage of apoptosis (annexin-FITC+/PI-). These results were in agreement with structure-activity relationships and morphological data.

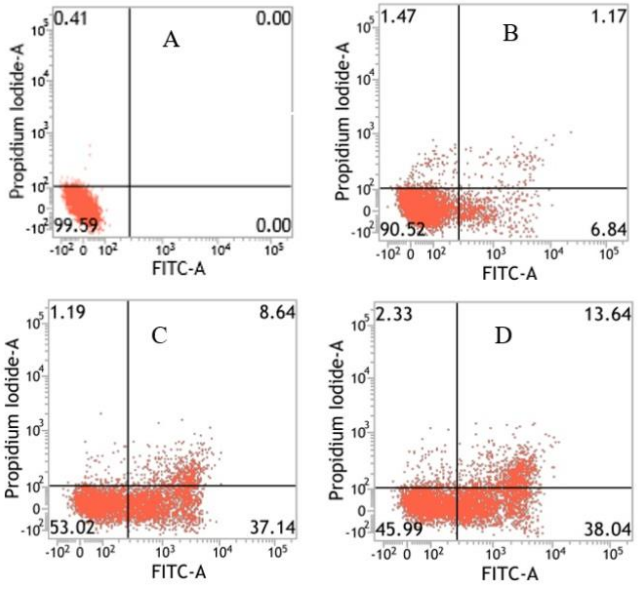


Figure 3 Effect of (3*R*,8*S*)-**2i** on apoptosis of Hccc-9810 cells determined by flow cytometric analysis. A: Control group; B, C, D: Treated group with 0.5, 1 and 2 μM (3*R*,8*S*)-**2i**.

It has been reported that the falcarindiol-induced cytotoxicity and cell death in the breast cancer and colorectal cancer cell lines were related with lipid peroxidation and endoplasmic reticulum stress[7-10]. The preliminary mechanism on the anti-proliferation effect of the falcarindiol analogue (3*R*,8*S*)-**2i** on Hccc-9810 cells was investigated by using Lactate dehydrogenase (LDH) release, malondialdehyde (MDA) content and superoxide dismutase (SOD) kit. LDH release is considered as a very sensitive biochemical index of cellular damage. As shown in Figure 4A, the falcarindiol analogue (3*R*,8*S*)-**2i** at different concentration from 0.5 to 2.0 μM could significantly increase the LDH release from 255 to 847 U/L compared to the control group (28 U/L, Table 2). The content of MDA is a good indicator for lipid peroxidation induced by oxygen free radicals, indirectly reflecting the degree of cell injury. As shown in Figure 4B, the treatment with 0.5, 1.0 and 2.0 μM of the falcarindiol analogue (3*R*,8*S*)-**2i** induced an increase of the MDA content to 12.75, 39.86 and 42.36 nmol/mL respectively (Table 2). SOD is an important free radical scavenger to protect biological systems from oxidative stress. The level of SOD was decreased from 35 to 19 U/mL as compared with those in control group (Table 2) when Hccc-9810 cells were treated with (3*R*,8*S*)-**2i** at a concentration of 0.5-2.0 μM (Figure 4C). These findings indicate that the anti-proliferation effect of the falcarindiol analogue (3*R*,8*S*)-**2i** may be correlated with intracellular free radical generation and oxidative stress, which might lead to apoptosis of Hccc-9810 cells. The detailed mechanistic study is in progress. This is the first investigation on the anticancer activity of falcarindiol and its analogues toward human CCA cells.

Table 2 Concentration effect of (3*R*,8*S*)-**2i** on LDH, MDA and SOD levels in Hccc-9810 cells. x ± s, n = 3. * P＜0.05, ** P＜0.01 vs control group

Group	LDH（U/L)	MDA(nmol/mL)	SOD（U/mL）
control	28.32±4.08**	0.96±0.44	35.52±1.57
0.5 mM	255.04±13.53**	12.75±0.68**	23.08±0.02*
1.0 mM	797.17±15.93**	39.86±0.80**	20.61±0.04*
2.0 mM	847.23±10.79**	42.36±0.54**	19.14±0.05*

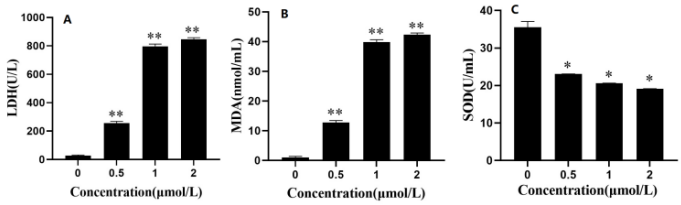


Figure 4 Effect of (3*R*,8*S*)-**2i** on LDH, MDA and SOD level in Hccc-9810 cells. x ± s, n = 3. * P＜0.05, ** P＜0.01 vs control group

Conclusions

In conclusion, we have synthesized 40 optically active falcarindiol analogues by using the simple and practical BINOL-based catalytic system under mild conditions. Their anticancer activities on Hccc-9810, HepG2, MDA-MB-231, Hela, MG-63

and H460 cells were screened and the structure-activity relationships were investigated. The most potent anti-proliferation effect on Hccc-9810 cells by using a falcarindiol analogue (3*R*,8*S*)-**2i** was observed for the first time in a dose-dependent manner. The falcarindiol analogue (3*R*,8*S*)-**2i** induced obvious apoptosis preferentially in Hccc-9810 cells as revealed by using Hoechst33258 staining and flow cytometry analysis. Mechanistic investigations including LDH release, MDA content and SOD activity suggest that the Hccc-9810 cell apoptosis induced by the falcarindiol analogue (3*R*,8*S*)-**2i** could involve intracellular free radical generation and oxidative stress. These findings indicate that the falcarindiol analogue (3*R*,8*S*)-**2i** could be considered as a promising therapeutic agent of human cholangiocarcinoma (CCA) for further study.

Experimental Section

General procedure and materials

All reactions were performed under inert gas conditions unless otherwise specified. The chemicals were commercially available and were used directly without purification. Methylene chloride, tetrahydrofuran, and diethyl ether were purified by MBRAUN SPS-800-Systems, and other solvents were dried by standard methods prior to use. The NMR data were recorded with a Bruker TM400 NMR spectrometer. High-resolution mass spectra (QTOF)were measured on SCIES-X500r. HPLC analyses were performed with a Waters 1525 by using Diacel Chiralcel OD-H, Chiracel AD-H, and Chiralcel AS-H columns, with detection at 254 or 220 nm by using a Waters 2487 instrument. Optical rotations were obtained by using a Hanon P810/P850 automatic polarimeter with a sodium 589.3 nm filter.

For anticancer activities of falcarindiol analogues, DMSO, Penicillin, Streptomycin was purchased from Sigma-Aldrich. DMEM medium with high glucose and RPMI1640 medium were purchased from Gibco. Human cholangiocarcinoma Hccc-9810, hepatocellular carcinoma HepG2, breast cancer MDA-MB-231, cervical carcinoma HeLa, osteosarcoma MG-63 and large cell lung cancer H460 cell lines were purchased from the Institute of Cell Biology, Academic Sinica (Shanghai, China). CCK-8 detection kit was obtained from Japanese colleagues. Fetal bovine serum was purchased from Hangzhou Sijiqing company. Trypsin was purchased from Bioder company. Malonaldehyde (MDA), superoxide dismutase (SOD) and lactic dehydrogenase (LDH) kit were purchased from Nanjing Jiancheng Institute of Biology. CKX53-SLP inverted phase contrast microscope, fluorescence inverted microscope (Olympus, Japan), Spectra Max M3 multi-function microplate reader (Molecular Devices, USA), TSX60086D refrigerator, HERACELL-150i CO_2 incubator and 481HP constant temperature shaker (U.S. Thermo Fisher Scientific company) were used.

Typical procedure for the asymmetric diyne addition to aldehydes by using ligand (*R*)- and (*S*)-BINOL

The first asymmetric addition: under argon atmosphere, Zn powder (5.1 mmol, 10.2 equiv.) and (*R*)- or (*S*)-BINOL (0.34 mmol, 0.68 equiv.) were added to a 25 mL flask equipped with a mechanical stirrer. Then EtI (10.2 mmol, 20.4 equiv.), $Ti(O^iPr)_4$ (0.85 mmol,1.70 equiv.) and buta-1,3-diynyl-TIPS (1 mmol, 2 equiv.) were added dropwise via syringe. After 5 min, THF (1 mL) was added and the mixture was stirred at room temperature (r.t.) for 24 h. Then diethyl ether (15 mL) was added, followed by the addition of 1-decanal (0.5 mmol, 1equiv.). The reaction mixture was stirred for 12 h, and then it was quenched with aqueous saturated NH_4Cl (5 mL). The solution was extracted with CH_2Cl_2 (4 × 50 mL), and the combined organic phase was dried over Na_2SO_4, filtered and concentrated under reduce pressure. The crude residue was purified by column chromatography on silica gel eluted with petroleum ether and ethyl acetate to afford the corresponding compound (*S*)-or (*R*)-**3**.

The second asymmetric addition: under argon atmosphere, Zn powder (3.06 mmol, 10.2 equiv.) and (*R*)- or (*S*)-BINOL (0.20 mmol, 0.68 equiv.) were added to a 25 mL flask equipped with a mechanical stirrer. Then EtI (6.12 mmol, 20.4 equiv), $Ti(O^iPr)_4$ (0.51 mmol, 1.70 equiv.), and (*S*)-or (*R*)-**5** (0.60 mmol, 2 equiv) were added dropwise by syringe. After 5 min, THF (1 mL) was added, and the mixture was stirred at r.t. for 24 h. Then diethyl ether (10 mL) was added, which was followed by the addition of an aldehyde (0.3 mmol, 1 equiv) after one hour. The mixture was stirred for 12 h, and then it was quenched with aqueous saturated NH_4Cl (5 mL). The solution was extracted with CH_2Cl_2 (3 × 20 mL), and the combined organic phase was dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel eluted with petroleum ether and ethyl acetate to afford the desired chiral falcarindiol analogues **2**.

Anticancer activities of the falcarindiol analogues 2

The viability of cells was measured by a colorimetric CCK8 assay. Cultured cells were counted and suspended in DMEM and RPMI1640 medium. 96-well plates were seeded at concentrations of 7000 and 5000 cells/well (180 μL medium) for Hccc-9810, HepG2, MDA-MB-231, HeLa, MG-63 and H460 cell lines respectively and then preincubated for 24 h. Each sample of the falcarindiol analogues **2** was dissolved in DMSO to produce a 0.2 mol/L stock solution. The falcarindiol **1** was selected as a positive control. Serial dilutions were performed (with the addition of DMEM and RPMI1640 medium for Hccc-9810, HepG2, MDA-MB-231, HeLa, MG-63 and H460 cell lines respectively) to produce final ligand concentrations ranging from 300-500 μmol/L. The testing sample was added at 20 μL per well, followed by incubation for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. Then the medium was removed and replaced with 100 μL 10% CCK8 (V/V) per well, followed by 30 min incubation at 37 °C. The absorbance was recorded at 450 nm by using microplate reader. The results were expressed as ratio of absorbance between treatments and control cells (solvent vehicle set at 100%). Four replicate wells were tested per assay which was repeated three times. IC_{50} values (concentration resulting in 50% inhibition) were calculated using GraphPad Prism (San Diego, CA).

Apoptosis morphology by Hoechst33258 staining

Hccc-9810 cells in the logarithmic growth phase were taken and inoculated in a 6-well culture plate at a density of about 1×10^5 cells per well. After cultured overnight at 37 °C and 5% CO_2 incubator, the medium was discarded, followed by treatment with 0.5 μM, 1.0 μM and 2.0 μM of the falcarindiol analogue (3*R*,8*S*)-**2i** (including control group). The cells were washed twice with PBS and fixed in 0.5 mL methanol per well for 30 min. The fixed cells were incubated with 1 mL Hoechest33258 staining solution for 10 min at 37 °C in the dark. After removal of the staining solution, the cells were washed twice with PBS, and then were observed by using a fluorescence microscope (FL CD12-002, AMG, USA) with excitation wavelength of 350 nm and emission wavelength of 461 nm. For cell counts, 5 random fields (about 100 cells each field) were observed per coverslip. Results were expressed as percentage of Hoescht-positive nuclei (condensed or fragmented) relative to the total number of nuclei counted per coverslip for each experimental condition.

Apoptosis assays by flow cytometric analysis

According to the Hoechst33258 staining procedure, Hccc-9810 cells (1×10^5) are grouped, followed by treatment with 0.5 μM, 1.0 μM and 2.0 μM of the falcarindiol analogues (3*R*,8*S*)-**2i** and (3*R*,8*S*)-**6** (including control group) for indicated times at 37 ºC. The treated cells were washed twice with PBS, and then harvested by centrifugation. After 200 μL of 1×Annexin V binding buffer was added, the treated cells were resuspended. The cell suspension was randomly taken 100 μL, and transferred to another 5 mL centrifuge tube, to which was added 5 μL Annexin V-FITC and 5 μL PI. After mixed well, the cell suspension was stained for 15 min in the dark at room temperature, and incubated with 400 μL 1×Annexin V binding buffer for 1 h, which was then detected by flow cytometry (BD PharmingenTM) and examined on Flow Jo software.

LDH Release, MDA Content and SOD Activity Assays

LDH release, MDA content and SOD activity assays were evaluated with commercial detection kits (NJJC Bio). In brief, the cells were broken by ultrasonic cell disruptor and centrifuged at 10, 000 rpm for 10 min at 4 ºC. The supernatant of each well was transferred to a fresh flat bottom 96 well culture plate and processed further for enzymatic analysis as per the manufacturer's instructions. Each experiment was repeated three times independently.

Acknowledgements

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Keywords: asymmetric synthesis; 1,1'-binaphth-2-ol (BINOL); falcarindiol analogues; anticancer activities; structure-activity relationships; mechanism.

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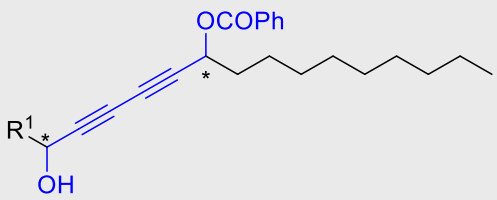

Falcarindiol analogue (3*R*, 8*S*)-**2i** inhibits Hccc-9810 cells with IC_{50} of 0.46 μM

2a R^1 = phenyl
2b R^1 = *meta*-fluoro phenyl
2c R^1 = *meta*-chloro phenyl
2d R^1 = *meta*-bromo phenyl
2e R^1 = *meta*-methoxyl pheny
2f R^1 = *ortho*-methyl phenyl
2g R^1 = *cyclo*-pentyl
2h R^1 = *trans*-styryl
2i R^1 = vinyl
2j R^1 = furanyll

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Synthesis, Anticancer Activity, Structure-Activity Relationship and Mechanism Investigation of Falcarindiol Analogues