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Synthesis and cytotoxic activities of 4,5-diarylisoxazoles

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Abstract—A series of 4,5-diarylisoxazoles related to combretastatin A-4 (**CA-4**) were synthesized and evaluated for cytotoxicity against three human cancer cell lines. Among them, compound **6e** showed better cytotoxic activity than **CA-4** in HeLa and HepG2 cell lines assayed with IC_{50} value as low as 0.022 and 0.065 nM, respectively.

Antimitotic agents continue to be an interesting target for medicinal chemist due to possible clinical application in cancer chemotherapy. Taxol is one successful example. Antimitotic agents inhibit cancer cell proliferation by interfering with microtubule polymerization or depolymerization process.[1,2]

Combretastatins, naturally occurring stilbenes, were isolated from *Combretum caffrum* by Pettit group.[3,4] Among them, combretastatin A-4 (**CA-4**) strongly inhibited the polymerization of tubulin by binding to the chochicine site and showed most potent cytotoxicity against a variety of human cancer cell lines including multiple drug-resistant cancer cell lines.[5,6] To date, many **CA-4** analogues and their anticancer activity have been extensively studied and a water-soluble sodium phosphate prodrug (**CA-4P**) is currently evaluated for clinical applications.[7,8]

From the previous comparative studies of the combretastatins it appears that the *cis* orientation of the two aromatic rings plays an important key role in cytotoxicity. However, during storage and administration *cis*-combretastatin analogues tend to isomerize to *trans* forms which show a dramatic decrease in their inhibitory effects on cancer cell growth and tubulin polymerization.[9,10] Accordingly, a number of *cis*-restricted analogues of **CA-4** were prepared using 1,2-substituted five-membered heterocycles such as imidazole,[11] oxazole,[11] pyrazole,[11,12] triazole,[12] tetrazole,[12] thiazole,[12] furanone,[13] cyclopentenone,[14] oxazolone,[15] dioxolane,[16] and furazan[17] to avoid the stability problem. Many of them showed potent cytotoxicity against various cancer cells compared to **CA-4**. In recent study, isoxazoline derivatives were reported to possess potent apoptosis-inducing activity in HL60 and in MDR cell lines.[18]

In our efforts to discover active antimitotic agents, we utilized isoxazole ring to mimic the *cis* double bond in **CA-4**. In this study, a series of 4,5-diarylisoxazoles (Fig. 1) were prepared and their cytotoxic activity was also evaluated against human cancer cell lines including human cervical epitheloid carcinoma (HeLa), human hepatocellular carcinoma (HepG2), and human ovarian adenocarcinoma (OVCAR-3).

The starting dithiane (**1a**) was prepared from 3,4,5-trimethoxybenzaldehyde by treatment with 1,3-propanedithiol in the presence of aniline hydrochloride. The dithiane (**1a**) reacted with *n*-BuLi and benzyl bromides (**2a**–**c**) at −78 °C to give the alkylated dithiane products (**3a**–**c**), respectively,[19] which were then converted to the corresponding ketone derivatives (**4a**–**c**) by reaction with HgO and $HgCl_2$. Treatment of the ketone derivatives (**4a**–**c**) with *N*,*N*-dimethylformamide dimethylacetal (DMFDMA) under reflux resulted in the corresponding enamino ketones (**5a**–**c**)[20] which were then reacted with

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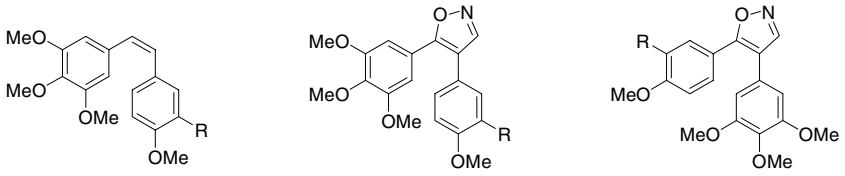


Figure 1. Chemical structures of **CA-4**, **CA-4P**, and 4,5-diarylisoxazoles.

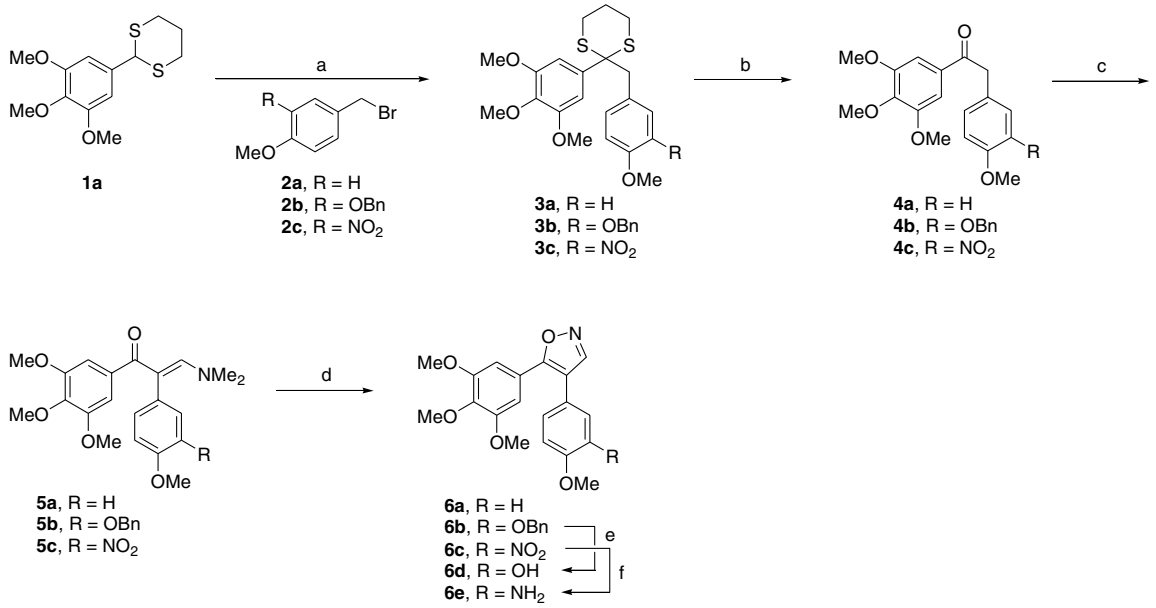
hydroxylamine hydrochloride to yield desired 4,5-diarylisoxazoles (**6a**–**c**), respectively.[21] Deprotection of the benzyl group of **6b** by hydrogenation gave **6d**. Reduction of nitro group of **6c** using acetic acid and zinc powder yielded **6e** (Scheme 1).[22]

Compounds **6f** and **6h** were prepared by using the same synthetic strategy as that for **6a** and **6d**, which is shown in Scheme 2 starting from 4-methoxybenzaldehyde (**1b**), benzylic derivative of 3-hydroxy-4-methoxybenzaldehyde (**1c**), and 3,4,5-trimethoxybenzyl bromide (**2d**).

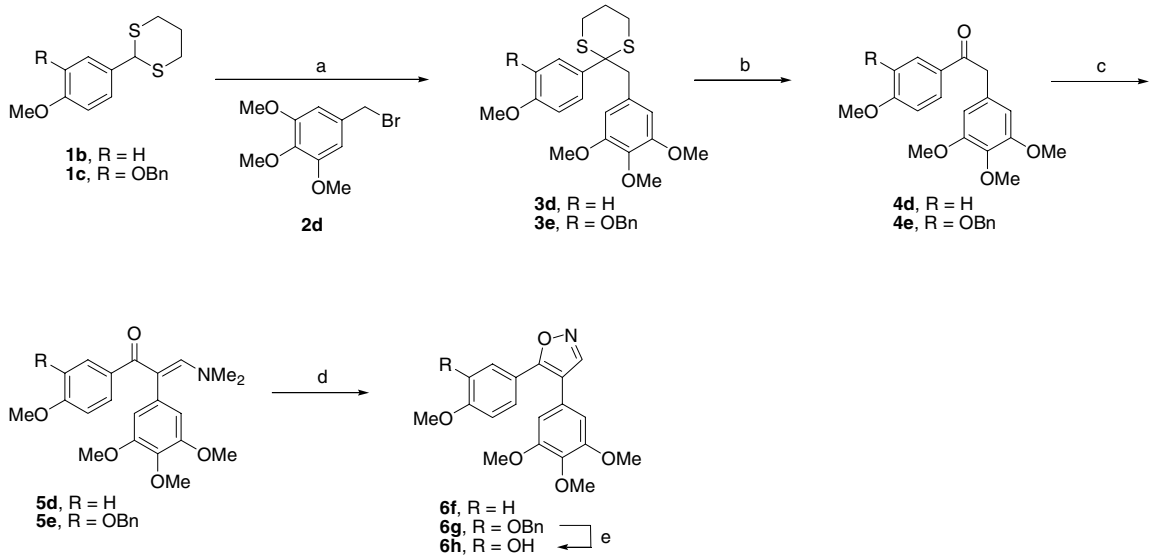
The structures of compounds (**6a**, **c**–**f**, **h**) were confirmed by detailed NMR analysis and elemental analysis. The cytotoxic activity of synthesized 4,5-diarylisoxazoles was evaluated against human cancer cell lines including human cervical epitheloid carcinoma (HeLa), human hepatocellular carcinoma (HepG2), and human ovarian adenocarcinoma (OVCAR-3). The results are summarized in Table 1. The cell line culture conditions[23] and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay for IC_{50} were carried out according to the procedures previously described.[24]

Previous reports indicated that 3,4,5-trimethoxy group on one of two aromatic rings was essential for strong cytotoxicity. Therefore, we chose 3,4,5-trimethoxyphenyl and 4-methoxyphenyl or 3-hydroxy-4-methoxyphenyl as two aromatic rings and first synthesized two isomers **6a** and **6d**. Assay results showed that **6a** and **6d** were active against three cancer cell lines, and **6d** was more potent than **6a**. Next, we switched two aromatic rings and synthesized two other isomers **6f** and **6h**. Compounds **6f** and **6h** showed moderate cytotoxic activity and less potent than **6a** and **6d**, which indicated the 3,4,5-trimethoxyphenyl group near the oxygen atom of isoxazole ring is important for strong cytotoxicity. The activity of **6d** and **6h** was greater than that of **6a** and **6f**, respectively, which indicated with the hydroxyl group in **6d** and **6h** is better than without the hydroxyl group in **6a** and **6d**. This result was in consistence with other reports.[12,14,16] Previous reports also indicated that replacement of a hydroxyl group with an amino group exhibited similar or greater activity.[13,15,17,25] Thus, **6c** and **6e** were prepared for activity comparison. Surprisingly, **6e** displayed excellent in vitro cytotoxicity against three cancer cell lines and showed better cytotoxicities than **CA-4** in HeLa and HepG2 cell lines assayed with IC_{50} value as low as 0.022 and 0.065 nM, respectively.

The effects of the two most active compounds **6d** and **6e** on the cell cycle were measured by flow cytometry



Scheme 1. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C, 30 min, then TMEDA, benzyl bromides, 25 °C, overnight; (b) HgO, $HgCl_2$, MeCN/H_2O, rt, 16 h; (c) DMFDMA, reflux, 14 h; (d) $NH_2OH{\cdot}HCl$, Na_2CO_3, MeOH, AcOH, reflux, 2 h; (e) H_2, 10% Pd/C, EtOAc, rt, 3 h; (f) Zn, AcOH, rt, 1 h.



Scheme 2. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C, 30 min, then TMEDA, benzyl bromides, 25 °C, overnight; (b) HgO, $HgCl_2$, MeCN/H_2O, rt, 16 h; (c) DMFDMA, reflux, 14 h; (d) $NH_2OH{\cdot}HCl$, Na_2CO_3, MeOH, AcOH, reflux, 2 h; (e) H_2, 10% Pd/C, EtOAc, rt, 3 h.

Table 1. Cytotoxicity of synthesized compounds in tumor cell lines[a]

Compound	Cytotoxicity (IC_{50},[b] nM)		
	HeLa	HepG2	OVCAR-3
6a	4.69	4.40	10.3
6c	11.6	13.7	9.6
6d	0.90	1.43	0.53
6e	0.022	0.065	0.135
6f	299	396	223
6h	29.4	33.9	14.8
CA-4	2.75	0.14	0.01

[a] HeLa, human cervical epitheloid carcinoma; HepG2, human hepatocellular carcinoma; OVCAR-3, human ovarian adenocarcinoma.
[b] Drug concentration required to inhibit the cell growth by 50%.

Table 2. Effects[a] of compounds **6d**, **6e**, and **CA-4** on cell cycle progression

Compound	G_0/G_1 (%)	S-phase (%)	G_2/M (%)
Control (DMSO)	50.0 ± 3.5	27.0 ± 4.8	23.0 ± 2.1
6d (10 nM)	17.1 ± 1.0	31.5 ± 2.8	51.4 ± 3.2
6e (10 nM)	2.81 ± 1.3	27.4 ± 2.4	69.8 ± 2.7
CA-4 (10 nM)	1.68 ± 0.3	19.0 ± 0.7	79.3 ± 1.0

[a] All experiments were independently performed three times.

against HepG2 human cancer cells after 24 h. The results are shown in Table 2. Compounds **6d** and **6e** caused significant arrest of the cells at the G_2/M phase relative to the untreated control, consistent with the behavior of tubulin-binding agents. Compound **6d** was recently reported to exhibit potent antitubulin activity.[26]

In conclusion, we have presented here the synthesis and evaluation of cytotoxicity of a series of 4,5-diarylisoxazoles. Compounds **6a**, **6c**, **6d**, and **6e** showed strong growth inhibitory activities against three human cancer cell lines, and **6e** was the most potent compound in this series. In addition, **6d** and **6e** caused G_2/M phase arrest of the cells and are considered to be potential new antimitotic agents for future clinical use.

Acknowledgments

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22. Typical procedure for the synthesis of **6e**. To a solution of **1a** (1.43 g, 5.0 mmol) in dry THF (40 mL) was added *n*-BuLi (3.4 mL, 1.6 M in hexane) at −78 °C under N_2. After stirring for 30 min, a solution of **2c** (1.23 g, 5.0 mmol) in THF (10 mL) and TMEDA (0.75 mL) were added at −78 °C, and the reaction mixture was allowed to stir at −25 °C overnight. Saturated NH_4Cl was then added and the mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was chromatographed over silica gel eluting with 20% EtOAc in hexane to give **3c** as a pale yellow solid (1.02 g, 45%). To a solution of **3c** (910 mg, 2.0 mmol) in CH_3CN/H_2O (50 mL, 85:15) were added HgO (866 mg, 4.0 mmol) and $HgCl_2$ (1.19 g, 4.4 mmol) at rt. After stirring for 16 h, the mixture was filtered through Celite and the filtrate was concentrated in vacuo. Water was added and the resulting mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was chromatographed over silica gel eluting with 20% EtOAc in hexane to give **4c** as a pale yellow solid (370 mg, 51%). A solution of **4c** (580 mg, 1.61 mmol) in DMFDMA (10 mL) was then refluxed under N_2 for 14 h. After cooling, the reaction mixture was concentrated in vacuo. Without purification, the resulting crude product **5c** was dissolved in MeOH/H_2O (12 mL, 2:1), and $NH_2OH{\cdot}HCl$ (120 mg, 1.77 mmol) and Na_2CO_3 (95 mg, 0.90 mmol) were added to the solution at rt. The mixture was adjusted to pH 4–5 by addition of acetic acid and then refluxed for 2 h. After cooling, the mixture was adjusted to pH 8 by addition of 25% NH_4OH and extracted three times with CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was chromatographed over silica gel eluting with 20% EtOAc in hexane to afford **6c** as a yellow solid (320 mg, 51%): mp 120–122 °C; IR (KBr) ν_{max} 2991, 2959, 2935, 2831, 1580, 1535, 1469, 1358, 1245, 1127, 1007 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.74 (s, 6H), 3.87 (s, 3H), 3.98 (s, 3H), 6.83 (s, 2H), 7.12 (d, J = 8.5 Hz, 1H), 7.56 (dd, J = 8.5, 2.0 Hz, 1H), 7.91 (d, J = 2.0 Hz, 1H), 8.34 (s, 1H); EI-MS *m*/*z* (%) 386 (100) [M^+], 371 (26), 343 (12), 324 (10); Anal. Calcd for $C_{19}H_{18}N_2O_7$: C, 59.07; H, 4.70; N, 7.25. Found: C, 58.97; H, 4.63, N, 7.25. To a solution of **6c** (116 mg, 0.30 mmol) in acetic acid (25 mL) was added zinc powder (4.70 g) at rt. After stirring for 1 h, the reaction mixture was filtered through Celite and the filtrate was concentrated in vacuo. Water was added and the resulting mixture was extracted three times with $CHCl_3$. The combined organic layer was washed with saturated $NaHCO_3$, brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was chromatographed over silica gel eluting with 30% EtOAc in hexane to give **6e** as a yellow solid (46 mg, 43%): mp 82–83 °C; IR (KBr) ν_{max} 3439, 3344, 3222, 3101, 2991, 2933, 2837, 1632, 1578, 1511, 1468, 1418, 1234, 1125, 1000, 845 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.72 (s, 6H), 3.85 (s, 3H), 3.86 (s, 3H), 6.80–6.82 (m, 3H), 6.92 (s, 2H), 8.26 (s, 1H); EI-MS *m*/*z* (%) 356 (100) [M^+], 195 (47), 173 (10); Anal. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. Found: C, 64.05; H, 5.88, N, 7.65.
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