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Original Article

Synthesis and biological activity of some novel Combretastatin analogues and related compounds

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ABSTRACT: CA-4 is a biologically very active compound by binding to the colchicine binding site which lead to the inhibition of microtubule polymerization as well as showing antiagiogenic and anticancer effects by selectively shutting down the tumor blood flow. To avoid the disadvantage of rather low in vivo efficiacy resulting from the isomerization of the cisstilbene derivative to the thermodynamically more stable trans-isomer, our research group started the project for CA-4 analogs synthesis. The incorporation of carbocycles with different ring sizes on the connecting carbon-bridge of the natural compound prevents the system to undergo cis-trans-isomerization. The synthesis of the cyclopropane derivative of CA-4 via the cyclopropanation reaction with diazomethane, and further analogs with incorporated moieties for better water solubility.

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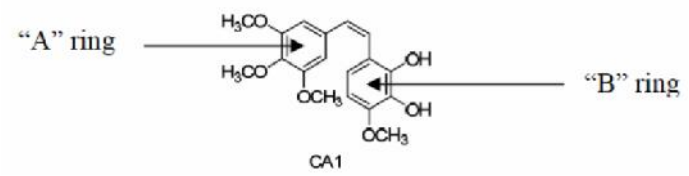
INTRODUCTION

Today cancer is accountable for about 20% of deaths in developing countries and for 18% of all deaths worldwide and therefore, cancer is one of the leading health problems in our society [1]. According to statistics of the International Agency Research on Cancer (IARC) of the World Health Organization (WHO) 15.2 million incident cases of cancer were expected in 2015 [2]. These impressive statistical data suggest that anti-tumor therapy is a very important research field today[3].

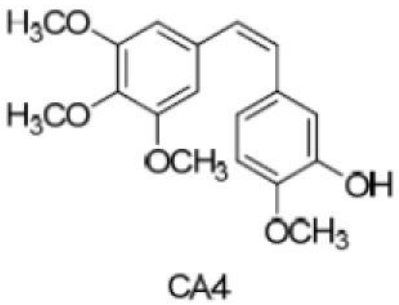
Combretastatinsshow promise for cancer treatment because of their pronounced potency in both inhibition of microtubule assembly assays and inhibition of cell growth assays [4]. Additionally, their simple structure allows for the total synthesis of analogues from commercially available starting materials to be completed quickly [5,6].

By subjecting analogues that vary at several moieties to *in vitro* and *in vivo* biochemical and biological activity, the structure-activity relationship profile for combretastatins can be expanded [7]. With an expanded library of combretastatin analogues, the key structural motifs necessary for their biological activity can be identified. Of the combretastatins, combretastatin A-1 (CA1) and combretastatin A-4 (CA4) are among the most biologically active in terms of microtubule depolymerization and cytotoxicity (**see Figures 4 and** 5)[8].

At their most basic level, CA1 and CA4 are comprised of two aromatic rings joined by a stilbene bridge. On both molecules, three methoxy functionalities populate one ring, labeled as ring "A." [9]. Additionally, CA1 has two hydroxyl functionalities and one methoxy functionality located on the other ring, labeled as ring "B." Combretastatin A-4 possesses only one hydroxyl functionality and one methoxy functionality on the "B" ring [10].



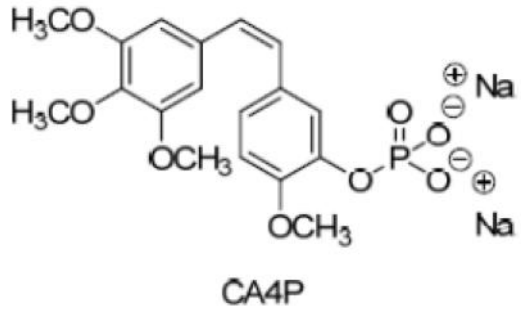
Combretastatin A-1 structure and ring



Combretastatin A-4

For both molecules, the functional groups on ring "A" and ring "B" directly alter the efficiency of microtubule depolymerization activity and cytotoxicity. The functional groups on the "A" ring are integral parts of the pharmacophore moiety that binds to the colchicine-binding site on β-tubulin, while the functional groups on the "B" ring stabilize the bound molecule by interacting with surrounding protein residues [11].

One of the most notable examples of such a modification can be seen in the structure of combretastatin A-4 phosphate (CA4P ; see Figure 9), in which a disodium phosphate salt is incorporated at the phenolic position [12]. The delivery of combretastatin analogues to tumors *in vivo* would be challenging without modifications such as these, unless harsh organic solvents are used. These organic solvents pose their own health hazards, so the use of water soluble compounds for treatment is necessary [13].

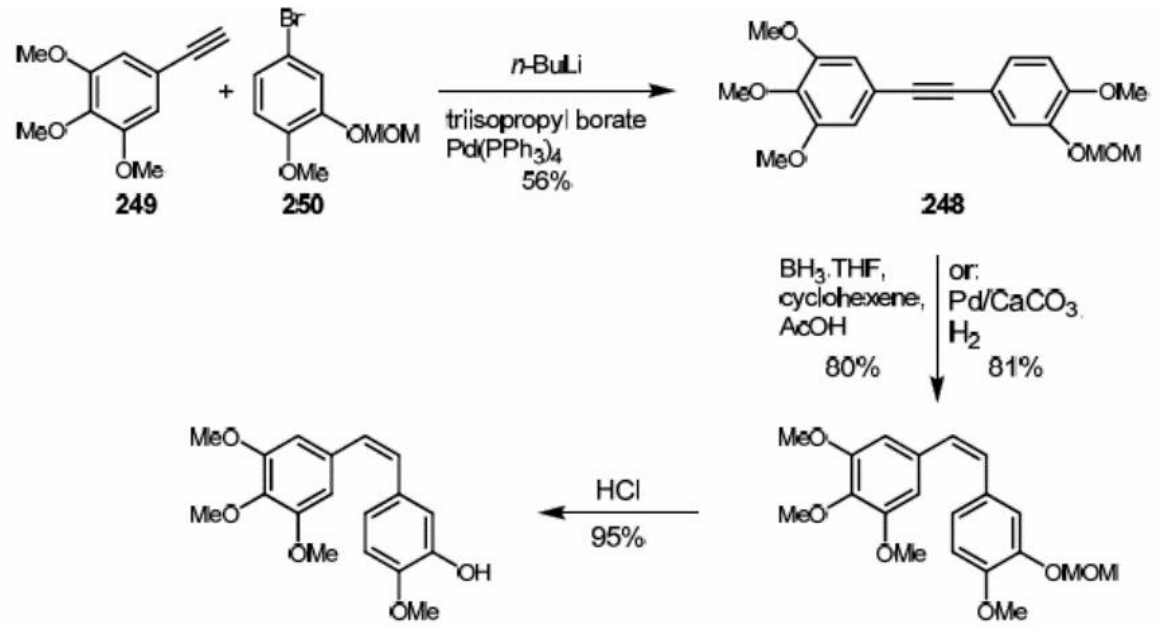


Combretastatin A-4 phosphate

Further drug development entails the incorporation of bioreductive triggers in the molecular structure[14]. While combretastatins do favor the destruction of tumor vasculature over normal blood vessels, high doses still have damaging side effects [15].Bioreductive triggers address this problem. Unlike other vascular disrupting agents, compounds with bioreductive trigger are introduced into the body in an inactive state. When these compounds encounter key bioreductive enzymes, the bioreductive trigger moiety is cleaved, leaving the active form of the compound [16,17].

As a result, elongated endothelial cells round up, causing disruption of endothelial cell layer surrounding blood vessel and exposing of underlying basement membrane [18]. This leads to bloodvessel congestion and loss of blood flow, loss of oxygen and nutrient supply to tumor cells. Therefore, tumor cells undergo necrosis. In view of strong anticancer/antivascular activity exhibited by CA-4, we have synthesized some novel combretastatin analogues, and tested for their antiangiogenic activity [19].

Synthesis of compounds



Synthesis of CA-4

Chemicals

All the chemicals and reagents used were from CDH, New Delhi. Glass wares used were from borosil. Ceric Ammonium Nitrate, Dimethoxyethane, Dimethylformamide, Dimethyl sulfoxide, Diethylether, Acetic acid etc.

MATERIALS AND METHODS

Chemistry

All compounds were purified by column chromatography and recrystallization and confirmatory establishment of structure was done by melting point, TLC, UV, IR and 1H NMR. Column chromatography was performed using silica gel (Qualigens, particle size 60-120 mm) [20]. TLC was performed on silica gel TLC plates. All melting points were recorded on a DECIBEL digital melting point apparatus. IR spectra were recorded on a 8400S SHIMADZU spectrometer. 1H NMR spectra were recorded on a dpx300 spectrometer (analysis laboratory, CDRI, LUCLNOW.).

Solvents and Chemicals Purification

The used solvents and chemicals were, if necessary, purified and dried according to common procedures as follows. Dry solvents were stored under an argon atmosphere over molecular sieve (4Å)[21]. Methylene chloride was distilled from P_2O_5, diethylether (Et2O) and tetrahydrofuran (THF) were freshly distilled from sodium/benzophenone under argon; Diisopropylamine (DIPA), diisopropylethylamine (DIPEA) and triethylamine (TEA), acetonitrile (MeCN), hexane and ethyl acetate were distilled from CaH2; toluene was refluxed over sodium and freshly distilled. All other solvents were HPLC grade.

Procedure of preparation of compounds.

2-Methoxyphenyl methanesulfonate (253).

To a solution of guaicol (252, 100.5 mmol, 11.3 mL) and NEt3 (150 mmol, 20.9 mL) in 100 mL CH_2Cl_2 was added Ms-Cl (126 mmol, 9.75 mL) dropwise over 5 min (126 mmol,N 9.75 mL) at 0 °C. The reaction mixture was allowed to stir at 0 °C for 90 min and further 12 hours at r.t. until total consumption of the starting material[22]. The solution was quenched with 100 mL water and the organic layer was separated. The aqueous phase was extracted with CH_2Cl_2 (2 x 100 mL) and the combined organic extracts were washed with saturated aq. $NaHCO_3$ (3 x 100 mL), water (2 x 150 mL) and brine (2 x 150 mL). The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure affording 19.8 g (97%) of 253 as yellow oil without further purification [23].

Rf 0.38 (hexanes/ethyl acetate 2:1);

5-Bromo-2-methoxyphenyl methanesulfonate (254).

To a solution of 253 (49 mmol, 10 g) in 25 mL DMF was added NBS (64 mmol, 11.4 g) in 25 mL DMF. The reaction mixture was stirred at room temperature until total consumption of the starting material (48 h). The reaction was quenched with saturated aqueous NH_4Cl (200 mL).

The resulting solution was diluted with diethyl ether (100 mL), the organic layer was separated and the aqueous phase was extracted with diethyl ether (4 x 100 mL). The combined organic extracts were washed with water (1 x 100 mL) and brine (1 x 100 mL), dried over Na2SO4, and the solvent was removed under reduced pressure affording 13.3 g of 254 as white crystals[24,25].

Rf 0.53 (hexanes/ethyl acetate 2:1);

5-Bromo-2-methoxyphenol (255).

A THF-solution of LDA (1.5 M, 69.5 mL, 53.4 mmol) was added dropwise to a solution of 254 (35.6 mmol, 10 g) in 36 mL THF at 0 °C. After being stirred for 30 min the reaction mixture was quenched with 5% aq. HCl. The product was extracted with diethyl ether (3 x 100 mL). The etheral extracts were washed successively with water (2 x 100 mL) and brine (2 x 100 mL), dried over Na2SO4, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexanes/ethyl acetate 5:1) affording 255 (6.7 g, 93%) as white crystals [26].

Rf 0.5 (hexanes/ethyl acetate 2:1);

(Z)-1,2,3-Trimethoxy-5-(4-methoxy-3-(methoxymethoxy)styryl)-benzene(258).

Cyclohexene (5.7 mmol, 0.58 mL) was added to borane-tetrahydrofurane complex (1 M in hexanes, 3 mmol, 3 mL) at 0 °C. After stirring for 90 min at this temperature 248 (0.56 mmol, 200 mg) dissolved in 4 mL THF was added. The reaction mixture was stirred at 0 °C until total consumption of the starting material (60 min, TLC), before being quenched with 1 mL AcOH. After that 10 mL ethyl acetate were added and the mixture was washed with saturated aqueous NaHCO3 (2 x 15 mL), water (2 x 15 mL) and brine (1 x 10 mL) before drying over Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (hexanes/ethyl acetate, 19:1) affording 161 mg (80%) of 258 [27].

Rf 0.4 (hexanes/ethyl acetate 5:1);

(Z)-2-Methoxy-5-(3,4,5-trimethoxystyryl)phenol (CA-4, 4).

To a solution of 258 (0.14 mmol, 50 mg) in 2 mL methanol 0.5 mL 3 M HCl were added. The reaction mixture was stirred for 24 h at room temperature. The solution was diluted with CH_2Cl_2. The organic layer was washed with water (2 x 8 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by HPLC affording 38 mg (95%) of 4[28].

Rf 0.37 (hexanes/ethyl acetate 2:1);

Spectral data;

2-Methoxyphenyl methanesulfonate (253).

^{1}H NMR (400 MHz, CDCl3) δ [ppm] 7.33-7.24 (m, 2H), 7.03-6.95 (m, 2H), 3.90 (s, 3H),3.18 (s, 3H);^{13}C NMR (400 MHz, CDCl3) δ [ppm] 128.41, 124.75, 121.32, 113.10, 56.13, 38.40.

5-Bromo-2-methoxyphenyl methanesulfonate (254).

^{1}H NMR (400 MHz, CDCl3) δ [ppm] 7.38 (d, J = 2.44 Hz, 1H), 7.31 (dd, J = 8.80, 2.44 Hz, 1H), 6.81 (d, J = 8.8 Hz, 1H), 3.81 (s, 3H), 3.13 (s, 3H);^{13}C NMR (400 MHz, CDCl3) δ [ppm] 151.32, 139.02, 131.47, 128.02, 114.59, 112.76, 56.68, 38.93.

5-Bromo-2-methoxyphenol (255).

^{1}H NMR (400 MHz, CDCl3) δ [ppm] 7.07 (d, J = 2.27 Hz, 1H), 6.96 (dd, J = 8.59, 2.37 Hz, 1H), 6.71 (d, J = 8.59 Hz, 1H), 5.63 (s, 1H), 3.87 (s, 3H);^{13}C NMR (400 MHz, CDCl3) δ [ppm] 146.67, 146.02, 122.94, 117.99, 113.44, 112.00, 56.24.

(Z)-1,2,3-Trimethoxy-5-(4-methoxy-3-(methoxymethoxy)styryl)-benzene(258).

^{1}H NMR (400 MHz, CDCl3) δ [ppm] 7.07 (d, J = 2.05 Hz, 1H), 6.92 (dd, J = 8.42, 2.03 Hz, 1H), 6.78 (d, J = 8.42 Hz, 1H), 6.50 (s, 2H), 6.46 (dd, J = 18.09, 11.98 Hz, 2H), 5.06 (s, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 3.70 (s, 6H), 3.41 (s, 3H);

^{13}C NMR (400 MHz, CDCl3) δ [ppm] 153.07, 149.21, 146.12, 137.21, 133.00, 130.15, 129.60, 129.15, 123.64, 117.52, 111.50, 106.01, 95.65, 60.95, 56.16, 56.01;IR ν 2937 2835 1579 1427 1078 1005 cm^{-1}.

(Z)-2-Methoxy-5-(3,4,5-trimethoxystyryl)phenol (CA-4, 4):

^{1}H NMR (400 MHz, CDCl3) δ [ppm] 6.92 (d, J = 2.07 Hz, 1H), 6.80 (dd, J = 8.26, 2.07 Hz, 1 H), 6.73 (d, J = 8.26 Hz, 1H), 6.44 (dd, J = 12.17, 23.67 Hz, 2H), 5.52 (s, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.69 (s, 6H);^{13}C NMR (400 MHz, CDCl3) δ [ppm] 153.01, 145.90, 145.39, 137.34, 132.84, 130,80, 129.62, 129.19, 121.25, 115.19, 110.47, 106.24, 61.06, 56.10, 56.08;IR ν 3752 3422 3152 2837 1419 1274 1005 cm^{-1}.

Pharmacology

CAM assay is used as a preliminary method to determine antiangiogenic effect of a compound. This assay is based upon the formation of a chorioallantoic membrane, in which takes place, in fertilized chicken eggs at a certain stage of the development of the embryo. pellets impregnated with the test compound are placed onto the vascular membrane of opened eggs, and the influence on angiogenesis is evaluated. For assay purpose, the fertile chicken eggs were procured from Kalchina hatchery,

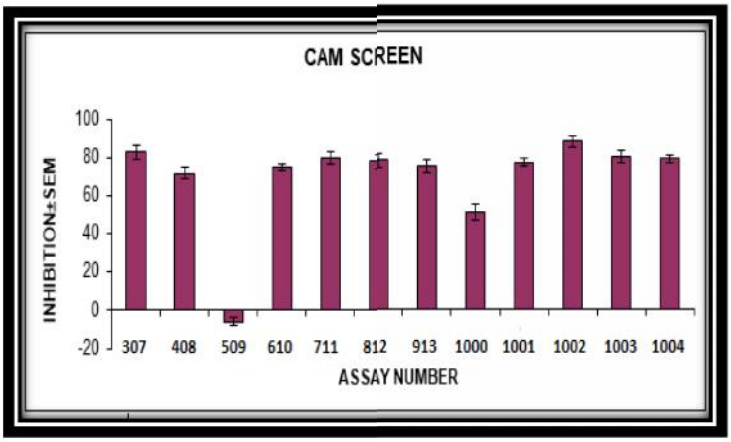
Antiangiogenesis study by (CAM) assay,

sixteen eggs were used per experiment to test one compound as a given dose [29]. The eggs were fertilized at 36.5^{o}C and 75 % relative humidity in ideal conditions. The shells of eggs were cleaned with 65% EtOH to avoid infections. After 70 hrs 6-8 ml of albumin was removed with a syringe at the lower side of the egg, and the hole was sealed with tape. Subsequently the upper part of the shell was removed, and the eggs were covered with a plastic film and incubated for another 70 hrs. At this point of time, when the diameter of CAM is between 1.7 and 2.5 cm, the pellets containing the test substances were placed on the CAM. Test substances were dissolved or suspended in a 2.4% agarose solution.

After gel formation, the volume of agarose gel corresponding to the dose of the test compound to be applied to the CAM was taken by means of a micropipette for viscous solutions. Therefore, the agarose pellets do not have a uniform size [30]. The half-cone-shaped agarose pellets are fixed becausev they slightly sink into the CAM. After 24 hrs the antiangiogenic effect was measured after addition of cream as a contrast fluid, by means of a stereomicroscope, by observing the avascular zone surrounding the pellet. Antiangiogenic activity is expressed as a score where 509 = no or weak effect, 103 = medium effect, and 1001 = strong effect (capillary free zone is at least twice as large as the pellet). Also, membrane irritation and embryotoxicity can be evaluated. B-1,4- galactan sulfate (LuPS S5) with an average molecular weight of 1500 was used as positive control 46 and an agarose pellet as a blank.

RESULTS AND DISCUSSION:

The concentration of the assays which were applied on the shell culture was 30 µM. The inhibition of shell proliferation within a certain time is investigated. The bars in Scheme 65 demonstrate that all compounds with just one exception, the alkyne derivative 261 (assay # 509) show potent biological activity. When the inhibition of a compound is less than 50% at 30 µM it is considered as inactive. It seems very interesting that also the alkyne derivatives are active at that concentration. For more detailed investigations the test was repeated with lower concentration to describe the dose-response relationship and the half maximal inhibitory concentration (IC50-value). The graph in Fig 1 demonstrates the IC50-value for CA-4 (12, assay # 711) for CAM cells.



The antiangiogenic activity of the test compounds is listed in table 1. All the compounds were tested at a 30 µM . because at higher dose most of compounds showed a toxic effect. Compound 509 showed an antiangiogenic score of more. Theresults of present study show that synthesized compounds have significant antiangiogenic activity. The most active analogues like alkyne derivative as bridge substituents while the least active analogues comparatively large groups as bridge substituents. Present study concludes that size of bridge substituents affects the antiangiogenic activity. Furthermore, most active analogues, of present study, are potential candidate for treatment of diseases related with angiogenesis.

The IC_{50}-concentrations for all tested compounds on CAM are listed in Table 1.

Table 1: IC_{50}-concentrations, CAM

Assay number	Compound number	Calculate IC_{50} Value (µM) CAM
307	260	0.98
408	216	24.11
509	261	>27
610	226	0.162
711	12	0.0003
812	237	1.07
913	227	0.199
1000	234	21.60
1001	7	0.027
1002	238	9.84
1003	235	8.99
1004	236	0.017

In this study, we found that all compounds with just one exception, the alkyne derivative 261 (assay # 509) show potent biological activity. When the inhibition of a compound is less than 50% at 30 µM it is considered as inactive. It seems very interesting that also the alkyne derivatives are active at that concentration.

CONCLUSION

CA-4 is a biologically very active compound by binding to the colchicine binding site which lead to the inhibition of microtubule polymerization as well as showing the antiangiogenic effects by selectively shutting down the tumor blood flow. To avoid the disadvantage of rather low in vivo efficacy resulting from the isomerization of the cisstilbene derivative to the thermodynamically more stable trans-isomer, our research group started the project for CA-4 analogs synthesis. The incorporation of carbocycles with different ring sizes on the connecting carbon-bridge of the natural compound prevents the system to undergo cis-trans-isomerization. The synthesis of the cyclopropane derivative of CA-4 (9) via the cyclopropanation reaction with diazomethane, and further analogs with incorporated moieties for better water solubility (268, 273) were achieved within this doctor of philosophy thesis. In cooperation with the central drug research institute,lucknowu.u.p. I was able to the biological activity (CAM and MTT) of the compounds prepared within this work.

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