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Research Article

Synthesis and Antiangiogenic Activity of some Novel Combretastatin A-4 analogues

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ABSTRACT: A series of Combretastatin A-4 analogues were synthesized in order to obtain new compounds with potential antiangiogenic activity. The structures of all synthesized compounds were confirmed by means of spectroscopical analytical techniques. All compounds were evaluated for their antiangiogenic activity by chorioallantoic membrane (CAM) assay method. The compounds showed significant antiangiogenic activity. Compound 5 showed maximum activity out of all synthesized compounds.

Keywords: Combretastatin A-4, antiangiogenic, cytotoxicity, anticancer, antitubulin.

INTRODUCTION

Angiogenesis or neovascularization is a complex process involving the activation, adhesion, proliferation, and transmigration of endothelial cells from pre-existing blood vessels. It plays a critical role in normal physiological processes such as wound healing, but also in a number of pathological processes, for instance diabetes retinopathy, arthritis, and the growth of solid tumors. Therefore angiogenesis is considered as a potential target for antitumor activity. Combretastatin A-4 is currently under investigation as an angiogenesis inhibitor (antiangiogenic). Combretastatin A-4 is a natural compound isolated by Pettit and co-workers (1982) from the bark of the South African bush willow tree *Combretum caffrum*. [1] Out of various stilbene derivatives (termed as combretastatins) isolated from the plant, combretastatin A-4 (CA-4, Fig. 1) was found to be most potent. [2] CA-4, *cis*-1-(3,4,5-trimethoxyphenyl)-2-(3'-hydroxy-4'-methoxy phenyl) ethene, is active in *cis* form. [3] From the structure-activity relationship (SAR) point of view, CA-4 belongs to the class of natural compounds related to biphenyls and contains, as a key structural feature, the *cis*-stilbene motif. CA-4 exerts a potent cytotoxicity against a variety of human cancer cells including multi drug resistant (MDR) cancer cell lines [4-11], and also displays potent antitumor effect in a wide variety of preclinical tumor models [12-16] as well as substantial antivascular (antiangiogenic) activity in tumor blood flow while causing no significant blood flow retention in normal tissues. [17-23] CA-4 does not show *in vivo* efficacy due to its poor pharmacokinetics resulting from its high lipophilicity and low aquous solubility [24] and also due to isomerization of *cis*-double bond to the more thermally stable *trans*-isomer, which is inactive. [2, 25] Till today various Combretastatin analogues have been synthesized and reported to posses cytotoxic activity against various cancer cell lines. [24, 26-33]

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CA-4 inhibits tubulin polymerization by binding to tubulin at colchicines binding site, resulting in disruption of dynamic equilibrium needed in formation of microtubules from α- and β- tubulin heterodimers, leading to formation of abnormal mitotic spindles. It results in cell cycle arrest in the M-phase, leading to apoptotic cell death. [34-39]

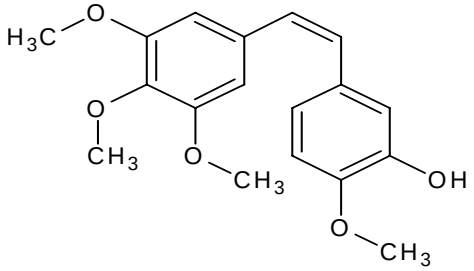

Fig. 1: Structural formula of CA-4

The IC_{50} (inhibitory concentration in 50% population) of CA-4 against tubulin polymerization is in range from 0.53 to 3.0 μM. [4, 10]

Recently, it has been found that CA-4 induces cell death primarily through mitotic catastrophe (formation of giant, multinucleated cells). CA-4 induces mitotic catastrophe by activation of a cysteine protease, called caspase-9. [40]

CA-4 is not a substrate of the MDR pump, a cellular pump that rapidly transports out foreign molecules, including many anticancer drugs. This is the major reason for its superior activity against MDR positive cancer cell lines. [41-42]

Antivascular effect of CA-4 is related to its antitubulin activity. The cellular microtubule network plays a major role in maintaining cell shape, particularly in the case of neovasculature. CA-4 causes microtubules to rapidly depolymerize. As a result elongated endothelial cells round up, causing disruption of endothelial cell layer surrounding blood vessel and exposing of underlying basement membrane. This leads to blood vessel congestion and loss of blood flow, loss of oxygen and nutrient supply to tumor cells. Therefore, tumor cells undergo necrosis. [7, 23, 43-44]

Table 1: Physical properties of the synthesized compounds

Comp. Code	R_1	R_2	Mol. Formula	M. Wt.	R_f[a]	m.p.
6a	H	$CSNH_2$	$C_{16}H_{12}ClN_3O_3S$	361.803	0.698	120
6b	H	C_5H_4N	$C_{20}H_{14}ClN_3O_3$	379.796	0.538	155
6c	H	$C_{10}H_7$	$C_{25}H_{17}ClN_2O_3$	428.867	0.702	95
6d	$R_1,R_2=C_5H_{10}$	C_5H_{10}	$C_{20}H_{19}ClN_2O_3$	370.829	0.407	132
6e	$R_1,R_2=C_4H_9N$	C_4H_9N	$C_{19}H_{18}ClN_3O_3$	371.818	0.585	162
6f	H	$C_6H_4OCH_3$	$C_{22}H_{17}ClN_2O_4$	408.834	0.519	92
6g	H	$C_{11}H_{11}N_2O$	$C_{26}H_{21}ClN_4O_4$	488.922	0.731	115

[a]Solvent System: [Dichloromethane : Carbon tetrachloride : Methanol (10:10:1)]

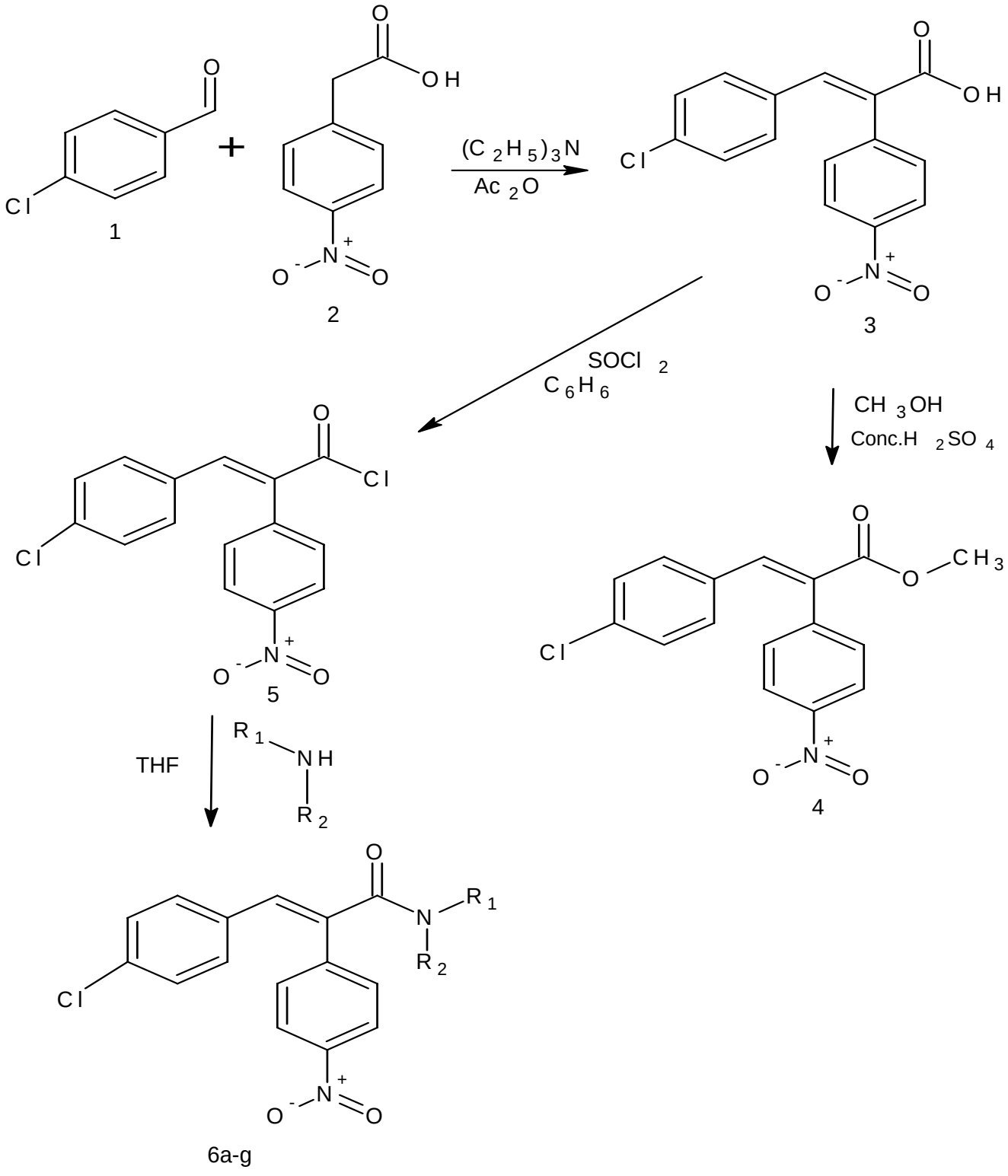

Fig. 2: Scheme of synthesis of compounds

Table 2: Antiangiogenic activity of compounds in the CAM assay

Test Compound	Concentration		Antiangiogenic score[b] ± SD (n = no. of experiment)
	(µg/pellet)	nmol/pellet	
3	10	33	1.2 ± 0.1 (n =3)
4	10	31	0.9 ± 0.1 (n =2)
5	10	31	1.5 ± 0.1 (n =3)
6a	10	28	0.8 ± 0.1 (n =2)
6b	10	26	0.3 ± 0.2 (n =2)
6c	10	23	0.2 ± 0.3 (n =2)
6d	10	27	0.6 ± 0.1 (n =2)
6e	10	27	0.5 ± 0.1 (n =2)
6f	10	24	0.3 ± 0.1 (n =2)
6g	10	20	0.6 ± 0.1 (n =2)
Agarose pellet			0.1 ± 0.1 (n =10)
β-1,4-galactan sulphate (LuPS S5)	50	25	1.4 ± 0.1 (n =10)

[b]0 = no or weak effect, 1 = medium effect, 2 = strong effect

In view of strong anticancer/antivascular activity exhibited by CA-4, we have synthesized some novel combretastatin analogues, and tested for their antiangiogenic activity.

MATERIALS AND METHODS

Chemistry

All compounds were purified by column chromatography and recrystallisation and confirmatory establishment of structure was done by melting point, TLC, UV, IR and ^{1}H NMR.Column chromatography was performed using silica gel (Qualigens, particle size 60-120 mm). 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. ^{1}H NMR spectra were recorded on a dpx300 spectrometer (analysis laboratory, IIT, New Delhi). Physical properties of the synthesized compounds are listed in Table 1 whereas scheme of synthesis is given in Fig. 2.

General procedure of preparation of compounds

Procedure of preparation of compound (3)

A mixture of p-nitrophenyl acetic acid **2** (2 mmol), benzaldehyde **1** (2 mmol), and triethylamine (0.5 ml) in acetic anhydride (5 ml) was refluxed for 12 hours, poured into hot saturated sodium carbonate solution (50 ml) and left over night. The mixture was extracted with diethyl ether (2×50 ml), and the ether extracts were discarded. The aquous solution was acidified with dilute HCl. The precipitated product was filtered with vaccum pump and dried. Product was subjected to column chromatography.

Procedure of preparation of compounds (4)

Concentrated H_2SO_4 (0.5 ml) was added to a stirred solution of carboxylic acid **3** (0.5 mmol) in absolute methanol (20 ml), and the mixture was heated under reflux for 6 hours. About 90% of excess methanol was removed by evaporation, and the residue was poured into ice-water (300 ml). The product was extracted with diethyl ether (2×40 ml), and the combined extracts were washed with 2 % NaOH solution (2×50 ml) followed by water (200 ml). Product was obtained from ether fraction.

Procedure of preparation of compounds (5)

A mixture of carboxylic acids **3** (0.5 mmol) and thionyl chloride (1ml) in benzene (10 ml) was refluxed for 6 hours. The excess thionyl chloride and benzene were removed under reduced pressure, and the residue was kept under vaccum for 30 minutes, and dried to give required product. Product was purified by recrystallization from EtOAc-hexane.

Procedure of preparation of compounds (6a-g)

A solution of appropriate amine (0.5 mmol) in THF (5 ml) was added to a solution of acid chlorides (prepared from 3 in 0.5 mmol scale, as described above) in THF (10 ml). The mixture was stirred for 3 hours. Solvents were romoved under reduced pressure, and the residue was poured onto ice (200 g). The product was extracted with diethyl ether (2×20 ml), washed with water and dried. Crude product was obtained from ether fraction. Product was purified by recrystallization from EtOAc-hexane.

Spectral data

(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)acrylic acid (3): FTIR (KBr) cm^{-1} 3025 and 856 (C-H), 1718 (C=O), 1633 (C=C), 1598 and 1412 (COO^-), 1520 and 1342 (C-NO_2), 749 (Ar-H), 688 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 10.00 (1H, bs), 8.26 (2H, d), 7.67 (1H, s), 7.20-7.61 (6H, m).

Methyl (2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)acrylate (4): FTIR (KBr) cm^{-1} 3027 and 857 (C-H), 2919 (CH_3), 1730 (C=O), 1643 (C=C), 1606 (C=C of Ar), 1520 and 1342 (C-NO_2), 1247 and 1103 (C-O), 750 (Ar-H), 678 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.26 (2H, d), 7.69 (1H, s), 7.21-7.61 (6H, m), 3.72 (3H, s).

(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)acryloyl chloride (5): FTIR (KBr) cm^{-1} 3050 and 856 (C-H), 1785 (C=O), 1643 (C=C), 1606 (C=C of Ar), 1519 and 1342 (C-NO_2), 750 (Ar-H), 694 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.92 (1H, s), 7.25-7.61 (6H, m).

(2*E*)-*N*-(aminocarbonothioyl)-3-(4-chlorophenyl)-2-(4-nitrophenyl)acrylamide (6a): FTIR (KBr) cm^{-1} 3474, 3349 and 1599 (NH_2), 3436 (N-H), 3054 and 851 (C-H), 1694 (C=O), 1656 (C=C), 1516 and 1345 (C-NO_2), 1117 (C=S), 748 (Ar-H), 699 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.92 (1H, s), 7.25-7.61 (6H, m), 5.06 (1H, bs), 1.28 (2H, s).

(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-*N*-pyridin-4-ylacrylamide (6b): FTIR (KBr) cm^{-1} 3446 (N-H), 3027 and 856 (C-H), 1688 (C=O), 1643 (C=N-C), 1600 (C=C of Ar), 1519 and 1342 (C-NO_2), 748 (Ar-H), 698 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.47 (2H, d), 8.26 (2H, d), 7.70 (1H, s), 7.25-7.61 (6H, m), 6.91 (2H, d), 9.78 (1H, bs).

(2*E*)-3-(4-chlorophenyl)-*N*-1-naphthyl-2-(4-nitrophenyl)acrylamide (6c): FTIR (KBr) cm^{-1} 3427 (N-H), 3063 and 763 (Ar-H), 3022 and 857 (C-H), 1685 (C=O), 1649 (C=C), 1599 (C=C of Ar), 1520 and 1344 (C-NO_2), 678 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.19-7.84 (14H, m), 9.56 (1H, bs).

1-[(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)prop-2-enoyl]piperidine (6d): FTIR (KBr) cm^{-1} 3008 and 857 (C-H), 2950 and 1455 (CH_2), 1675 (C=O), 1634 (C=C), 1599 (C=C

of Ar), 1518 and 1342 (C-NO_2), 1198 (NR_3), 751 (Ar-H), 691 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.82 (1H, s), 7.25-7.61 (6H, m), 2.91 (4H, t), 1.55 (6H, p).

1-[(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)prop-2-enoyl]piperazine (6e): FTIR (KBr) cm^{-1} 3441 (N-H), 3041 and 857 (C-H), 2951 and 1454 (CH_2), 1666 (C=O), 1598 (C=C of Ar), 1520 and 1341 (C-NO_2), 1197 (NR_3), 748 (Ar-H), 691 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.26 (2H, d), 7.72 (1H, s), 7.25-7.61 (6H, m), 3.03 (4H, t), 2.81 (4H, t), 1.84 (1H, s).

(2*E*)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-*N*-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)acrylamide (6f): FTIR (KBr) cm^{-1} 3421 (N-H), 3029 and 856 (C-H), 2915 and 1473 (CH_3), 1760, 1713 and 1675 (C=O), 1601 (C=C of Ar), 1519 and 1342 (C-NO_2), 1188 (NR_3), 750 and 704 (Ar-H), 681 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.78 (1H, s), 7.61 (2H, d), 7.25-7.61 (8H, m), 7.15 (1H, t), 9.65 (1H, bs), 2.50 (3H, s), 1.76 (3H, s).

(2*E*)-3-(4-chlorophenyl)-*N*-(2-methoxyphenyl)-2-(4-nitrophenyl)acrylamide (6g): FTIR (KBr) cm^{-1} 3434 (N-H), 3031 and 858 (C-H), 2935 and 1465 (CH_3), 1695 (C=O), 1601 (C=C of Ar), 1519 and 1341 (C-NO_2), 1239 and 1009 (C-O), 750 (Ar-H), 679 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.26 (2H, d), 7.75 (1H, s), 7.65 (1H, d), 7.25-7.61 (7H, m), 7.03 (2H, m), 9.76 (1H, bs), 3.92 (3H, s).

Pharmacology

CAM assay is routinely 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 neovascularization takes place, in fertilized chicken eggs at a certain stage of the development of the embryo. Agarose pellets impregnated with the test compound are placed onto the vascular membrane of opened eggs, and the influence on angiogenesis is evaluated. [45] For assay purpose the fertile chicken eggs were procured from Kalchina hatchery, Modinagar.

Antiangiogenesis study by chorioallantoic membrane (CAM) assay

Twelve eggs were used per experiment to test one compound as a given dose. The eggs were fertilized at 37°C and 80 % relative humidity in ideal conditions. The shells of eggs were cleaned with 70 % ethanol to avoid infections. After 72 hrs 8-10 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 72 hrs. At this point of time, when the diameter of CAM is between 1.8 and 2.6 cm, the pellets containing the test substances were placed on the CAM. Test substances were dissolved or suspended in a 2.5 % 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. 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 0 = no or weak effect, 1 = medium effect, and 2 = 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 20000 was used as positive control [46] and an agarose pellet as a blank.

RESULTS

The antiangiogenic activity of the test compounds is listed in Table 2. All the compounds were tested at a dose of 10 µg/pellet, corresponding to approximately 27 nmol/pellet, because at higher dose most of compounds showed a toxic effect. Compound **3** and **5** showed an antiangiogenic score of more than 1. Compound **5** was found to be most potent with a score of 1.5 ± 0.1.

DISCUSSION

The result of present study shows that synthesized compounds have significant antiangiogenic activity. The most active analogues **3** and **5** have smaller groups like COOH or COCl as bridge substituents while the least active analogues **6b**, **6c** and **6f** have comparatively large groups as bridge substituents. Present study concludes that size of bridge substituent affect the antiangiogenic activity.

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