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SYNTHESIS AND ANTIANGIOGENIC ACTIVITY OF SOME NOVEL ANALOGUES OF COMBRETASTATIN A-4

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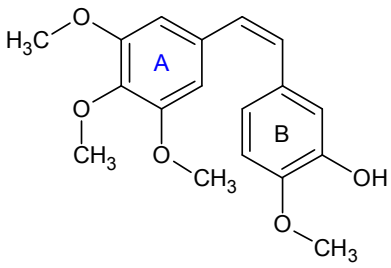
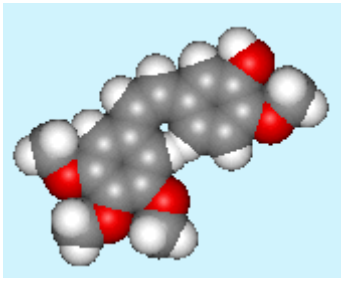
ABSTRACT: A series of Combretastatin A-4 analogues were synthesized in order to obtain new compounds with potential antiangiogenic activity. The structures of all synthesized compounds were confirmed by means of spectroscopical analytical techniques. All compounds were evaluated for their antiangiogenic activity by chorioallantoic membrane (CAM) assay method. The compounds showed significant antiangiogenic activity. Compound 5a 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] 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] 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] In view of strong anticancer/antivascular activity exhibited by CA-4, we have synthesized some novel combretastatin analogues, and tested for their antiangiogenic activity.

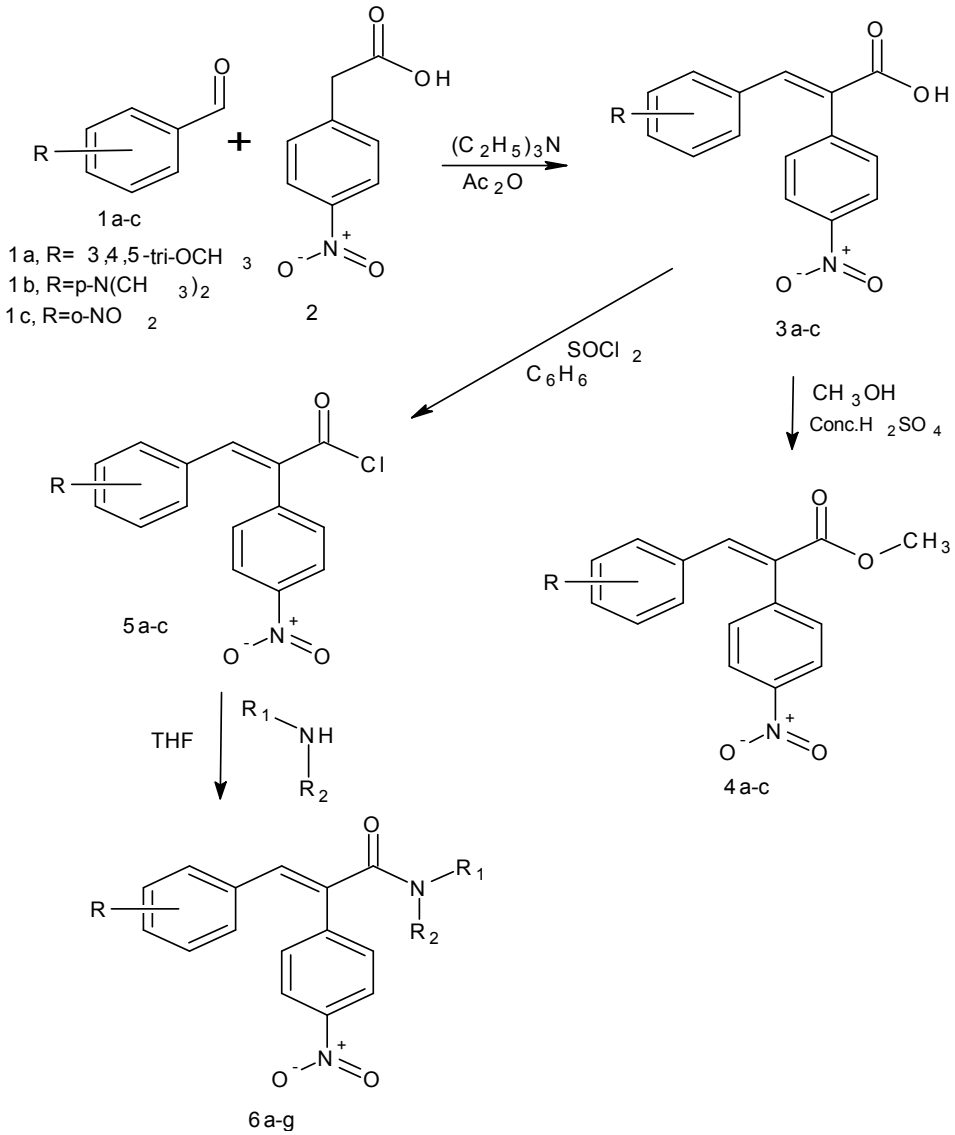




(a) **(b)**

Fig. 1 (a) Structural formula and (b) 3D-structure of CA-4.

Fig. 2 Scheme of synthesis of compounds



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 compounds 3a-c

A mixture of p-nitrophenyl acetic acid **2** (2 mmol), benzaldehyde **1a** or **1b** or **1c** (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 ovenight. The mixture was extracted with diethyl ether (2x50 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 4a-c

Concentrated H_2SO_4 (0.5 ml) was added to a stirred solution of carboxylic acid **3a** or **3b** or **3c** (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 (2x40 ml), and the combined extracts were washed with 2% NaOH solution (2x50 ml) followed by water (200 ml). Product was obtained from ether fraction.

Procedure of preparation of compounds 5a-c

A mixture of carboxylic acids **3a** or **3b** or **3c** (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 **3a** or **3b** or **3c** 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 (2x20 ml), washed with water and dried. Crude product was obtained from ether fraction. Product was purified by recrystallization from EtOAc-hexane.

Spectral data

(2E)-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acrylic acid (3a):

FTIR (KBr) cm^{-1} 3061 and 855 (C-H), 2935 and 1456 (CH_3), 1707 (C=O), 1661 (C=C), 1598 and 1418 (COO^-), 1517 and 1342 (C-NO_2), 1241 and 1003 (C-O), 751 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 9.98 (1H, bs), 8.26 (2H, d), 7.87 (1H, s), 7.33 (2H, d), 6.47 (2H, s), 3.78 (6H, s), 3.77 (3H, s).

Methyl (2E)-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acrylate (4a):

FTIR (KBr) cm^{-1} 3068 and 853 (C-H), 2939 and 1453 (CH_3), 1728 (C=O), 1661 (C=C), 1603 (C=C of Ar), 1519 and 1342 (C-NO_2), 1241, 1129 and 1000 (C-O), 750 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.73 (1H, s), 7.37 (2H, d), 6.61 (2H, s), 3.88 (3H, s), 3.79 (3H, s), 3.76 (6H, s).

(2E)-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acryloyl chloride (5a):

FTIR (KBr) cm^{-1} 3069 and 857 (C-H), 2938 and 1459 (CH_3), 1755 (C=O), 1655 (C=C), 1604 (C=C of Ar), 1515 and 1339 (C-NO_2), 1251 and 1012 (C-O), 739 (Ar-H), 701 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.23 (2H, d), 7.77 (1H, s), 7.35 (2H, d), 6.55 (2H, s), 3.81 (3H, s), 3.77 (6H, s).

(2E)-N-ethyl-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acrylamide (6a):

FTIR (KBr) cm^{-1} 3434 and 1563 (N-H), 3068 and 852 (C-H), 2937 and 1382 (CH_3), 1691 (C=O), 1649 (C=C), 1600 (C=C of Ar), 1522 and 1342 (C-NO_2), 1458 (CH_2), 1252 and 1003 (C-O), 750 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.77 (1H, s), 7.30 (2H, d), 6.46 (2H, s), 5.58 (1H, bt), 3.80 (6H, s), 3.76 (3H, s), 3.36 (2H, q), 1.11 (3H, t).

(2E)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-N-(2-chlorophenyl)acrylamide (6b):

FTIR (KBr) cm^{-1} 3433 (N-H), 3079 and 859 (C-H), 2924 (CH_3), 1683 (C=O), 1631 (C=C), 1600 (C=C of Ar), 1518 and 1345 (C-NO_2), 1239 and 1007 (C-O), 750 (Ar-H), 707 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.24 (2H, d), 7.79 (1H, s), 7.60 (1H, d), 7.32 (4H, m), 7.15 (1H, t), 6.46 (2H, s), 9.61 (1H, bs), 3.81 (6H, s), 3.76 (3H, s).

(2E)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-N-(4-fluorophenyl)acrylamide (6c):

FTIR (KBr) cm^{-1} 3430 (N-H), 3075 and 851 (C-H), 2934 and 1467 (CH_3), 1689 (C=O), 1657 (C=C), 1599 (C=C of Ar), 1512 and 1339 (C-NO_2), 1231 and 1004 (C-O), 1121 (C-F), 750 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.89 (1H, s), 7.33 (2H, d), 7.60 (2H, d), 6.96 (2H, d), 6.47 (2H, s), 5.65 (1H, bs), 3.79 (6H, s), 3.78 (3H, s).

(2E)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-N-(2-methylphenyl)acrylamide (6d):

FTIR (KBr) cm^{-1} 3432 (N-H), 3065 and 851 (C-H), 2934 and 1461 (CH_3), 1683 (C=O), 1663 (C=C), 1599 (C=C of Ar), 1519 and 1345 (C-NO_2), 1244 and 1004 (C-O), 750 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.28 (2H, d), 7.77 (1H, s), 7.60 (1H, d), 7.30 (4H, m), 7.16 (1H, t), 6.44 (2H, s), 9.47 (1H, bs), 3.78 (6H, s), 3.77 (3H, s), 2.34 (3H, s).

(2E)-N-(aminocarbonothioyl)-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acrylamide (6e):

FTIR (KBr) cm^{-1} 3505, 3378 and 1600 (NH_2), 3424 and 1555 (N-H), 3057 and 859 (C-H), 2933 and 1456 (CH_3), 1690 (C=O), 1648 (C=C), 1519 and 1342 (C-NO_2), 1249 and 1001 (C-O), 1126 (C=S), 751 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.23 (2H, d), 7.77 (1H, s), 7.32 (2H, d), 6.44 (2H, s), 9.55 (1H, bs), 3.78 (6H, s), 3.77 (3H, s), 1.25 (2H, s).

(2E)-3-(2-nitrophenyl)-2-(4-nitrophenyl)acrylic acid (3b):

FTIR (KBr) cm^{-1} 3099 and 840 (C-H), 1721 (C=O), 1661 (C=C), 1592 (COO^-), 1522 and 1342 (C-NO_2), 745 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 10.02 (1H, bs), 8.28 (2H, d), 8.23 (1H, d), 7.75 (1H, s), 7.65 (1H, t), 7.52 (2H, m), 7.33 (2H, d).

Methyl (2E)-3-(2-nitrophenyl)-2-(4-nitrophenyl)acrylate (4b): FTIR (KBr) cm^{-1} 3048 and 880 (C-H), 2925 and 1470 (CH_3), 1716 (C=O), 1671 (C=C), 1521 and 1340 (C-NO_2), 1247 and 1108 (C-O), 749 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.24 (2H, d), 8.19 (1H, d), 7.81 (1H, s), 7.70 (1H, t), 7.47 (2H, m), 7.32 (2H, d), 3.95 (3H, s).

(2E)-3-(2-nitrophenyl)-2-(4-nitrophenyl)acryloyl chloride (5b):

FTIR (KBr) cm^{-1} 3052 and 856 (C-H), 1761 (C=O), 1689 (C=C), 1610 (C=C of Ar), 1522 and 1345 (C-NO_2), 750 (Ar-H), 708 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.28 (2H, d), 8.22 (1H, d), 7.77 (1H, s), 7.62 (1H, t), 7.50 (2H, m), 7.33 (2H, d).

(2E)-N-ethyl-3-(2-nitrophenyl)-2-(4-nitrophenyl)acrylamide (6f):

FTIR (KBr) cm^{-1} 3428 and 1565 (N-H), 3036 and 855 (C-H), 2928 (CH_3), 1673 (C=O), 1522 and 1342 (C-NO_2), 1464 (CH_2), 749 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d), 8.23 (1H, d), 7.79 (1H, s), 7.67 (1H, t), 7.57 (2H, m), 7.29 (2H, d), 5.63 (1H, bt), 3.33 (2H, q), 1.15 (3H, t).

(2E)-3-[4-(dimethylamino)phenyl]-2-(4-nitrophenyl)acrylic acid (3c):

FTIR (KBr) cm^{-1} 3065 and 857 (C-H), 2882 and 1438 (CH_3), 1715 (C=O), 1665 (C=C), 1600 and 1412 (COO^-), 1520 and 1348 (C-NO_2), 1195 (NR_3), 756 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 10.00 (1H, bs), 8.23 (2H, d), 7.75 (1H, s), 7.33 (2H, d), 7.17 (2H, d), 6.70 (2H, d), 2.97 (6H, s).

Methyl (2E)-3-[4-(dimethylamino)phenyl]-2-(4-nitrophenyl)acrylate (4c):

FTIR (KBr) cm^{-1} 3036 and 856 (C-H), 2881 (CH_3), 1731 (C=O), 1661 (C=C), 1601 (C=C of Ar), 1526 and 1344 (C-NO_2), 1248 and 1114 (C-O), 1191 (NR_3), 752 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.28 (2H, d), 7.83 (1H, s), 7.35 (2H, d), 7.15 (2H, d), 6.71 (2H, d), 3.88 (3H, s), 3.11 (6H, s).

(2E)-3-[4-(dimethylamino)phenyl]-2-(4-nitrophenyl)acryloyl chloride (5c):

FTIR (KBr) cm^{-1} 3036 and 856 (C-H), 2882 (CH_3), 1787 (C=O), 1688 (C=C), 1606 (C=C of Ar), 1521 and 1341 (C-NO_2), 1189 (NR_3), 751 (Ar-H), 718 (C-Cl); ^{1}H NMR ($CDCl_3$) δ 8.26 (2H, d), 7.77 (1H, s), 7.32 (2H, d), 7.19 (2H, d), 6.68 (2H, d), 2.93 (6H, s).

(2E)-3-[4-(dimethylamino)phenyl]-N-ethyl-2-(4-nitrophenyl)acrylamide (6g):

FTIR (KBr) cm^{-1} 3424 and 1561 (N-H), 3021 and 856 (C-H), 2888 (CH_3), 1678 (C=O), 1601 (C=C of Ar), 1522 and 1347 (C-NO_2), 1467 (CH_2), 1196 (NR_3), 749 (Ar-H); ^{1}H NMR ($CDCl_3$) δ 8.26 (2H, d), 7.75 (1H, s), 7.33 (2H, d), 7.18 (2H, d), 6.70(2H, d), 5.59 (1H, bt), 3.31 (2H, q), 3.04 (6H, s), 1.08 (3H, t).

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 37^0C and 80% relative humidity in ideal conditions. The shells of eggs were cleaned with 70% EtOH 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 AND DISCUSSION

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 30 nmol/ pellet approximately, because at higher dose most of compounds showed a toxic effect. Compound **3a**, **5a**, **5b** and **5c** showed an antiangiogenic score of more than 1. Compound **5a** was found to be most potent with a score

of 1.6±0.1. The results of present study shows that synthesized compounds have significant antiangiogenic activity. The most active analogues **3a**, **5a**, **5b** and **5c** have smaller groups like COOH, $COOCH_3$ or COCl as bridge substituents while the least active analogues **6e**, **6f** and **6g** comparatively large groups as bridge substituents. Present study concludes that size of bridge substituents affect the antiangiogenic activity. Furthermore, most active analogues, of present study, are potential candidate for treatment of diseases related with angiogenesis.

Table 1 Physical properties of the synthesized compounds

Comp.	R, R_1, R_2	Molecular formula	R_f Value[a]	M.P. (^{O}C)
3a	R=3,4,5-tri-OCH_3	$C_{18}H_{17}NO_7$	0.792	90
4a	R=3,4,5-tri-OCH_3	$C_{19}H_{19}NO_7$	0.717	122
5a	R=3,4,5-tri-OCH_3	$C_{18}H_{16}ClNO_6$	0.792	107
6a	R=3,4,5-tri-OCH_3 R_1=H R_2=$CSNH_2$	$C_{19}H_{19}N_3O_6S$	0.700	117
6b	R=3,4,5-tri-OCH_3 R_1=H R_2=C_6H_4F	$C_{24}H_{21}FN_2O_6$	0.646	102
6c	R=3,4,5-tri-OCH_3 R_1=H R_2=C_2H_5	$C_{20}H_{22}N_2O_6$	0.569	112
6d	R=3,4,5-tri-OCH_3 R_1=H R_2=$C_6H_4CH_3$	$C_{25}H_{24}N_2O_6$	0.708	97
6e	R=3,4,5-tri-OCH_3 R_1=H R_2= C_6H_4Cl	$C_{21}H_{21}ClN_2O_6$	0.669	77
3b	R=p-$N(CH_3)_2$	$C_{17}H_{16}N_2O_4$	0.792	210
4b	R=p-$N(CH_3)_2$	$C_{18}H_{18}N_2O_4$	0.880	200
5b	R=p-$N(CH_3)_2$	$C_{17}H_{15}ClN_2O_3$	0.804	152
6f	R= p-$N(CH_3)_2$ R_1=H R_2=C_2H_5	$C_{19}H_{21}N_3O_3$	0.610	162
3c	R= o-NO_2	$C_{15}H_{10}N_2O_6$	0.280	80
4c	R= o-NO_2	$C_{16}H_{12}N_2O_6$	0.760	Decomposed
5c	R= o-NO_2	$C_{15}H_9ClN_2O_5$	0.816	92
6g	R= o-NO_2 R_1=H R_2=C_2H_5	$C_{17}H_{15}N_3O_5$	0.782	62

[a]Solvent system: [Dichloromethane:Carbontetrachloride:Methanol (10:10:1)]

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	
3a	10	28	1.4 ± 0.1 (n =3)
4a	10	27	1.0 ± 0.1 (n =2)
5a	10	26	1.6 ± 0.1 (n =3)
6a	10	24	0.9 ± 0.1 (n =2)
6b	10	22	0.4 ± 0.2 (n =2)
6c	10	26	0.7 ± 0.1 (n =2)
6d	10	22	0.3 ± 0.3 (n =2)
6e	10	21	0.6 ± 0.1 (n =2)
3b	10	32	1.0 ± 0.1 (n =2)
4b	10	31	0.8 ± 0.1 (n =2)
5b	10	30	1.2 ± 0.1 (n =2)
6f	10	29	0.6 ± 0.1 (n =2)
3c	10	32	0.8 ± 0.1 (n =2)
4c	10	30	0.6 ± 0.2 (n =2)
5c	10	30	1.1 ± 0.1 (n =2)
6g	10	29	0.3 ± 0.4 (n =2)
Agarose pellet			0.1 ± 0.1 (n =10)
β-1,4-galactan sulphate (LuPS S5)	50	2.5	1.4 ± 0.1 (n =10)

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

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