Notes

Antiangiogenic Activity of Synthetic Dihydrobenzofuran Lignans

Sandra Apers,[†] Dietrich Paper,[‡] Jutta Bürgermeister,[‡] Slavka Baronikova,[†] Stefaan Van Dyck,[§] Guy Lemière,[§] Arnold Vlietinck,[†] and Luc Pieters*[,†]

Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium, Department of Pharmacy, University of Regensburg, Universitätsstrasse 31, D-93040 Regensburg, Germany, and Department of Chemistry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

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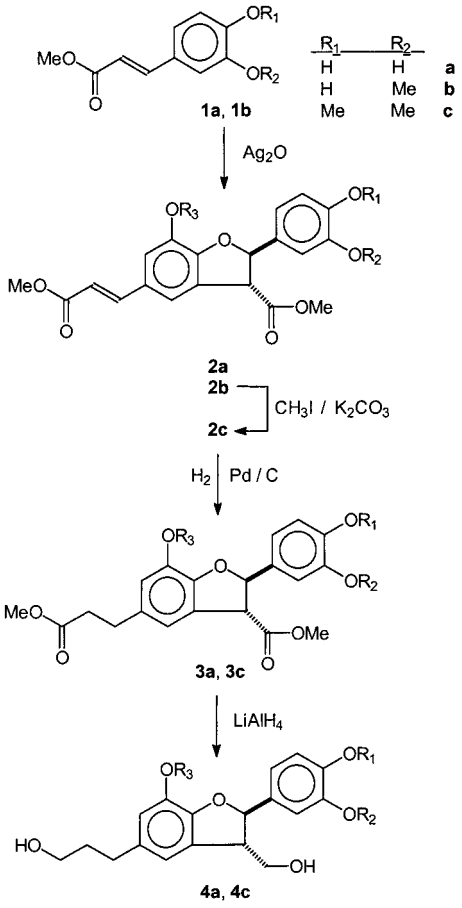
A series of synthetic dihydrobenzofuran lignans, obtained by biomimetic oxidative dimerization of caffeic or ferulic acid methyl ester followed by derivatization reactions, was tested for its antiangiogenic activity in the CAM (chorioallantoic membrane) assay. The dimerization product of caffeic acid methyl ester (**2a**) (methyl (*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]prop-2-enoate) showed a pronounced antiangiogenic activity, especially the 2*R*,3*R*-enantiomer.

Angiogenesis or neovascularization is a complex process involving the activation, adhesion, proliferation, and transmigration of endothelial cells from preexisting 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, diabetic retinopathy, arthritis, and the growth of solid tumors. Therefore angiogenesis is considered as a potential target for antitumoral therapy. Some endogenic angiogenesis inhibitors that are currently being assessed in clinical trials include angiostatin and endostatin, which both are fragments of larger circulatory proteins (plasminogen and collagen XVIII, respectively); inhibitors of vascular endothelial growth factor (VEGF); platelet factor 4 (PF4), a protein present in α-granules of platelets; and recombinant human PF4. Nonendogenous angiogenesis inhibitors that have entered clinical trials include CM 101, a group B streptococcus polysaccharide toxin that is responsible for pulmonary disease in infected human neonates; TNP-470, a derivative of the microbial antibiotic fumagillin; genistein, an isoflavonoid with a selective tyrosine kinase inhibitory activity; pentosan polysulfate, a xylan polysulfate; and tecogalan, a sulfated polysaccharide−peptidoglycan complex isolated from the cell wall of an *Arthrobacter* species.[1−5] Characterization of new antiangiogenic leads may yield new therapeutic agents in this area. Among the known angiogenesis inhibitors, natural compounds such as sulfated carbohydrates or triterpenoids play a prominent role.[2] In the present study the antiangiogenic activity of synthetic 3′,4-di-*O*-methylcedrusin **4c**, a dihydrobenzofuran lignan originally isolated from South American *Croton* species,[6] and some synthetic precursors and analogues (**2**−**4**) was evaluated in the CAM (chorioallantoic membrane) assay, an in vivo model for angiogenesis.[7]

Synthesis of dihydrobenzofuran lignans **2**−**4** from caffeic acid methyl ester **1a** or ferulic acid methyl ester **1b** has been described before. Briefly, the monomeric precursors

Scheme 1



are dimerized in the presence of silver oxide, generating the dihydrobenzofuran skeleton with a 2,3-*trans*-configuration (Scheme 1). The 4′-OH group of **2b** is methylated with methyl iodide in the presence of potassium carbonate to yield **2c**. The double bond in the C_3 side chain is hydrogenated in the presence of Pd−C, giving **3a** and **3c**. $LiAlH_4$ reduction of the ester functionalities to primary alcohols yields **4a** and **4c** (Scheme 1). All compounds were

* To whom correspondence should be addressed. Tel and fax: +32-3-820 27 09. E-mail: luc.pieters@ua.ac.be.
† Department of Pharmaceutical Sciences, University of Antwerp.
‡ Department of Pharmacy, University of Regensburg.
§ Department of Chemistry, University of Antwerp.

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Table 1. Antiangiogenic Activity of Compounds **2**−**4** in the CAM Assay

test compound	concentration (μg/pellet)	concentration (nmol/pellet)	antiangiogenic score[a] ± sd (n = no. of expt)
2a (*rac*)	20	51	1.1 ± 0.1 (n=2)
2a (2*R*,3*R*)	20	51	1.5 ± 0.1 (n=2)
	5	13	0.8 ± 0.1 (n=3)
2a (2*S*,3*S*)	20	51	0.7 ± 0.2 (n=3)
	5	13	0.2 ± 0.1 (n=2)
2b (*rac*)	20	48	0.4 ± 0.1 (n=2)
2c (2*S*,3*S*)	20	47	0.3 ± 0.4 (n=2)
2c (2*R*,3*R*)	20	47	0.5 ± 0.1 (n=2)
3a (*rac*)	20	51	0.5 ± 0.3 (n=2)
4a (*rac*)	20	60	0.3 ± 0.4 (n=2)
4c (*rac*)	20	53	0.6 ± 0.1 (n=2)
agarose pellet (blank)			0.1 ± 0.1 (n>10)
β-1,4-galactan sulfate (LuPS S5)	50	2.5	1.4 ± 0.2 (n>10)

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

obtained as racemic mixtures. Compounds **2a** and **2c** were resolved into their enantiomers by preparative-scale chiral HPLC.[8]

The antiangiogenic activity of the test compounds is listed in Table 1. The dihydrobenzofuran lignans were tested at a dose of 20 μg/pellet, corresponding to about 50 nmol/pellet, because at a dose of 40 μg some of the lignans showed a toxic effect. Only **2a**, the dimerization product of caffeic acid methyl ester, showed an antiangiogenic score of more than 1. When evaluating both enantiomers of **2a** separately, it turned out that especially the (2*R*,3*R*)-isomer exhibited a pronounced antiangiogenic activity, with a score of 1.5 ± 0.1. However, also a membrane-irritating effect (about 50%) was observed at 20 μg/pellet. Compound **2a**-(2*R*,3*R*) still showed antiangiogenic properties (score 0.8) at 5 μg/pellet. Methylation and reduction of the parent compound reduced the antiangiogenic activity, as observed before for the antimitotic activity and the inhibition of tubulin polymerization.[8] Indeed, in the same series of dihydrobenzofuran lignans, also **2a**-(2*R*,3*R*) was the most potent antimitotic compound and inhibitor of tubulin polymerization. Also other antimitotic agents and inhibitors of tubulin polymerization, such as colchicine or vincristine, inhibited angiogenesis in the CAM-assay.[2] However, they did not inhibit the FGF-2 (fibroblast growth factor-2) or VEGF (vascular endothelial growth factor) induced corneal neovascularization in mice, in contrast to 2-methoxyestradiol or paclitaxel, which also act on tubulin.[9] Since the proliferation of endothelial cells is involved in angiogenesis, the antiangiogenic effect of antimitotic agents may be related to their antiproliferative action on endothelial cells. Dihydrobenzofuran **2a** can be considered as a new lead for antiangiogenic agents, which deserves further exploration.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi B-545 melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian Unity 400 spectrometer. Additional HETCOR and long-range HETCOR measurements to verify the proposed assignments were performed on the same spectrometer. Chemical shifts are reported in δ (ppm). DCI mass spectra were obtained on a Ribermag R-10-10B mass spectrometer. Column chromatography was performed on Merck silica gel 60, 0.040−0.063 mm, 230−400 mesh ASTM. Precoated silica gel plates (Kieselgel 60, F_{254}, 0.2 mm) were used for TLC analysis. All products and reagents were puchased from Acros, Belgium.

Chiral separations were performed on a column (5 × 21 cm) packed with 0.25 kg of Chiralpack AD with EtOH as the mobile phase. Peak shaving and closed loop recycling techniques were used during the process. Determination of their absolute configuration was based on their CD spectra.[8] Analytical HPLC using two independent systems was performed to check the purity of the products. Column A: Alltech Econosil C8 (4.6 × 250 mm); column B: BIO-RAD Bio-Sil C18 (4.6 × 150 mm). Experimental details of the HPLC analysis are reported for all test compounds in the following form: column; mobile phase; retention time. The flow rate was 1 mL/min.

Methyl cinnamates (1a,b) were prepared from a mixture of the corresponding cinnamic acid (4 g) and Dowex 50 W × 8200−400 (0.4 g) in 25 mL of absolute methanol. After heating under reflux for 1 night the mixture was filtered and evaporated under reduced pressure to afford the product as a solid (100%), which was used without further purification.

1a: amorphous, mp 158 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8]

1b: amorphous, mp 148 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8]

Methyl (*E*)-3-[2-(3,4-dihydroxyhenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]prop-2-enoate (2a) was prepared according to the method of Lemière et al.[10] using 1.905 g (9.8 mmol) of methyl caffeate, 826 g (3.5 mmol) of silver(I) oxide, 40 mL of anhydrous benzene, and 20 mL of anhydrous acetone. The product was purified by column chromatography (3.8 × 30 cm, silica gel 60, 0.040−0.063 mm) with ethyl acetate−*n*-heptane, 1:1, as the eluent. After evaporation and lyophilization a white foam was obtained (33%): amorphous, mp 159 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] Chiral HPLC analysis: Chiralpak AD, length 5 cm; *n*-hexane−ethanol, 60:40. CD spectra of both enantiomers [**2a**-(2*R*,3*R*) and **2a**-(2*S*,3*S*)] have been recorded and published before.[8] Anal. ($C_{20}H_{18}O_6$) C, H.

Methyl (*E*)-3-[2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]-prop-2-enoate (2b) was prepared according to the method of Lemière et al.[10] using an improved workup. After evaporation the residual brown oil was dissolved in methanol. The solution was left to stand overnight. Crystals (white, mp 151 °C) were formed, filtered, and washed with cold methanol (50%): ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water; 65:35; 5.01 min; column B, methanol−water, 50:20; 4.93 min. Anal. ($C_{22}H_{22}O_8$) C, H.

(2*S*,3*S*)- and (2*R*,3*R*)-Methyl (*E*)-3-[2-(3,4-dimethoxyphenyl)-7-methoxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]prop-2-enoate (2c) were prepared using the method of Lemière et al.,[10] including preparative scale HPLC, using an improved workup. After the evaporation of the reaction mixture, the resulting yellow oil was dissolved in methanol. The solution was left to stand overnight. Crystals (75%) were formed, filtered, and washed with cold methanol: white crystals, mp 135 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water, 65:35; 5.06 min; column B, methanol−water, 50:20, 5.61 min. Anal. ($C_{23}H_{24}O_8$) C, H.

Methyl (*E*)-3-[2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]propanoate (3a) was prepared from **2a** by dissolving 418 mg (1.18 mmol) of **2a** in 30 mL of acetone, to which 220 mg of 5% Pd/C was added. The mixture was put in a Parr apparatus under 60 psi hydrogen pressure and was shaken for 20 min. After filtration and evaporation the crude oil was purified by column chromatography (3 × 20 cm, silica gel 60, 0.040−0.063 mm) with ethyl acetate−*n*-heptane, 1:2, as the eluent, resulting in a colorless oil (95%): ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water, 65:35; 6.25 min; column B, methanol−water, 60:40; 3.45 min.

Methyl 3-[2-(3,4-dimethoxyphenyl)-7-methoxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5-yl]propanoate (3c) was prepared according to the method of Lemière

et al.[2] The reaction was improved by using a Parr apparatus at 60 psi hydrogen pressure for 20 min. Workup was performed identically, resulting in a white powder (81%): mp 97−99 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water, 65:35; 4.50 min; column B, methanol−water, 50:20; 4.26 min.

3-[2-(3,4-Dihydroxyphenyl)-3-hydroxymethyl-7-hydroxy-2,3-dihydro-1-benzofuran-5-yl]propan-1-ol (4a) was prepared from **3a** according to the method of Lemière et al.[10] In the workup the crude oil was dissolved in 3 mL of ethyl acetate and left to stand overnight at 0 °C, resulting in the formation of white crystals (20%): mp 123 °C; ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water, 65:35; 3.01 min; column B, methanol−water, 60:40; 2.41 min.

3-[2-(3,4-Dimethoxyphenyl)-3-hydroxymethyl-7-methoxy-2,3-dihydro-1-benzofuran-5-yl]propan-1-ol (4c) was prepared from **3c** according to the method of Lemière et al.[10] without further modification (56%). ^{1}H NMR, ^{13}C NMR, DCI-MS (NH_3).[8] HPLC analysis: column A, methanol−water, 65:35; 3.43 min; column B, methanol−water, 50:20; 2.01 min.

Chorioallantoic Membrane (CAM) Assay. 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.[7] Twelve eggs were used per experiment to test one compound at a given dose. The eggs were fertilized at 37 °C and 80% relative humidity in ideal conditions. The shells of the eggs were cleaned with 70% EtOH to avoid infections (e.g., *Salmonella*). After 72 h, 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 h. At this point of time, when the diameter of the 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 micropipet for viscous solutions. Therefore the agarose pellets do not have a uniform size. The half-cone-shaped agarose pellets are fixed because they slightly sink into the CAM. After 24 h 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. β-1,4-Galactan sulfate (LuPS S5) with an average molecular weight of 20 000 was used as positive control,[11] and an agarose pellet as a blank.

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