Brief Articles

Novel A-Ring and B-Ring Modified Combretastatin A-4 (CA-4) Analogues Endowed with Interesting Cytotoxic Activity

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Received April 30, 2008

A novel class of combretastatins, modified at A-ring or both A- and B-rings, mainly by replacement with benzofuran or benzo[*b*]thiophene, were synthesized. The new heterocombretastatins showed good cytotoxic activity on BMEC and H-460 cell lines. The aminocombretastatin **9f** potently inhibits cell growth of BMEC and combretastatin-resistant HT-29 cell lines, with potential interest to treat colon carcinoma. Heterocombretastatins **9a**,**b** inhibit tubulin polymerization similarly to CA-4 by having a binding to colchicine site five times stronger.

Introduction

Combretastatins are natural antimitotic agents isolated from the bark of the South African tree *Combretum caffrum*. Among these compounds, combretastatin A-4 (CA-4,[a] **1**, Figure 1) possesses the most potent and interesting antitumor activity.[1,2] CA-4 is an exceptionally strong inhibitor of tubulin polymerization and potently cytotoxic against murine lymphocytic leukemia and human ovarian and colon cancer cell lines.[3] Its mechanism of action is believed to be related to tubulin-binding properties that result in rapid tumor endothelial cell damage, neovascular shutdown, and subsequent hemorrhagic necrosis.[4] CA-4P, a combretastatin-A4 phosphate prodrug, is a vascular disrupting agent (VDA) that was tested in clinical studies.[5] Interestingly, CA-4P does not induce the common side effects of existing chemotherapy such as alopecia and bone marrow toxicity. However, cardiovascular toxicity and neurotoxicity were dose limiting for CA-4P.[6] These significant side effects currently represent the main obstacles to broad clinical application of CA-4P. For this reason, it is necessary to develop other CA-4 structurally related compounds with more specificity for tumor endothelial cells than normal endothelial cells to avoid cardiac toxicity from endothelial damage. In this context, from a medicinal chemistry point of view, the structural simplicity of CA-4 offers a stimulating premise for the design of new and more potent compounds with improved pharmacological properties.[3]

In an ongoing project aimed at developing novel apoptosis inducers incorporating the stilbene motif, we recently started a study for the purpose of evaluating the apoptotic activity of natural and synthetic stilbenoids. Several active stilbenes were identified and, among them, the *cis*-3,4′,5-trimethoxy-3′-aminostilbene (**3**) proved highly potent against various tumor cells, selectively suppressing tumor vascular perfusion without damaging normal vascular perfusion in a DCE-MRI study.[7] Most importantly, mice treated with five daily injections of stilbene **3** did not show any compromise in heart function.[8,9] Recently, we also examined the effects regarding replacement of the B-ring of CA-4 with different benzoheterocycles.[10,11]The phosphate prodrugs ST2495 (**5**) and ST2496 (**6**) demonstrated high antitumor activity in in vitro and in vivo models, notwithstanding a minimal effect on microtubule organization, with respect to CA-4. Interestingly, despite a lower in vitro cytotoxic potency of the precursor drugs, compared to CA-4, the prodrugs were active in vivo in a manner comparable to CA-4P. The prodrugs **5** and **6** were also effective after oral administration, thus suggesting an additional mitotic molecular target or, alternatively, a different binding site or mode of interaction with tubulin for ST molecules. In a more recent work, aminoindole cycles have been also introduced into phenstatin derivatives active as tubulin assembly inhibitors.[12] In this study, we further investigated stilbenes by extending our

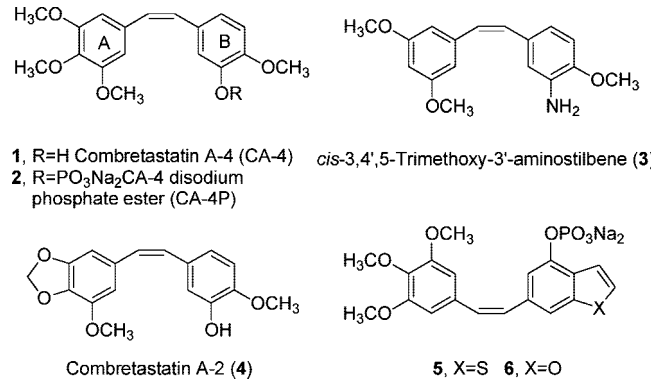

Figure 1. Natural and synthetic combretastatins.

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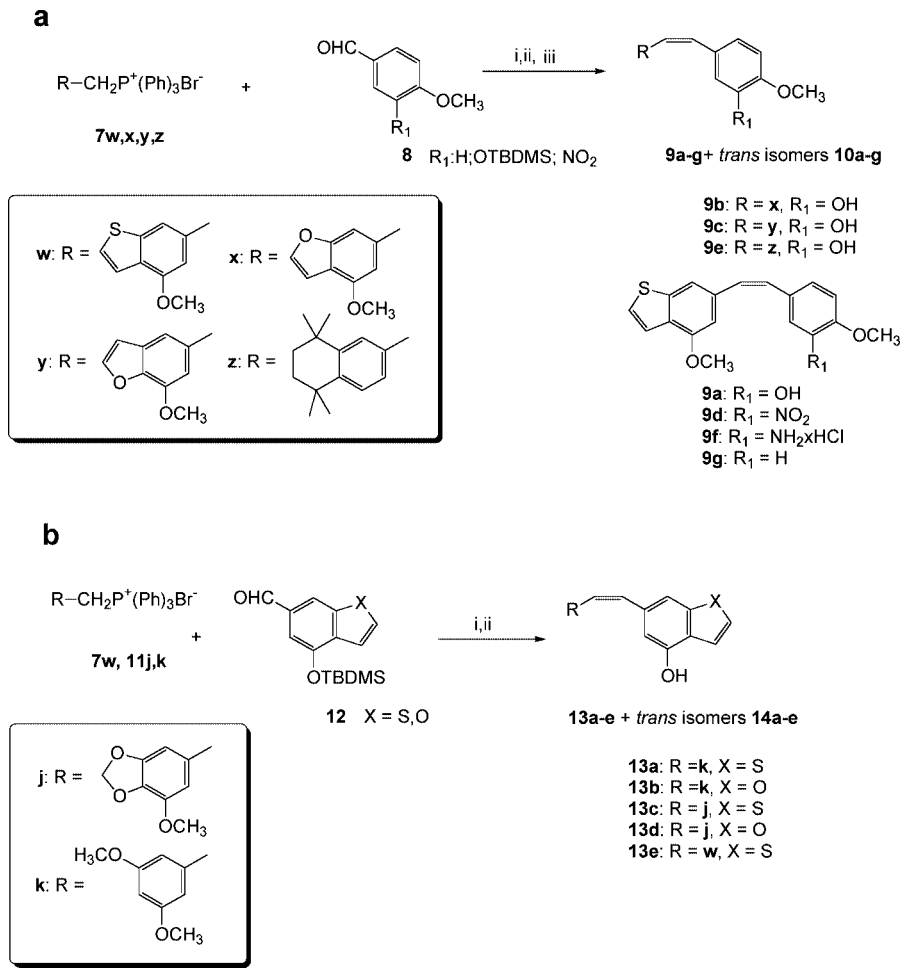
[a] Abbreviations: CA-4, combretastatin A-4; CA-4P, combretastatin-A4 phosphate prodrug; VDA, vascular disrupting agent; CTBC, colchicine tubulin binding competition assay; TPI, tubulin polymerization inhibition assay.

10.1021/jm8005004 CCC: $40.75
Published on Web 09/11/2008

Scheme 1[a]



[a] Reagents and conditions: (i) NaH, THF; (ii) TBAF, CH_2Cl_2; (iii) AcOH, Zn, MeOH, 1N HCl.

earlier studies on benzoheterocyle-based combretastatins. We describe the effects due to replacement of the A-ring of CA-4 with different benzoheterocycles, concentrating mainly on benzofuran and benzo[*b*]thiophene rings (compounds **9a**−**c**,**f**). The novel structural modifications are, in part, in agreement with a pharmacophoric model recently described in the literature.

Additionally, compounds structurally related to **5** and **6** (derivatives **13a**−**d** and **14a**–**d**) were obtained by replacement of the A-ring with the 3,4-methylendioxy ring system, the characteristic moiety of natural combretastatin A-2 (**4**), as well as with the dimethoxy group, characteristic of **3**. To corroborate our SAR investigation, we further explored combretastatins where both the A- and B-rings were replaced with the benzo[*b*]thiophene ring (compounds **13e**, **14e**) and derivatives **9e** and **10e** bearing the 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene hydrophobic system.

We describe the evaluation of a primary screening of cytotoxicity on BMEC cell lines for all synthesized compounds. Moreover, preliminary screening was carried out by analyzing the activity of the new active compounds in both in vitro tubulin polymerization inhibition (TPI) and colchicine tubulin binding competition assay (CTBC).

The interesting biological results obtained with compounds **9a**−**c** and **9f**, the high cytotoxic activity as well as tubulin polymerization inhibition properties, further demonstrated the importance of the benzofuran and benzo[*b*]thiophene heteroaromatic rings in conferring activity on combretastatin molecules, proving them ideal bioisosteres not only for replacement of the B-ring of CA-4 but also, and possibly more important, as bioisosters of the A-ring.

Chemistry. The synthetic route followed for the synthesis of the desired novel combretastatins is outlined in Scheme 1a,b. The phosphonium salts **11j**,**k**[13,14] and the (1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalen-6-yl)methyl-triphenylphosphonium bromide (**7z**)[15] were prepared as reported in the literature. The phosphonium salts **7w**,**x**,**y** were obtained by starting from the corresponding benzyl alcohols by reaction with carbon tetrabromide and triphenylphosphine to obtain the corresponding benzyl bromides, which were, in turn, reacted with triphenylphosphine in acetonitrile at 100 °C.[11] The aldehydes **12** were achieved through a previously communicated procedure.[11]

Stilbenes **9a**−**g**, **13a**−**e** and the relative *trans* isomers **10a**−**g** and **14a**−**e** were prepared in good yields through a Wittig reaction between the appropriate aldehyde and phosphonium salt in the presence of NaH. The phenolic moieties of the crude stilbenes were, in turn, revealed by treatment with TBAF. The 1:1 mixture of the *Z* and *E* stilbene isomers was finally separated by careful silica gel column chromatography and the single isomers identified by NMR for their different coupling constants between alkenyl proton signals (about 12 Hz for *Z* and 16 Hz for *E* isomers). The nitro groups of derivatives **9d** and **10d** were reduced with zinc in acetic acid to give amines **9f** and **10f** in good yields.

Biological Results. A pharmacophore model for colchicine site inhibitors was recently described, indicating for combretastatins a positive electrostatic potential region favoring activity

Table 1. Cytototoxicity and Tubulin Interactions of Synthesized Compounds

		IC$_{50}$ [nM] ± SD					
compd	*E*/*Z*	IC$_{50}$ [nM] ± SD	BMEC[a]	HT-29[b]	H-460[c]	TP	CTBC
colchicine							1.7 ± 0.6
CA-4	*Z*		3.7 ± 0.3	118 ± 33	1.2 ± 0.2	4.9 ± 0.2	0.1 ± 0.02
CA-2	*Z*		48 ± 1.3	>1000	40 ± 0.4		
9a	*Z*		17 ± 0.2	260 ± 32	13 ± 2.5	2.9 ± 0.09	0.02 ± 0.002
9b	*Z*		35 ± 1.8	420 ± 57	19 ± 5	5.1 ± 0.07	0.02 ± 0.003
9c	*Z*		35 ± 0.3	590 ± 39	12 ± 0.8	3.9 ± 0.1	0.05 ± 0.002
9e	*Z*		>10000			21.5 ± 1.9	NT
9f	*Z*		20 ± 2.3	21 ± 1.3	19 ± 1.3	3.6 ± 0.28	6.2 ± 0.12
9g	*Z*		123 ± 33		110 ± 8.5		
10b	*E*		>10000			>100	6.2 ± 0.3
13a	*Z*		110 ± 11			12.3 ± 0.5	3.8 ± 0.6
13b	*Z*		800 ± 40			7.1 ± 0.08	4.0 ± 0.09
13c	*Z*		880 ± 52			14.2 ± 0.9	4.9 ± 0.07
13d	*Z*		>3000			16.6 ± 0.7	7.7 ± 0.1
13e	*Z*		250 ± 0.5		312 ± 53	24.3 ± 0.8	NT

[a] Bovine microvascular endothelial cells. [b] Colon carcinoma cells. [c] Non-small-cell lung carcinoma cells. TP: tubulin polymerization; CTBC: colchicine tubulin binding competition; IC$_{50}$: concentration of drug required to produce an inhibitory effect of 50%; SD: standard deviation; NT: not tested.

in A- and B-rings. This region is located in the outer zone of the trimethoxy groups in the A-ring,[16,17] suggesting that other hydrogen bond acceptors could replace the trimethoxy groups giving active compounds. Therefore, heteroaromatic rings, such as **9a**−**c**, **9f**,**g**, and **13c**−**e**, containing electron-rich atoms, may contribute to increase the activity of novel combretastatins. At the same time, the benzoheterocycle skeleton could also be favorable for steric interaction with a large hydrophobic pocket at the active site, as described in the pharmacophoric model. In this context, we synthesized a novel class of heterocombretastins bearing different substitutions at the A-ring as well as at both A- and B-rings. In particular, we synthesized compounds where:

(a) The A-ring is replaced by a benzofuran ring, **9b** (ST2897),[18] **10b**, **9c**, **10c**; the benzo[*b*]thiophene ring, **9a** (ST2899), **10a**, **9f**,**g**, **10f**,**g**, as well as the 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene portion, **9e** and **10e**.

(b) The B-ring is replaced by a benzofuran or a benzo[*b*-]thiophene heterocycle, whereas the A-ring is replaced by a benzo[*b*]thiophene, a methylendioxobenzene ring as well as the dimethoxy phenyl group: **13a** (ST2900), **14a**, **13b** (ST2902), **14b**, **13c** (ST2892), **14c** (ST2891), **13d** (ST2933), **14d** (ST2934), **13e**, **14e**.

All the synthesized compounds were tested in a preliminary cytotoxicity assay on a BMEC cell line (see Table 1).[19] Some of the most active compounds were also tested on lung H-460 and colon HT-29 carcinoma cells, the latter naturally resistant to CA-4. At first glance, it is evident that there is a difference in activity between *E* and *Z* stereoisomers, the latter being unambiguously more potent, which is unsurprising because it is well documented in the literature and also by us.[20,21] Compounds **9a**−**c**, in which the A-ring is replaced by a benzofuran or benzo[*b*]thiophene ring system, proved of great importance. The new heterocombretastatins showed cytotoxic activity at a low nanomolar level on the BMEC cell line as well as on H-460 cell lines. Interestingly, **9a** activity on the combretastatin-resistant HT-29 cell line is comparable to the natural CA-4. The activity of **9a**−**c** also does not seem greatly influenced by the position of the endocyclic heteroatom because the two regioisomers **9b** and **9c** possess the same IC$_{50}$ inhibition on BMEC cell lines. The benzo[*b*]thiophene derivative **9a** therefore, has the lowest IC$_{50}$ on both BMEC and HT-29 cell lines, making it a lead for further investigations.

Substitution of the A-ring with the 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene gave derivative **9e**, lacking any activity notwithstanding the potential improvement due to hydrophobic interactions at the active site. A further dramatic loss of activity was observed by replacement of the characteristic isovanillic moiety of compounds **9a**−**c** and of CA-2 with a benzoheterocycle, as Table 1 shows. The only exception is compound **13e**, bearing the double benzo[*b*]thiophene substitution, which, although less potent than the nonactive compounds, shows cytotoxic activity against BMEC and H460 cell lines (IC$_{50}$ value about 250 and 312 nM, respectively). Accordingly, the compound still affects tubulin polymerization (IC$_{50}$ = 24.3 nM).

The most active compounds described above, **9a**−**c**, were further examined for their effects on tubulin assembly. Preliminary screening was carried out analyzing the activity of the new compounds in both in vitro tubulin polymerization inhibition (TPI) and colchicine tubulin binding competition assay (CTBC). Interestingly, the heterocombretastatins **9a**−**c** potently inhibit tubulin polymerization with an IC$_{50}$ similar to or better than that of CA-4 and, quite surprisingly, the binding of compounds **9a**−**b** to the colchicine binding site resulted five times higher than that of natural CA-4. Regarding the dimethoxylated derivatives **13a** and **13b**, these are less active than the parent trimethoxy derivatives. This was also quite surprising, since we previously demonstrated the importance of the dimethoxy group for compound **3**. Because both compounds showed inhibition to tubulin polymerization and to binding at the colchicine site, the lack of cytotoxic activity may be due to the difficulty in reaching the cellular target.

Finally, further SAR information was obtained by structural alterations of the isovanillic portion of **9a** by removal of the OH moiety as well as by its replacement with an amino group as in compounds **9f**,**g**. The amino compound **9f** seems of great importance in inhibiting cell growth of BMEC and H-460 at low nanomolar level, with a profile of activity partly comparable with the amino derivatives of CA-2, recently described by Pettit,[22] and, quite surprisingly, the compound is also active on the HT-29 cell line. Colon carcinoma cells are not particularly responsive toward antimitotic agents, including combretastatins, and generally this class of chemotherapic agents is not used for the treatment of colon carcinoma. As displayed in Table 1, combretastatin A-4 showed in HT-29 cells an IC$_{50}$ of 118 nM and the most active compounds of the series **9a**−**c** were still less active than combretastatin A-4. In contrast, **9f** displayed an IC$_{50}$ of only 21 μM, which was similar to that obtained on non-small-cell lung carcinoma H-460 cell line. Compound **9f** showed a tubulin polymerization IC$_{50}$ similar to that of CA-4 (4.9 μM vs 3.6 μM), although the colchicine tubulin binding

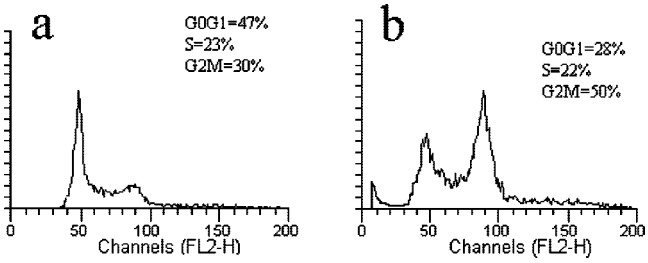


Figure 2. Effects of compound **9f** on DNA content/cell. Cells were cultured without (a), or with 0.5 μM **9f** (b). Cell cycle distribution was analyzed after 24 h treatment by the standard propidium iodide procedure as described in the Experimental Section of Supporting Information. Sub-G_0-G_1 (A), G_0-G_1, S, and G_2-M cells are indicated in the control panel (a).

competition (CTBC) IC_{50}s of **9f** and CA-4 were markedly different (0.1 μM vs 6.2 μM). This might suggest that **9f** acts on tubulin polymerization like combretastatins but on a site different from colchicine. To confirm that the main mechanism of action of **9f** was antimitotic, we evaluated its effect on cell cycle. As shown in Figure 2, compound **9f** induced a prevalent block of cells in G_2/M phase of cell cycle. Therefore, **9f** is an unique analogue displaying a profile of activity comparable to combretastatin but endowed with therapeutical potential in colon carcinoma.

In conclusion, we describe in this paper a novel class of combretastatins bearing the replacement of the A-ring or both A- and B-rings by a benzoheterocycle (benzofuran or benzo[*b*]-thiophene) and methylenedioxobenzene as well as dimethoxybenzene or 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene fragments. Our data evidence that derivatives of CA-4, bearing a benzofuran or benzo[*b*]thiophene heterocycle, which replaces the A-ring, possess potent cytotoxic activity while also showing potent binding to tubulin and inhibition of tubulin polymerization.

Moreover, compounds bearing the double benzo[*b*]thiophene substitution appears of interest as it produces good cytotoxic activity. It appears to be particularly interesting that the amino derivative **9f** is able to inhibit growth of colon carcinoma HT-29 cells at low nM concentration.

The interesting biological results obtained with structural changes at A-ring of CA-4, a region of the natural compound hitherto scarcely considered, demonstrate that structural alteration at this portion of the natural compound offers interesting premises for the design of novel and potent CA-4 analogues.

Experimental Section

General Procedure for the Synthesis of Stilbenes 9a−e,g, 10a−e,g, 13a−e, 14a−e. To a solution of aldehyde **8**, **12** (2 mmol), solubilized in 10 mL of anhydrous THF, the opportune triphenylphosphonium salt **7w**,**x**,**y**,**z**, **11j**,**k** (2.2 mmol) was added. The suspension thus obtained was cooled in an ice bath, then NaH (50% in mineral suspension, 2.2 mmol, 110 mg) was added. The reaction was stirred at room temperature for 24 h, filtered on a celite bed and washed with THF. After solvent evaporation, the residue was extracted with methylene chloride (15 mL), the extracts washed with water (5 mL) and brine (5 mL), then dried and concentrated in vacuo.

For derivatives in which the phenolic moiety is protected as TBDMS ether, the residue was dissolved in methylene chloride (10 mL) and tetrabutyl ammonium fluoride (6 mmol) was added. After 1 h at room temperature, the solution was diluted with methylene chloride (5 mL), washed with water (3 × 5 mL) and brine (5 mL), then dried. After concentration, *E* and *Z* stereoisomers were separated and purified by careful flash chromatography on silica gel (eluent: 5−20% ethyl acetate/light petroleum). Stereoisomeric purity of separated compounds was confirmed through NMR.

2-Methoxy-5-[(*Z*)-2-(4-methoxy-benzo[*b*]thiophen-6-yl)-ethenyl]-phenol (9a). Yield 43%, oil. ^{1}H NMR δ 3.74 (s, 3H), 3.87 (s, 3H), 5.50 (s, 1H), 6.52 (d, J = 12 Hz, 1H), 6.60 (d, J = 12. Hz, 1H), 6.68- 6.72 (m, 2H), 6.78 (d, J = 2 Hz, 1H), 6.91 (d, J = 2 Hz, 1H), 7.29 (d, J = 5.4 Hz, 1H), 7.37−7.44 (m, 2H). Anal. ($C_{18}H_{16}O_3S$) C, H, S.

2-Methoxy-5-[(*Z*)-2-(4-methoxy-benzofuran-6-yl)-ethenyl]-phenol (9b). Yield 40%, oil. ^{1}H NMR δ 3.76 (s, 3H), 3.86 (s, 3H), 5.52 (br, 1H), 6.50 (d, J = 12 Hz, 1H), 6.56−6.64 (m, 2H), 6.73 (s, 1H), 6.78−6.80 (m, 2H), 6.91 (d, J = 2 Hz, 1H), 7.01 (s, 1H), 7.49 (d, J = 2 Hz, 1H). MALDI-TOF: 297.2 [M+]. Anal. ($C_{18}H_{16}O_4$) C, H.

2-Methoxy-5-[(*Z*)-2-(7-methoxy-benzofuran-5-yl)-ethenyl]-phenol (9c). Yield 38%, oil. ^{1}H NMR δ 3.81 (s, 3H), 3.86 (s, 3H), 5.47 (s, 1H), 6.48 (d, J = 12 Hz, 1H), 6.60 (d, J = 12.2 Hz, 1H), 6.68 (d, J = 2 Hz, 1H), 6.72 (s, 1H), 6.75−6.76 (m, 2H), 6.88 (d, J = 2 Hz, 1H), 7.1 (s, 1H), 7.57 (d, J = 2 Hz, 1H). Anal. ($C_{18}H_{16}O_4$) C, H.

6-[(*Z*)-2-(4-Methoxyphenyl)-ethenyl]-4-methoxybenzo[*b*]thiophene (9g). Yield 40%. ^{1}H NMR δ 3.72 (s, 3H), 3.78 (s, 3H), 6.57 (d, J = 12.0 Hz, 1H), 6.60 (d, J = 12.0 Hz, 1H), 6.67−6.69 (m, 1H), 6.75−6.79 (m, 2H), 7.22−7.23 (m, 1H), 7.24−7.26 (m, 1H), 7.29 (d, J = 5,6 Hz, 1H), 7.38 (s, 1H), 7.42 (dd, J = 5.6 Hz, J = 0.8 Hz 1H). ^{13}C NMR δ 55.3, 105.0, 113.7, 115.4, 120.5, 124,9, 129.0, 129.6, 129.8, 130.4, 135.1, 141.1, 158.8. Anal. ($C_{18}H_{16}O_2S$) C, H, S.

General Procedure for the Synthesis of Amino Derivatives 9f and 10f. Zinc powder (100 mmol, 6.5 g) was added portionwise to a solution of nitrostilbenes **9d**, **10d** (1 mmol) in acetic acid (15 mL). The suspension was stirred for 2 h at room temperature. The reaction mixture was filtered over celite and concentrated. The crude material was dissolved in ethyl acetate (15 mL) and washed with sodium bicarbonate 5% (5 mL) and brine (5 mL), dried, and concentrated to afford the desired crude amino compound used for the next reaction without any purification. To a suspension of amino stilbene derivative solubilized in methanol, 1 N HCl was added, the mixture stirred at room temperature for 10 min and concentrated to afford the solid stilbene salt.

6-[(*Z*)-2-(3-Amino-4-methoxyphenyl)-ethenyl]-4-methoxybenzo[*b*]thiophene Hydrochloride (9f). Yield 70%, mp 159−161 °C. ^{1}H NMR (CD_3OD) δ 3.75 (s, 3H), 3.95 (s, 3H), 6.62 (d, J = 12.4 Hz, 1H), 6.67 (s, 1H), 6.77 (d, J = 12.4 Hz, 1H), 7.13 (d, J = 8.4 Hz, 1H), 7.25 (d, J = 2.0 Hz, 1H), 7.34 (d, J = 1.2 Hz, 1H), 7.36−7.37 (m, 1H), 7.11−7.15 (m, 1H), 7.43 (d, J = 5.6 Hz, 1H). ^{13}C NMR (CD_3OD) δ 55.7, 56.9, 105.8, 113.2, 116.1, 121.3, 125.6, 126.4, 129.1, 131.1, 132.1, 132.3, 135.7, 153.1, 156.0. Anal. ($C_{18}H_{18}ClNO_2S$) C, H, N, S.

Acknowledgment. This work was financially supported in part by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (PRIN 2006), Rome, Italy.

Supporting Information Available: Synthetic procedures and characterizations of intermediates and less active compounds, experimental procedures for biological assays, and elemental analysis of target derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM8005004