Heterocyclic and Phenyl Double-Bond-Locked Combretastatin Analogues Possessing Potent Apoptosis-Inducing Activity in HL60 and in MDR Cell Lines

Daniele Simoni,*,† Giuseppina Grisolia,† Giuseppe Giannini,‡ Marinella Roberti,§ Riccardo Rondanin,† Laura Piccagli,† Riccardo Baruchello,† Marcello Rossi,† Romeo Romagnoli,† Francesco Paolo Invidiata,|| Stefania Grimaudo,⊥ M. Katherine Jung,# Ernest Hamel,▽ Nicola Gebbia,O Lucia Crosta,O Vincenzo Abbadessa,⊥ Antonietta Di Cristina,⊥ Luisa Dusonchet,¶ Maria Meli,¶ and Manlio Tolomeo⊥

Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17-19, 44100 Ferrara, Italy, R & D, Sigma-Tau, Pomezia, Italy, Dipartimento di Scienze Farmaceutiche, Università di Bologna, Bologna, Italy, Dipartimento Farmacochimico Tossicologico e Biologico, Università di Palermo, Palermo, Italy, Cattedra di Ematologia e Servizio AIDS, Policlinico di Palermo, Palermo, Italy, SAIC-Frederick, Inc., Frederick, Maryland 21702, Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland 21702, Consorzio di Ricerca sul Rischio Biologico in Agricoltura (Co.Ri.Bi.A.), and Dipartimento di Scienze Farmacologiche, Università di Palermo, Palermo, Italy

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Two new series of combretastatin (CA-4) analogues have been prepared. The alkenyl motif of CA-4 was replaced either by a five-membered heterocyclic (isoxazoline or isoxazole) or by a six-membered ring (pyridine or benzene). The new compounds have been evaluated for their effects on tubulin assembly and for cytotoxic and apoptotic activities. Five compounds (**18b**, **20a**, **21a**, **34b**, and **35b**) demonstrated an attractive profile of cytotoxicity (IC_{50} < 1 μM) and apoptosis-inducing activity but poor antitubulin activity. The isoxazoline derivatives **18b**, **20a**, and **21a**, demonstrated potent apoptotic activity different from that of natural CA-4. Their ability to block most cells in the G2 phase suggests that these compounds could act on targets different from the mitotic spindle. This would indicate activation of both the intrinsic and the extrinsic apoptotic pathways. The data suggest unambiguously that structural alteration of the stilbene motif of CA-4 can be extremely effective in producing potent apoptosis-inducing agents.

Introduction

Apoptosis, also known as programmed cell death, is a physiologic cell suicide mechanism that controls cell number in the tissues of metazoans.[1] Apoptosis is involved in embryo development, during morphogenesis, and in the adult animal, during tissue turnover. Moreover, it plays an important role in the development of the immune response. Because apoptosis is a major modality by which tumor cells can be eliminated, the identification of new drugs able to induce the death program in different tumor cell types is an important goal in cancer therapy and may provide new useful tools for the treatment of patients with drug-resistant malignancies.[2,3]

After inhibition of cell proliferation by inducing a block in one of the cell cycle phases, most anticancer drugs induce programmed cell death through a mechanism involving cell cycle checkpoint proteins such as p53. This process includes activation of key elements of apoptosis, the cell's intrinsic death program. A failure of this mechanism or the activation of apoptosis-inhibitor proteins (Bcl-2, Bcl-XL) in neoplastic cells can induce resistance to anticancer chemotherapy.[4,5] Genetic and epigenetic alterations that are important in carcinogenesis often disable the cell death pathway, thereby affecting the way that cancer cells respond to therapeutic insult. Therefore, factors affecting apoptosis activation might be important determinants of drug sensitivity.[2,3,6,7] New drugs able to modulate the expression of molecules involved in the apoptotic pathway and able to induce apoptosis in multidrug-resistant or apoptosis-resistant tumor cell lines are of great importance in cancer chemotherapy.

Combretastatins are natural antimitotic agents isolated from the bark of the South African tree *Combretum caffrum*. Among these compounds, combretastatin A-4 (CA-4, **1**, Figure 1) possesses the most potent and interesting antitumor activity.[8−14] From a structure−activity relationship 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 is an exceptionally strong inhibitor of tubulin polymerization and is potently cytotoxic against murine lymphocytic leukemia and against human ovarian and colon cancer cell lines.[15−20] Its mechanism of action is thought to be related to the tubulin-binding properties that result in rapid tumor endothelial cell damage, neovascular shutdown, and subsequent haemorrhagic necrosis.[21−23] It has been recently demon-

* Author to whom correspondence should be addressed. Phone: +39-0532-291291. Fax: +39-0532-291296. E-mail: smd@dns.unife.it.
† Dipartimento di Scienze Farmaceutiche, Università di Ferrara.
‡ R & D, Sigma-Tau.
§ Dipartimento di Scienze Farmaceutiche, Università di Bologna.
|| Dipartimento Farmacochimico Tossicologico e Biologico, Università di Palermo.
⊥ Cattedra di Ematologia e Servizio AIDS, Policlinico di Palermo.
SAIC-Frederick, Inc.
▽ National Cancer Institute at Frederick, National Institutes of Health.
O Consorzio di Ricerca sul Rischio Biologico in Agricoltura (Co.Ri.Bi.A.).
¶ Dipartimento di Scienze Farmacologiche, Università di Palermo.

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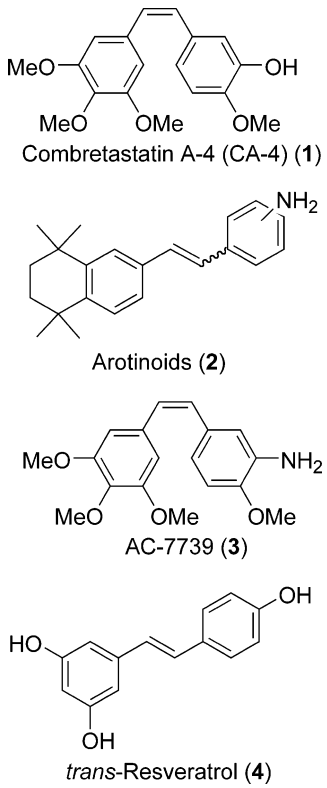


Figure 1. Natural and synthetic stilbenes.

strated that CA-4P, a combretastatin-A4 prodrug, induces cell death primarily through mitotic catastrophe in a panel of human B-lymphoid tumors.[24] Mitotic catastrophe appears to be a cell death modality different from apoptosis. Indeed, it has been reported that CA-4 was able to activate caspase-9, but the inhibition of caspase-9 by the use of the specific inhibitor *Z*-LEHD-fmk did not inhibit combretastatin-induced cell death. This indicates that apoptosis is a secondary mechanism of death in a small proportion of cells treated with CA-4.[24]

We recently started a study aimed at evaluating the apoptotic activity of natural and synthetic stilbene−arotinoids (**2**) structurally related to vitamin A.[2,7,25,26] Because some derivatives demonstrated potent apoptotic activity in both normal and multidrug-resistant (MDR) cell lines, we decided it would be informative to explore novel stilbenoids as a logical starting point in the quest for anticancer chemotherapeutics. We were further encouraged in this direction by our own discovery that the amino derivative AC7739 (**3**)[27] and compounds structurally related to *trans*-resveratrol (**4**)[28] possess potent apoptosis-inducing activity. In this context, we previously reported that the cis or trans stereochemistry of the double bond of biologically active stilbenes was the major discriminating factor affecting their apoptotic activity. These findings, together with the observation that CA-4 kills cells with a modality different from apoptosis, prompted us to synthesize novel combretastatin analogues characterized by a steric conformational block at the ethylene bridge.

Here, we will describe the synthesis of combretastatin analogues bearing five-membered heterocyclic systems that replace the alkenyl bridge of the natural CA-4. The alkenyl motif of **1** was replaced by an isoxazoline (**9b**, **13b**, **18b**, **19b**, **20a**−**b**, and **21a**−**b**) or an isoxazole ring system (**10b** and **14b**), yielding compounds that incorporate the two phenyl rings of the starting CA-4 at the 3,5- or 4,5-positions. Furthermore, as previously communicated, terphenyl analogues of CA-4 **27a**−**c**, **31b**, **34b**, and **35b**) were synthesized as novel, conformationally restricted stilbene mimetics of natural combretastatin.[29] We hypothesized that the terphenyl-based architectures, which incorporate a phenyl bridge or a pyridine ring as a bioisosteric substitution for an alkenyl portion of the stilbenes, could constitute interesting carbon frameworks in obtaining conformationally restricted stilbene mimetics. Thus, combretastatins bearing a phenyl or a pyridine ring that substitutes for the alkenyl bridge portion were prepared. Because stereochemistry of the vinyl portion of stilbenes is an aspect of molecular structure that deeply influences biological activity, the availability of the *ortho*-, *meta*-, and *para*-terphenyl isomers was considered essential for determination of the structure−activity relationship of this novel class of compounds. We will therefore also detail the synthesis and biological activity of these previously communicated terphenyl analogues.[29]

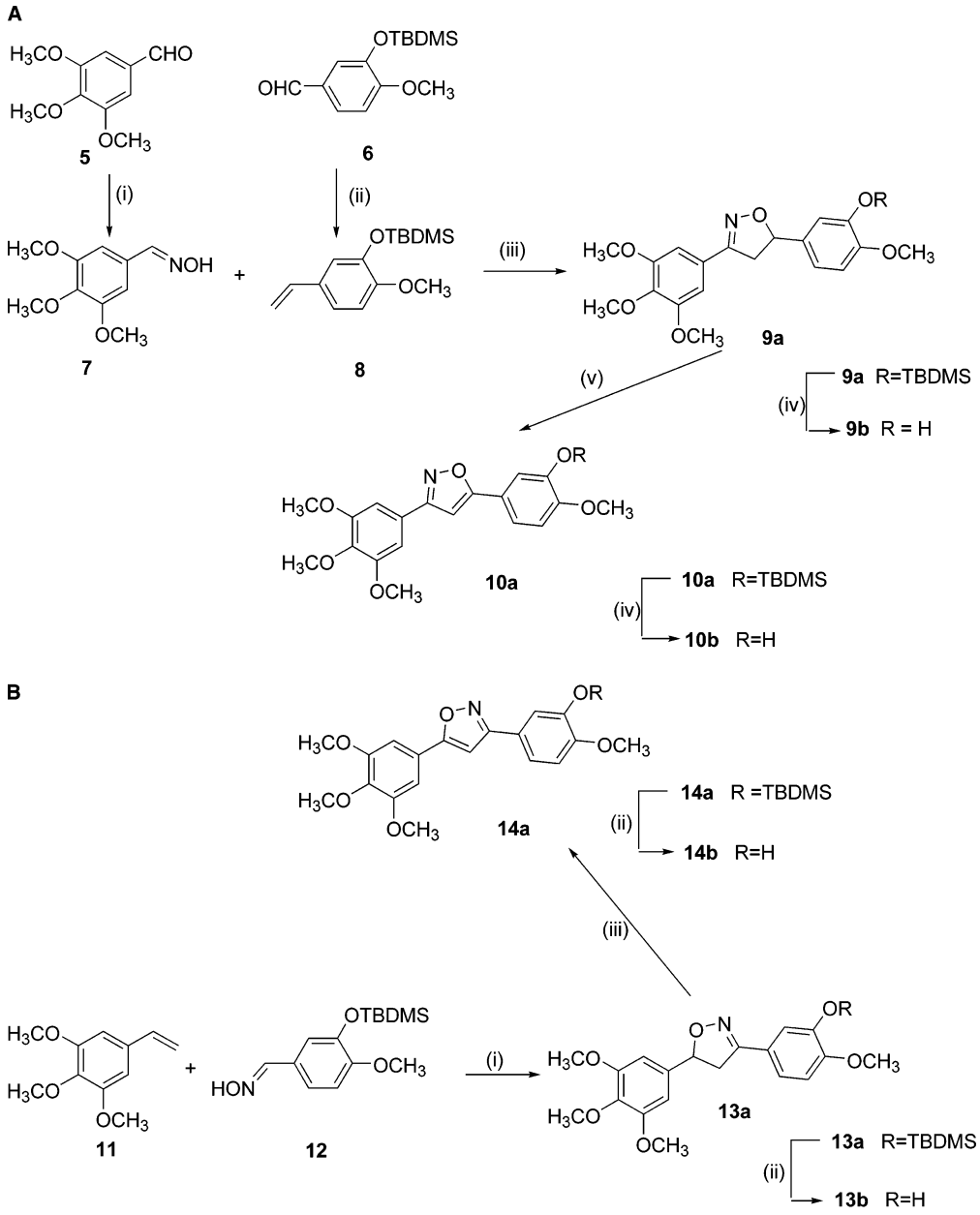
The antitumor activity of each compound was tested in vitro on different leukemia cell lines sensitive or resistant toward several chemotherapeutic agents. Some of the heterocyclic-bridged combretastatins demonstrated an attractive profile of cytoxicity and were able to induce apoptosis at concentrations lower than 1 μM in HL60 cells. A number of the newly synthesized compounds were also evaluated for potential interactions with tubulin. An attempt to rationalize the different biological activity of terphenyls **27a,b**, **31b**, and **35b** will be described through a molecular modeling investigation.

Chemistry

The syntheses of the isoxazoline and isoxazole derivatives **9b**, **10b**, **13b**, and **14b** are outlined in Scheme 1. We took advantage of known procedures for the preparation of aldehyde **6**,[30] whereas **5** was commercially available. Reaction of **5** and **6** with hydroxylamine in a MeOH/H_2O (3:1) solution produced the corresponding oximes **7** and **12** in high yields. The Wittig reaction between **6** and methyltriphenylphosphonium bromide, in the presence of NaH, gave olefin **8** in good yield. Olefin **11** was obtained through a similar approach starting from **5**. The nitrile oxide generated from oxime **7** following Torssell's procedure[31] underwent a [3 + 2] regioselective cycloaddition with olefin **8**, affording isoxazoline **9a** in good yield. In the same manner, isoxazoline **13a** was obtained from the nitrile oxide generated from oxime **12** and olefin **11**. Oxidation of the isoxazoline rings of **9a** and **13a** was easily accomplished by means of MnO_2 in benzene solution at reflux for 2 h, and the corresponding isoxazoles **10a** and **14a** were obtained, respectively, in 99% and 64% yield. Upon treatment with tetrabutylammonium fluoride in methanol, **9a** and **13a** as well as **10a** and **14a** underwent facile removal of the TBDMS group, providing the desired **9b**, **13b**, **10b**, and **14b** in good yields.

For the preparation of the isoxazoline derivatives **18b**, **19b**, **20a,b**, and **21a,b** (Scheme 2), we took advantage, to some extent, of chemistry based on the 3-sulfonyl-4,5-diphenylisoxazolines. The utility of the 3-sulfonyl-

Scheme 1[a]

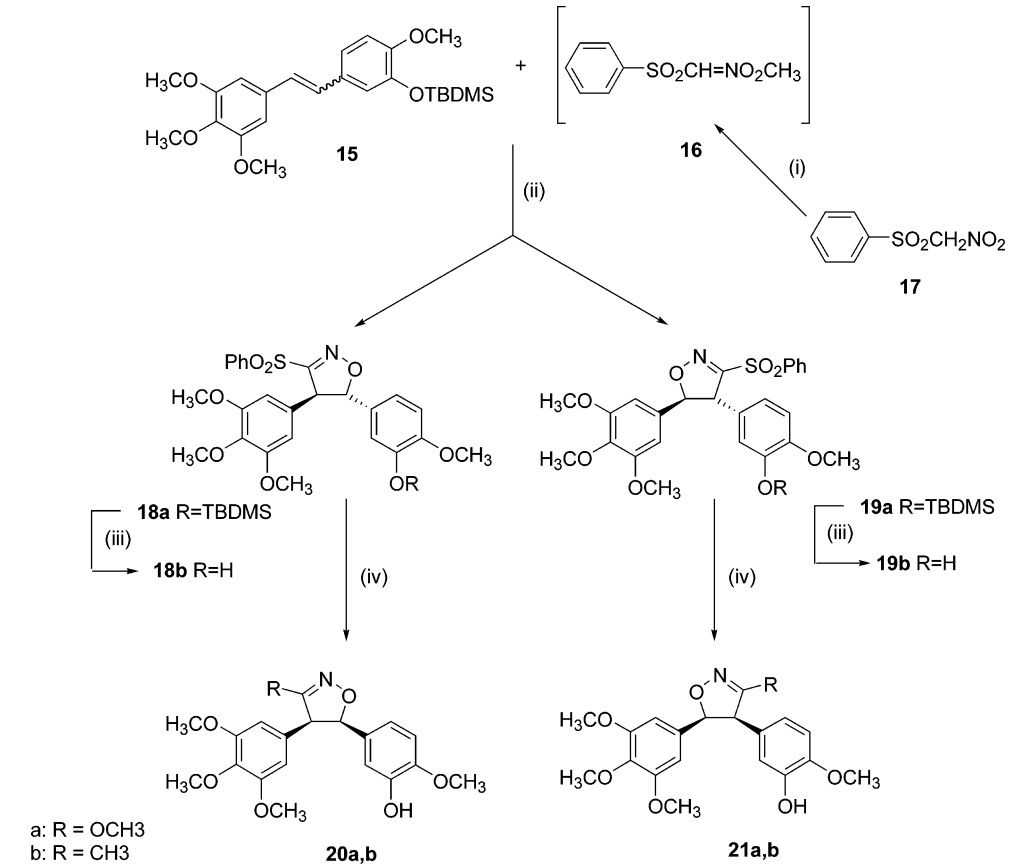


(i) $CHCl_3$, Pyridine, *N*-Chlorosuccinimide, TEA; (ii): TBAF, CH_2Cl_2; (iii): MnO_2, benzene, rfx.

[a] Reagents and conditions for A: (i) $NH_2OH \times HCl$, $NaHCO_3$, $MeOH/H_2O$ 3:1; (ii) $CH_3P(Ph)_3^+Br^-$, NaH, THF; (iii) $CHCl_3$, pyridine, *N*-chlorosuccinimide, TEA; (iv) TBAF, CH_2Cl_2; (v) MnO_2, benzene, reflux. Reagents and conditions for B: (i) $CHCl_3$, pyridine, *N*-chlorosuccinimide, TEA; (ii) TBAF, CH_2Cl_2; (iii) MnO_2, benzene, reflux.

isoxazolines as synthetic intermediates for the preparation of certain 3-substituted 4,5-diphenylisoxazolines has been widely documented by Wade et al.[32] The 3-(benzenesulfonyl)isoxazolines, readily obtained from the cycloaddition of benzenesulfonylcarbonitrile oxide and alkenes, undergo a variety of nucleophilic substitutions, which includes reactions with sodium alkoxides as well as with alkyl and aryllithium reagents. Such an approach was especially appealing to us because of the large number of substituents that could be incorporated at the 3-position of the isoxazoline ring system. Accordingly, the methyl nitronic ester **16** was prepared by treatment of the corresponding nitro compound **17** with ethereal diazomethane. The nitronic ester **16** reacted with both the cis and trans TBDMS-protected combretastatin derivatives **15,** in the presence of *p*-toluenesulfonic acid and in refluxing methylene chloride, to give the isoxazolines **18a** and **19a** in low yield (the stereochemistry was confirmed by a nuclear Overhauser spectroscopy (NOESY) experiment). The NMR spectrum of the crude reaction products indicated the trans stereochemistry of the isoxazolines **18a** and **19a** as the only diastereoisomers. Because the conditions of the cycloaddition are rather drastic, we had advanced the hypothesis that isomerization of the (*Z*)-stilbene **15** to the E isomer would occur quantitatively and rapidly so that no trace of the cis isoxazoline isomer was observed. Attempts to affect the nucleophilic substitution of the sulfonyl group of **18a** and **19a** with appropriate nucleophiles presented difficulties. Because simple 3-benzenesulfonyl-4,5-diphenyl-isoxazolines reacted easily with alkoxides and alkyl or aryllithium derivatives,

Scheme 2[a]



[a] Reagents and conditions: (i) Diazomethane, Et_2O; (ii) $CHCl_2$, *p*-Ts-OH, reflux; (iii) TBAF, CH_2Cl_2; (iv) Na, CH_3OH or MeLi, THF.

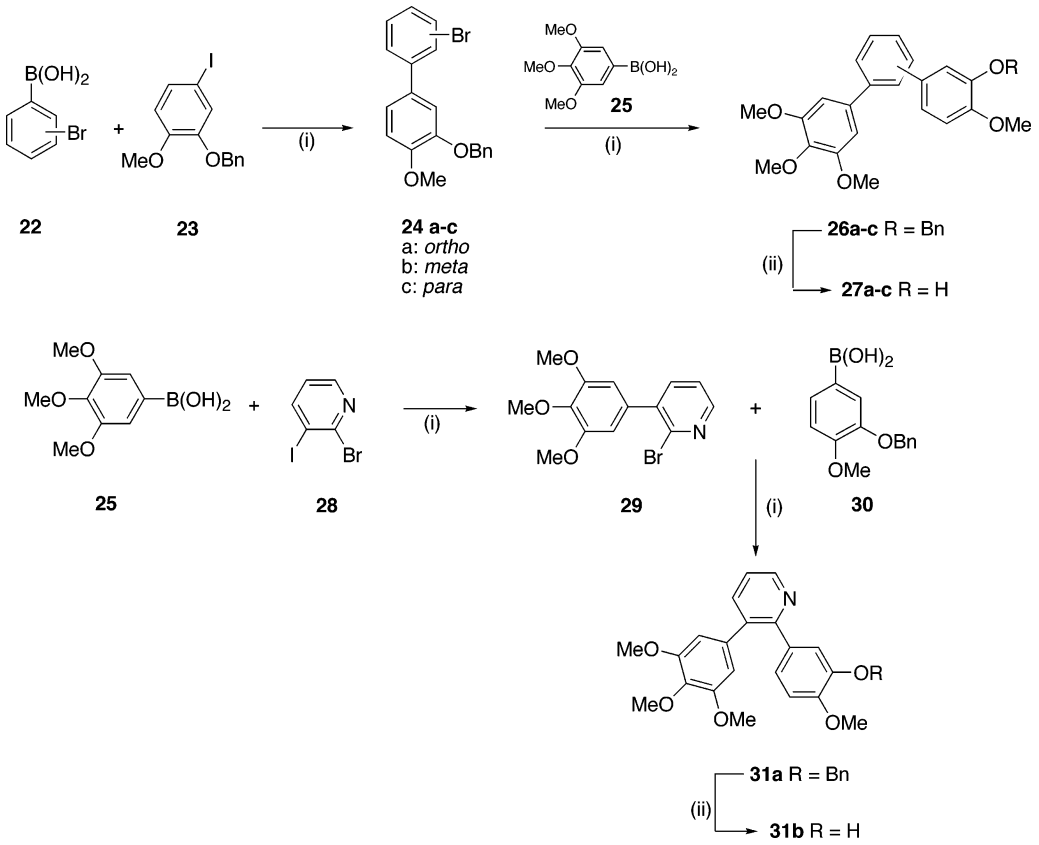
compounds **18a** and **19a** were resistant to nucleophilic substitution with phenyllithium, and methyllithium gave only low yields. However, when treated with sodium methoxide, **18a** and **19a** were converted to the 3-alkoxyisoxazolines **20a** and **21a**, respectively, in 65% and 69% yield. Interestingly, both of the starting trans diastereoisomers **18a** and **19a** gave only the *cis*-4,5-diphenylisoxazoline derivatives **20a** and **21a** without detectable amounts of the trans isomers. Because it has been reported that *trans*-4,5-diphenylisoxazolines, when reacted with nucleophiles, only occasionally result in formation of traces of the cis isomers, our results are quite surprising.[32] However, the coupling constants between 4H and 5H of **20a** and **21a** unambiguously demonstrate the cis stereochemistry for these derivatives. Similar results were obtained with the methyl derivatives **20b** and **21b**, but yields of the reaction were much lower.

The synthesis of terphenyls **27a**−**c** and **31b** was achieved through a double Suzuki cross-coupling strategy, as depicted in Scheme 3 and previously communicated.[29] Full experimental details for these compounds are included in the Experimental Section and were not previously described. As palladium-catalyzed cross-coupling reactions between arylboronic acids and aryl halides or triflates (Suzuki coupling reaction)[33] have become a common method for the synthesis of biaryls, we envisaged the possibility that a highly convergent and flexible double Suzuki coupling approach could be used to prepare the desired terphenyls. Accordingly, the Suzuki coupling between **22** (commercially available) and iodo derivative **23** afforded in good yields the desired diphenyl derivatives **24a**−**c.** The coupling was achieved in toluene, tetrakis(triphenylphosphine)−palladium(0) was employed as the catalyst for the reaction, and aqueous Na_2CO_3 provided the required basic environment. The subsequent Suzuki cross coupling between the bromodiphenyl derivatives **24a**−**c** and boronic acid **25** (commercially available) was achieved under the standard conditions described above. In a similar manner, the pyridine derivative **31a** was also obtained. Hydrogenation of **26a**−**c** and **31a** in the presence of Pd−C provided the desired **27a**−**c** and **31b** in good yields. By means of a different but more productive strategy, the pyridine analogue **35b** and its chloro derivative **34b** were obtained as outlined in Scheme 4. The intermediate **32** was reacted with the vinamidinium hexafluorophosphate salt (CDT-phosphate) **33** according to the Nair procedure in the presence of *tert*-BuOK, ammonium acetate, and an equimolar amount of DABCO.[34,35] DABCO accelerated the reaction and led to complete consumption of the preformed potassium enolate. Hydrogenation of **34a** in the presence of 10% palladium over $CaCO_3$ yielded **35a**. Upon treatment with TFA in dichloromethane, the intermediates **34a** and **35a** underwent facile removal of the MEM protecting group, resulting in good yields of derivatives **34b** and **35b.**

Biological Results and Discussion

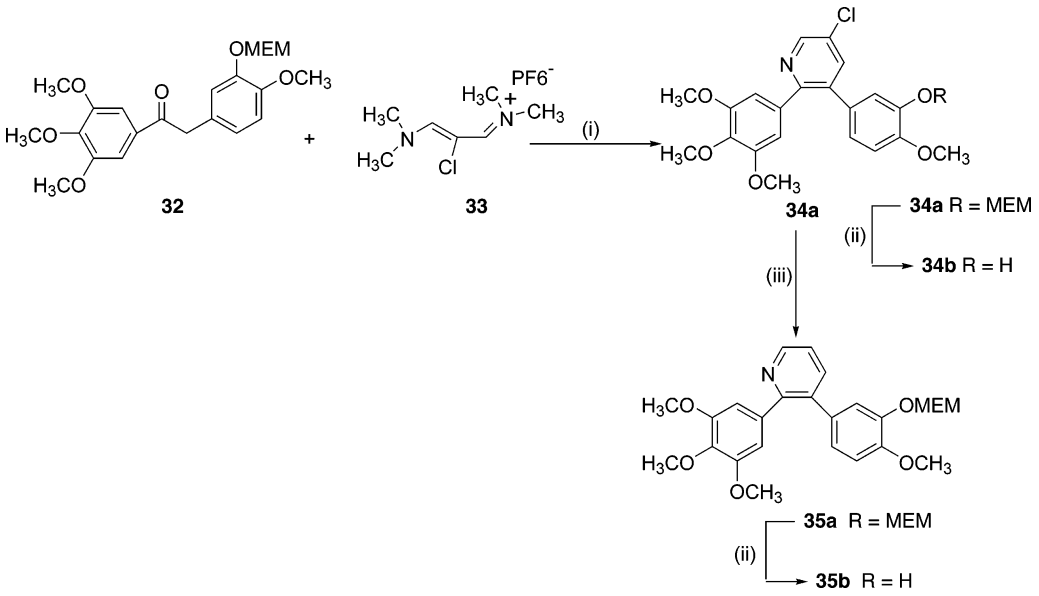
The premises for our work were formulated by preliminary studies from our laboratories in which we demonstrated that natural and synthetic stilbenoids possess potent apoptosis-inducing activity. Because the *cis*-stilbene motif represents the key structural feature of CA-4 and, moreover, it is well-known that the ability

Scheme 3[a]



[a] Reagents and conditions: (i) $Pd(Ph_3P)_4$, aqueous Na_2CO, toluene/EtOH; (ii) H_2, Pd/C, EtOH.

Scheme 4[a]



[a] Reagents and conditions: (i) *tert*-ButOK, DABCO, NH_4OAc, THF, 6 h reflux; (ii) TFA, CH_2Cl_2, 5 h at room temperature, (iii) H_2, 10% $Pd/CaCO_3$, 1 N NaOH/EtOH 1:1, overnight at room temperature.

of natural stilbenes to elicit a wide range of physiological effects often stems from changes in olefin configuration, our idea was that the synthesis of double-bond-locked analogues of natural CA-4 could afford compounds endowed with apoptotic activity. The main objective was to investigate the importance of the alkenyl bridge of CA-4 in conferring such activity.

Table 1 shows the cytotoxic activity values of 16 new CA-4-like analogues on HL60 cells, expressed as IC_{50} values (concentration able to inhibit cell growth 50%) and AC_{50} values (concentration able to induce apoptosis in 50% of cells). In all cases, the two activities seems to be closely linked, with the AC_{50} value generally about 2-fold higher than the IC_{50} value. On the basis of their activities, they can be split into three classes: (a) compounds active at concentrations over 10 μM, **19b**, **27a**−**c**; (b) compounds active at concentrations between 1 and 10 μM, **9b**, **10b**, **13b**, **14b**, **18b**, **20b**, **21b**, and

Table 1. Antiproliferative Activity (Evaluated as IC_{50}) and Apoptotic Activity (Evaluated as AC_{50}) of Combretastatin Analogues in HL60 Cells

compound	IC_{50} (μM)	AC_{50} (μM)	compound	IC_{50} (μM)	AC_{50} (μM)
9b	3.2	5.5	**21a**	0.25	0.5
10b	3	4.5	**21b**	2	3.5
13b	1.5	2.2	**27a**	20	30
14b	3	4	**27b**	12	22
18b	0.9	2	**27c**	30	35
19b	15	25	**31b**	5	10
20a	0.1	0.3	**34b**	0.5	0.8
20b	2	3.5	**35b**	0.3	0.6

31b; (c) compounds active in both assays at concentrations between 0.1 and 0.8 μM, **20a**, **21a**, **34b**, and **35b**.

Our results indicate that introduction of a isoxazoline or an isoxazole heterocyclic bridge, as in compounds **9b**, **10b**, **13b**, **14b**, **19b**, **20b**, and **21b**, leads to analogues with limited cytotoxic and apoptotic activity, although compounds structurally related to our derivatives, but containing a pyrazole or other heterocycle, were reported to be as potent as the natural compound.[14,18,36−39] Although we verified the inactivity of the terphenyl structures **27a**−**c** and **31b,** we found that the corresponding pyridine derivatives **34b** and **35b** possess interesting apoptotic activity. Thus, the results obtained with the terphenyl derivatives suggest that introduction of an electronegative component next to the trimethoxyphenyl of CA-4 is important in conferring apoptotic activity to this class of compounds.

Equally interesting results were obtained with the 4,5-disubstituted heterocycle-bridged derivatives **20a** and **21a**, which demonstrated an attractive profile of cytoxicity and apoptosis-inducing activity. Compound **18b** appears also to be of some interest in view of its unusual structure. The presence of a third phenyl ring located at the double-bond region of CA-4 makes this compound a new lead for the possible discovery of novel apoptotic agents. Indeed, diarylindoles and analogues have shown potent antitubulin activity and cytoxicity.[18,37−39] The biological activities of **20a** and **21a** were markedly greater than those of all of the other isoxazoline and isoxazole derivatives. Of note, by locating the phenyl substituents at the 4,5-positions of the isoxazoline scaffold, as in compounds **20a** and **20b**, we greatly influenced the activity of the compounds, given that the activity of the 3,5-diphenyl-substituted isomers **9b** and **13b** were decidedly lower. Thus, the right location of the phenyl substituents on the isoxazoline scaffold seems critical for the development of effective apoptotic agents.

Most of the compounds described here were examined for effects on tubulin assembly, as described.[40] Compounds **9b**, **10b**, **13b**, **14b**, **18b**, **19b**, **20b**, **21b**, and **27a**−**c** were essentially inactive, yielding IC_{50} values >40 μM (the IC_{50} being defined as the concentration of compound required to inhibit extent of assembly of 10 μM tubulin by 50% after a 20 min incubation at 30 °C). Two of the most cytotoxic compounds, **20a** and **21a**, did inhibit tubulin assembly, yielding IC_{50} values of 9.9 ± 1 and 8.7 ± 0.3 μM, respectively. These values are about 5-fold higher than the IC_{50} value obtained for CA-4 (1.8 ± 0.3 μM). Because CA-4 is a particularly potent inhibitor of the binding of [^{3}H]colchicine to tubulin, routinely completely inhibiting colchicine binding in reaction mixtures containing 1 μM tubulin, 5 μM CA-4, and 5 μM [^{3}H]colchicine, we also examined **20a** and **21a** for their effects on colchicine binding to tubulin.[41] The two compounds were much less potent than CA-4. At 5 μM, less than 25% inhibition was observed with both compounds. Greater inhibition occurred when their concentrations were increased to 50 μM. At this higher inhibitor concentration, **20a** inhibited [^{3}H]colchicine binding by 77 ± 3% and **21a** by 68 ± 3%. Of the basis of these comparisons with CA-4, the interaction with tubulin is negatively impacted by the alterations of the alkenyl bridge that occur in compounds **20a** and **21a**.

Table 2. Therapeutic Index (T.I.) of CA-4, **18b**, **20a**, and **21a**[a]

compound (IC_{90})	% CFU-GEMM inhibition	therapeutic index
CA-4 (**1**) (3.0 nM)	37	2.4
18b (2.0 μM)	42	2.1
20a (0.3 μM)	36	2.5
21a (0.5 μM)	34	2.6

[a] Bone marrow cells were treated with each drug used at a concentration corresponding to the IC_{90} value calculated for HL60 cells. After 14 days the percentage of CFU-GEMM was determined. T.I. was calculated from the formula IC_{90}/percentage of CFU-GEMM inhibition.

Compounds **18b**, **20a**, and **21a** were further investigated for their mechanisms of action. These compounds were less cytotoxic in leukemia HL60 cells than the natural CA-4 (Table 2). However, the therapeutic index of **18b**, **20a**, and **21a,** calculated from the ratio between cytotoxicity on neoplastic cells and cytotoxicity on normal bone marrow cells, was similar to the therapeutic index of CA-4 (Table 2). As shown in Figure 2 and Figure 3, **18b**, **20a**, and **21a** induced a recruitment of cells into the G2-M phase of the cell cycle after 8 h of treatment. As the flow cytometric analysis of the cell cycle does not distinguish cells blocked in G2 from cells blocked in M phase, a fluorescent microscopy analysis was performed after staining the cells with ethidium bromide and acridine orange. The control drug, CA-4 induced nearly a complete block in the M phase of the cell cycle (Table 3). In contrast, as shown in Table 3, the percentage of mitotic cells evaluated after 24 h of treatment with **18b**, **20a**, and **21a** was only 8−12%, indicating that these drugs may act primarily by inducing a G2 block of the cell cycle. These data, showing a low-mitotic block, indicate that the new analogues **18b**, **20a**, and **21a** may act with a mechanism different from that of CA-4. Additionally, we observed that **18a**, **20a**, and **21a** are potent proapoptotic agents. Apoptosis appeared after 6 h of drug exposure and increased in the following hours to reach a value of 80−90% after 48 h. Apoptosis can be activated by specific cell death receptors that are located on the cell surface membrane (extrinsic pathway) and by proapoptotic factors released from the mitochondria (intrinsic pathway).

Most of the known anticancer agents induce apoptosis by activating the intrinsic pathway by means of the release of cytochrome-c, preceded by depolarization of mitochondrial membranes. The extrinsic pathway is not involved in drug-induced apoptosis, being mainly activated in immunologic processes. Nevertheless, we haveevaluated the possible activation of both the extrinsic and intrinsic pathways in apoptosis induced by **18b**, **20a**, and **21a**.

To evaluate the activation of the intrinsic apoptotic pathway, the $\Delta\Psi$ dissipation was measured by flow

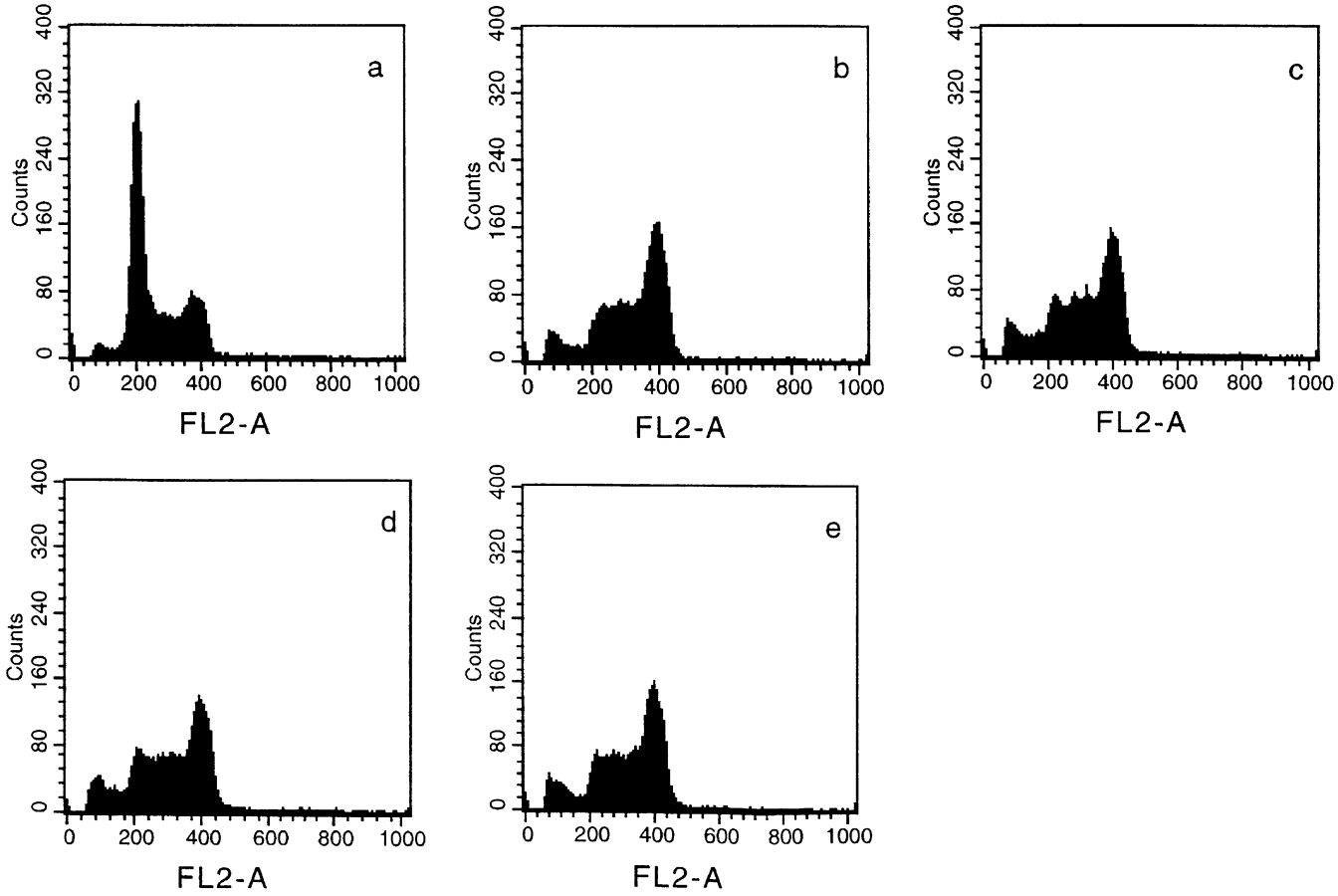


Figure 2. Flow cytometric analysis of cell cycle distribution in HL60 cells treated with (b) 2.5 nM CA-4, (c) 2 μM **18b**, (d) 1 μM **21a**, (e) 1 μM **20a**; a = control. Cell cycle distribution was evaluated after 8 h of treatment.

cytometry by exposing cells to $DiOC_6$ after 8 h of drug treatment. The polarization status of the mitochondrial membrane is determined by an electrochemical gradient ($\Delta\Psi$). This gradient is essential for driving the ATP synthase that phosphorylates ADP to ATP. Activation of the intrinsic apoptotic pathway can be assessed indirectly by determining a whether the $\Delta\Psi$ is reduced. For this, cells are incubated with a lipophilic cationic fluorochrome, such as $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide), which accumulates in the mitochondrial matrix driven by the $\Delta\Psi$. A reduction in fluorescence intensity, as measured by cytofluorometry, indicates a $\Delta\Psi$ dissipation and, therefore, the activation of the intrinsic apoptosis pathway. As shown in Figure 4, compounds **18b**, **20a**, and **21a** induced a marked decrease in $DiOC_6$ fluorescence intensity.

The activation of the intrinsic apoptotic pathway was confirmed by experiments using the caspase 9 inhibitor *Z*-LEHD. After $\Delta\Psi$ dissipation, proapoptotic factors, such as cytochrome-c, are released, and these specifically activate caspase 9. As shown in Table 4, when HL60 cells were exposed to **18b**, **20a**, and **21a** in combination with *Z*-LEHD (5 μM), the percentage of apoptotic cells was reduced about 50−90%.

For our initial evaluation of the extrinsic pathway, HL60 cells were treated with **18b**, **20a**, or **21a** in the presence of a Fas-blocking monoclonal antibody (ZB4). Table 5 shows that inhibition of the apoptosis induced by the compounds occurred when the Fas-blocking monoclonal antibody ZB4 was also present. This implies that **18b**, **20a**, and **21a** have the ability to activate the extrinsic pathway as well as the intrinsic pathway.

Activation of the extrinsic pathway was confirmed by studying two human T-cell lymphoma cell lines, HUT78 and HUT78B1. The latter line was derived from HUT78 by exposure of the cells to gradually increasing concentrations of a Fas-agonistic monoclonal antibody. HUT78 cells express Fas and are sensitive to Fas-ligand-induced apoptosis, whereas HUT78B1 cells express a nonfunctional death domain deletion of Fas. The HUT78B1 cells are therefore resistant to Fas-ligand-induced apoptosis. As shown in Table 6, **18b**, **20a**, and **21a** were less active as apoptosis-inducing agents in the Fas-resistant HUT78B1 cell line than in the HUT78 line.

On the basis of these results, it appears that **18b**, **20a**, and **21a** act by activating both the intrinsic and the extrinsic pathways. Considering that the most commonly used chemotherapeutic agents are unable to affect the extrinsic pathway, it would be worthwhile examining the novel double-bond-locked stilbene derivatives **18b**, **20a**, and **21a** for in vivo antitumor activity. Interestingly, compounds **18b**, **20a**, and **21a** were also active in the apoptosis resistant and BCR-ABL-expressing K562 cell line (blastic crisis of chronic myelogenous leukemia), as well as in two multidrug resistant (MDR), P-glycoprotein expressing-cell lines, HL60R and K562ADR (Table 7). Thus, these molecules could also be useful for the treatment of malignancies expressing the MDR phenotype.

In conclusion, structural alteration of the alkenyl bridge of CA-4 seems of particular importance in confer-

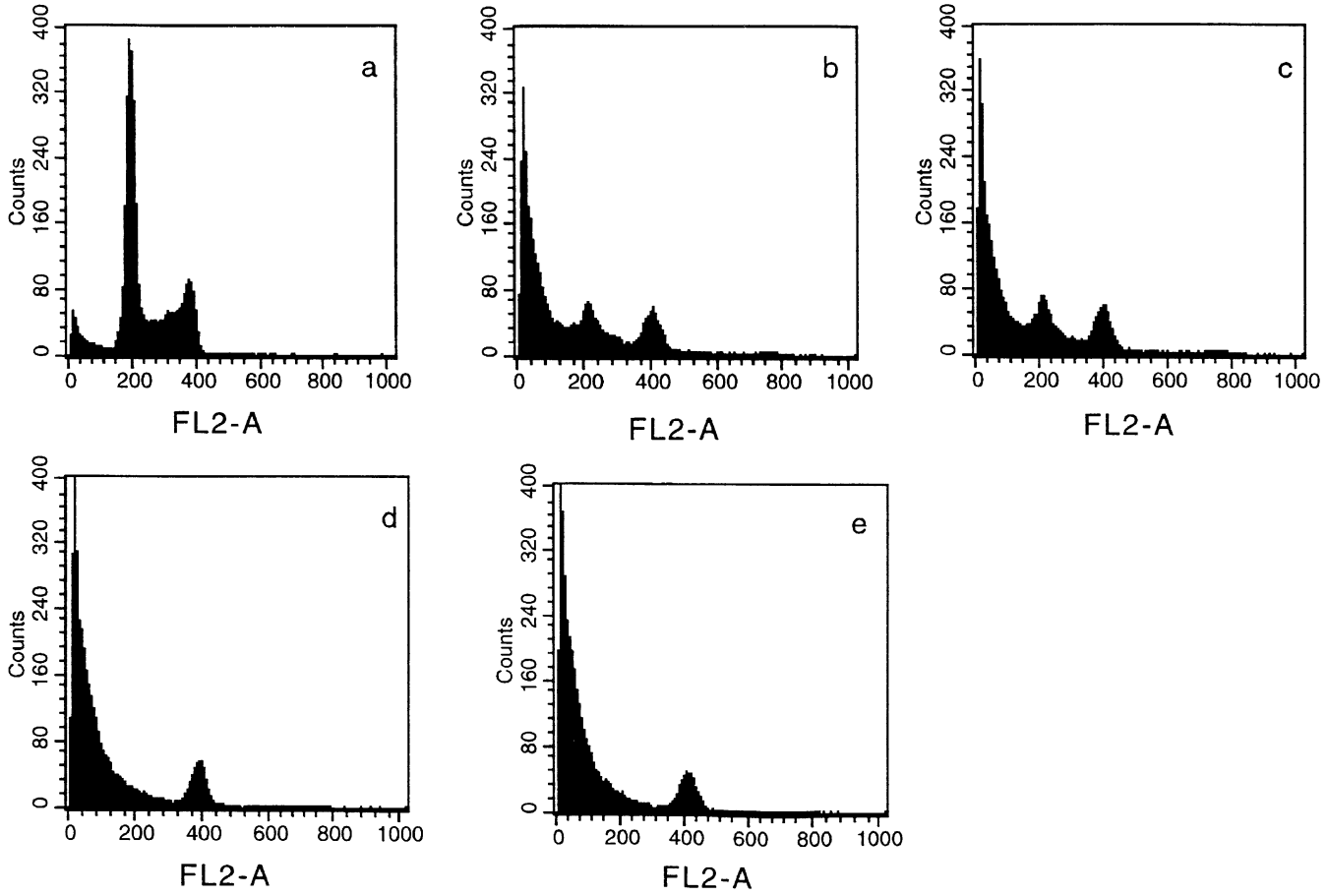


Figure 3. Flow cytometric analysis of cell cycle distribution in HL60 cells treated with (b) 2.5 nM CA-4, (c) 2 μM **18b**, (d) 1 μM **21a**, (e) 1 μM **20a**; a = control. Cell cycle distribution was evaluated after 48 h; most of the drug treated cells were in sub-G_0-G_1 phase (apoptosis).

Table 3. Percentage of Mitotic Cells after 8 h Exposure of HL60 Cells to 2 μM **18b**, 1 μM **20a**, 1 μM **21a**, and 2.5 nM CA-4[a]

compound	% mitosis
CA-4	88
18b	18
20a	17
21a	15

[a] Evaluation was performed by fluorescence microscopy after staining of cells with acridine orange and ethidium bromide

ring apoptotic or mitotic catastrophe activity on these molecules. The introduction of a double-bond-locked motif confers on the structurally restricted stilbene mimetics **18b**, **20a**, and **21a** a potent apoptotic activity different from that of natural CA-4. Their ability to block most cells in the G2 phase of the cell cycle suggests that these compounds could act on targets different from the mitotic spindle. This may confer on these molecules a wider spectrum of action than the parent CA-4, which arrests cells in the M phase of the cell cycle. Of note, our data suggest unambiguously that structural alteration of the stilbene motif of CA-4 can be extremely effective in producing potent apoptosis-inducing agents by activating both the intrinsic and the extrinsic pathways. We are continuing to explore other structurally restricted derivatives of natural CA-4.

Molecular Modeling. The different apoptotic activity found between the pyridine derivative **35b** and the other terphenyls **27a,b** and **31b** prompted us to investigate possible explanations. A preliminary molecular modeling investigation was performed aimed at exploring the importance of the conformational features of compounds CA-4, **27a**, **31b**, and **35b** on receptor affinity. Considering that all ligands likely bind at the same site at the receptor level, they should adopt a common three-dimensional geometry that is responsible for their affinity and functional activity. Thus, the conformational space of each molecule was sampled by Monte Carlo methods in which a number of conformations is generated by randomly rotating selected bonds.

A conformational analysis was carried on six rotatable bonds without any solvent. The energy cut off was set 50 kcal/mol above the estimated total energy of the molecule, and the maximum number of attempts was 8000. The lowest-energy conformations obtained for each compound were superimposed on the template molecule CA-4. The two phenyl rings and the oxygen were used as the common substructure in this alignment. The results of the superimpositions, shown in Figure 5, reveal that only compound **35b** has a comparable conformation and similar orientation in space as CA-4. These preliminary data seem therefore to confirm that, beside the different biological activity, a conformational difference exists between the terphenyl architectures investigated. Other molecular modeling studies are currently in progress.

Materials and Methods

Chemistry. Melting points were obtained in open capillary tubes and are uncorrected. Reactions and product mixtures were routinely monitored by thin-layer chromatography (TLC)

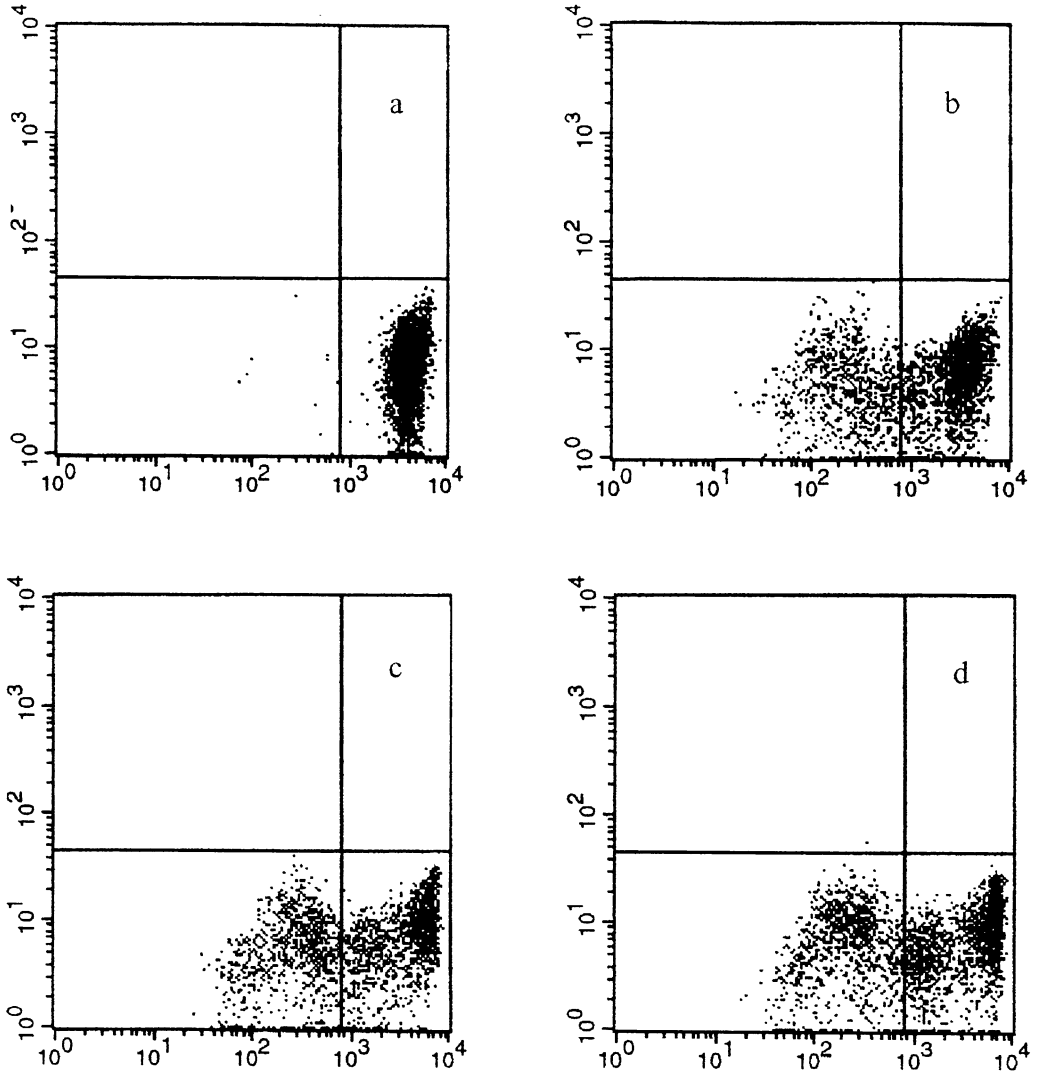


Figure 4. HL-60 cells were cultured for 8 h in the presence of (b) 2 μM **18b**, (c) 1 μM **21a**, or (d) 1 μM **20a**, followed by staining of the cells with $DiOC_6$; mitochondrial membrane potential was studied by flow cytometry (a = control).

Table 4. Effects of the Caspase 9 Inibitor *Z*-LEHD-fmk on Apoptosis Induced by 2 μM **18b** (2 μM), **20a** (0.3 μM), and **21a** (0.5 μM)

compound	% apoptosis inhibition
18b + Z-LEHD-fmk	71.5
20a + Z-LEHD-fmk	50
21a + Z-LEHD-fmk	91.4

Table 5. Effects of the Fas-Blocking MoAb ZB4 on Apoptosis Induced by **18b** (2 μM), **20a** (0.3 μM), and **21a** (0.5 μM)

compound	% apoptosis inhibition
18b + ZB4	25
20a + ZB4	36
21a + ZB4	57

Table 6. Percentage of Apoptosis in HUT78 and HUT78B1 Cells after 48 h of Exposure to **18b**, **20a**, and **21a**

compound	% apoptosis in HUT78	% apoptosis in HUT78B1
18b (2.0 μM)	70	46
20a (0.3 μM)	80	60
21a (0.5 μM)	75	40

Table 7. Antiproliferative Activity (Evaluated as IC_{50}) and Apoptotic Activity (Evaluated as AC_{50}) of Compounds **18b**, **20a**, and **21a** in HL60R, K562, and K562ADR Cell Lines

	HL60R		K562		K562ADR	
compound	IC_{50} (μM)	AC_{50} (μM)	IC_{50} (μM)	AC_{50} (μM)	IC_{50} (μM)	AC_{50} (μM)
18b	3	5	1	2	2	8
20a	0.25	0.75	0.2	0.4	0.5	2
21a	0.75	2	0.2	0.4	0.5	2

on Merck silica gel precoated F254 plates. Infrared spectra (IR, cm^{-1}) were measured on a Perkin-Elmer 257 instrument. Nuclear magnetic resonance (^1H NMR, δ) spectra were determined, when not specified, in $CDCl_3$ solution using a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as the internal standard; *J* values are expressed in hertz (Hz). Light petroleum ether refers to the 40−60 °C boiling range fractions. Column chromatographies were performed with Merck 60−200 mesh silica gel. All drying operations were performed over anhydrous $MgSO_4$. Column chromatography (medium pressure) was carried out by using the "flash" technique. Microanalysis of all new synthesized compounds agreed within ±0.4% of calculated values.

General Procedure for the Preparation of Oximes 7 and 12. To a solution of hydroxylamine hydrochloride (250 mg, 3.7 mmol) dissolved in water (7 mL), $NaHCO_3$ (470 mg, 5.6 mmol) was added portionwise at 0 °C, and the mixture was stirred for 30 min at room temperature. The appropriate

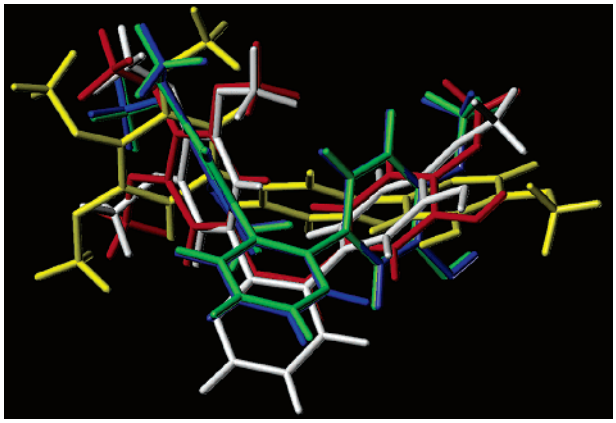


Figure 5. Superposition of compounds **27a** (blue), **27b** (yellow), **31b** (green), and **35b** (white) onto template CA-4 (red).

aldehyde **5** or **6** (3.1 mmol), dissolved in methanol (5 mL), was then added to the solution, and stirring was continued for an additional 6 h. Methanol was evaporated in vacuo, and the residue extracted with diethyl ether. The organic extracts were washed with brine, dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel.

3,4,5,-Trimethoxy-benzaldehyde Oxime (7). Eluent ethyl acetate/light petroleum 1:3; mp 95−97 °C; yield 56% (366 mg); ^{1}H NMR 3.98 (s, 9H), 7.97 (s, 1H), 8.07 (s, 1H). Anal.-($C_{10}H_{13}NO_4$) C, H, N.

3-(*tert*-Butyl-dimethyl-silanyloxy)-4-methoxy-benzaldehyde Oxime (12). Eluent ethyl acetate/light petroleum 1:9; oil; yield 47% (410 mg); ^{1}H NMR 0.17 (s, 6H), 1.00 (s, 9H), 3.84 (s, 3H), 6.84 (d, J = 8.3, 1H), 7.08 (s, 1H), 7.11−7.15 (m, 2H). Anal. ($C_{14}H_{23}NO_3Si$) C, H, N.

General Procedure for the Preparation of Olefins 8 and 11. NaH (55%) (145 mg, 6 mmol), previously washed with dry hexane, was added to a stirred suspension of methyltriphenylphosphonium bromide (1.07 g, 3 mmol) in dry THF (15 mL) containing the appropriate aldehyde **5** or **6** (3 mmol). After the suspension was stirred for 5 h at room temperature, diethyl ether (30 mL) was added, and the mixture was poured into ice−water and extracted with Et_2O. The combined organic extracts were dried and evaporated, and the residue was chromatographed on silica gel.

***tert*-Butyl-(2-methoxy-5-vinyl-phenoxy)-dimethyl-silane (8).** Eluent diethyl ether/light petroleum 1:9; oil; yield 67% (530 mg); ^{1}H NMR 0.16 (s, 6H), 1.00 (s, 9H), 3.80 (s, 3H), 5.10 (d, J = 10.8, 1H), 5.56 (d, J = 17.5, 1H), 6.60 (dd, J = 10.9, J = 17.5, 1H), 6.78 (d, J = 8.8, 1H), 6.94−6.97 (m, 2H). Anal. ($C_{15}H_{24}O_2Si$) C, H.

1,2,3-Trimethoxy-5-vinyl-benzene (11). Eluent diethyl ether/light petroleum 1:4; oil; yield 85% (495 mg); ^{1}H NMR 3.85 (s, 3H), 3.88 (s, 6H), 5.20 (d, J = 11.0, 1H), 5.62 (d, J = 18.1, 1H), 6.62 (dd, J = 11.0, J = 18.1, 1H), 6.65 (s, 2H). Anal. ($C_{11}H_{14}O_3$) C, H.

General Procedure for the Preparation of Isoxazolines 9a and 13a. A mixture of *N*-chlorosuccinimide (174 mg, 1.3 mmol), pyridine (2 drops), and oxime **7** or **12** (1.3 mmol) in anhydrous $CHCl_3$ (15 mL) was stirred for 1 h at 50−60 °C. Olefin **8** or **11** (1.4 mmol) was then added, followed by triethylamine (0.27 mL, 1.95 mmol) solubilized in $CHCl_3$ (5 mL). After the mixture was stirred at 25 °C for 20 min, water was added (10 mL). The organic phase was washed with 2.5% HCl (15 mL), water (15 mL), and brine (15 mL), then dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel.

5-[3-(*tert*-Butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-3-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazole (9a). Eluent diethyl ether/light petroleum 2:3; oil; yield 67% (410 mg); ^{1}H NMR 0.15 (s, 6H), 1.00 (s, 9H), 3.28 (dd, J = 8.7, J = 16.4, 1H), 3.69 (dd, J = 10.7, J = 16.4, 1H), 3.80 (s, 3H), 3.87 (s, 9H), 5.63 (dd, J = 8.6, J = 10.7, 1H), 6.81−6.97(m, 5H). Anal. ($C_{25}H_{35}NO_6Si$) C, H, N.

3-[3-(*tert*-Butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazole (13a). Eluent diethyl ether/light petroleum 3:7; oil; yield 75% (462 mg); ^{1}H NMR 0.17 (s, 6H), 1.00 (s, 9H), 3.33 (dd, J = 8.2, J = 16, 1H), 3.78 (dd, J = 10.3, J = 16.1, 1H), 3.83 (s, 6H), 3.87 (s, 6H), 5.70 (dd, J = 8.2, J = 10.3, 1H), 6.62 (s, 2H), 6.85 (d, J = 8.3, 1H), 7.17 (dd, J = 2.1, J = 8.2, 1H), 7.28 (d, J = 2.1, 1H). Anal. ($C_{25}H_{35}NO_6Si$) C, H, N.

General Procedure for Preparation of Isoxazoles 10a and 14a. The appropriate isoxazoline **9a** or **14a** (48 mg, 0.1 mmol) solubilized in dry benzene (or a 10:1 benzene/dioxane solution) containing active manganese dioxide (450 mg, 5.17 mmol) was heated under reflux, and the water formed was removed by means of a Dean−Stark apparatus. The end of the reaction was monitored by TLC. The solid was filtered through Celite and washed carefully with the same solvent. After evaporation of the filtrate, the residue obtained was chromatographed on silica gel.

5-[3-(*tert*-Butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-3-(3,4,5-trimethoxy-phenyl)-isoxazole (10a). Eluent ethyl acetate/light petroleum 1:4; oil; yield 99% (47 mg); ^{1}H NMR 0.12 (s, 6H), 1.20 (s, 9H), 3.89 (s, 3H), 3.93 (s, 3H), 3.97 (s, 6H), 6.66 (s, 1H), 6.96 (d, J = 8.0, 1H), 7.15 (s, 2H), 7.34 (d, J = 2.0, 1H), 7.44 (dd, J = 2.0, J = 8.0, 1H). Anal. ($C_{25}H_{33}NO_6Si$) C, H, N.

3-[3-(*tert*-Butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-5-(3,4,5-trimethoxy-phenyl)-isoxazole (14a). Eluent ethyl acetate/light petroleum 1:4; oil; yield 64% (30 mg); ^{1}H NMR 0.20 (s, 6H), 1.03 (s, 9H), 3.87 (s, 3H), 3.91 (s, 3H), 3.95 (s, 6H), 6.68 (s, 1H), 6.93 (d, J = 8.2, 1H), 7.05 (s, 2H), 7.38−7.44 (m, 2H). Anal. ($C_{25}H_{33}NO_6Si$) C, H, N.

General Procedure for the Preparation of the Isoxazoline Derivatives 18a and 19a. The freshly prepared solution of the crude nitronic ester **16** (1.4 mmol) in CH_2Cl_2 was treated with compound **15** (600 mg, 1.4 mmol) and *p*-TsOH·H_2O (270 mg, 1 mmol). The resulting solution was refluxed for 30 min, cooled, diluted with CH_2Cl_2 (20 mL), washed with 5% aqueous NaOH (10 mL) followed by water, then dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel.

3-Benzenesulfonyl-5-[3-(*tert*-butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-4-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazole (18a). Eluent ethyl acetate/light petroleum 3.5:6.5; oil; yield 15% (130 mg); ^{1}H NMR 0.15 (s, 6H), 1.00 (s, 9H), 3.68 (s, 6H), 3.82 (s, 6H), 4.57 (d, J = 6.6, 1H), 5.55 (d, J = 6.6, 1H), 6.14 (s, 2H), 6.71 (s, 1H), 6.83 (s, 2H), 7.35−7.41 (m, 2H), 7.55−7.65 (m, 3H). Anal. ($C_{31}H_{39}NO_8SSi$) C, H, N, S.

3-Benzenesulfonyl-4-[3-(*tert*-butyl-dimethyl-silanyloxy)-4-methoxy-phenyl]-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazole (19a). Eluent ethyl acetate/light petroleum 3.5:6.5; oil; yield 15% (130 mg); ^{1}H NMR 0.16 (s, 6H), 0.99 (s, 9H), 3.79 (s, 3H), 3.82 (s, 6H), 3.85 (s, 3H), 4.54 (d, J = 6.6, 1H), 5.53 (d, J = 6.6, 1H), 6.39 (s, 2H), 6.55−6.67 (m, 3H), 7.42 (d, J = 8.0, 2H), 7.53−7.62 (m, 1H), 7.67−7.72 (m, 2H). Anal. ($C_{31}H_{39}NO_8SSi$) C, H, N, S.

General Procedure for Preparation of the Isoxazoline Derivatives 20a and 21a. A freshly prepared solution of Na metallic (23 mg, 1 mmol) solubilized in dry CH_3OH (15 mL) was treated with the appropriate isoxazoline derivative, **18a** or **19a** (613 mg, 1 mmol). The resulting solution was stirred at room temperature for 6 h, concentrated in vacuo, diluted with CH_2Cl_2 (30 mL), and worked up by washing the organic layer with water (20 mL), drying over anhydrous sodium sulfate, and concentrating at reduced pressure. The residue was chromatographed on silica gel.

2-Methoxy-5-[3-methoxy-4-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]-phenol (20a). Eluent ethyl acetate/light petroleum 2:3; oil; yield 65% (253 mg); ^{1}H NMR 3.86 (s, 9H), 3.91 (s, 6H), 4.14 (d, J = 9.1, 1H), 5.40 (d, J = 9.1, 1H), 5.68 (br, 1H), 6.43 (s, 2H), 6.84 (s, 2H), 6.95 (s, 1H). Anal. ($C_{20}H_{23}NO_7$) C, H, N.

2-Methoxy-5-[3-methoxy-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-4-yl]-phenol (21a). Eluent ethyl acetate/

light petroleum 2:3; oil; yield 69% (270 mg); ^{1}H NMR 3.84 (s, 9H), 3.91 (s, 6H), 4.19 (d, J = 9.2, 1H), 5.38 (d, J = 9.2, 1H), 5.69 (s, 1H), 6.54 (s, 2H), 6.69−6.74 (m, 1H), 6.84−6.88 (m, 2H). Anal. ($C_{20}H_{23}NO_7$) C, H, N.

General Procedure for Preparation of the Isoxazoline Derivatives 20b and 21b. To a solution of appropriate isoxazoline **18a** or **19a** (40 mg, 0.07 mmol) in THF (7 mL), methyllithium (1.6 M in Et_2O) (63 mL, 0.1 mmol) was added portionwise at −70 °C. After the mixture was stirred at −70 °C for 30 min, THF (4 mL) and water (0.5 mL) were added, and the mixture was allowed to warm to room temperature. The reaction mixture was added to water (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The organic phases were washed with brine, dried, and evaporated under reduced pressure. The residue was chromatographed on silica gel.

2-Methoxy-5-[3-methyl-4-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]-phenol (20b). Eluent ethyl acetate/light petroleum 4:6; oil; yield 65% (170 mg); ^{1}H NMR 1.86 (s, 3H), 3.84 (s, 9H), 3.92 (s, 3H), 4.15 (d, J = 7.8, 1H), 5.32 (d, J = 7.8, 1H), 5.69 (s, 1H), 6.50 (s, 2H), 6.68 (dd, J = 2.2, J = 8.2, 1H), 6.81 (d, J = 2.2, 1H), 6.86 (d, J = 8.2, 1H). Anal. ($C_{20}H_{23}NO_6$) C, H, N.

2-Methoxy-5-[3-methyl-3-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-4-yl]-phenol (21b). Eluent ethyl acetate/light petroleum 4:6; oil; yield 60% (160 mg); ^{1}H NMR 1.88 (s, 3H), 3.85 (s, 9H), 3.94 (s, 3H), 4.03 (d, J = 7.0, 1H), 5.32 (d, J = 7.0, 1H), 5.66 (br, 1H), 6.39 (s, 2H), 6.83 (s, 2H), 6.89 (s, 1H). Anal. ($C_{20}H_{23}NO_6$) C, H, N.

General Procedure for Removal of the TBDMS Protecting Group. A solution of the appropriate silyl ether **9a, 10a, 13a, 14a, 18a,** or **19a** (0.11 mmol) in dry tetrahydrofuran (6 mL) was treated with tetrabutylammonium fluoride (37.8 mg, 0.12 mmol). Stirring was continued for 20 min, and then ice (10 g) was added, followed by diethyl ether. The ethereal layer was washed with water (3 × 40 mL) and dried. Removal of solvent in vacuo was followed by filtration through a short column of silica gel.

2-Methoxy-5-[3-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]-phenol (9b). Eluent ethyl acetate/light petroleum 1:1; white solid, mp 141−144 °C; yield 90% (36 mg); ^{1}H NMR 3.30 (dd, J = 8.2, J = 16.2, 1H), 3.74 (dd, J = 10.9, J = 16.2, 1H), 3.89 (s, 12H), 5.65 (dd, J = 8.2, J = 10.9, 1H), 5.63 (br, 1H), 6.85−6.95 (m, 5H). Anal. ($C_{19}H_{21}NO_6$) C, H, N.

2-Methoxy-5-[3-(3,4,5-trimethoxy-phenyl)-isoxazol-5-yl]-phenol (10b). Eluent ethyl acetate/light petroleum 1:4; white solid, mp 183−184 °C; yield 99% (39 mg); ^{1}H NMR 3.91 (s, 3H), 3.95 (s, 9H), 5.80 (br, 1H), 5.82 (s, 1H), 6.94 (d, J = 8.9, 1H), 7.08 (s, 2H), 7.37−7.41 (m, 2H). Anal. ($C_{19}H_{19}NO_6$) C, H, N.

2-Methoxy-5-[5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-3-yl]-phenol (13b). Eluent diethyl ether/light petroleum 2:3; white solid, mp 129−133 °C; yield 90% (36 mg); ^{1}H NMR 3.30 (dd, J = 8.4, J = 16.4, 1H), 3.75 (dd, J = 10.4, J = 16.4, 1H), 3.86 (s, 3H), 3.90 (s, 6H), 3.96 (s, 3H), 5.65 (dd, J = 8.4, J = 10.4, 1H), 5.68 (br, 1H), 6.62 (s, 2H), 6.90 (d, J = 8.1, 1H), 7.22 (dd, J = 2.1, J = 8.1, 1H), 7.28 (d, J = 2.1, 1H). Anal. ($C_{19}H_{21}NO_6$) C, H, N.

2-Methoxy-5-[5-(3,4,5-trimethoxy-phenyl)-isoxazol-3-yl]-phenol (14b). Eluent ethyl acetate/light petroleum 1:4; white solid, mp 197−200 °C; yield 64% (25 mg); ^{1}H NMR 3.90 (s, 3H), 3.95 (s, 9H), 5.80 (br, 1H), 6.70 (s, 1H), 6.93 (d, J = 8.3, 1H), 7.04 (s, 2H), 7.36 (d, J = 1.9, 1H), 7.43 (dd, J = 1.9, J = 8.3, 1H). Anal. ($C_{19}H_{19}NO_6$) C, H, N.

5-[3-Benzenesulfonyl-4-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-5-yl]-2-methoxy-phenol (18b). Eluent ethyl acetate/light petroleum 3.5:6.5; oil; yield 99% (55 mg); ^{1}H NMR 3.67 (s, 6H), 3.82 (s, 3H), 3.91 (s, 3H), 4.58 (d, J = 6.5, 1H), 5.56 (d, J = 6.5, 1H), 5.62 (br, 1H), 6.15 (s, 2H), 6.79−6.84 (m, 3H), 7.37−7.43 (m, 2H), 7.55 (d, J = 8.1, 1H), 7.61−7.65 (m, 2H). Anal. ($C_{25}H_{25}NO_8S$) C, H, N, S.

5-[3-Benzenesulfonyl-5-(3,4,5-trimethoxy-phenyl)-4,5-dihydro-isoxazol-4-yl]-2-methoxy-phenol (19b). Eluent ethyl acetate/light petroleum 3.5:6.5; oil; yield 69% (38 mg); ^{1}H NMR 3.82 (s, 6H), 3.84 (s, 3H), 3.89 (s, 3H), 4.56 (d, J = 6.6, 1H), 5.55 (d, J = 6.6, 1H), 5.57 (br, 1H), 6.39 (s, 2H), 6.56−6.58 (m, 2H), 6.62 (d, J = 2.1, 1H), 6.71 (d, J = 8.1, 1H), 7.37−7.44 (m, 2H), 7.55−7.59 (m, 1H), 7.70 (dd, J = 2.1, J = 8.1, 1H). Anal. ($C_{25}H_{25}NO_8S$) C, H, N, S.

Suzuki Coupling: General Procedure for the Preparation of Biphenyls 24a−c and Terphenyl Derivatives (26a−c). To a suspension of $Pd[(PPh)_3]_4$ (10 mg, 0.08 mmol) in toluene, the appropriate aryl halide derivative **23** or **24a−c** (0.3 mmol) was added, and the mixture was stirred for 10 min at room temperature. To this suspension was added sequentially the arylboronic acid **22** or **25** (0.3 mmol) solubilized in a minimum volume of EtOH containing Na_2CO_3 (260 mg, 2.4 mmol), and the mixuture was refluxed for 3 h, cooled, and filtered. The filtrate was evaporated to dryness, and the residue was diluted with CH_2Cl_2 (30 mL) and washed with a saturated NaCl (15 mL) solution. Standard workup, followed by column chromatography, gave biphenyls **24a−c** and terphenyls **26a−c.**

3-Benzyloxy-2′-bromo-4-methoxy-biphenyl (24a). Eluent diethyl ether/light petroleum 0.2:9.8; oil; yield 90% (100 mg); ^{1}H NMR 3.93 (s, 3H), 5.17 (s, 2H), 6.95 (s, 2H), 7.25−7.39 (m, 9H), 7.62 (d, J = 8.0, 1H). Anal. ($C_{20}H_{17}BrO_2$) C, H.

3-Benzyloxy-3′-bromo-4-methoxy-biphenyl (24b). Eluent diethyl ether/light petroleum 1:4; oil; yield 60% (66 mg); ^{1}H NMR 3.91 (s, 3H), 5.19 (s, 2H), 6.95 (d, J = 8.4, 1H), 7.09 (s 2 H), 7.26 (d, J = 7.8, 1H), 7.38−7.46 (m, 7 H), 760 (s, 1H). Anal. ($C_{20}H_{17}BrO_2$) C, H.

3-Benzyloxy-4′-bromo-4-methoxy-biphenyl (24c). Eluent diethyl ether/light petroleum 1:4; oil; yield 63% (70 mg); ^{1}H NMR 3.92 (s, 3H), 5.20 (s, 2H), 6.97−7.03 (m, 1H), 7.08 (d, J = 1.9, 1H), 7.25−7.52 (m, 10H). Anal. ($C_{20}H_{17}BrO_2$) C, H.

3-Benzyloxy-4,3″,4″,5″-tetramethoxy-[1,1′,2′,1″]terphenyl (26a). Eluent diethyl ether/light petroleum 1:4; oil; yield 55% (75 mg); ^{1}H NMR 3.62 (s, 6H), 3.83 (s, 3H), 3.88 (s, 3H), 4.89 (s, 2H), 6.37 (s, 2H), 6.80−6.87 (m, 3H), 7.28−7.45 (m, 9H). Anal. ($C_{29}H_{28}O_5$) C, H.

3-Benzyloxy-4,3″,4″,5″-tetramethoxy-[1,1′,3′1″]terphenyl (26b). Eluent diethyl ether/light petroleum 1:4; oil; yield 96% (131 mg); ^{1}H NMR 3.93 (s, 3H), 3.97 (s, 9H), 5.13 (s, 2H), 6.83 (s, 2H), 7.02 (d, J = 8.0, 1H), 7.21−7.30 (m, 2H), 7.31−7.44 (m, 3H), 7.44−7.51 (m, 5H), 7.53−7.65 (m, 1H). Anal. ($C_{29}H_{28}O_5$) C, H.

3-Benzyloxy-4,3″,4″,5″-tetramethoxy-[1,1′,4′,1″]terphenyl (26c). Eluent diethyl ether/light petroleum 1:4; oil; yield 79% (108 mg); ^{1}H NMR 3.90 (s, 3H), 3.93 (s, 9H), 5.23 (s, 2H), 6.80 (s, 2H), 6.98 (d, J = 8, 1H), 7.18 (s, 2H), 7.22−7.45 (m, 9H). Anal. ($C_{29}H_{28}O_5$) C, H.

2-Bromo-3-(3,4,5-trimethoxy-phenyl)-pyridine (29). Compound **28** (300 mg, 1.1 mmol) was added to a suspension of $Pd[(PPh)_3]_4$ (10 mg, 0.08 mmol) in toluene, and the mixture was stirred for 10 min at room temperature. To this solution were added sequentially **25** (250 mg, 1.2 mmol) solubilized in a minimum volume of EtOH and Na_2CO_3 (260 mg, 2.4 mmol), and the mixuture was refluxed for 3h, cooled, and filtered. The filtrate was evaporated to dryness, and the residue was diluted with CH_2Cl_2 (30 mL) and washed with saturated NaCl solution (15 mL). Standard workup was followed by column chromatography .

Eluent ethyl acetate/light petroleum 1:2; oil; yield 53% (189 mg); ^{1}H NMR 3.88 (s, 6H), 3.91 (s, 3H), 6.62 (s, 2H), 7.30 (dd, J = 4.8, J = 7.6, 1H), 7.63 (dd, J = 1.9, J = 7.5, 1H), 8.36 (dd, J = 2.0, J = 4.8, 1H). Anal. ($C_{14}H_{14}$ $BrNO_3$) C, H, N.

3-Benzyloxy-4-methoxy-phenyl-boronic Acid (30). To a 1 M THF solution of the 5-iodo-2-methoxy-benzyl-phenol (**23**) (350 mg, 1 mmol) under argon at −78 °C was added dropwise a 1.6 M solution of *n*-BuLi (0.7 mL, 1.1 mmol) in hexanes over 15−30 min. The reaction mixture was stirred for an additional 15 min at −78 °C, treated with $B(OiPr)_3$ (0.4 mL, 3 mmol), and allowed to warm to room temperature over 12 h. The reaction was cooled to 0 °C and acidified to pH 6.5 with 5% HCl, then extracted with CH_2Cl_2 (2 × 20 mL). The organic layer was washed with saturated brine (20 mL) solution, dried (Na_2SO_4), and evaporated to dryness to yield a foamy material, which was used immediately in the cross-coupling reaction.

2-(3-Benzyloxy-4-methoxy-phenyl)-3-(3,4,5-trimethoxy-phenyl)-pyridine (31a). To a suspension of $Pd[(PPh)_3]_4$ (10 mg, 0.08 mmol) in toluene was added **29** (190 mg, 0.6 mmol), and the mixture was stirred for 10 min at room temperature. To the solution were added sequentially **30** (160 mg, 0.7 mmol) solubilized in a minimum volume of EtOH and Na_2CO_3 (163 mg, 1.5 mmol), and the mixuture was refluxed for 3 h, then cooled, and filtered. The filtrate was evaporated to dryness, and the residue was diluted with CH_2Cl_2 (30 mL) and washed with a saturated NaCl (15 mL) solution. The organic layer was dried and evaporated under reduced pressure, and the residue purified by column chromatography to yield **31a**.

Eluent ethyl acentate/light petroleum 1:1; oil; yield 15% (44 mg); ^{1}H NMR 3.67 (s, 6H), 3.85 (s, 3H), 3.87 (s, 3H), 4.93 (s, 2H), 6.36 (s, 2H), 6.80 (d, J = 8,3, 1H), 6.97−7.04 (m, 2H), 7.24−7.38 (m, 6H), 7.70 (dd, J = 1.5, J = 7.5, 1H), 8.64 (dd, J = 1.6, J = 4.8, 1H). Anal. ($C_{28}H_{27}NO_5$) C, H, N.

General Procedure for Removal of the Benzyl-Protecting Group (27a−c, 31b). Pd−C (10 mg) was added carefully under Ar to a solution of the appropriate terphenyl derivative (**26a−c**, **31a**) (0.2 mmol) in anhydrous THF (5−6 mL). The resulting mixture was hydrogenated at 45−50 psi on a Parr apparatus for 24 h. The catalyst was removed by filtration using a pad of Celite. Evaporation of the filtrate under reduced pressure, followed by purification on a silica gel column, gave compounds **27a−c** and **31b**.

4,3″,4″,5″-Tetramethoxy-[1,1′,2′,1″]terphenyl-3-ol (27a). Eluent diethyl ether/light petroleum 1:4; mp 140−142 °C; yield 100% (73 mg); ^{1}H NMR 3.65 (s, 6H), 3.83−3.85 (m, 6H), 5.52 (s, 1H), 6.37 (s, 2 H), 6.55 (dd,, J = 2.1, J = 8.2, 1H), 6.70 (d, J = 8.2, 1H), 6.80 (d, J = 2.1, 1H), 7.39−7.42 (m, 4H). Anal. ($C_{22}H_{22}O_5$) C, H.

4,3″,4″,5″-Tetramethoxy-[1,1′,3′,1″]terphenyl-3-ol (27b). Eluent diethyl ether/light petroleum 1:4; mp 43−45 °C; yield 100% (73 mg); ^{1}H NMR 3.89 (s, 3H), 3.92 (s, 9H), 5.71 (s, 1H), 6.81 (s, 2H), 6.93 (d, J = 8.2, 1H), 7.13 (dd, J = 1.7, J = 8.2, 1H), 7.24 (d, J = 1.7, 1H), 7.49−7.50 (m, 3H), 7.69 (s, 1H). Anal. ($C_{22}H_{22}O_5$) C, H.

4,3″,4″,5″-Tetramethoxy-[1,1′,4′,1″]terphenyl-3-ol (27c). Eluent diethyl ether/light petroleum 1:4; mp 97−100 °C; yield 96% (70 mg); ^{1}H NMR 3.90 (s, 3H), 3.94 (s, 9H), 5.74 (br, 1H), 6.81 (s, 2H), 6.93 (d, J = 8.5, 1H), 7.14 (dd, J = 2.2, J = 8.5, 1H), 7.24 (d, J = 2.2, 1H), 7.61 (s, 4H). Anal. ($C_{22}H_{22}O_5$) C, H.

2-Methoxy-5-[3-(3,4,5-trimethoxy-phenyl)-pyridin-2-yl]-phenol (31b). Eluent ethyl acetate/light petroleum 2:3; oil, yield 65% (48 mg); ^{1}H NMR 3.69 (s, 6H), 3.86 (s, 6H), 5.62 (br, 1H), 6.40 (s, 2H), 6.72 (d, J = 8.2, 1H), 6.82 (dd, J = 2.0, J = 8.2, 1H), 7.04 (d, J = 2.0, 1H), 7.25−7.29 (m, 1H), 7.72 (dd, J = 1.7, J = 7.7, 1H), 8.64 (dd, J = 1.5, J = 4.6, 1H). Anal. ($C_{21}H_{21}NO_5$) C, H, N.

5-Chloro-3-[-4-methoxy-3-(2-methoxy-ethoxymethoxy)-phenyl]-2-(3,4,5-trimethoxy-phenyl) pyridine (34a). To a suspension of the ketone **32** (200 mg, 0,6 mmol) in dry THF (5−6 mL) at 0 °C was added dropwise a 20% solution of *t*-BuOK (70 mg, 0.63 mmol) solubilized in anhydrous THF. The slurry was stirred at room temperature for 45 min, and the vinamidinium hexafluorophosphate salt **33** (280 mg, 0.9 mmol) was added in one portion. The resulting mixture was stirred at 45 °C for 3 h. DABCO (70 mg, 0.6 mmol) and ammonium acetate (100 mg, 1.2 mmol) were added in one portion. The resulting dark solution was heated at reflux for 6 h and concentrated under reduced pressure. The residue was directly purified by chromatography on silica gel.

Eluent ethyl acetate/petroleum eather 3:2; oil; yield 35% (103 mg); ^{1}H NMR 3.35 (s, 3H), 3.47−3.50 (m, 2H), 3.65 (s, 6H), 3.75−3.90 (m, 8H), 5.16 (s, 2H), 6.58 (s, 2H), 6.83 (s, 2H), 7.07 (s, 1H), 7.69 (d, J = 2.3, 1H), 8.58 (d, J = 2.4, 1H). Anal. ($C_{25}H_{28}ClNO_7$) C, H, N.

3-[4-Methoxy-3-(2-methoxy-ethoxymethoxy)-phenyl]-2-(3,4,5-trimethoxy-phenyl)-pyridine (35a). The chloro derivative **34a** (28 mg, 0.057 mmol) was suspended in a mixture of ethanol (1 mL) and 1 N NaOH (1 mL), and the reaction mixture was shaken with hydrogen in the presence of 10% palladium on calcium carbonate. The resulting mixture was stirred at room temperature for 12 h. Ethanol was evaporated in vacuo, and the residue was extracted with diethyl ether (3 × 10 mL). The organic extracts were washed with brine (10 mL) and dried, and the organic solvents removed by evaporation under reduced pressure. The residue was chromatographed on silica gel.

Eluent diethyl ether/light petroleum 3:2; oil; yield 90% (23 mg); ^{1}H NMR 3.34 (s, 3H), 3.45−3.50 (m, 2H), 3.66 (s, 6H), 3.75−3.79 (m, 2H), 3.82 (s, 3H), 3.87 (s, 3H), 5.16 (s, 2H), 6.63 (s, 2H), 6.84 (s, 2H), 7.07 (s, 1H), 7.30 (dd, J = 4.9, J = 8.0, 1H), 7.72 (dd, J = 1.5, J = 7.7, 1 H), 8.65 (dd, J = 1.8, J = 4.7, 1H). Anal. ($C_{25}H_{29}NO_7$) C, H, N.

General Procedure for Removal of the MEM Protecting Group (34b, 35b). A solution of the appropriate derivative **34a** or **35a** (16 mg, 0.033 mmol) in CH_2Cl_2 (3 mL) was treated with 2−3 drops of trifluoroacetic acid at 23 °C for 4−5 h. The resulting mixture was diluted with CH_2Cl_2 (15 mL), washed with $NaHCO_3$ (10 mL), and dried, and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel.

5-[5-Chloro-2-(3,4,5-trimethoxy-phenyl)-pyridin-3-yl]-2-methoxy-phenol (34b). Eluent ethyl acetate/petroleum ether 3:2; mp 163−165 °C; yield 95% (13 mg); ^{1}H NMR 3.66 (s, 6H), 3.83 (s, 3H), 3.89 (s, 3H), 5.69 (br, 1H), 6.60−6.64 (m, 3H), 6.77 (d, J = 8.3, 1H), 6.82 (d, J = 2.1, 1H), 7.68 (d, J = 2.6, 1H), 8.58 (d, J = 2.3, 1H). Anal. ($C_{21}H_{20}ClNO_5$) C, H, Cl, N.

2-Methoxy-5-[2-(3,4,5-trimethoxy-phenyl)-pyridin-3-yl]-phenol (35b). Eluent ethyl acetate/petroleum ether 3:2; mp 188−190; yield 97% (12 mg); ^{1}H NMR 3.66 (s, 6H), 3.83 (s, 3H), 3.88 (s, 3H), 5.77 (br, 1H), 6.59−6.63 (m, 3H), 6.76 (d, J = 8.2, 1H), 6.83 (d, J = 2.0, 1H), 7.29 (dd, J = 4.1, J = 7.4, 1H), 7.68 (dd, J = 1.8, J = 7.6, 1H), 8.63 (d, J = 1.8, J = 4.7, 1H). Anal. ($C_{21}H_{21}NO_5$) C, H, N.

Biology. Cell Culture. Neoplastic cells (HL60, HL60R, K562, and K562ADR) were grown in continuous culture RPMI 1640 (Gibco Grand Island, NY) containing 10% FCS (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 2mM L-glutamine (Sigma Chemical Co., St Louis, MO) in a 5% CO_2 atmosphere at 37°C.

Chemicals. The caspase inhibitor *Z*-LEHD-fmk (*Z*-Leu-Glu(Ome)-His-Asp(Ome)-fmk) was purchased from Alexis Biochemicals (Laufelfingen, Switzerland). The anti-Fas Ab ZB4 was purchased from Upstate Biotechnology (Lake Placid, NY). All other reagents were analytical grade.

Drug Preparation. Each combretastatin derivative compound was dissolved in dimethyl sulfoxide (DMSO) as a 20 mM stock solution, which was stored at −20 °C and protected from light. In experiments with cells, DMSO never exceeded 0.5%, and this amount did not interfere with cell growth.

Cytotoxicity Assays. To evaluate the number of live and dead neoplastic cells, the cells were stained with trypan blue and counted on a hemocytometer. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, U.K.) in 1 mL of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was determined and expressed as percent of control proliferation.

To evaluate the cytotoxic effects on hemopoietic progenitor cells, mononucleated cells obtained from bone marrow aspirates of five normal volunteers were treated with different concentrations of each drug. Briefly, 3−5 mL of bone marrow was diluted in RPMI 1640, layered over Ficoll−Hypaque gradients (density, 1.077), and centrifuged at 400*g* for 30 min, and the interface mononuclear cells were collected. The interface cells were washed 3 times in PBS, counted, and resuspended at a concentration of 1×10^5 cells/mL in MEM containing 0.9% methylcellulose, 30% FCS, 10^{-5} M 2-mercaptoethanol, 5% PHA-LCM, and 1 IU human erythropoietin in 15 mm plastic dishes. After 14 days of culture at 37 °C in an environment of 5% CO_2 and 100% humidity, the number of CFU-GEMM, CFU-GM, and CFU-E was determined.

Flow Cytometric Analysis of Cell Cycle and Apoptosis. Cells were washed once in ice-cold PBS and resuspended at 1

$\times 10^6$/mL in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μg/mL in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After a 30 min incubation, fluorescence was analyzed as single-parameter frequency histograms using a FACScan flow cytometer (Becton Dickinson). Apoptosis was determined by evaluating the percentage of hypodiploid nuclei accumulated in the sub-G0G1 peak after labeling with propidium iodide.

Morphological Evaluation of Apoptosis and Necrosis. Drug-induced apoptosis and necrosis were determined morphologically after labeling with acridine orange and ethidium bromide. The cells (2×10^5) were centrifuged (300*g*), and the pellet was resuspended in 25 μL of the dye mixture. A 10 μL aliquot of the mixture was examined in oil with a 100× objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labeling with ethidium bromide.

Cytofluorimetric Analysis of $\Delta\Psi$. The cationic lipophilic fluorochrome $DiOC_6$ (Sigma) was used to measure the $\Delta\Psi$. Cells were adjusted to a density of 0.5×10^6/mL and incubated at 37 °C for 30 min in the presence of $DiOC_6$ (40 nM), followed by immediate analysis of fluorochrome incorporation in a Becton Dickinson cytofluorometer. $DiOC_6$ fluorescence was recorded in FL1.

Inhibition of Fas and Caspase 9. Cells (2×10^5) were exposed to the Fas-blocking MoAb ZB4 (Alexis) or the caspase 9 inhibitor *Z*-LEHD (Alexis) for 2 h, after which the compounds being evaluated were added. The percentage of apoptosis was determined after 48 h by morphological assay after staining with ethidium bromide and acridine orange.

Tubulin Assays. Bovine brain tubulin was purified as described previously.[42] Assays for the evaluation of tubulin assembly[40] and for the binding of [^{3}H]colchicine to tubulin[40] were performed as described previously. The tubulin assembly assay included a preincubation of potential inhibitor, at varying concentrations, with 10 μM tubulin prior to addition of the GTP required for the assembly reaction. After GTP addition, reactions were followed turbidimetrically for 20 min at 30 °C, and the IC_{50} value was defined as the compound concentration, obtained by interpolation between the data points, that inhibited the extent of assembly by 50%. In the colchicine binding assay, reaction mixtures contained 1.0 μM tubulin, 5.0 μM [^{3}H]colchicine, and potential inhibitor at 5 or 50 μM. Reaction mixtures were incubated for 10 min at 37 °C, a time point at which the colchicine binding reaction is about 50% complete.

Computational Methods. All modeling work was carried out on an Octane R12000 Silicon Graphics workstation. The three-dimensional models of the molecules were built starting from a crystal structure core and by assembling fragments from the SYBYL 6.9 software package standard library. (The Sybyl program (Version 6.9) was supplied by Tripos Associates, 11699 South Hanley Road, Suite 303, St. Louis, MO 63144.) Energy minimizations were performed with the Tripos force field without including electrostatic terms, using the method of Powell. Semiempirical molecular orbital calculations were done using the AM1 Hamiltonian[43] (module MOPAC implemented in SYBYL). A conformational analisys was carried out on six rotatable bonds, using the Monte Carlo options implemented in MacroModel 8.1.[44] The energy cutoff was set 100 kcal/mol above the estimated total energy of the molecule, and the maximum number of attempts was 8000. The lowest-energy conformers were reoptimized with the semiempirical quantum mechanics calculations AM1. Resulting geometries were superimposed on the oxygen atoms and phenyl rings of CA-4.

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Supporting Information Available: Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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