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Synthesis, In Vitro, and In Vivo Evaluation of Phosphate Ester Derivatives of Combretastatin A-4

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Abstract—Combretastatin A-4 disodiumphosphate (CA4P), a prodrug formulation of the natural product combretastatin A-4 (CA4), is currently in clinical investigation for the treatment of cancer. In vivo, CA4P is rapidly enzymatically converted to CA4, a potent inhibitor of tubulin polymerization (IC_{50} = 1–2 μM), and rapidly causes bloodflow shutdown in tumor tissues. A variety of alkyl and aryl di- and triesters of CA4P have been synthesized and evaluated as potential CA4 prodrugs and/or stable CA4P analogues.

An emerging area of cancer chemotherapy research is focused upon the development of agents, both small molecules as well as biologics, that selectively destroy tumor vasculature.[1] These compounds are commonly referred to as vascular targeting agents (VTAs). Several small molecules that show anti-tumor effects based on this novel mechanism have been identified and are in various stages of clinical development.[2] These compounds are prodrug formulations of tubulin-binding anilines or phenols that, in prodrug form, do not bind to tubulin. These drugs function biologically by selectively shutting down blood flow to neoplastic cells while leaving the blood supply to healthy cells intact.[3] The selectivity of VTAs for the microvessels of tumors may reflect, in part, variability in the cytoskeletal make-up of rapidly proliferating endothelial cells inherent to microvessels feeding tumor cells versus the normally proliferating endothelial cells of microvessels serving healthy cells.[4] Combretastatin A-4 prodrug (CA4P) is a remarkable VTA that is a simple phosphate monoester of the phenolic natural product combretastatin A-4 (CA4), which is isolated from the Combretum caffrum willow tree in South Africa.[5]

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Preclinical and clinical studies have revealed an extremely short circulation half-life for the CA4P phosphate prodrug.[6] Accordingly, the synthesis of longer-lived CA4 prodrugs are attractive, as they could potentially shed light on the role of pharmacokinetics in CA4P's VTA activity. Additionally, although CA4P is widely recognized as a prodrug of CA4, it is possible that CA4P itself might exhibit some biological activity, perhaps against a different target than CA4. In order to address these questions we have synthesized and characterized a variety of phosphate esters of CA4. The syntheses and activities of these compounds are presented herein.

Combretastatin A-4, which was the common starting material for the synthesis of each of the derivatives reported herein, was synthesized following the method reported by Pettit et al.[7] The CA4 dialkylphosphate triesters **1**–**3** (Scheme 1) were then readily available via the carbon tetrachloride-mediated oxidative phosphorylation of CA4 with the appropriate dialkyl H-phosphonate.[8,9] These reactions afforded the desired products in good yield, with minimal isomerization of the somewhat labile *cis*-stilbene bond.

A second set of CA4 derivatives synthesized were the anionic alkyl and aryl phosphate diesters **4**–**8** (Scheme 2). Once again starting with CA4, these compounds

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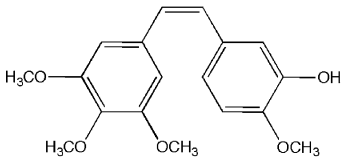
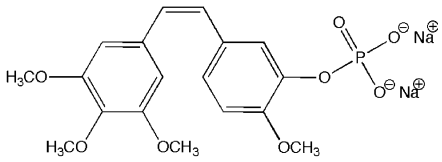
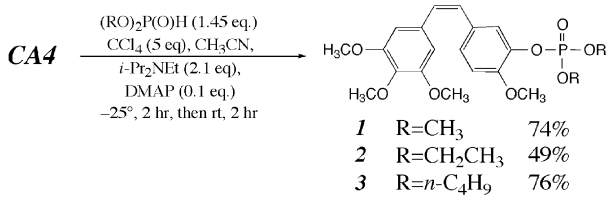


CA4



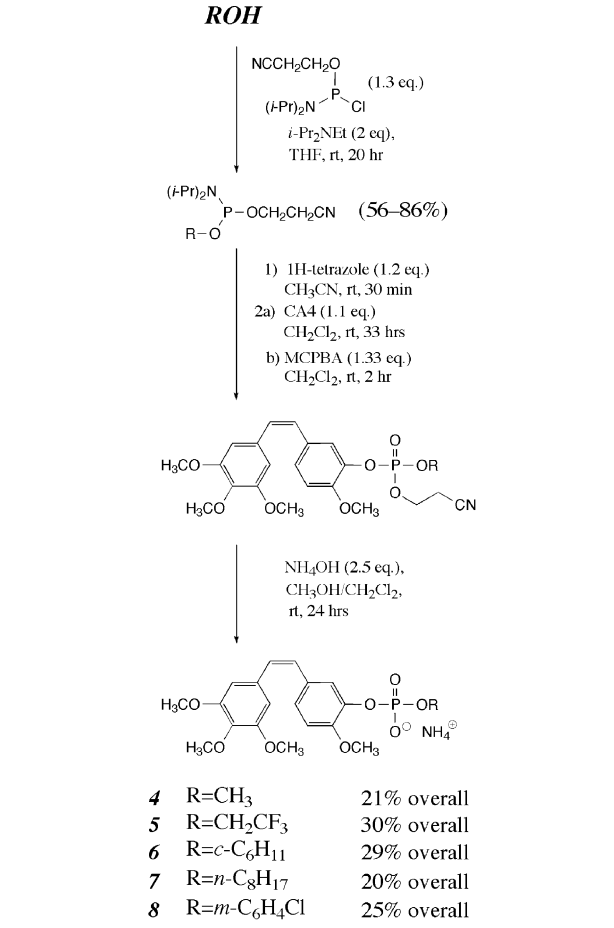
CA4P (disodium salt)



Scheme 1. Synthesis of phosphate triesters **1–3**.

were accessible in reasonable yield using standard phosphoramidite coupling chemistry.[10]

Each target alcohol was initially converted into its 2-cyanoethyl-*N*,*N*-diisopropylamino phosphoramidite. The phosphoramidites were then coupled with CA4 using 1H-tetrazole as an activator, followed by MCPBA oxidation in situ to produce the 2-cyanoethyl protected phosphate triesters. In the final step, alcoholic ammonium hydroxide was used to remove the 2-cyanoethyl protecting groups, affording the phosphate diesters **4–8** in reasonable overall yields (as calculated from the starting alcohols; 35–45% overall based on CA4 consumption).



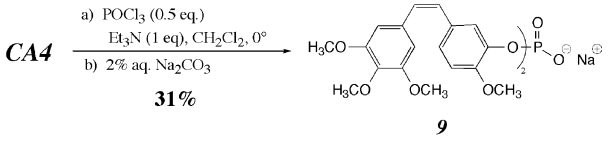
Scheme 2. Synthesis of phosphate diesters **4–8**.

A special phosphate diester, the phosphate-linked CA4 dimer **9**, was also synthesized (Scheme 3). This compound was readily available via the condensation of two equivalents of CA4 with $POCl_3$, followed by hydrolysis of the resulting acid chloride.

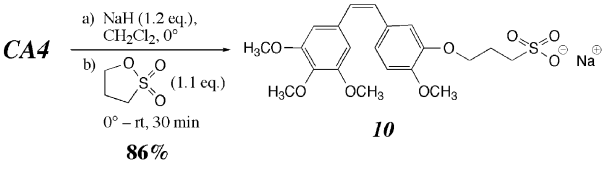
One additional compound prepared for comparison to the phosphate esters was the ether-linked alkyl sulfonate salt **10** (Scheme 4). This compound was isolated in good yield from the ring opening of 1,3-propane sultone by a phenoxide anion derived from CA4.

The biological activity of each of the compounds synthesized was then assayed. A standard in vitro MTT assay was used to determine the relative cytotoxicity of the compounds (Table 1).[11,12] Two different periods of drug exposure—1 h and 5 days–were examined to determine the rapidity of the cytotoxic activity of the compounds.

In comparison to CA4 or CA4P the analogues described in this paper exhibited unremarkable cytotoxicities,[13] although the phosphate-linked dimer did exhibit relatively good activity ($IC_{50}=0.2$ μM) against a colon cancer cell line (HT-29) that is resistant to other combretastatins. Compound **10**, a non-hydrolyzable anionic ether derivative of CA4, was completely inactive in the cytotoxicity assay. This is not altogether surprising as it is known[14] that, unlike CA4, this compound does not interfere with tubulin polymerization.



Scheme 3. Synthesis of CA4 phosphate dimer **9**.



Scheme 4. Synthesis of CA4 trimethylenesulfonate **10**.

Table 1.

Compd	IC_{50} (1 hr; μM)	IC_{50} (5 day; μM)	Vascular shutdown[a] (100 mg/kg)
CA4	1.05	0.004	71
CA4P	0.8	0.002	88
1	35	0.13	20
2	nt	nt	nt
3	8	0.26	0
4	38	0.07	0
5	10	0.06	0
6	>25	3.0	0
7	25	0.07	89
8	>25	3.0	90
9	>25	0.51	0
10	>25	>25	10

[a]Vascular shutdown is expressed as vessel density decrease in percent of the control.

Our primary interest, however, lies in developing new VTAs that destroy tumors indirectly, by cutting off tumor blood flow rather than by being inherently cytotoxic. Accordingly, the ability of these CA4P analogues to shut down blood flow in an endotheliaoma tumor model was also examined.[15] This in vivo assay quickly provides a direct indication of the vascular targeting ability of a given compound, and additional value is inherent in the in vivo nature of the assay. We were therefore encouraged to discover that the diester derivatives **7** and **8** (at 100 mg/kg) were able to shut down tumor vasculature at levels comparable to CA4P. Figure 1 is an example from a vascular shutdown experiment that demonstrates the dramatic difference in vascular volume found in treated and control tumor slices. In this experiment fluorescence is found in actively vascularized tissue; the lack of fluorescence in the treated tissue demonstrates the decrease in functional vasculature.[15]

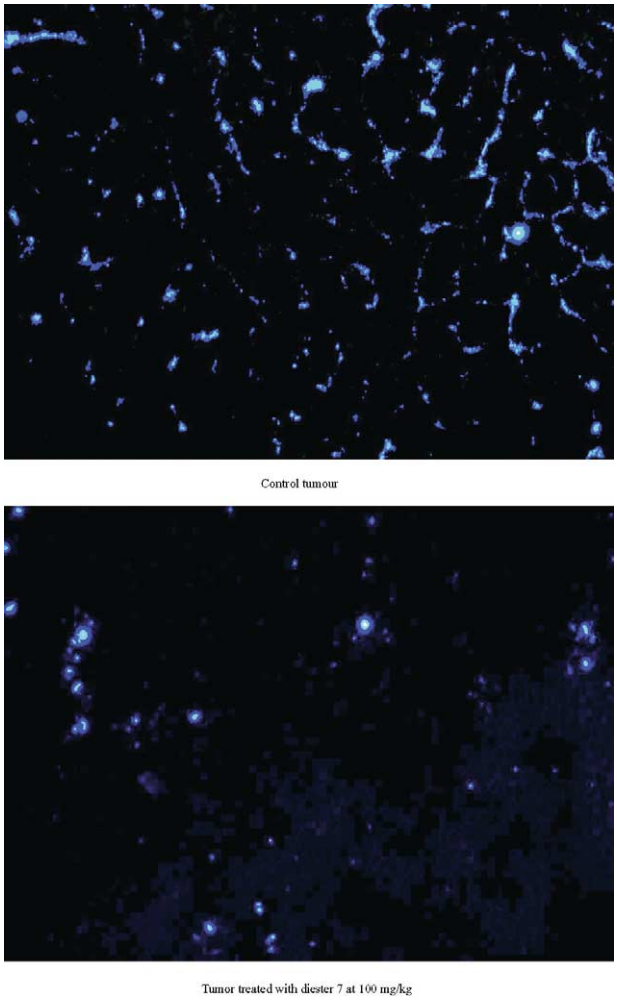


Figure 1. Vascular shutdown by diester **7** at 100 mg/kg.

Due to the activity compound **7** exhibited in this assay, along with its limited cytotoxicity, it was subsequently tested at 10 mg/kg, where it was found to cause a 46% shutdown in tumor vasculature. This is significantly greater activity than is found for CA4P at the same dose (10% decrease in vascular volume at 10 mg/kg).

In conclusion, we have synthesized nine novel ester derivatives of the phosphate prodrug CA4P as well as a non-hydrolyzable anionic CA4 derivative. Two of the phosphate diesters (**7** and **8**) accomplish vascular shutdown that is equivalent or greater than CA4P, while at the same time exhibiting decreased in vitro cytotoxicity. Additional studies exploring the vascular targeting potential of CA4P diesters are currently underway, as well as stability studies aimed at clarifying the source of the desired vascular activity.

Acknowledgements

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12. Mouse heart endotheliaoma cells (MHEC5-T) growing in the exponential phase were plated at a final concentration of 1×10^5 cells/mL. Cell lines were exposed to serial dilutions of the drugs. Cell viability was determined after incubation for 1 h or 5 days at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diplenyl-tetrazolium bromide (MTT) method. IC_{50} was defined as the drug concentration that reduced cell viability 50% in comparison with control.
13. Relative cytoxicities were examined in several cell lines in addition to the MHEC5-T endotheliaoma model (data not shown).
14. Unpublished data, M. L. Trawick, Baylor University.
15. Experiments were performed in the MHEC5-T tumor model established by sc injection of 0.5×10^6 cultured MHEC5-T cells into the right flank of SCID mice. When the transplanted tumor reached the size of 300 mm^3 (a size without development of necrosis), mice received ip doses of either 10 mg/kg or 100 mg/kg of the various compounds. Twenty-four h later 0.25 mL of diluted FluoSphere beads (1:6 in physiological saline) was injected in the tail vein. The animals were sacrificed after 3 min and the tumor was removed. Cryosections (8 μm thick) were directly examined under a fluorescent microscope. Blood vessels were indicated by blue fluorescence from injected microbeads. For quantification, three sections from three tumors treated in each group were examined and in each section, more than 70% of the area was automatically recorded with a microscopic digital camera at ×100 magnification. The computer program Stage Pro (Media Cybernetics, MD) was used to control the picture recording. Image analysis was performed with Image Plus software (Media Cybernetics, MD). The results were expressed as vessel density decrease in percent of the control.