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Examination of the 1,4-disubstituted azetidinone ring system as a template for combretastatin A-4 conformationally restricted analogue design

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Abstract—A series of novel 1,4-diaryl-2-azetidinones was prepared by stereospecific Staudinger reaction as conformationally restricted analogues of combretastatin A-4 because molecular modeling studies suggested close geometric similarities. They were evaluated for cytotoxicity against a number of human tumor and normal cell lines. Strong potencies were observed, with the best compounds exhibiting IC_{50}'s of 25–74 nM against human neuroblastoma IMR 32 cell growth and a variety of other cell lines. Compounds inhibited tubulin polymerization with potencies commensurate with their cytotoxic activity and a more soluble anilino-containing analogue was very effective in inhibiting the growth of AR42J rat pancreatic tumors transplanted into in nude mice. Further studies on this interesting group of compounds as anti-cancer agents appear warranted.

Combretastatin A-4 (CA4) is a potent naturally occurring anti-mitotic agent and functions by inhibiting cellular tubulin polymerization by binding to the colchicine site.[1] It possesses strong cytotoxicity against a variety of human cancer cells and is not subject to the multiple drug resistance phenomenon. Thus, given its favorable biological profile, many structure–activity relationships have been reported for CA4 itself in an effort to improve upon the natural substance.[2–5] A general synthesis of CA4 and analogues was developed by Pettit and co-workers,[6–8] which utilized the Wittig reaction to produce both the *Z*- and *E*-isomers in an average ratio of about 1:1.5 so that a separation step was required to isolate pure *cis*-CA4. Also, these studies revealed that the *Z*-geometry of two aromatic rings linked by the olefinic group was an essential feature for biological activity. Unfortunately, *Z*-CA4 analogues are prone to isomerization to the more stable but inactive *E*-form during storage and administration. CA4 is also poorly soluble in aqueous systems possibly leading to enhanced systemic toxicity after injection. In an effort to avoid these problems, several attempts have successfully been made to replace the double bond in the CA4 structure with a number of mostly five-membered ring heterocyclic moieties.[9–12] A recent report by Banik et al.[13] suggested to us that a four-membered ring β-lactam (or azetidinone) structure might be an interesting substrate for attaching substituted aromatic ring systems normally associated with CA4 and herein we describe the synthesis and evaluation of a number of *cis*-β-lactam analogues of CA4.

The general synthetic strategy employed for the preparation of the new β-lactam derivatives was based on the Staudinger reaction as previously reported for 1,4-diarylazetidinones[13] and it is shown in Figure 2. This reaction scheme involves the cycloaddition of an acid chloride to an imine in the presence of a tertiary base. Numerous investigations of the stereochemistry of the resulting β-lactam have revealed that dropwise addition of the acyl chloride to a solution of the imine and tertiary amine gave the *cis*-cycloadduct exclusively,[14,15] although the reaction products are still present as a equimolar mixture of (*R*,*S*) and (*S*,*R*) enantiomers. Thus, for an active substance in Table 1, true potency could be twice as high, presuming that only one isomer would be active. In Figure 2, a 1.5 molar excess of acyl chloride in methylene chloride was added dropwise to a solution of 1 mmol of imine and 1.5 mmol of DIPEA in 20 mL methylene chloride. Stirring was continued for 2–18 h until the reaction was complete by TLC monitoring. The reaction mixture was then washed with

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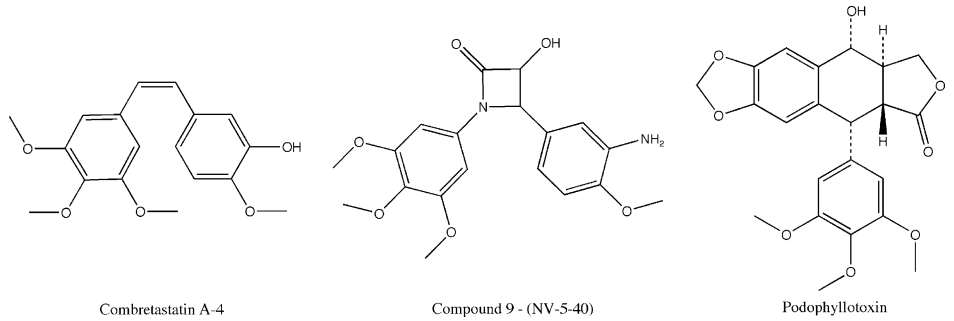


Figure 1. Structures of CA4, podophyllotoxin, and new 1,4-diaryl-2-azetidinones.

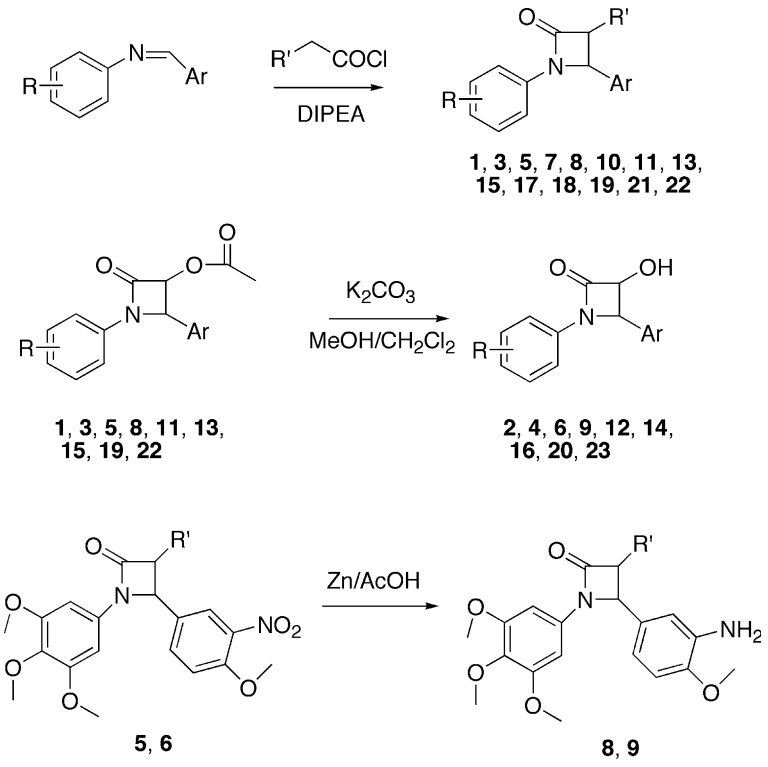
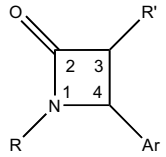


Figure 2. General synthetic strategy for the preparation of 1,4-diaryl-2-azetidinones.

saturated $NaHCO_3$, 1 N HCl, and brine, dried over Na_2SO_4, and concentrated under reduced pressure. Products were purified either by crystallization (ethyl acetate–ether) or chromatographically (eluent–ether) to give the target β-lactams in 75–90% yield. Structural integrity was demonstrated by proton NMR and elemental analysis. The acetoxy group in **1**, **3**, **5**, **8**, **11**, **13**, **15**, **19**, and **22** was hydrolyzed to the hydroxy group by treatment with potassium carbonate in a mixture of methanol/methylene chloride (1:3) to give **2**, **4**, **6**, **9**, **12**, **14**, **16**, **20**, and **23**, respectively. The nitro group in compounds **6** and **15** was reduced in excellent yield to an amino group using zinc dust in glacial acetic acid to afford **9** and **17**.

All synthesized compounds **1**–**23** were tested for cytotoxic activity against human neuroblastoma cells (IMR 32), which respond well to CA4 itself,[16] using a standard MTT assay kit (Promega Corporation, Madison, WI). Compounds, **5**, **6**, **7**, **8**, **9**, **19**, and **20** were additionally tested against a panel of 12 human and rat tumor cells and normal CHO cells (Table 2). All data are shown in Table 1 and it is noteworthy that all of these compounds retained strong cytotoxic activity against most cancer cells and concentrations necessary for activity were about 5–10 times lower than for normal CHO cells. Since 3,4,5-trimethoxy substituents in one of the aromatic rings have been demonstrated to be essential for the biological activity of CA4s and some of its heterocyclic ring analogues,[11,12] we opted to maintain this substitution strategy in this new β-lactam series. While the stilbene-like structure of CA4 is geometrically very symmetrical, structures of 1,4-diaryl-2-azetidinones contain two chemically nonequivalent positions for

Table 1. Cytotoxic activity of β-lactam analogues of CA4



	R	Ar	R′	Cytotoxicity, IC_{50}, nM			
				IMR32	NCI-H69	MCF-7	CHO-K
1	3,4,5-Trimethoxyphenyl	4-Methoxyphenyl	$OCOCH_3$	251			
2			OH	33	47	62	509
3	4-Methoxyphenyl	3,4,5-Trimethoxyphenyl	$OCOCH_3$	>1000			
4			OH	>1000			
5	3,4,5-Trimethoxyphenyl	4-Methoxy-3-nitrophenyl	$OCOCH_3$	123			
6			OH	38	74	97	416
7			OMe	>1000			
8		3-Amino-4-methoxyphenyl	$OCOCH_3$	73	41	200	509
9			OH	37	25	81	411
10			OMe	>1000			
11		6-Methoxy-pyridin-3-yl	$OCOCH_3$	>1000			
12			OH	>1000			
13		6-Methoxy-naphthalen-2-yl	$OCOCH_3$	>1000			
14			OH	89			
15		5-Nitro-furan-2-yl	$OCOCH_3$	811			
16			OH	>1000			
17		5-Amino-furan-2-yl	$OCOCH_3$	>1000			
18		4-Dimethylaminophenyl	$OCOCH_3$	520			
19		Benzo[1,3]dioxol-5-yl	$OCOCH_3$	910			
20			OH	473			
21			OMe	>1000			
22	Benzo[1,3]dioxol-5-yl	3,4,5-Trimethoxyphenyl	$OCOCH_3$	>1000			
23			OH	>1000			

Table 2.

Cell code	Tumor types	IC_{50} values (nM)						
		5	**8**	**6**	**9**	**7**	**20**	**22**
CHO-K1	Chinese hamster ovary	473	461.6	235.7	543.5	>10,000	891.3	>10,000
ACHN	Human renal adenocarcinoma	595.4	149.9	29.19	91.22	>10,000	2120	>10,000
DU-145	Prostate carcinoma	479.2	430.2	91.87	379.7	>10,000	3159	>10,000
IMR32	Human neuroblastoma	123.3	73.09	41.4	38.3	>10,000	444.7	>10,000
NCI-H460	Human lung carcinoma	392.8	449.3	425.4	184.2	>10,000	1439	>10,000
MCF-7	Human breast carcinoma	105.3	70.46	37.09	55.02	>10,000	544.3	>10,000
HCT-116	Human colon carcinoma	526.3	367.9	68.75	139.6	>10,000	950.3	>10,000
T98G	Human glioblastoma	143.7	145	49.84	109.1	>5000	799.7	>10,000
Hela	Human cervix adenocarcinoma	201.8	261.7	39.98	63.86	>10,000	546.2	>10,000
CFPAC-1	Human pancreatic cancer	196.8	204.3	42.15	50.22	>10,000	885.3	>10,000
DLD-1	Human colon adenocarcinoma	326.3	393.7	59.07	130	>10,000	904.7	>10,000
AR42J	Rat pancreatic tumor	581.70	219.30	211.50	62.07	>10,000	1481.0	>10,000
MOLT-4	Human leukemia	350.20	371.60	74.02	71.73	>10,000	397.8	>10,000

aromatic ring placement. In order to evaluate whether these positions are equivalent (or not) for cytotoxic activity, we designed several compound 'pairs' (compounds **1** and **3**, **2** and **4**, **19** and **22**, and **20** and **23** in Table 1) in which the aromatic rings were reversed. Comparing the biological activities of these pairs, it can be readily seen that the 3,4,5-trimethoxyphenyl ring preferably has to be in position 1 in which the azetidinone carbonyl group is directed toward 3,4,5-trimethoxyphenyl ring. Interestingly, another CA4-like compound, podophyllotoxin (Fig. 1), also contains a carbonyl group directed toward its 3,4,5-trimethoxyphenyl ring and a podophyllotoxin analogue lacking such a carbonyl group possessed reduced cytotoxic activity.[17]

While retaining the 3,4,5-trimethoxyphenyl moiety in position 1, we next examined several variations of the second aromatic group in position 4 as well as substituents in position 3 of the azetidinone ring. The latter substituents had unpredictable effects on the activity. Replacement of acetoxy group with a hydroxy group in some cases improved activity (**3**, **6**) or even converted inactive compound into active ones (**14**), but in the other

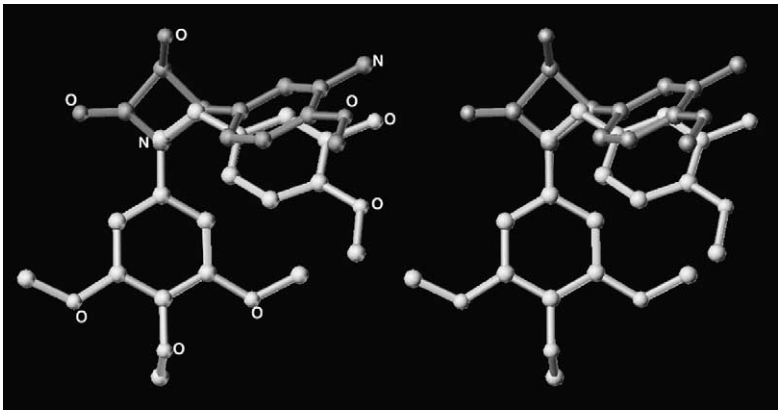


Figure 3. A stereopair illustrating the differences in three-dimensional structures of combretastatin and compound **9**. The analogues are aligned by a superimposition of trimethoxyphenyl moieties, which is shown in the plane of the paper. Hydrogen atoms and multiple bonds have been omitted, and only the heteroatoms are labeled for clarity.

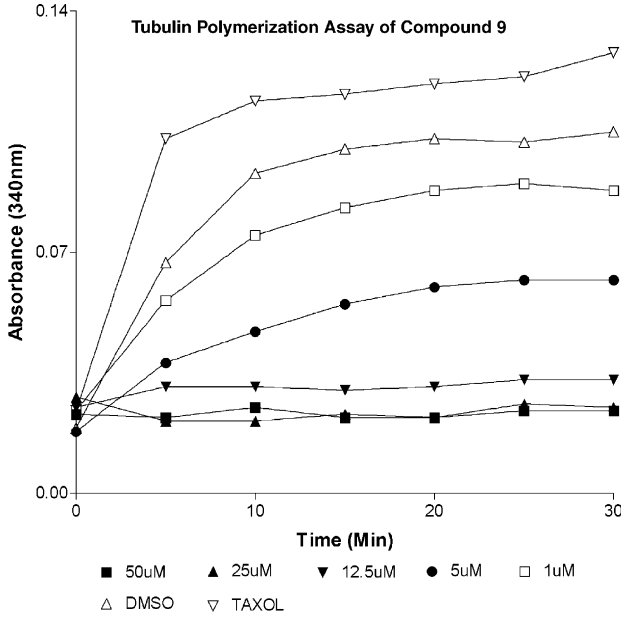


Figure 4. Inhibitory effects and dose responses of compound **9** on tubulin polymerization. Taxol, a tubulin polymerization promoter, was included for comparison.

cases had a very weak effect (**9**, **20**). A smaller 3-methoxy substituent always led to inactive compounds (**7**, **10**, and **21**). Eight aromatic groups, comprising both substituted phenyl and heterocycles, were tested as substituents in position 4. Among them, the best activity was obtained with 4-methoxyphenyl (**2**), 4-methoxy-3-nitrophenyl (**6**), and 4-methoxy-3-aminophenyl (**9**). These data are in general agreement with much SAR data on CA4s[18,19] itself and its constrained analogues.[11,12]

Molecular modeling studies comparing CA4 and the most potent azetidinone (compound **9**) were carried out. The models were built in Sybyl 6.9 (Tripos Inc., St. Louis, MO) and the 3-D structures optimized by the semi-empirical Mopac method using AM1 Hamiltonian. The structures were superimposed by a least squares fit of the aromatic carbon atoms in the trimethoxybenzene moieties and the results are shown in Figure 3. As anticipated, the crucial orientation and spacing of the two aromatic rings was essentially retained in the new compounds whilst the additional groups present on the β-lactam ring generally point away from the purported binding 'face' of the molecule thus reducing the possibility of steric interference.

Since many cytotoxic agents, including colchicine, CA4, and podophyllotoxin, act through inhibition of tubulin polymerization[20,21] we chose seven of the most potent and tested their abilities to inhibit bovine brain tubulin polymerization at different concentrations using kits purchased from Cytoskeleton (Denver, CO). The results are shown in Figure 4 and a more in-depth dose–response study of potent compound **9** confirmed that it had strong inhibitory activity with an IC_{50} of 5 μm, which was only about ×2 less than the IC_{50} value previously obtained for CA4[16] itself in this assay. Taxol (a tubulin polymerization promoter) was included in the assay as an additional control.

Encouraged by the promising in vitro properties of compound **9**, its ability to inhibit transplanted tumor growth in nude mice was then examined. It was determined that a maximum tolerated i.p. dose for this compound in normal mice was in the region of 100 mg/kg body weight and this dose was administered i.p. to mice growing rat pancreatic AR42J tumors ×3 per week for a total of 12 times. Excellent inhibition of tumor growth was obtained (Fig. 5) with a 66% decrease in tumor volume compared to control animals at the end of the 4 week treatment cycle. Some nonlethal toxicity was seen at this dose level as evidenced by significant overall loss of body weight in treated animals (Fig. 5) compared to controls.

The stability of **2**, **8**, **9**, and **10** in rat serum and phosphate buffer was also investigated. Solutions of the β-lactams were incubated in serum or phosphate buffer at 37 °C and, after separation of serum proteins, examined by analytical HPLC at 15 min intervals. Complete stability was observed in buffer (data not shown), however, rapid degradation of the compounds was ob-

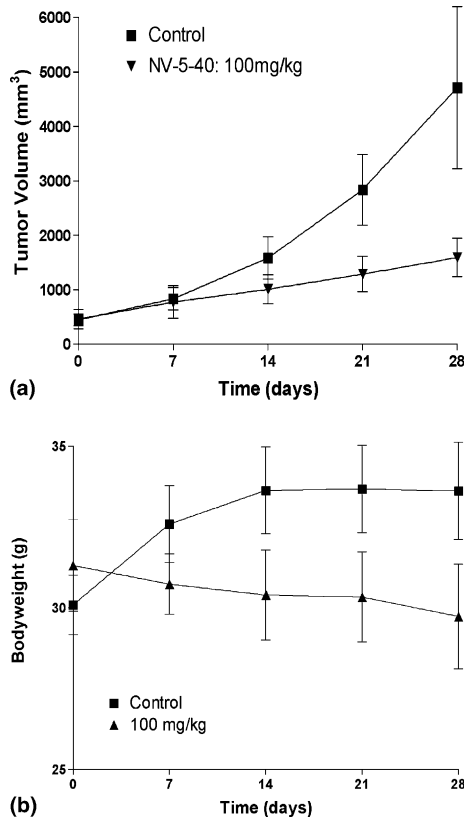


Figure 5. Anti-tumor activity of 1,4-diarylazetidinone compound **9** against AR42J tumors on nude mice. (a) Tumor bearing mice were treated with propylene glycol (100 μL, i.p.) as control or compound **9** (100 mg/kg in 100 μL of propylene glycol i.p.) three times per week for four weeks. (b) Animal weights demonstrate some toxicity.

served in rat serum (Fig. 6) with half-lives of around 15 min. This short serum half-life could result in lower toxic side effects depending on the route of administration.

Previous studies on 1,4-diarylazetidinones[13] demonstrated the possibility of using the system for designing cytotoxic compounds by employing a variety of aromatic and heterocyclic ring systems in positions 1 and 4. The most potent compounds displayed cytotoxicity against several human cancer cell lines in the 5–10 μM IC_{50} dose range. The present study demonstrates that incorporation of standard aromatic ring systems present in CA4 and its analogues onto the azetidinone backbone can increase these potencies in many tumor cell lines by two orders of magnitude into the low nM range. This and the ability of compound **9** to effectively inhibit tumor growth in vivo make them interesting potential therapeutic candidates.

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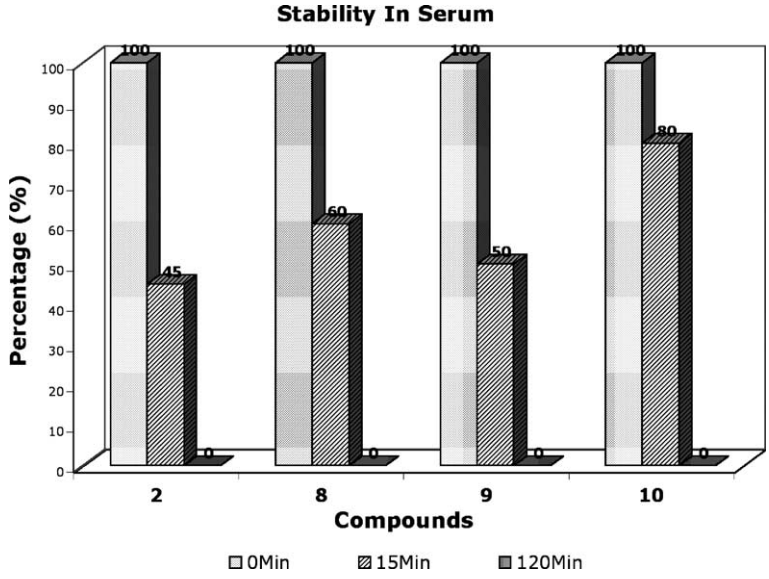

Figure 6. Rat serum stability over time of azetidinone compounds **2**, **8**, **9**, and **10**.

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