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## CONCISE ARTICLE

## An amino-benzosuberene analogue that inhibits tubulin assembly and demonstrates remarkable cytotoxicity†

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The recent discovery of a small-molecule benzosuberene-based phenol that demonstrates remarkable picomolar cytotoxicity against selected human cancer cell lines and strongly inhibits tubulin polymerization (1–2  $\mu$ M) inspired the design and synthesis of a variety of new, structurally diverse benzosuberene derivatives. An efficient synthetic route to functionalized benzosuberenes was developed. This methodology utilized a Wittig reaction, followed by a selective alkene reduction and ring-closing cyclization to form the core benzosuberone structure. This synthetic route facilitated the preparation of a 4-nitro-1-(3',4',5'-trimethoxyphenyl) benzosuberene derivative and its corresponding 4-amino analogue in good yield. The 4-amino analogue was a strong inhibitor of tubulin polymerization (1.2  $\mu$ M), demonstrated enhanced cytotoxicity against the human cancer cell lines examined ( $GI_{50}$  = 33 pM against SK-OV-3 ovarian cancer, for example), and exhibited a concentration dependent disruption of a pre-established capillary-like network of tubules formed from human umbilical vein endothelial cells.

## Introduction

The discovery of small-molecule anticancer agents that demonstrate nanomolar to subnanomolar cytotoxicity against human cancer cell lines is noteworthy, and such compounds have the potential to become drug candidates through an appropriately focused development program. The discovery of compounds that demonstrate picomolar cytotoxicity is even more exciting. In previous studies,<sup>1–3</sup> we identified a functionalized benzosuberene-based phenol **1** as one such compound (Fig. 1). Its structure is reminiscent of both colchicine<sup>4,5</sup> and combretastatin A-4 (**CA4**),<sup>6</sup> which are natural products that are potent inhibitors of tubulin assembly.<sup>7</sup> Both compounds interact with tubulin at a small-molecule binding site named the colchicine site (Fig. 1). Compound **1** also bears structural similarity to

a dihydronaphthalene analogue **2** (discovered previously in our laboratory) that is strongly cytotoxic and inhibitory against tubulin assembly.<sup>1,8</sup> In addition to its antiproliferative mechanism of action, both **CA4** and compound **2** damage tumor vasculature.<sup>8,9</sup> Preliminary studies with the benzosuberene phenol **1**, in appropriate prodrug form, also suggest vascular damage as one component of its overall mechanism of action as an antitumor agent.<sup>2</sup> This makes it likely that the benzosuberene amino derivative **3** would function through a similar mechanistic pathway.

Small-molecule anticancer agents that target solid-tumor vasculature represent an important emerging area of research significance.<sup>9,10</sup> Solid tumors require nutrients and oxygen provided by a network of vasculature, which has distinct morphological differences compared with a corresponding vascular network feeding normal healthy tissue.<sup>11</sup> Tumor vasculature is highly disorganized with abnormal bulges, blind ends, and shunts.<sup>12</sup> It is also characterized as leaky and discontinuous. It is these physiological dissimilarities that collectively offer a therapeutic advantage for the selective targeting and disruption of tumor vasculature through small-molecule drug intervention.

Tumor vasculature can be efficiently targeted through two distinct mechanistic pathways.<sup>13</sup> Angiogenic inhibiting agents (AIAs) prevent new blood vessel growth.<sup>14</sup> Vascular disrupting agents (VDAs), on the other hand, damage existing tumor vasculature through a series of cell-signaling pathways.<sup>15,16</sup> One important class of small-molecule VDAs bind to tubulin

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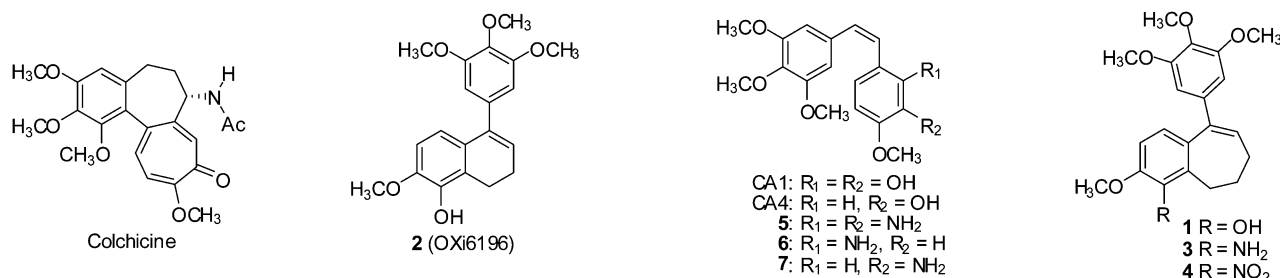


Fig. 1 Representative small-molecule inhibitors of tubulin assembly.

heterodimers at the colchicine site and thereby potentially inhibit tubulin assembly. The colchicine site is located on the  $\beta$ -subunit of the  $\alpha\beta$ -tubulin heterodimer, near the interface between the two subunits.<sup>17</sup>

In the late 1970's, the combretastatins were discovered by Pettit and co-workers in the South African bush willow tree, *Combretum caffrum*. Combretastatin A-1 (CA1)<sup>18–20</sup> and combretastatin A-4 (CA4)<sup>6</sup> are two of the most potent compounds isolated from *C. caffrum*, and both have pronounced biological activity as VDAs and as inhibitors of tubulin polymerization. In prodrug forms, both of these compounds, as well as the synthetic combretastatin amino-analogue 7, are currently in human clinical trials.<sup>21–25</sup>

Our original synthetic methodology towards the benzosuberene molecular scaffold utilized a cyanogen azide ring expansion reaction,<sup>1,26–28</sup> and, while reliable, the yield for this overall process was relatively low (Scheme 1). Accordingly, we developed a new, efficient synthetic strategy towards the functionalized six-seven fused ring system. This new method facilitated the preparation of the nitro-benzosuberene analogue 4 and its corresponding amino derivative 3. These two new benzosuberene analogues were evaluated for their ability to inhibit tubulin assembly and for their cytotoxicity against three human cancer cell lines.

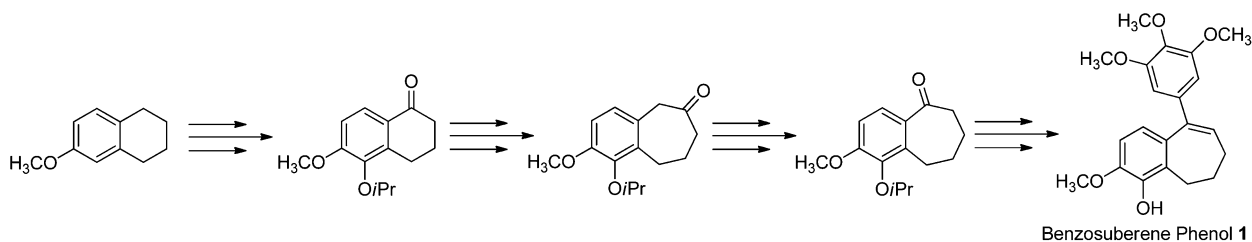
## Results and discussion

Our improved synthetic approach to the target benzosuberene analogues relied on a sequential Wittig reaction, selective reduction, cyclization strategy to assemble the requisite 6,7-ring fusion. Key features of this overall synthetic route include both improved yields and fewer reaction steps. While other synthetic approaches to this type of 6,7-fused ring system are known<sup>1,29,30</sup> (including Friedel–Crafts cyclization<sup>31</sup>), the strategy that we employed proved to be highly efficient. A Wittig reaction using 3-(carboxypropyl)triphenylphosphonium bromide, potassium *tert*-

butoxide, and 3-methoxy-2-nitrobenzaldehyde resulted in compound 8 in good yield (Scheme 2, Route 1). A selective reduction of the alkene moiety to afford compound 9 was achieved using a 10% Pd/C catalyst with 1,4-cyclohexadiene in excess. Reduced analogue 9 underwent cyclization upon treatment with Eaton's reagent (7.7 wt% P<sub>2</sub>O<sub>5</sub> in methanesulfonic acid)<sup>32</sup> to afford nitrobenzosuberone 10 (ref. 33) in good yield. Alternatively, nitrobenzosuberone 10 was prepared by nitration of benzosuberone 15. The regioisomeric mixture obtained (10, 37% and 12, 49%) was separated chromatographically (Scheme 2, Route 2).<sup>33</sup> Dropwise addition of nitrobenzosuberone 10 to 3,4,5-trimethoxyphenyllithium (prepared *in situ* from the corresponding aryl bromide) gave tertiary alcohol 11, which underwent elimination upon exposure to acetic acid to generate nitrobenzosuberene 4. The nitro group in compound 4 was reduced to its corresponding amine 3 upon treatment with zinc dust in acetic acid. Eaton's reagent has been successfully employed for cyclization to form tetralone (6,6-fused) and benzosuberone (6,7-fused) ring systems,<sup>32,37,38</sup> and we were encouraged by the efficient extension of this methodology to these functionalized benzosuberene core structures.

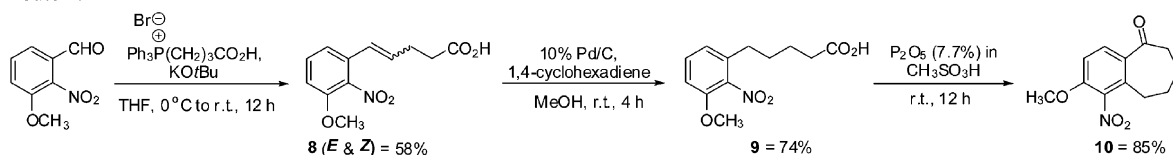
## Inhibition of tubulin assembly

The two new benzosuberene analogues 3 and 4 were found to be potent inhibitors of tubulin assembly (IC<sub>50</sub> = 1.2  $\mu$ M and 2.5  $\mu$ M, respectively (Table 1)), comparable to CA1, CA4, and the benzosuberene phenol 1. A binding assay utilizing tritium-labeled colchicine indicated that amino-benzosuberene 3, at a concentration of 5  $\mu$ M, inhibited colchicine binding by 91% (Table 1). At the lower concentration of 1  $\mu$ M, amine 3 inhibited colchicine binding by 65%. This is comparable with the activity of CA4 (100% inhibition at 5  $\mu$ M and 88% inhibition at 1  $\mu$ M). The nitro analogue 4 was less active as an inhibitor of colchicine binding.

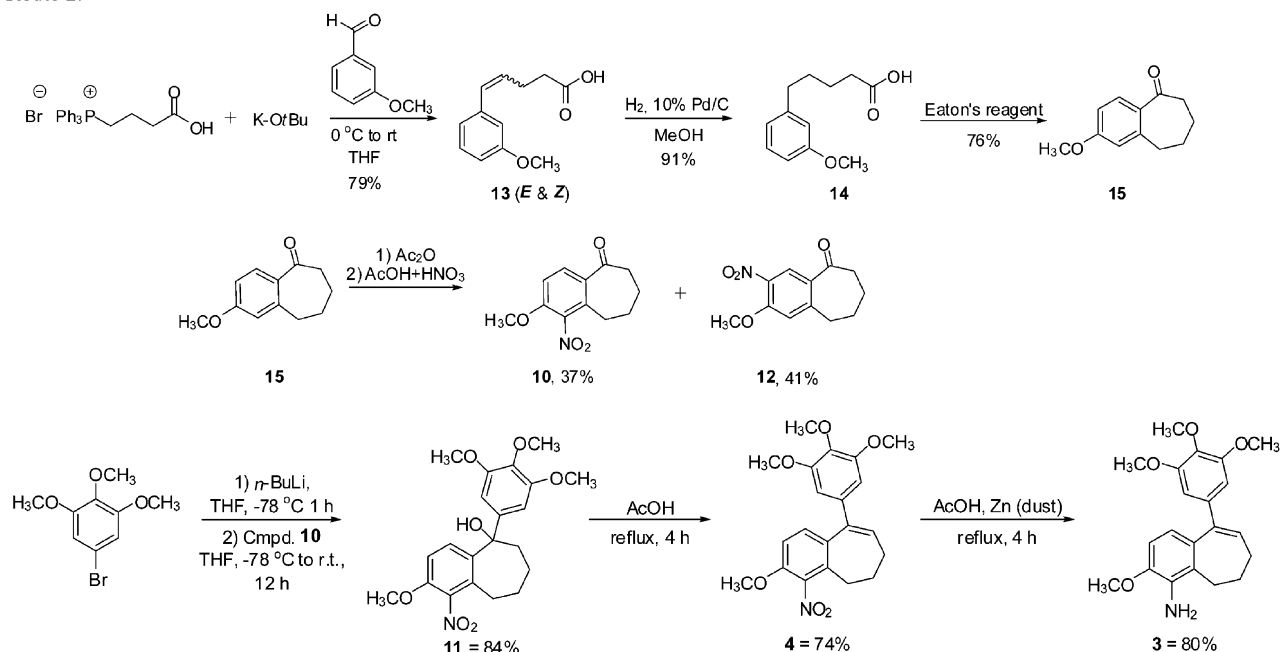


Scheme 1 Original synthetic route to the benzosuberenes.<sup>1–3</sup>

## Route 1:



## Route 2:



Scheme 2 Improved ring-cyclization methodologies for the construction of benzosuberene-based compounds.

Table 1 Inhibition of tubulin polymerization and colchicine binding

Compound	Inhibition of tubulin polymerization $\text{IC}_{50}$ ( $\mu\text{M}$ )	Inhibition of colchicine binding <sup>a</sup> (%)	
		5 $\mu\text{M}$	1 $\mu\text{M}$
<b>CA1</b>	1.9 <sup>b</sup>	nd <sup>c</sup>	nd
<b>CA4</b>	1.2 <sup>b,d</sup>	100	88
<b>1</b>	1.7 <sup>e</sup>	nd	nd
<b>3</b>	1.2	91	65
<b>4</b>	2.5	63	nd

<sup>a</sup> Reaction mixtures contained 1  $\mu\text{M}$  tubulin, 5  $\mu\text{M}$  [ $^3\text{H}$ ]colchicine, and inhibitor as indicated. <sup>b</sup> See ref. 34. <sup>c</sup> nd = not determined in this study. <sup>d</sup> See ref. 35. <sup>e</sup> See ref. 1.

## Cell line studies

In addition, the compounds were evaluated for their cytotoxicity against human non-small cell lung (NCI-H460), prostate (DU-145), and ovarian (SK-OV-3) cancer cell lines. The amino-benzosuberene analogue demonstrated remarkable cytotoxicity against ovarian cancer with a  $\text{GI}_{50}$  value of 32.9  $\mu\text{M}$ . In addition, the compound was strongly cytotoxic against the non-small cell lung and prostate cell lines. While somewhat less active than the parent benzosuberene phenol **1**, the amino derivative **3** was more active against each cell line than the natural products **CA4** and **CA1**. The nitrobenzosuberene analogue **4** was significantly less cytotoxic than any of the comparison compounds (Table 2).

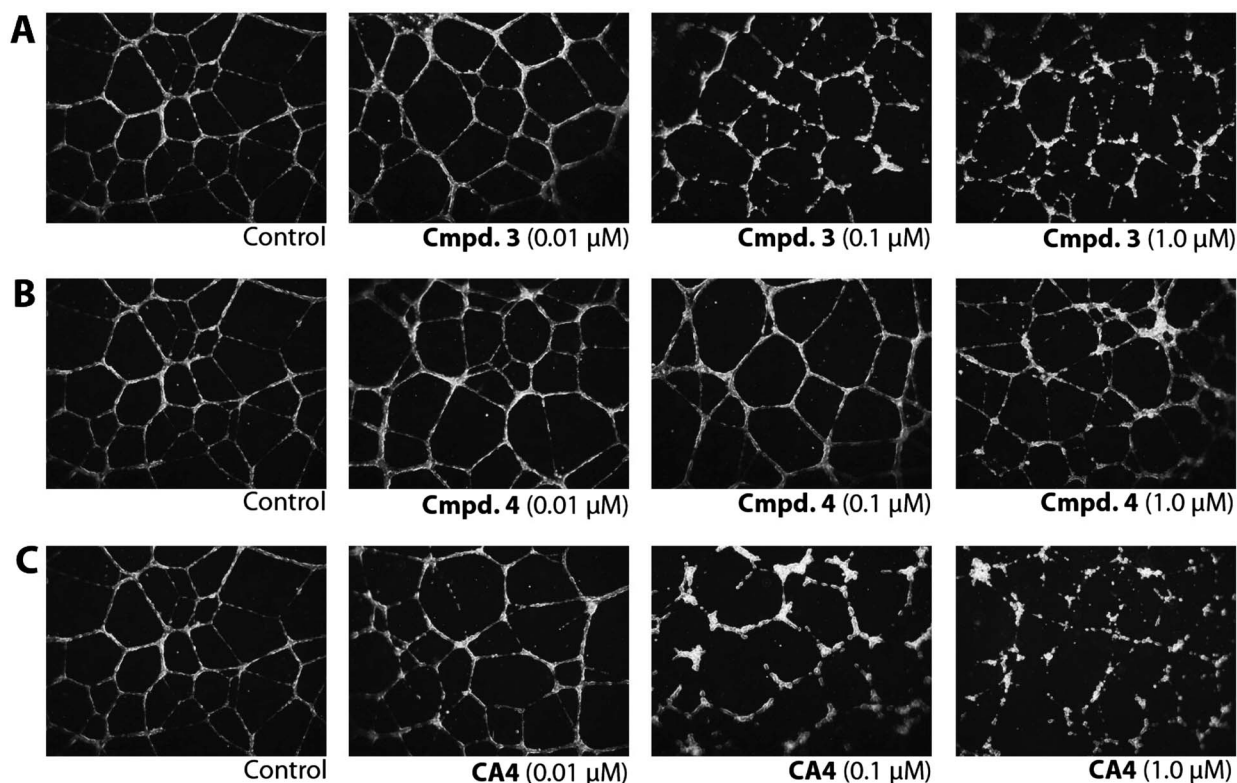
## Endothelial tube disruption

HUVECs, plated in medium supplemented with growth factors onto Matrigel™ as a model for the extracellular matrix, form a network of capillary-like tubules (Fig. 2, Control). The disruption of these tubules by individual compounds provides an *in vitro* assessment for vascular disrupting agents (VDAs). After 16 h, the endothelial tubule network was treated for 2 h with varying concentrations of **3**, **4**, or **CA4** (for comparison as a positive control), or vehicle (medium containing 1% DMSO, negative control). Representative photographs from a minimum of three independent experiments are shown in Fig. 2. Tubule disruption and cell rounding was observed for **3** at 0.10  $\mu\text{M}$ , and

Table 2 Cytotoxicity studies against human cancer cell lines NCI-H460, DU-145, SK-OV-3

Compound	$\text{GI}_{50}$ ( $\mu\text{M}$ ) SRB assay <sup>a</sup>		
	NCI-H460	DU-145	SK-OV-3
<b>CA1</b>	0.015 <sup>b</sup>	0.033 <sup>b</sup>	0.038 <sup>b</sup>
<b>CA4</b>	0.0028 <sup>b</sup>	0.00054 <sup>b</sup>	0.00042 <sup>b</sup>
<b>1</b>	0.000028 <sup>c</sup>	0.0000032 <sup>c</sup>	0.00003 <sup>c</sup>
<b>3</b>	0.00469	0.00111	0.0000329
<b>4</b>	0.216	0.211	0.152
<b>16</b>	nd <sup>d</sup>	0.00353	nd

<sup>a</sup> Average of  $n \geq 3$  independent determinations. <sup>b</sup> For additional data see ref. 36. <sup>c</sup> See ref. 1. <sup>d</sup> nd = not determined in this study.



**Fig. 2** HUVEC tube disruption. Effects of a 2 h treatment with (A) **Cmpd. 3**, (B) **Cmpd. 4**, and (C) **CA4** on a pre-established network of capillary-like tubules formed from HUVECS seeded onto Matrigel™ in a growth factor rich medium over a period of 16 h. **CA4** was used as a positive control. All experiments including the vehicle control contained a final concentration of 1% DMSO. These results are representative of the photographic record that was obtained with a minimum of three independent experiments for each compound concentration and observing 9 fields per well (40× objective).

this effect was greatly increased at a concentration of 1  $\mu\text{M}$  (Fig. 2A). Compound **4** was much less active and demonstrated no significant effects at concentrations of 0.01 or 0.10  $\mu\text{M}$ . Only minor disruption of tubules was observed at 1.0  $\mu\text{M}$  compound **4**, although a substantial number of cells were beginning to round (Fig. 2B). In comparison, tubule disruption activity was detected at 0.01  $\mu\text{M}$  for **CA4**, and this activity was more pronounced with increasing concentrations (Fig. 2C).

### Hydrochloride salt formation

To facilitate planned *in vivo* experiments in mice, a hydrochloride salt **16** was prepared synthetically from amine **3** in an effort to obtain a more water-soluble derivative (Scheme 3). Although the salt derivative **16** demonstrated limited solubility in water, it was possible to obtain complete dissolution (NMR study used approximately 3 mg of compound **16** in approximately 1.0 mL of

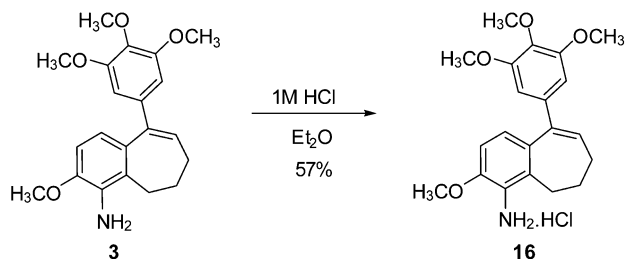
$\text{D}_2\text{O}$ ). Obtaining a clear (to the naked eye) solution took several days. A more robust solubilization strategy involved adding DMSO (with vigorous vortexing and/or ultrasound to achieve a clear solution) followed by the addition of water (to achieve a favorable formulation for *in vivo* experiments in mice). The amine salt **16** demonstrates cytotoxicity (*in vitro*) in the DU-145 prostate cancer cell line comparable to its parent amino-benzosuberene analogue **3** (Table 1).

### Conclusions

In summary, we have developed a sequential Wittig reaction, selective reduction, Eaton's reagent-mediated cyclization strategy as an efficient synthetic route for the preparation of functionalized benzosuberene derivatives. An amino-benzosuberene analogue **3**, prepared by this method, demonstrated pronounced cytotoxicity against human cancer cell lines. In addition, this compound was a potent inhibitor of tubulin polymerization and effectively disrupted pre-established endothelial tubes. In a collective sense, these data position compound **3** as an excellent candidate for further evaluation (through additional biochemical/biological studies and *in vivo* tumor imaging) as an anticancer, vascular disrupting agent.

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**Scheme 3** Synthesis of the HCl salt **16** of amine benzosuberene **3**.



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