Anti-Cancer Drugs 1998, 9, pp. 545–550

Research paper

Antimitotic activity of diaryl compounds with structural features resembling combretastatin A-4

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Series of diaryl ethers, amines and amides have been synthesized and tested for antitumor activity. These diaryl compounds possess some of the structural features of combretastatin A-4 (a potent antimitotic agent). They were designed to discover whether transferring these structural motifs from stilbenes to heterosubstituted diaryl compounds would enhance their biochemical activities. Molecular modeling studies suggested that these diaryl compounds could adopt conformations similar to combretastatin A-4. However, although some agents (5–7) were cytotoxic and others (10 and 12) could interact with tubulin, none were as potent as combretastatin A-4. [© 1998 Lippincott-Raven Publishers.]

Key words: Antitumor, antimitotic, combretastatin, molecular modeling.

Introduction

Combretastatin A-4 (**1**), a stilbene isolated from the African shrub *Combretum caffrum*, is one of the most potent antimitotic agents discovered.[1] It shows strong cytotoxicity in several cancer cell lines, is able to inhibit the assembly of tubulin at low concentration and can displace colchicine from its binding site on tubulin. Previous studies on analogs of combretastatin A-4 (**1**) have suggested which structural features might be important for antimitotic activity.[2–6] These include (i) a 3,4,5-trimethoxy aryl unit, (ii) a small group on the 4′-position and (iii) two aryl rings tilted with respect to each other. Studies on stilbenes have indicated that a *cis* configuration is preferable for antimitotic activity although some *trans* compounds do show biological activities. Similarly it has been shown that replacement of the *cis*-alkene with a sulfur atom can retain antimitotic activity of the diaryl system. We have therefore undertaken the synthesis and evaluation of molecules substituting the *cis*-alkene bond of combretastatin with different heteroatoms. Herein we describe the synthesis and biochemical evaluation of 12 diarylethers and diarylamines consisting of some of the molecular features described above.

Financial support from the Cancer Research Campaign is gratefully acknowledged.

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Materials and methods

Chemistry

^{1}H NMR spectra were determined on a Hitachi Perkin Elmer R-600 High Resolution Spectrometer in deuterochloroform at 60 MHZ and are expressed in δ values relative to tetramethylsilane. Coupling constants (J) were measured in Hz. Melting points are uncorrected. Microanalyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of Manchester. Electron impact mass spectra were determined on a VG Trio 2 mass spectrometer at an ionization energy of 70 eV.

3,4,5-Trimethoxydiphenyl ether (2)

A mixture of 3,4,5-trimethoxyphenol (1 g, 5 mmol), bromobenzene (1.8 g, 11 mmol), potassium carbonate (0.7 g, 5 mmol) and cuprous iodide (0.09 g, 0.5 mmol) was heated under reflux for 1.5 h, cooled and extracted with hot toluene (40 ml). The toluene was evaporated and the excess bromobenzene removed by Kugelrohr distillation. The residue was dissolved in boiling methanol, treated with charcoal and the solvent evaporated. The product was purified by flash chromatography using ethyl acetate/petrol 40/60 (4:1) as eluent to afford the title compound (**2**) as a

white powder (0.98g, 70%) m.p. 83–84 C (from aqueous ethanol) (lit.[7] m.p. 84–86 C). *m/z* 260 (M^+, 40%); 245 ($M-CH_3$, 100).

4-Methyl-3′,4′,5′-trimethoxydiphenyl ether (**3**)

By the method described for ether (**2**) the title compound (**3**) was prepared from 3,4,5-trimethoxyphenol (1 g, 5 mmol) and 4-bromotoluene (1.8 g, 10 mmol) as white crystals (0.80 g, 71%) m.p. 75–76 C (from petrol 60/80) (lit.[8] m.p. 73–75 C). (Found: C, 70.2; H, 6.8. $C_{16}H_{18}O_4$ requires C, 70.1; H, 6.6%.) δ_H 2.32 (3 H, s, $ArCH_3$); 3.75, 3.78 (9 H, 2 s, $3 \times OCH_3$); 6.20 (2 H, s, Hs *ortho* to OCH_3); 6.84 (2 H, d, *J* 8, Hs *ortho* to CH_3); 7.14 (2 H, d, *J* 8, Hs *meta* to CH_3). *m/z* 274 (M^+, 82%); 259 ($M-CH_3$, 100).

4-Ethyl-3′,4′,5′-trimethoxydiphenyl ether (**4**)

By the method described for ether (**2**) the title ether (**4**) was prepared from 3,4,5-trimethoxyphenol (1 g, 5 mmol) and 1-bromo-4-ethylbenzene (2 g, 10 mmol) as white crystals (1.02 g, 65%) m.p. 49–50 C (from petrol 60/80). (Found: C, 71.1; H, 7.0. C_1-$H_{20}O_4$ requires C, 70.8; H, 7.0%.) δ_H 1.21 (3 H, t, *J* 7, CH_3CH_2); 2.65 (2 H, q, *J* 7, CH_2); 3.74, 3.77 (9 H, $3 \times OCH_3$); 6.20 (2 H, s, Hs *ortho* to OCH_3); 6.85 (2 H, d, *J* 8, Hs *ortho* to CH_3); 7.14 (2 H, d, *J* 8, Hs *meta* to CH_3). *m/z* 288 (M^+, 73%); 273 ($M-CH_3$, 100).

4-Methoxy-3′,4′,5′-trimethoxydiphenyl ether (**5**)

By the method described for ether (**2**) the title compound (**5**) was prepared from 3,4,5-trimethoxyphenol (1 g, 5 mmol) and 4-bromoanisole (1.9 g, 10 mmol) as white crystals (1.43 g, 90%) m.p. 62–64 C (from aqueous methanol) (lit.[9] m.p. 63 C). δ_H 3.73, 3.74, 3.75 (12 H, $4 \times OCH_3$); 6.15 (2 H, s, Hs *ortho* to OCH_3); 6.80–7.08 4 H, m, 2-H, 3-H, 5-H, 6-H). *m/z* 290 (M^+, 80%); 275 ($M-CH_3$, 100).

N-Acetyl-3,4,5-trimethoxydiphenylamine (**6**)

By the method described for ether (**2**) the title amine (**6**) was prepared from 3,4,5-trimethoxyacetanilide (0.94 g, 4.2 mmol) and bromobenzene (1.8 g, 11 mmol) with heating under reflux for 13.5 h as white crystals (1.07 g, 85%) m.p. 85–86 C (from petrol 40/60). (Found: C, 68.0; H, 6.8; N, 4.7. C_1-$H_{19}NO_4$ requires C, 67.8; H, 6.4; N, 4.7%.) δ_H 2.08 (3 H, s, $COCH_3$); 3.78 (9 H, s, $3 \times OCH_3$); 6.49 (2 H, s, Hs *ortho* to OCH_3); 7.30 (5 H, s, ArHs). *m/z* 301 (M^+, 92%); 244 ($M-NCOCH_3$, 100).

N-Acetyl-4-methyl-3′,4′,5′-trimethoxydiphenylamine (**7**)

By the method described for amide (**6**) the title compound (**7**) was prepared from 3,4,5-trimethoxyacetanilide (1 g, 4.4 mmol) and 4-bromotoluene (1.8 g, 10 mmol) with heating under reflux for 18 h as white crystals (1.23 g, 88%) m.p. 109–111 C (from hexane). (Found: C, 68.1; H, 7.1; N, 4.3. $C_{18}H_{21}NO_4$ requires C, 68.6; H, 6.7; N, 4.5%.) δ_H 2.04 (3 H, s, $COCH_3$); 2.33 (3 H, s, $ArCH_3$); 3.77 (9 H, s, $3 \times OCH_3$); 6.45 (2 H, s, Hs *ortho* to OCH_3); 7.14 (4 H, s, Hs *ortho* and *meta* to CH_3). *m/z* 315 (M^+, 65%); 258 ($M-NCOCH_3$, 100).

N-Acetyl-4-ethyl-3′,4′,5′-trimethoxydiphenylamine (**8**)

By the method described for amide (**6**) the title amide (**8**) was prepared from 3,4,5-trimethoxyacetanilide (0.98 g, 4.4 mmol) and 1-bromo-4-ethylbenzene (2 g, 10 mmol) with heating under reflux for 14 h as white crystals (1.3 g, 91%) m.p. 131–13 C (from hexane). (Found: C, 69.2; H, 7.0; N, 3.9. $C_{19}H_{23}NO_4$ requires C, 69.3; H, 7.0; N, 4.3%.) δ_H 1.25 (3H, t, *J* 7, CH_3CH_2); 2.06 (3 H, s, $COCH_3$); 2.68 (2 H, q, *J* 7, CH_2); 3.80 (9 H, s, $3 \times OCH_3$); 6.48 (2 H, s, Hs *ortho* to OCH_3); 7.18 7.14 (4 H, s, Hs *ortho* and *meta* to CH_2). *m/z* 329 (M^+, 97%); 272 ($M-NCOCH_3$, 100).

N-Acetyl-4-methoxy-3′,4′,5′-trimethoxydiphenylamine (**9**)

By the method described for amide (**6**) the title compound (**9**) was prepared from 3,4,5-trimethoxyacetanilide (0.99 g, 4.4 mmol) and 4-bromoanisole (1.9 g, 10 mmol) as a white powder (1.31 g, 90%) m.p. 122–123 C (from hexane). (Found: C, 65.4; H, 6.4; N, 4.0. $C_{18}H_{21}NO_4$ requires C, 65.2; H; 6.4, N, 4.2%.) δ_H 2.01 (3, s, $COCH_3$); 3.75 (9 H, s, $3 \times OCH_3$); 6.43 (2 H, s, HS *ortho* to OCH_3); 6.82 (2 H, d, *J* 8, 3,5-Hs); 7.20 (2 H, d, *J* 8, 2,6-Hs). *m/z* 331 (M^+, 68%); 274 ($M-NCOCH_3$, 100).

3,4,5-Trimethoxydiphenylamine (**10**)

A solution of the amide (**6**) (1.21 g, 4 mmol) in ethanol

(6 ml) containing potassium hydroxide (1 g, 20 mmol) was heated under reflux for 2 h. After evaporation of the ethanol the crude product was extracted with hot toluene (60 ml), cooled and washed with saturated aqueous brine, and dried ($MgSO_4$). Evaporation of the solvent afforded the title amine (**10**) as colorless prisms (0.64 g, 56%) m.p. 81-83°C (from petrol 40/60). (Found: C, 69.3; H, 7.0; N, 5.4. $C_{15}H_{17}NO_3$ requires C, 69.5; H, 6.6; N, 5.4%.) δ_H 3.77 (9 H, s, $3 \times OCH_3$); 5.57 (1H, br s, NH); 6.30 (2 H, s, Hs *ortho* to OCH_3); 6.80-7.40 (5 H, m, ArHs). *m/z* 259 (M^+, 100%); 244 ($M-CH_3$, 98).

4-Methyl-3',4',5'-trimethoxydiphenylamine (**11**)

By the method described above from amide (**7**) (1.25 g, 3.97 mmol) the title amine (**11**) was obtained as yellow prisms (0.94 g, 76%) m.p. 81-83°C (from petrol 40/60). (Found: C, 70.0; H, 7.0; N, 5.3. $C_{16}H_{19}NO_3$ requires C, 70.3; H, 1.0: N, 5.1%.) δ_H 2.30 (3 H, s, $ArCH_3$); 3.76 (9 H, s, $3 \times OCH_3$); 5.50 (1 H, br s, NH); 6.25 (2 H, s, Hs *ortho* to OCH_3); 6.84-7.20 (4 H, m, ArHs). *m/z* 273 (M^+, 93%); 258 ($M-CH_3$, 100).

4-Ethyl-3',4',5'-trimethoxydiphenylamine (**12**)

By the method described above from amide (**8**) (1.25 g, 3.80 mmol) the title amine (**12**) was obtained as white crystals (0.78 g, 60%) m.p. 74-76°C (from diethyl ether/petrol 40/60) after flash chromatography using ethyl acetate/petrol 40/60 (1:2) as eluent. (Found: C, 71.5; H, 7.6; N, 4.8. $C_{17}H_{21}NO_3$ requires C, 71.1; H, 7.4; N, 4.9%.) δ_H 1.20 (3 H, t, *J* 7, CH_3CH_2); 2.60 (2 H, q, *J* 7, CH_2); 3.75 (9 H, s, $3 \times OCH_3$); 5.50 (1 H, br s, NH); 6.23 (2 H, s, Hs *ortho* to OCH_3); 6.90-7.20 (4 H, m, ArHs). *m/z* 287 (M^+, 55%); 272 ($M-CH_3$, 100).

4-Methoxy-3',4',5'-trimethoxy-diphenylamine (**13**)

By the method described above from amide (**9**) (1.38 g, 4.38 mmol) the title amine (**13**) was obtained as a white powder (1.08 g, 83%) m.p. 110-112°C (from methanol) after flash chromatography using ethyl acetate/petrol 40/60 (1:2) as eluent. (Found: C, 66.6; H, 6.7; N, 4.8. $C_{16}H_{19}NO_3$ requires C, 66.4; H, 6.6; N, 4.8%.) δ_H 3.74, 3.76 (12 H, $4 \times OCH_3$); 5.40 (1 H, br s, NH); 6.10 (2 H, s, Hs *ortho* to OCH_3); 6.78 (2 H, d, *J* 8, 3,5-Hs); 7.05 (2 H, d, *J* 8, 2,6-Hs). *m/z* 289 (M^+, 60%); 274-CH_3, 100).

Biochemistry

Cytotoxicity testing

A P388 mouse leukemia cell line was cultured as described previously.[10] The cell line was mycoplasma free and cytotoxicity tests were carried out using the MTT assay.[11] The ID_{50} concentration was calculated by reference to a standard curve constructed for control cells.

Tubulin assembly

The assembly of microtubules from isolated porcine tubulin was carried out spectrophotometrically at 350 nm and utilized the increase in turbidity which is associated with microtubule formation. Assembly was initiated by temperature increase from 10 to 35°C. The effect of drugs on the increase of light absorption was carried out as described previously.[6] Drugs were dissolved in dimethyl sulfoxide (below 4%) which did not affect control assembly.

Competitive binding assays

The ability of agents to compete with colchicine for binding to tubulin was examined by the spun column method.[12] Briefly, tubulin (5 μM) was incubated with a test compound and colchicine (10 μM, spiked with [^{3}H]colchicine, 20 nCi/ml) for 90 min in buffer (0.1 M Mes, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, pH 6.8). The mixture was loaded on to previously prepared columns of 1 ml G50 Sephadex (in 40 mM Mes, 40 mM Tris, 1 mM $MgSO_4$, pH 7.5, 11.5 ml/g Sephadex). These were centrifuged (900 *g*, 2 min) and the eluent analyzed by liquid scintillation counting. When tubulin was not present negligible levels of [^{3}H]colchicine were detected indicating that the free (non-protein bound colchicine) compound is not absorbed by the Sephadex. Thus, all radioactivity arises from tubulin-bound colchicine. All experiments were performed in triplicate.

Cell cycle analysis

P388 cells were incubated at a concentration corresponding to $10 \times ID_{50}$ of the drug for 24 h before fixation [acetone:ethanol (1:1) as described previously[2]].

Cytotoxicity, competitive binding, tubulin assembly-disassembly, cell cycle analysis and immunocyto-

chemistry experiments were carried out as previously described.[2]

Molecular modeling

Molecular modeling was performed using the Quanta and Charmm programs (Molecular Simulations, Burlington, MA). The compounds (**5**, **6**, **10**, **14** and **15**) were built using Chemnote, imported into Quanta and minimized using the Steepest Descents program (50 iterations) followed by minimization using the Newton-Raphson algorithm (50 iterations). A conformational search of the two C-N-C or C-O-C (diphenyl ether) or C-S-C torsions was performed using the conformational search program in Quanta. For each structure the torsion angles of the two bonds were altered stepwise by 30° over 360° and energy minimized at these fixed positions using the Adopted Basis Newton-Raphson minimization procedure following each step. This process produced a two-dimensional contour plot which was analyzed to yield the minimum energy conformations associated with the selected angles of the structures. For combretastatin A-1 (**14**) the X-ray crystal structure[13] was downloaded into Quanta from the Cambridge Structural Database (via Daresbury Laboratories, Warrington, UK). This structure (**14**) was treated in a similar manner to that described above for compounds (**5**, **6**, **10** and **15**). Structures were overlaid using the Molecular Similarity module in Quanta.

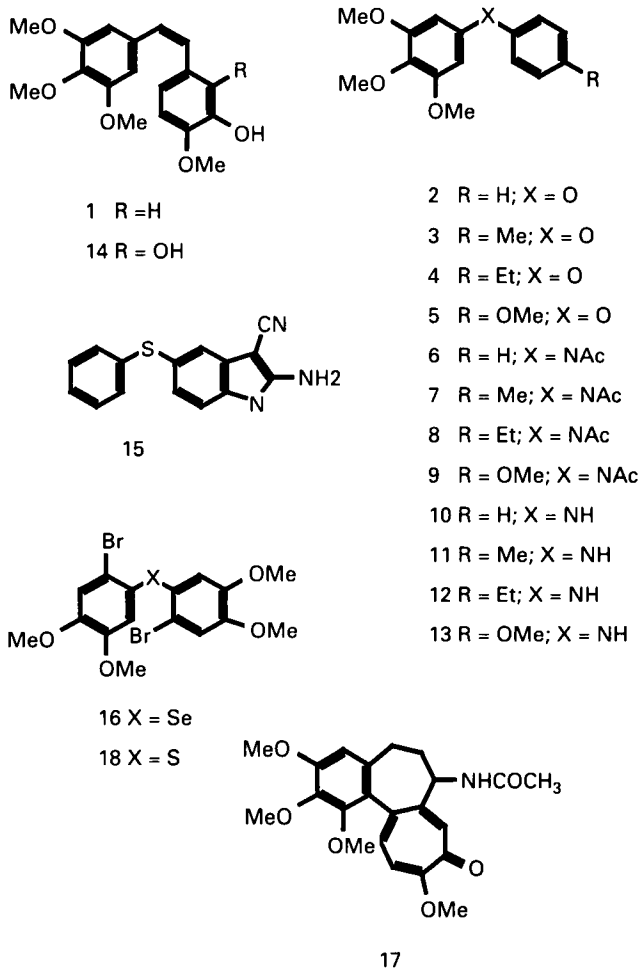

Figure 1. Structures of compounds.

Results

The ethers (**2–5**) were prepared in moderate yield by the reaction of potassium 3,4,5-trimethoxyphenoxide with an appropriately substituted bromobenzene in the presence of copper iodide. In a similar manner the amides (**6–9**) were prepared in good to excellent yields. Deacetylation using ethanolic potassium hydroxide afforded the secondary amines (**10–13**) in good yields.

The compounds (**2–13**) were tested for cytotoxicity in P388 murine leukemia cells. The most toxic compound was the tetramethoxy ether (**5**) (ID_{50}=4.1 μM). The two amides (**6** and **7**) also showed strong cytotoxicities (ID_{50}s < 9 μM) whilst the ethers (**2** and **4**) were moderately cytotoxic (ID_{50}s=15.9 μM).

Only the unsubstituted amine (**10**) and ethyl substituted amine (**12**) had the ability to displace colchicine from tubulin. However, none of the ethers (**2–5**), amines (**10–13**) or amides (**6–9**) showed good ability to inhibit the assembly of tubulin. The ethyl substituted ether (**4**) showed the best ability of this series to inhibit the assembly of tubulin (IC_{50}=100 μM).

Flow cytometric studies showed that the tetramethoxy ether (**5**) possessed a strong ability to cause cell arrest in the G_2/M phase of the cell cycle. Eighty percent of the cells accumulated in the G_2/M phase compared to controls. The ethers (**2** and **3**) showed a moderate ability (30–40%) to cause cells to arrest in the G_2/M phase as did the methoxy substituted amine (**13**).

Overlaying the minimum energy structures of amphethinile (**15**) with combretastatin A-1 (**14**), ether (**5**), amide (**6**), and amines (**10**) and (**12**) gave RMS deviations of 0.42, 0.27, 0.27, 0.34 and 0.35 Å, respectively, over five atoms (ring and hetero atoms). The angles between the aryl rings of combretastatin A-1 (**14**), ether (**5**), amide (**6**) and amine (**10**) were 62, 78, 78 and 84°, respectively.

Discussion

Combretastatin A-4 (**1**) binds strongly to tubulin, is a powerful inhibitor of tubulin assembly and is a potent cytotoxic agent *in vitro*. The structural features which are considered to be important[2] for the antimitotic activity of combretastatin A-4 (**1**) include a trimethoxy unit, two aryl rings tilted with respect to each other and a small alkyl or methoxy unit on the 4′-position.

The dibromoselenide (**16**) and dibromosulfide (**18**) have shown biochemical effects[14] similar to those of known anti-mitotic agents (binding to tubulin, increased percentage of cells in the G_2/M phase of the cell cycle). The phenylthio substituted indole, amphethinile (**15**), inhibits the assembly of tubulin and can displace colchicine (but not vinblastine) from tubulin.[15] The thioether (**15**) can also induce a G_2/M block in L1210 murine leukemia cells[16] and has undergone phase I clinical trials.[17]

The aryl ethers (**2–5**), amides (**6–9**) and amines (**10–13**) described herein possess structural features of combretastatin A-4 (**1**) and of the heteroether substituted biaryls (**15**, **16** and **18**), and were synthesized to discover whether they also possessed antitumor and antimitotic properties.

Three compounds (**5–7**) showed cytotoxicities with an ID_{50} of less than 10 μM. Of these three compounds only the tetramethoxy substituted ether (**5**) had a strong ability to block cells in the G_2/M phase of the cell cycle. An ability to arrest cells in the G_2/M phase suggested that the agent (**5**) could be antimitotic. However, this agent (**5**) neither inhibited the assembly of tubulin nor displaced colchicine from its binding site on tubulin. The ethers (**2** and **3**) and amine (**13**) showed some ability to block cells in the G_2/M phase of the cell cycle but were again unable to inhibit the assembly of tubulin or to displace colchicine from tubulin. The two amines (**10** and **12**) were able to displace colchicine from tubulin but were not particularly cytotoxic and were not able to inhibit the assembly of tubulin.

These results suggest that trimethoxy substitution on one aryl ring with a small alkyl/alkoxy group on the 4′-postion of another aryl ring in the same molecule is not sufficient to produce an antimitotic agent. Previous experiments have shown that the number of atoms between the two aryl rings affects antimitotic properties. Optimal inhibition of tubulin occurred when the two aryl rings were separated by two carbons.[4] However, some diaryl methanes,[3–4] amphethinile (**15**), the sulfide (**18**) and the selenide (**16**)—which all only possess a one atom unit between their aryl rings—do exhibit antimitotic activity. The agents (**2–13**) described herein contain a single atom (N or O) separating their two aryl rings. However, their antimitotic activity is moderate at best.

The antimitotic agents amphethinile (**15**), combretastatin A-4 (**1**) and colchicine (**17**) possess two aryl rings which are tilted with respect to each other. The ring systems of these agents (**1**, **15** and **17**) are

Table 1. Growth inhibition in P388 murine leukemia cells

Compound	ID_{50} (μM)	Inhibition of tubulin assembly (%)	Colchicine displacement (%)	Accumulation of cells in G_2/M phase (%)
2	15.9	82	54	38
3	38.2	–	82	30
4	15.9	50	66	13
5	4.1	82	82	78
6	8.6	–	95	4
7	7.2	–	92	6
8	>152	–	74	NT
9	>151	–	85	11
10	106	–	22	12
11	46.9	–	90	15
12	28.4	–	37	13
13	83.4	–	92	32
15	0.5	26	50	76

Inhibition of tubulin assembly (%) at a dose of 100 μM.
Percentage of [^{3}H]colchicine bound to tubulin after drug competition with a drug:protein ratio=10:1.
Accumulation of cells in the G_2/M phase of the cell cycle 24 h after administration of drugs at 10 × ID_{50} concentration.

superimposable on each other and the planar aryl rings of each molecule are at an angle of approximately 60 to each other.[6] In its minimal energy conformations the angle between the planes of the aromatic rings of combretastatin A-1 (**14**) is 62 . However, the ether (**5**) and amines (**10** and **12**) both have angles of 78 between their aryl rings, whilst the same angle for amide (**6**) is 84 . Yet the ether (**5**), the amines (**10** and **12**) and the amide (**6**) structures in their minimum energy conformations can overlap well with amphethinile (**15**) with RMS deviations of 0.42 Å or less. This three-dimensional complementarity between the antimitotic amphethinile (**15**) and agents (**5**, **6**, **10** and **12**) would perhaps suggest that the latter compounds could possess antimitotic activity. However, these agents (**5**, **6**, **10** and **12**), whilst displaying some cytotoxicity and anti-mitotic activity (Table 1), are not as potent as combretastatin A-4 (**1**) or amphethinile (**15**).

Conclusion

Obviously structural similarities between molecules will enhance the possibility of them having similar biochemical effects. However, the whole chemistry of the molecules will also play a role in their biological activity. In the cases described here the important biochemistry may be derived from both molecular shape and from the inherent chemistry of the substrates. Differences in size, charge, *d*-orbital availability between the sulfur and oxygen and nitrogen heteroatoms may provide a reason for the differing biochemistries of amphethinile (**15**) and the ethers (**2–5**), amines (**6–9**) and amides (**10–13**) described here.

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(Received 9 April 1998; revised form accepted 23 April 1998)