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Cytotoxic Mannich Bases of 1-Arylidene-2-tetralones

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Various 1-arylidene-2-tetralones 1 had been shown previously to possess moderate cytotoxic properties unaccompanied by murine toxicity. The objective of the present investigation was to undertake different molecular modifications of representative members of series 1 with a view to discerning those structural features leading to increased potencies. All compounds were evaluated using human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells. The Mannich bases 2, 4, 5 and 7 possessed increased potencies compared to the corresponding unsaturated ketones 1 and in general were potent cytotoxics having IC_{50} values in the 0.2–10 μM range. QSAR using the cytotoxicity data for 2a–e suggested that potency was positively correlated with the size of the substituents in the arylidene aryl ring. Compounds 2a–f were evaluated using a panel of approximately 53 human tumour cell lines and, when all cell lines were considered, were more potent than the reference drug melphalan. In particular, marked antileukemic activity was displayed. Molecular modeling was utilized in order to evaluate whether the shapes of the different compounds contributed to the varying potencies observed. Representative compounds demonstrated minimal or no inhibiting properties towards human N-myristoyltransferase (NMT) and did not bind to calf thymus DNA. This study has revealed a number of unique lead molecules as candidate antineoplastic agents serving as prototypes for future development.

Keywords: Mannich bases; 2-Tetralones; Cytotoxicity; N-myristoyltransferase

INTRODUCTION

One of the principal aims of this laboratory is the discovery of novel cytotoxic agents that are thiol alkylators. This goal is based on the premise that compounds demonstrating an exclusive or preferential avidity for cellular thiols, in contrast to amino or hydroxy groups which are found in nucleic acids, should be bereft of the mutagenic and carcinogenic properties displayed by a number of currently available anticancer drugs.[1] A group of compounds possessing such properties are α,β-unsaturated ketones[2,3] and these molecules form the basis of the current investigation.

Very recently, a series of 1-arylidene-2-tetralones **1** has been described which displayed cytotoxic properties.[4] These molecules were designed on the following basis. First, these compounds possess an α,β-unsaturated keto group. Second, 1-arylidene-2-tetralones contain the structural motifs of two groups of antineoplastic agents, namely 2-arylidenecyclohexanones[5] and stilbenes.[6] Examination of representative members of this series of compounds against a panel of human tumour cell lines revealed that their average potency was more than three times that of the clinically used alkylating agent melphalan. The cytotoxic activity was shown to be caused in part by

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interference with macromolecular syntheses and probably the redox potentials in neoplastic cells. In addition, approximately half of the unsaturated ketones **1** were examined for toxicity in mice using doses up to and including 300 mg/kg. Neither mortalities nor other signs of toxicity were observed. Furthermore, doses of 30 mg/kg of two representative members of series **1** administered orally to rats did not lead to toxic symptoms. Hence the potent cytotoxicity towards various neoplastic cells and the absence of marked toxic *in vivo*, as well as the novelty of the structures, dictated further explorations of this cluster of unsaturated ketones as candidate cytotoxics.

A further reason for preparing thiol alkylators as candidate cytotoxic agents was as follows. The enzyme myristoyl-CoA:protein N-myristoyltransferase (NMT) catalyzes the attachment of myristate onto the N-terminal glycine residues of certain polypeptides.[7] A number of studies, which revealed that the activity of NMT in certain colonic and gall bladder cancers was greater than in the corresponding normal cells, have been reviewed recently.[8] Hence NMT represents an important molecular target in oncology research. According to the model of the mode of action of this enzyme, proposed by Peseckis and Resh, myristoylation involves the thiol group of the cysteine-169 residue of this enzyme.[9] Various cytotoxic Mannich bases of conjugated unsaturated ketones, designed to undergo electrophilic attack with cellular thiols, inhibit human NMT (hNMT).[10] Hence, the evaluation of representative compounds prepared in this study towards hNMT was planned.

A number of organic bases bind to DNA.[11] Therefore the examination of selected basic compounds (and their corresponding non-basic analogues) for this property was proposed in order to determine whether DNA binding contributed to any of the observed cytotoxicity.

The objectives of the present investigation included the syntheses of a small number of prototypic molecules designed to possess increased potencies compared to analogues in series **1**. In addition, the examination of certain physicochemical and biochemical properties of the new compounds was proposed. The data obtained should enable the formulation of guidelines for the expansion of lead molecules. The decisions regarding the specific compounds to be prepared were based on the considerations given below.

The conversion of a number of the enones into the corresponding Mannich bases was proposed for the following reasons. First, a previous study revealed that the rates of reaction with a thiol of various Mannich bases of a number of conjugated styryl ketones were approximately 240 times faster than was found with the precursor enones.[12] In addition, where biodata were available, these Mannich bases displayed significantly greater cytotoxicity than the styryl ketones from which they were derived.[13] Thus a comparison of the cytotoxicity between representative 1-arylidene-2-tetralones **1** and the corresponding Mannich bases **2** was planned. The results obtained would permit a decision to be reached as to the utility of this molecular modification of series **1**.

In addition, two further approaches were planned. The recent observation of the potent cytotoxicity of 3,5-bis(phenylmethylene)-4-piperidone **3** and related compounds has been described.[14] Compound **3** is a 3-aminoketone and thus may be regarded as a cyclic Mannich base. The attachment of **3** to a 1-arylidene-2-tetralone via an amidic linkage was considered, suggesting the syntheses of **4** and **5**. The compounds could be cytotoxic per se and/or demonstrate bioactivity by undergoing hydrolysis to liberate the corresponding 1-arylidene-2-tetralone, the cytotoxicity of which would be augmented by the release of **3**. Finally, in view of the cytoprotective effect of various cysteamine derivatives,[15] the thioester **7** was considered, which could undergo hydrolysis liberating N-acetylcysteamine and a candidate cytotoxic. Since a number of small molecular weight thiols afford protection to normal cells without interfering with the antineoplastic effect of cytotoxic drugs,[16,17] **7** may display greater toxicity to tumours than to corresponding normal cells.

EXPERIMENTAL PROCEDURES

Chemistry

Melting points in Celsius degrees are uncorrected and yields are expressed as percentages. Elemental analyses (C, H, N) were undertaken on **2a–f**, **4**, **5**, **6a**, **b** and **7** by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan and were within 0.4% of the calculated values. Compounds **2a**, **c**, **d**, **f**, **4**, **5**, **6a** and **7** were obtained as hemihydrates. ^{1}H NMR spectra were determined using a Bruker AM 500 FT NMR machine (500 MHz).

Synthesis of 2a–f

The 1-arylidene-2-tetralones **1a–f** were prepared by a literature procedure.[4] In addition, **1d** was also prepared by an alternate route as follows. Hydrogen chloride was passed into a suspension of 4-formylbenzoic acid (10 mmol) and 2-tetralone (10 mmol) in diethyl ether (25 ml) for 0.5 h. The resultant suspension was stirred at room temperature for 12 h. The precipitate was collected, washed with diethyl ether (2 × 15 ml) and recrystallized from methanol:chloroform (9:1) to give **1d** in 76% yield.

1-Methylenepiperidinium chloride was freshly prepared by adding acetyl chloride (1.5 mmol) to a solution of dipiperidinomethane (1.5 mmol) in diethyl ether (20 ml) at 0°C. The mixture was stirred for 0.25 h, the resultant precipitate was collected and added to a solution of the 1-arylidene-2-tetralone (1.0 mmol) in dry acetonitrile (15 ml). After stirring at room temperature (25°C) for 16 h, the solvent was removed in vacuo and the residue was recrystallized from a mixture of ether and methanol (2:8). The melting points and yields were as follows: **2a**: 194 (dec.), 54; **2b**: 230 (dec.), 56; **2c**: 234–236, 48; **2d**: 200 (dec.), 32; **2e**: 228 (dec.), 38; **2f**: 179–180, 46. The Mannich base **2d** was also prepared by an alternative route. A mixture of **1d** (5 mmol), paraformaldehyde (5 mmol), piperidine hydrochloride (5 mmol) and acetic acid (30 ml) was heated at 75–80°C for 1 h. On cooling, the precipitate was collected, washed with acetic acid (2 × 10 ml) and recrystallized from methanol to give **2d** in 58% yield. The ^{1}H NMR spectrum of a representative compound, namely **2e**, was as follows. The numbering of the carbon atoms in the tetralin, heterocyclic and aryl rings are unprimed, single primed and double primed, respectively. δ ($CDCl_3$): 1.40–1.46 (m, 1H, C4′H), 1.82–1.89 (m, 3H, C3′H, C4′H, C5′H), 2.17–2.26 (m, 1H, C3′H), 2.32–2.40 (m, 1H, C5′H), 2.69–2.79 (m, 2H, 2 × C4H), 3.01–3.10 (m, 3H, C2′H, C6′H, C3H), 3.49–3.58 (m, 3H, NCH_a, C2′H, C6′H), 3.62–3.65 (dd, 1H, NCH_b), 3.79 (s, 3H, OCH_3), 6.76–6.78 (d, 2H, C2″H, C6″H), 7.01–7.04 (t, 1H, C6H), 7.18–7.21 (t, 1H, C7H), 7.39–7.41 (d, 2H, C5H, C8H), 7.43–7.44 (d, 2H, C3″H, C5″H), 7.67 (s, 1H, CH =), 12.07 (br s, 1H, $NH^{(+)}$).

Synthesis of 4 and 5

Oxalyl chloride (12 mmol) was added to a suspension of 4-formylbenzoic acid (10 mmol) in chloroform (15 ml). The mixture was heated under reflux for 12 h and then in vacuo leading to 4-formylbenzoyl chloride which was used without further purification.

Triethylamine (20 mmol) was added to a suspension of 3,5-bis(phenylmethylene)-4-piperidone (10 mmol), which was prepared by a literature procedure,[14] in chloroform (25 ml). The mixture was cooled to 0–5°C to which a solution of 4-formylbenzoyl chloride vide supra in chloroform (5 ml) was added. After stirring at room temperature for 12 h, the organic solution was washed with water (20 ml) and hydrochloric acid (7.5% w/v, 25 ml). Evaporation of the solvent led to a residue which was purified using silica gel (60–200 mesh) and an eluting solvent of ethyl acetate in hexane (2% v/v) to give 1-(4-formylbenzoyl)-3,5-bis(phenylmethylene)-4-piperidone in 47% yield.

Acetic acid (50 mg), piperidine (50 mg) and 4 Å molecular sieves (3.0 g) were added sequentially to a solution of 2-tetralone (5 mmol) in chloroform (20 ml). Subsequently 1-(4-formylbenzoyl)-3,5-bis(phenylmethylene)-4-piperidone (4.67 mmol) was added and the mixture was stirred at room temperature for 24 h and filtered. The sieves were washed with chloroform (50 ml) and the organic phase was treated with sodium metabisulphite solution (5% w/v, 20 ml) for 0.5 h. The chloroform extract was separated, washed with water (25 ml) and saturated sodium chloride solution (25 ml). After removal of the solvent, the residue was chromatographed using a column of silica gel (60–200 mesh) and an eluting solvent of ethyl acetate in hexane (2% v/v). The product isolated was recrystallized from methanol: chloroform (9:1) to give **4**, mp 201–202°C in 61% yield.

An alternative route to compound **4** was accomplished as follows. A mixture of **1d** (10 mmol) and dicyclohexylcarbodiimide (10 mmol) in chloroform (20 ml) was stirred at room temperature for 0.5 h to which was added over 0.25 h a solution of **3** (10 mmol) in chloroform (15 ml). The resultant mixture was stirred at room temperature for 8 h and the solvent reduced to approximately 8–10 ml. On cooling to 10–15°C, dicyclohexylurea was removed by filtration and washed with chloroform (2 × 5 ml at 5–6°C). The organic solvent was removed in vacuo to give a residue which was recrystallized from methanol:chloroform (9:1) to give **4** in 72% yield. For the ^{1}H NMR spectrum, the carbon atoms of the tetralin ring, the aryl ring attached to the carbonyl group and the aryl rings attached to the heterocycle are unprimed, single primed and double primed, respectively. δ ($CDCl_3$): 2.63 (t, 2H, C4H, J = 6.5 Hz), 3.02 (t, 2H, C3H, J = 6.5 Hz), 4.64 (bs, 2H, NCH_2), 4.71 (bs, 2H, NCH_2), 6.94 (t, 1H, C6H), 7.08–7.11 (m, 6H, 2 × C3″H, 2 × C4″H, 2 × C5″H), 7.23 (t, 1H, C7H), 7.29 (d, 1H, C5H), 7.30–7.48 (m, 9H, C8H, C2′H, C3′H, C5′H, C6′H, 2 × C2″H, 2 × C6″H), 7.51 (s, 1H, CH =), 7.91 (s, 2H, 2 × CH =).

A suspension of **4** (1 mmol) in acetonitrile (20 ml) was heated under reflux to which was added 1-methylenepiperidinium chloride vide supra. The mixture was stirred at room temperature for 36 h. The solvent was removed in vacuo and the residue was recrystallized from methanol to give **5**, mp 180°C (dec.) in 42% yield. For the ^{1}H NMR spectrum, the carbon atoms on the tetralin, piperidine, aryl rings attached to the piperidone group and the aryl ring attached to the carbonyl group are designated as unprimed, single primed, double primed and triple primed, respectively. δ ($CDCl_3$): 1.43 (m, 1H, C4′H), 1.87 (m, 3H, C3′H, C4′H, C5′H), 2.23 (m, 1H, C3′H), 2.39 (m, 1H, C5′H), 2.69–2.79 (m, 2H, C4H), 3.01–3.14 (m, 3H, C3H, C2′H, C6′H), 3.45–3.58

(m, 3H, NCH_a, C2′H, C6′H), 3.67 (dd, 1H, NCH_b), 4.5–5.3 (br s, 4H, 2 × NCH_2), 6.93 (t, 1H, C6H), 7.06–7.13 (m, 6H, 2 × C3″H, 2 × C4″H, 2 × C5″H), 7.24 (t, 1H, C7H), 7.30–7.43 (m, 9H, C5H, C8H, 2 × C2″H, 2 × C6″H, C2‴H, C3‴H, C5‴H, C6‴H), 7.53 (s, 1H, CH =), 7.91 (s, 2H, 2 × CH =), 12.25 (s, 1H, NH +).

Synthesis of 6a and 6b

Oxalyl chloride (23.6 mmol) was added to a suspension of 4-formylbenzoic acid (20 mmol) in chloroform (30 ml). The mixture was heated under reflux for 12 h and then in vacuo to give a solid, which was used without further purification. The acid chloride was added to a solution of piperidine (20 mmol) and N,N-dimethylaminopyridine (20 mmol) in chloroform (30 ml), which was cooled with an ice bath. The mixture was stirred at room temperature for 12 h and the organic phase was washed with hydrochloric acid (7.5% w/v) and water. Evaporation of the solvent afforded a residue which was purified by column chromatography using silica gel (60–200 mesh) and an eluting solvent of ethyl acetate in hexane (20% v/v) to give 4-(1-piperidinylcarbonyl)-benzaldehyde in 41% yield. For the ^{1}H NMR spectrum, the number of the carbon atoms of the piperidine ring are primed. δ ($CDCl_3$): 1.50 (br s, 2H, C4′H), 1.67 (br s, 4H, C3′H, C5′H), 3.27 (br s, 2H, C2′H), 3.71 (br s, 2H, C6′H), 7.52 (d, 2H, C3H, C5H, J = 8.00 Hz), 7.90 (d, C2H, C6H, J = 8.00 Hz), 10.02 (s, 1H, CHO).

4-(1-Piperidinylcarbonyl)benzaldehyde (5 mmol), piperidine (50 mg), acetic acid (50 mg) and 4 Å molecular sieves (2.0 g) were added to a solution of 2-tetralone (5 mmol) in chloroform (20 ml). The mixture was stirred at room temperature for 12 h and filtered. The sieves were washed with chloroform (25 ml) and after treatment with sodium metabisulfite solution (5% w/v, 25 ml), the organic phase was evaporated to produce a residue which was purified by column chromatography using silica gel (60–200 mesh) and a solvent system of ethyl acetate in hexane (10% v/v) to give a product which was recrystallized from ether: chloroform (8:2) yielding **6b**, mp 122–123°C in 58% yield. In the ^{1}H NMR spectrum, the carbon atoms on the tetralin, aryl and heterocyclic rings were designated as unprimed, single primed and double primed, respectively. δ ($CDCl_3$): 1.56 (br s, 2H, C4″H), 1.65 (br s, 4H, C3″H, C5″H), 2.62 (t, 2H, C4H), 3.03 (t, 2H, C3H), 3.32 (br s, 2H, C2″H), 3.68 (br s, 2H, C6″H), 7.00 (t, 1H, C6H), 7.18–7.22 (t, 1H, C7H), 7.23–7.32 (m, 3H, C5H, C3′H, C5′H), 7.38 (d, 1H, C8H, J = 8.11 Hz), 7.48 (d, 2H, C2′H, C6′H, J = 8.11 Hz), 7.62 (s, 1H, CH =).

The experiment was repeated using 4-oxopiperidine in place of piperidine. After column chromatography, the product obtained was recrystallized from methanol to produce **6a**, mp 161–162°C, in 22% yield. For the ^{1}H NMR spectrum, the numbers of the carbon atoms of the tetralin, aryl and heterocyclic rings are unprimed, single primed and double primed, respectively. δ ($CDCl_3$): 2.35–2.56 (m, 4H, C3″H, C5″H), 2.63 (t, 2H, C4H), 3.04 (t, 2H, C3H), 3.73 (br s, 2H, C2″H), 3.99 (br s, 2H, C6″H), 7.01 (t, 1H, C6H), 7.28 (t, 1H, C7H), 7.20–7.26 (m, 2H, C5H, C8H), 7.36 (d, 2H, C2′H, C6′H, J = 8.19 Hz), 7.46 (d, 2H, C3′H, C5′H, J = 8.11 Hz), 7.62 (s, 1H, CH =).

Synthesis of 7

A solution of 4-formylbenzoic acid (10 mmol), thionyl chloride (20 mmol), dimethylformamide (0.02 ml) and chloroform (20 ml) was heated under reflux for 3 h. The solvent and excess thionyl chloride were removed in vacuo to give 4-formylbenzoyl chloride, which was used without further purification. A solution of this acid chloride in chloroform (15 ml) was added over 10 min to a solution of N-acetylcysteamine (10 mmol), which was prepared by a literature method,[18] triethylamine (20 mmol) and chloroform (20 ml) at 0–5°C. After stirring at room temperature for 4 h, water (15 ml) was added. The organic layer was separated, washed with saturated sodium bicarbonate solution (15 ml), and saturated sodium chloride solution (15 ml). Removal of the solvent in vacuo gave 4-(2-acetylaminoethylthiocarbonyl)benzaldehyde. ^{1}H NMR: δ (DMSO-d_6): 1.90 (s, 3H, CH_3), 3.23 (t, 2H, SCH_2), 3.53 (q, 2H, NCH_2), 6.11 (br s, 1H, NHCO), 7.95 (d, 2H, aryl H, J = 8.26 Hz), 8.07 (d, 2H, aryl H, J = 8.90 Hz), 10.15 (s, 1H, CHO).

A solution of the thioester (3 mmol), 2-tetralone (3 mmol), acetic acid (20 mg), piperidine (20 mg), 4 Å molecular sieves (1.0 g) and chloroform (10 ml) was stirred at room temperature for 12 h. After filtration, the molecular sieves were washed with chloroform (10 ml) and the organic solution was washed with sodium metabisulfite solution (5% w/v), water and saturated sodium chloride solution. After evaporation, the resultant solid was purified using a column of silica gel (60–100 mesh) and an eluting solvent of ethyl acetate in hexane (3% v/v) to give 1-[4-(2-acetylaminoethylthiocarbonyl)phenylmethylene]-2-tetralone. In the ^{1}H NMR spectrum, the carbon atoms in the tetralin and aryl rings are unprimed and single primed, respectively. δ ($CDCl_3$): 1.96 (s, 3H, CH_3), 2.63 (t, 2H, C4H), 3.05 (t, 2H, C3H), 3.21 (t, 2H, SCH_2), 3.52 (q, 2H, NCH_2), 5.88 (br s, 1H, NHCO), 7.00 (t, 1H, C6H), 7.20–7.34 (m, 3H, C5H, C7H, C8H), 7.45 (d, 2H, C3′H, C5′H, J = 8.30), 7.62 (s, 1H, CH =), 7.83 (d, 2H, C2′H, C6′H, J = 8.37 Hz).

Reaction of *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (2 mmol) with acetyl chloride (2 mmol) in diethyl ether (10 ml) at 0–5°C led to the formation of methylenedimethylammonium chloride, which was

collected and used without further purification. This product was added to a mixture of the 1-arylidene-2-tetralone vide supra (1.5 mmol) in acetonitrile (25 ml) and heated under reflux for 10 h. The solvent was removed in vacuo and the residue was recrystallized from a mixture of ether and methanol (9:1) to give **7**, mp 142–143 in 30% yield (based on the quantity of 1-arylidene-2-tetralone used). For the ^{1}H NMR spectrum, the carbon atoms of the tetralin and aryl rings are unprimed and single primed, respectively. δ ($CDCl_3$): 1.96 (s, 3H, CH_3), 2.83 (d, 3H, NCH_3), 2.90 (d, 3H, NCH_3), 2.95–3.01 (m, 1H, C3H), 3.11–3.20 (m, 4H, CH_2S, C4H), 3.47 (q, 2H, NHCH_2), 3.61–3.70 [m, 2H, CH_2N(CH_3)$_2$], 6.10 (s, 1H, CONH), 6.99 (t, 1H, C6H), 7.15 (d, 1H, C5H), 7.24 (t, 1H, C7H), 7.42 (merged dd, 3H, C8H, C3′H, C5′H), 7.67 (s, 1H, CH =), 7.82 (d, 2H, C2′H, C6′H), 12.53 (s, 1H, NH^+).

Statistical Analysis

The σ, π and MR values of the aryl substituents in **2a–e** were taken from the literature[19] and the figures for both the R^1 and R^2 groups were combined. Linear and semilogarithmic plots were obtained using a commercial software package.[20] The following correlations and trends towards significance were noted [physicochemical constant, assay, linear(l) or semilogarithmic (sl) plots, Pearson's correlation coefficient and p value] namely: MR, P388, l, −0.838, 0.076; MR, P388, sl, −0.826, 0.085; MR, L1210, l, −0.747, 0.147; MR, Molt 4/C8, l, −0.871, 0.055; MR, Molt 4/C8, sl, −0.885, 0.046; MR, CEM, l, −0.799, 0.105; MR, CEM, sl, −0.752, 0.143. The correlations and trends to significance found with the SI values of the human (SI_h) or murine (SI_m) cell lines were as follows [physicochemical constant, SI, linear (l) or semilogarithmic (sl) plots, Pearson's correlation coefficient and p value]: MR, SI_h, l, −0.785, 0.116; MR, SI_h, sl, −0.761, 0.135; σ, SI_h, l, −0.815, 0.093; σ, SI_h, sl, −0.843, 0.073; σ, SI_m, l, 0.765, 0.135; σ, SI_m, sl, 0.775, 0.123.

Molecular Modeling

Models of **1a**,**c**, **2a**,**c** and **3–6** were built with a MacroModel 8.0 programme[21] followed by a Monte Carlo search for the lowest energy conformations using an Amber force field of 1000 initial conformations. Compounds **2a**,**c**, **3** and **5** were modeled as the free bases and also as the corresponding protonated species. Measurements were made on the lowest energy conformations.

Cytotoxicity Evaluations

The compounds were evaluated for cytotoxicity in the Molt 4/C8, CEM and L1210 screens using a literature procedure.[22] In brief, solutions of at least three different concentrations of compounds in suitable solvents were incubated with the cells at 37°C. After 48 h, the percentage survival of the cells was recorded. Control experiments were undertaken whereby the solvents and neoplastic cells were also incubated at 37°C for 48 h. The assays were carried out in triplicate at each concentration of the compounds. A similar procedure was adopted using P388 cells.[23] The method for evaluating **2a–f** against 53(51–58) cell lines and melphalan has been reported previously.[24] In brief, the human tumour cell lines were exposed to at least five different concentrations of the compounds. After 48 h, cell viability and growth were determined by a protein assay using sulphorhodamine B. The MG MID figures quoted in Table IV are IC_{50} values for **2a–c**, **e** and melphalan. In the case of **2d**, 3/58 cell lines had MG MID figures greater than 10^{-4} M including 2/8 breast cancer cell lines. In addition, evaluation of **2f** revealed that 2/51 cell lines had MG MID values of greater than 10^{-4} M. The figure of 10^{-4} M was included in the calculations of the MG MID values.

hNMT Assays

The evaluation of **1d**, **2d**, **4** and **5** as candidate inhibitors of hNMT was undertaken by a literature procedure.[4] In brief, *Escherichia coli* DH5α with recombinant pT7-7 ·hNMT was grown to stationary phase in LB medium and the cells were harvested after 2 h incubation at 37°C. The recombinant hNMT was purified by a literature procedure[25] and the assays were carried out in the presence of cAMP-dependent protein kinase derived peptide.[26,27]

DNA Binding Study

The ketones **1d**, **2d**, **4** and **5** were examined for DNA binding properties using an ethidium displacement assay.[28] In brief, excess of the drug was incubated with 50 μg/ml of calf thymus DNA at 20°C for 24 h in a tromethamine hydrochloride buffer, pH 8. An aliquot of the DNA was removed and assessed for its ability to bind ethidium.

RESULTS

Series **1** was prepared by the Knoevenagel method using piperidine as the catalyst.[4] (*E*), (*E*)-3,5-bis (Phenylmethylene)-4-piperidone **3** was prepared by acid catalyzed condensation between benzaldehyde and 4-piperidone.[14] The remaining compounds were synthesized by the routes outlined in Figure 1. Subsequently, successful attempts were made to improve the yields of some of the compounds as

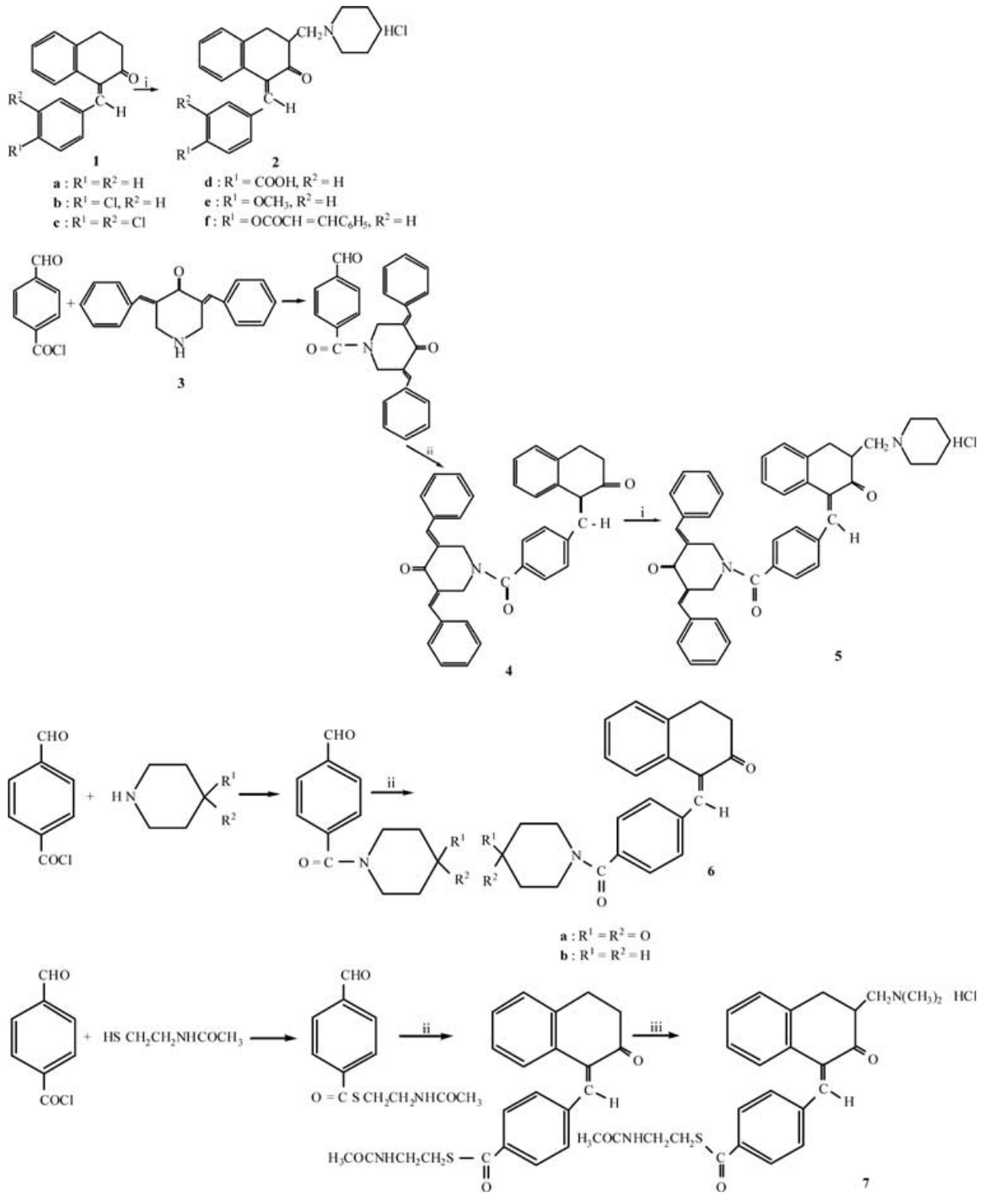

FIGURE 1 Synthetic routes employed in preparing compounds **2**, **4**–**7**. The reagents used were as follows: (i): 1-methylenepiperidinium chloride, (ii): 2-tetralone and (iii): methylenedimethylammonium chloride.

follows. Compound **1d** was also prepared from 4-carboxybenzaldehyde and 2-tetralone using acidic catalysis, whereby the percentage yield rose to 76 from 40. It was converted into the corresponding Mannich base **2d** using paraformaldehyde and piperidine hydrochloride in 58% yield, rather than 1-methylenepiperidinium chloride in which case the yield was 32%. In addition, **4** was prepared by dicyclohexyldiimide coupling between **1d** and **3** in 72% yield, compared to 29% when the initial procedure was followed.

The compounds in series **2**–**7** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 cells. These data

TABLE I Evaluation of the compounds in series **2**–**7** against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells

	IC_{50} (μM)[a]			IC_{50} (μM)[a]		
Compound	Molt 4/C8	CEM	SI[b]	P388	L1210	SI[b]
2a	28.1 ± 15.8	15.8 ± 5.4	1.79	0.379 ± 0.05	10.4 ± 0.7	27.4
2b	9.38 ± 0.42	7.15 ± 0.10	1.31	0.234 ± 0.02	7.38 ± 0.49	31.5
2c	8.49 ± 0.8	8.11 ± 0.46	1.05	0.242 ± 0.01	7.55 ± 0.44	31.2
2d	8.83 ± 1.54	9.08 ± 0.26	1.03	0.262 ± 0.03	7.10 ± 0.13	27.1
2e	9.20 ± 1.99	6.11 ± 1.02	1.51	0.269 ± 0.01	5.89 ± 0.04	21.9
2f	24.0 ± 14.7	10.2 ± 0.9	2.35	0.240 ± 0.01	7.80 ± 0.03	32.5
3[c]	1.67 ± 0.15	1.70 ± 0.02	1.02	0.77 ± 0.02	7.96 ± 0.11	10.3
4	9.65 ± 0.63	7.68 ± 0.34	1.26	0.355 ± 0.03	15.5 ± 0.8	43.7
5	5.49 ± 3.03	1.64 ± 0.12	3.35	0.085 ± 0.01	2.05 ± 0.16	24.1
6a	38.2 ± 5.2	19.1 ± 8.8	2.00	15.3 ± 1.1	40.3 ± 3.7	2.63
6b	40.0 ± 0.3	24.1 ± 10.3	1.66	9.40 ± 1.6	35.2 ± 1.0	3.75
7	7.46 ± 0.06	8.83 ± 0.40	1.18	0.294 ± 0.03	7.04 ± 0.31	24.0
Melphalan[d]	3.24 ± 0.79	2.47 ± 0.30	1.31	0.22 ± 0.01	2.13 ± 0.03	9.68

[a] The IC_{50} value indicates the concentration of compound required to inhibit the growth of the malignant cell lines by 50%. [b] The letters SI indicate the selectivity index, i.e. the ratios of the IC_{50} values generated using either the T-lymphocytes or murine leukemic cells. [c] Reprinted in part with permission from *J. Med. Chem.* **44**, 586. Copyright (2001) American Chemical Society. [d] The IC_{50} figures are reproduced from *Eur. J. Med. Chem.* (2000) **35**, 970.

are presented in Table I. A quantitative structure-activity relationship (QSAR) study of **2a**–**e** revealed that the size of the aryl substituents probably had a dominant effect on potency. Molecular modeling was employed with a view to discerning whether the shapes of representative compounds were related to the different potencies. In addition, **2a**–**f** were examined for cytotoxic activity towards approximately 53 human tumour cell lines. These results are given in Table IV. Four representative compounds **1d**, **2d**, **4** and **5** were examined for inhibitory effects on of hNMT. At the highest concentrations utilized, **4** and **5** inhibited 29% and 10% of the activity of the enzyme, while **1d** and **2d** had no effect. None of these four compounds bound to calf thymus DNA.

DISCUSSION

The enones prepared in this study were shown by ^{1}H NMR spectroscopy to be isomerically pure. Previous investigations using X-ray crystallography revealed that the olefinic bond of representative members of series **1** as well as the hydrochloride salt of **3** adopted the *E* configuration.[29,30] The compounds **2** and **4**–**7** prepared in this study were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells. These data are portrayed in Table I. Two human cell lines were chosen in order that a comparison of the potencies of the compounds **2**–**7** for these neoplasms could be made. Similarly, two murine cell lines were also employed. These comparisons were based on the assumption that compounds displaying varying potencies may display some selectivity for tumour cells rather than the corresponding normal tissue.

The data in Table I revealed that **2** and **4**–**7** were, in general, potent cytotoxics with 71% of these enones having IC_{50} values of less than 10 μM. P388 leukemic cells were the most sensitive to these molecules and, with the exception of **6a** and **6b**, the IC_{50} values of all of these compounds were less than 0.4 μM in this screen. The first consideration of the structure-activity relationships was whether incorporation of an aminoalkyl group into the tetralin ring would lead to potency increases. The data in Table II revealed that the conversion of each of the tetralones **1** into the corresponding Mannich bases **2** led to

TABLE II The ratios of the potencies and SI values of **2a**–**f** and **5** and the analogues without a 3-(1-piperidylmethyl) hydrochloride group[a]

	Human T-lymphocytes			Murine leukemic cells		
Compound	Molt 4/C8	CEM	SI[b]	P388	L1210	SI[b]
2a	1.39	2.48	1.77	38.5	4.27	9.01
2b	3.34	3.55	1.07	29.9	5.37	5.57
2c	1.67	3.13	0.587	14.8	5.14	2.86
2d	7.29	5.57	0.811	73.7	9.49	7.77
2e	3.58	3.06	0.858	27.8	8.49	3.28
2f	>20.8	39.3	>1.21	73.3	55.9	0.883
5	1.76	4.68	2.66	4.18	7.56	0.552

[a] These potency ratios were computed by dividing the IC_{50} values of the nonbasic analogues **1a**–**f** and **4** by the IC_{50} figures of the Mannich bases **2a**–**f** and **5**, respectively. The cytotoxicity data of **1a**–**f** were described in Ref. [4]. The SI ratios were calculated by dividing the SI figures for **2a**–**f** and **5** by the data for **1a**–**f** and **4**, respectively. [b] The letters SI refer to the selectivity index.

increased cytotoxicity. The IC_{50} value of **1f** towards Molt 4/C8 cells was $>500\,\mu M$.[4] Hence a specific increase in the potency of **2f** compared to **1f** could not be computed for this cell line. After omitting this datum, the average increase in potencies of **2a–f** compared to **1a–f** was 18.3 fold when all four cell lines were taken into consideration.

The figures in Table II revealed that the largest average increase in potencies of **2a–f** compared to **1a–f** was 43-fold, which was found in the P388 screen. In addition, conversion of **4** into the corresponding Mannich base **5** led to an average increase in potency of 4.55, although in this case the greatest increase in potency was noted in the L1210 screen. Thus the formation of the Mannich bases **2** and **5** from the corresponding α,β-unsaturated ketones **1** and **4**, respectively, invariably led to increases in cytotoxicity, indicating the importance and usefulness of this molecular modification.

In addition to the insertion of an aminomethyl group into the tetralone ring, the following observations were noted pertaining to other molecular modifications. Both **1d** and **2d** were coupled with the cytotoxin **3**, which led to **4** and **5**, respectively. While **2d** and **5** were equiactive in the Molt 4/C8 screen, the remaining seven comparisons revealed that **4** and **5** were significantly more potent than **1d** and **2d**, respectively. In order to obtain an insight into the contributions to cytotoxicity of some of the different groups of **4**, molecular simplification[31] was undertaken. Thus, in both **6a** and **6b**, the arylidene rings attached to the heterocycle in **4** were excised and, in addition, the 4-oxo atom was removed in **6b**. The data in Table I revealed that **6a** and **6b** had similar IC_{50} values and both compounds were significantly less potent than **4**. Thus the two phenylmethylene groups attached to the heterocycle of **4** contributed markedly to the potency displayed by **4**. In addition, since both **6a** and **6b** had greater potencies than **1d**, the heterocycle ring of **4** likely contributed to the observed cytotoxicity. Molecular modification of **1d** gave rise to **7** leading to increases in potencies in all four screens.

Melphalan is an alkylating agent used in cancer chemotherapy and a comparison was made between this drug and the compounds prepared in this study. The potencies of the Mannich base **5** were 0.6, 1.5, 2.6 and 1.0 times that of melphalan in the Molt 4/C8, CEM, P388 and L1210 screens, respectively. This observation revealed compound **5** to be an important lead molecule. In addition, **2a–f**, **4** and **7** possessed an average 80 (58–94)% of the potency of melphalan in the P388 screen.

Thus expansion of the 1-arylidene-2-tetralones as candidate cytotoxics should bear in mind the results of this study which demonstrate clearly the importance of the insertion of an aminoalkyl group at position 3 of the tetralin nucleus and the attachment of cytotoxic amines to the arylidene aryl ring via a potentially labile group.

As stated earlier, a valuable feature of a cytotoxic agent is the ability to display differential potencies towards different cancer cell lines. The selectivity index (SI) figures were computed for both the human T-lymphocytes and murine leukemic cells, and the results are presented in Table I. A SI figure of 10 was arbitrarily chosen as evidence of noteworthy selectivity. The data in Table I revealed that all of the compounds in series **2–5** and **7** not only met this criteria when murine leukemic cells were considered, but had greater SI values than melphalan. The highest figure was obtained with **4** revealing it to be a useful lead molecule. Half of the compounds **2–7** had greater SI figures than melphalan, when the data for human T-lymphocytes were reviewed, and maximum activity was noted with **5**. However, the SI values were lower than those found using murine cells. The figures in Table II pertaining to SI ratios showed that, in general, conversion of the enones **1a–f** and **4** into the corresponding Mannich bases **2a–f** and **5**, respectively, resulted in increased selectivity. In summary, the SI data showed that most comparisons resulted in greater selectivity than melphalan and also that the data from murine leukemic cells provided ample evidence for developing these novel prototypic cytotoxic agents.

The possibility that cytotoxicity was influenced by one or more physicochemical properties of the aryl substituents in series **2** was addressed as follows. The electronic, hydrophobic and steric properties of the R^1 and R^2 groups may be described by the Hammett sigma (σ), Hansch pi (π) and molar refractivity (MR) constants, respectively. Linear and semilogarithmic plots were constructed between each of these figures. for **2a–e** and the IC_{50} values generated in the four cytotoxicity screens, as well as the SI data obtained from the human and murine cell lines. (Compound **2f** was not included in these analyses due to the apparent unavailability of the substituent constants for the (*E*)-3-phenyl-2-propenoyloxy group). A negative correlation was noted between the MR values of the aryl substituents and the IC_{50} figures in the Molt 4/C8 ($p < 0.05$) screen. In addition, trends were noted towards negative correlations between the MR constants and the IC_{50} figures obtained in the P388 ($p < 0.1$), L1210 ($p < 0.15$) and CEM ($p < 0.15$) tests, as well as the SI values using human T-lymphocytes ($p < 0.15$). Both negative and positive trends towards significance were observed between the σ constants and the human ($p < 0.1$) and murine ($p < 0.15$) values, respectively. No other correlations ($p > 0.15$) were observed. The conclusion to be drawn from this analysis is that in planning expansion of series **2**, increase in the size of the aryl substituents is likely to

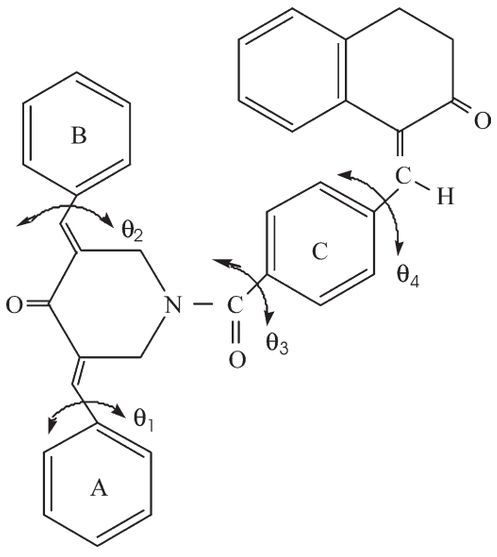

FIGURE 2 Torsion angles $\theta_1-\theta_4$ measured for compounds **3**–**6**.

TABLE III The torsion angles $\theta_1-\theta_4$ found in compounds **3**–**6**[a]

Compound	θ_1°	θ_2°	θ_3°	θ_4°
3	−47.8, −85.9	47.7, 85.8	–	–
4	−51.3	46.4	43.8	−87.0
5	77.5, −59.2	55.0, 56.1	54.8, 69.4	−105.5, −87.6
6a	–	–	44.1	−38.1
6b	–	–	44.4	−37.3

[a] The positive and negative figures refer to determinations of the torsion angles being clockwise and anticlockwise, respectively. In the case of **3** and **5**, the two numbers refer to the figures generated for the free base and protonated species, respectively.

lead to potency increases. On the other hand, variation of the σ and π constants of the R^1 and R^2 groups is likely to play a much smaller role in governing potencies.

The greater cytotoxicity of the compounds in series **2**, compared to the analogues in series **1**, could have been due to the 1-piperidylmethyl group altering the locations of those groups which interact at a binding site. For example, if cytotoxicity was influenced by the positions of the arylidene aryl ring and olefinic and oxygen atoms in relation to each other, then the introduction of the 1-piperidylmethyl group in **2** may have led to differences between the relative positions of these groups in the compounds **1** and **2**. In order to explore this possibility, the molecular models of **1a**, **2a** (free base) and **2a** (protonated species) were built. The C1, C2, C3 and C4 atoms were superimposed and atoms that were common to all three molecules occupied very similar positions in space. In fact the root mean square (RMS) for the fit was 0.017 Å. The experiment was repeated with **1c** and **2c** as the free base and protonated form in which case a similar observation was made (RMS = 0.008 Å). Thus, the increased cytotoxicity of the compounds in series **2** is likely due to the presence of the 1-piperidylmethyl group per se and not to it causing differences between the location of potential pharmacophoric atoms or groups in series **1** and **2**.

A review of the IC_{50} values of **3**, **4** and **5** in Table I revealed that, overall, the relative potencies were **5** > **3** > **4**. The question posed was whether differences in the shapes of the molecules accounted for the disparity in cytotoxicity. On occasions, bioactivity has been correlated with the interplanar angles between aryl rings and the adjacent unsaturated linkages.[32–34] Molecular models of **3**–**5** were built as well as the much weaker cytotoxic agents **6a** and **6b**. The torsion angles $\theta_1-\theta_4$ were measured as illustrated for the enone **4** in Figure 2. The $\theta_1-\theta_3$ values refer to the lack of coplanarity of the aryl rings A, B and C, respectively, with the adjacent unsaturated groups, while the θ_4 figures refer to the angles between ring C and the attached olefinic moiety. The data are presented in Table III.

Comments will be made initially when **3** and **5** were modeled as the free bases. The torsion angles θ_1 and θ_2 in **3** and **4** are virtually identical and the differences in cytotoxicity between these two compounds are therefore not attributable to the topography at the potential sites of electrophilic attack on cellular constituents, namely the olefinic atoms attached to the heterocyclic ring. In the case of **6a** and **6b**, the θ_3 and θ_4 values were approximately 44° and 38°, respectively. The θ_3 figure for **4** was similar, namely 44° approximately, but the θ_4 value revealed that the tetralin ring was almost perpendicular to ring C, indicating that the two phenylmethylene groups present in **4**, but not **6a** or **6b**, exerted a marked effect on the shape of the molecule. The introduction of a 1-piperidylmethyl substituent into **4**, leading to **5**, caused marked increases in the $\theta_1-\theta_4$ values, which may be a contributing factor to its greater overall potency than either **3** or **4**. The structures of **4** and **5** are illustrated in Figure 3. Thus, while introducing a 1-piperidylmethyl group into **1a**,**c**, forming the analogues **2a**,**c**, did not alter the shapes of the structural features common to these molecules, its inclusion in **5** had a profound effect on the stereochemistry of this molecule. In addition, **3** and **5** contain basic nitrogen atoms which are capable of ionisation in biological milieu. Both compounds were modeled as the protonated species. In the case of **3**, the θ_1 and θ_2 angles of the protonated form were almost doubled which could have been caused by increased nonbonded interactions between the equatorial hydrogen atoms at positions 2 and 6 of the piperidine ring with one of the ortho protons on the aryl rings.[35] The 1, 2 and 6 atoms of the piperidinone ring of **5** were inverted on protonation which contributed to the differences in the $\theta_1-\theta_4$

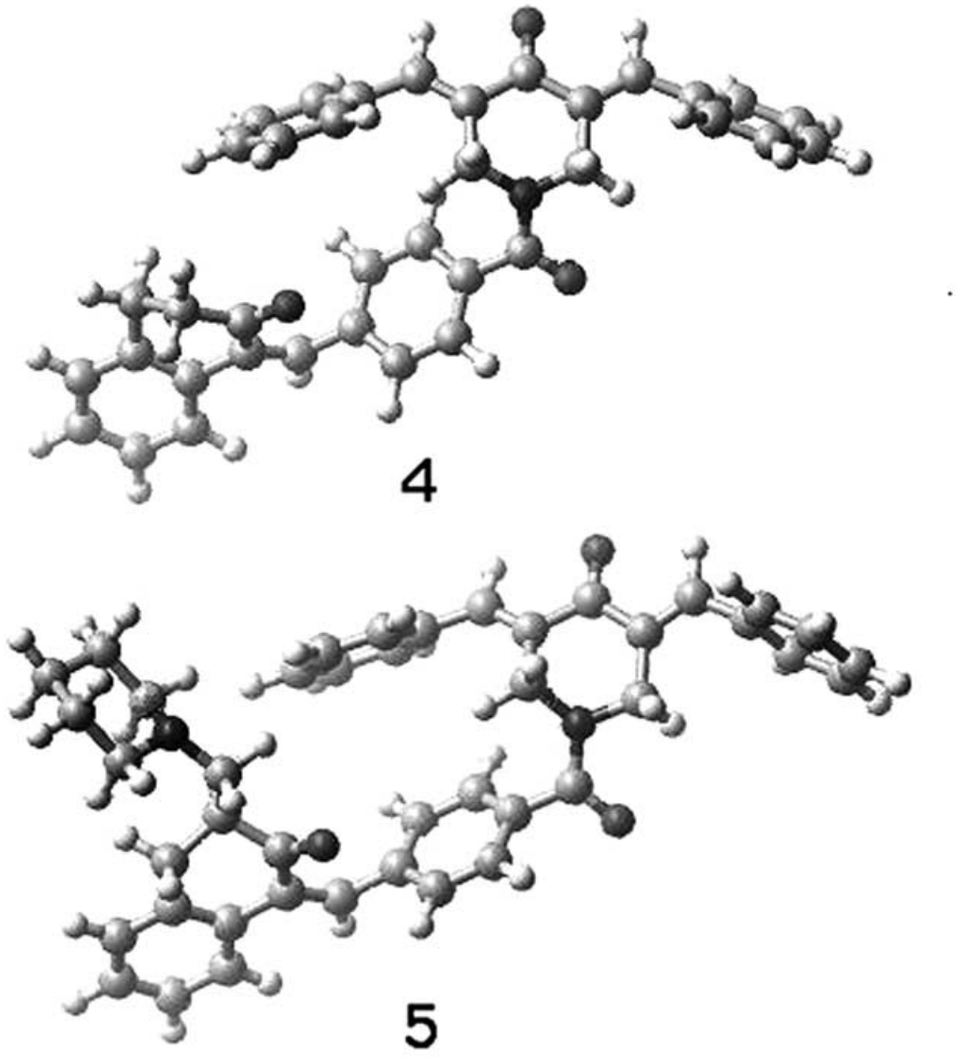

FIGURE 3 Shapes of **4** and **5** (free base) determined by molecular modeling.

values from the free base. One may conclude from the molecular modeling studies that the introduction of both the 3,5-bis(phenylmethylene) groups into the heterocyclic ring and a 1-piperidinylmethyl substituent on the tetralin ring may cause marked changes in the shapes of the molecules which could have influenced the potencies which were observed.

Thus guidelines for analogue development have been discerned based on studies involving quantitative structure-activity relationships and molecular modeling. However since the aim of this study was to detect lead molecules, only a small number of compounds were involved in these two physicochemical investigations and expansion of the different series is necessary in order to confirm or negate the conclusions drawn.

All compounds in series **2** were assessed against approximately 53 human tumour cell lines from nine different types of cancer, namely leukemia, melanoma, non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers.[24] In this assay, compounds were screened using concentrations of 10^{-4} M to 10^{-8} M. If the growth of a cell line is not inhibited by 50% at the highest concentration used, namely 10^{-4} M, the figure of 10^{-4} M is still employed in calculating the average toxicity to all cell lines. Thus the term MG MID (mean graph midpoint) is used rather than IC_{50}. The MG MID values of **2a–f** and melphalan are presented in Table IV.

The Mannich bases **2a–f** displayed greater cytotoxicity than melphalan when all cell lines were considered. The most potent compound was

TABLE IV Evaluation of **2a–f** against a panel of human tumor cell lines

	MG MID[a] (μM)					
Compound	All cell lines	Leukemic	Colon	Renal	Breast	SI[b]
2a	12.9	2.50	16.3	10.8	8.71	16.6
2b	8.71	1.84	11.2	8.24	5.99	39.8
2c	13.2	3.18	18.6	11.8	12.2	22.9
2d	3.80	0.962	2.42	2.51	5.42	>891
2e	5.89	1.70	6.10	7.56	4.35	977
2f	21.9	8.16	19.5	19.4	21.1	35.5
Melphalan	24.6	3.98	49.9	19.7	30.7	178

[a] The letters MG MID indicate the mean graph midpoint which is explained in the text. [b] The letters SI indicate the selectivity index which is the ratio of the IC_{50} values of the compound towards the least and most sensitive of all the cell lines.

2d, which possessed 6.5 times the potency of melphalan. In continuation with the aim of detecting compounds with selectivity for certain tumours, a review of the mean graphs[36] was undertaken. Increased sensitivity of certain cell lines was displayed towards the compounds, particularly the leukaemia, colon, renal and breast subpanels. These observations are presented in Table IV. The data reveal that the compounds in series **2** are all potent antileukemic agents, particularly **2d** which also demonstrated marked toxicity to the important neoplastic diseases of colon, renal and breast tumours. The SI values indicated the huge differential in sensitivity of the cell lines to both **2d** and **2e**, which were far greater than the figure generated for melphalan. These results demonstrate further the viability of incorporating the 3-(1-piperidylmethyl) group into the cyclohexene ring of 1-arylidene-2-tetralones.

In an attempt to discern the mode(s) of action of these novel cytotoxics, four representative compounds **1d**, **2d**, **4** and **5** were evaluated as inhibitors of hNMT and also as DNA binding agents. These compounds were chosen since, in all four screens, the Mannich bases **2d** and **5** were more potent than the precursor unsaturated ketones **1d** and **4**; consequently, differences in hNMT inhibiting properties and DNA binding might account for the greater cytotoxicity of **2d** and **5**. Using concentrations up to 200 μM, **1d** and **2d** had no effect on hNMT, while the percentage inhibitions of **4** and **5** at this concentration were 29 and 10, respectively. None of the compounds bound to calf thymus DNA. Thus the principal site(s) of action of the compounds prepared in this study are neither hNMT nor DNA.

CONCLUSIONS

Molecular modifications of various 1-arylidene-2-tetralones **1** were undertaken in three different ways; namely, first, formation of the corresponding Mannich bases **2**, second, coupling with the cytotoxic enone **3** to produce **4** and **5** and third, formation of the Mannich base **7** which contained a thioester group. These synthetic strategies led, in general, to compounds with greater potencies than the analogues in series **1**, when the Molt 4/C8, CEM, P388 and L1210 screens were utilized. In addition, the compounds in series **2** demonstrated significant potencies towards human tumour cell lines and, in particular, the antileukemic effect is noteworthy. In most instances, the compounds prepared in this study demonstrated selective toxicity to different cells, which further enhanced their potential utility. The conclusion may be drawn that the modifications undertaken of various 1-arylidene-2-tetralones led to novel, potent, prototypic cytotoxics and, in particular, the further development of compound **5** is clearly warranted.

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