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Original article

Cytotoxic 2,6-bis(arylidene)cyclohexanones and related compounds

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Abstract – A number of 2-arylidenecyclohexanones **1**, 2,6-bis(arylidene)cyclohexanones **2** and related Mannich bases **3**–**5** were prepared. Various torsion angles as well as atomic charges on olefinic carbon atoms were determined by molecular modelling on all compounds. These molecules showed cytotoxicity towards murine P388 and L1210 cells as well as to human Molt 4/C8 and CEM T-lymphocytes. The average cytotoxicity of the dienones **2** was more than three times greater than was found with the monoarylidene analogues **1**, and, in general, were slightly more cytotoxic than the Mannich bases **3**–**5**. A number of the compounds displayed potency towards a panel of human tumour cell lines and most of the representative compounds in series **2**–**5** were selectively toxic to colon cancers and leukaemic cells. © 2000 Éditions scientifiques et médicales Elsevier SAS

unsaturated ketones / Mannich bases / molecular modelling / cytotoxicity

1. Introduction

The principal aim of research in this laboratory is the discovery of novel cytotoxic and anticancer alkylating agents. The currently available antineoplastics that act by alkylation of cellular nucleophiles suffer from a number of significant disadvantages, many of which are related to their interactions with nucleic acids [1]. In contrast, various α,β-unsaturated ketones react preferentially or exclusively with thiols but not with amino or hydroxy groups [2, 3]. Hence, since thiols are not part of the nucleic acid structures, conjugated enones may be significantly less mutagenic and carcinogenic than conventional drug strategies using alkylating agents [4]. In addition, the mode of action of enones by thiol alkylation should enable cytotoxicity to be displayed towards cancers which are resistant to established alkylating agents. Support for this viewpoint was obtained when two cell lines which were resistant to the antineoplastic agent melphalan were shown to be virtually free from cross resistance to a number of Mannich bases of conjugated styryl ketones [5]. Thus, the preparation of a number of prototypic enones and related compounds as candidate cytotoxic and anticancer agents was considered a profitable avenue to pursue.

A number of years ago, the cytotoxicity of *E*-2-benzylidenecyclohexanone **1a** towards an epidermoid carcinoma of the nasopharynx (KB screen) was described in which this enone had an ED_{50} of 1.34 μM [6]. Thus, elaboration to give series **1** was planned in which the aryl substituents were chosen with divergent Hammett σ, Hansch π and molecular refractivity (MR) values with a view to finding out whether cytotoxicity was related to the electronic, hydrophobic or steric properties of these groups. Furthermore, the theory of sequential cytotoxicity, which originated

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in this laboratory [7], states that successive chemical attacks of cellular constituents may be highly deleterious to malignant cells and, thus, the preparation and bioevaluation of the potential bis alkylators **2** was considered. In fact, several studies have shown that various neoplasms are more vulnerable to multiple chemical insults than the corresponding normal cells [8, 9], and hence compounds with selective toxicity for neoplastic tissues may evolve using this approach.

Another design of compounds with potential preferential lethality to cancers was as follows. The pH of a number of tumours is lower than the corresponding normal cells [10] and hence an increase in the electrophilicity of the olefinic carbon atoms in enones under acidic conditions should enable greater thiolation in cancerous cells to occur. An examination of available Hammett σ_m values revealed that the figure for the dimethylaminomethyl group is 0.00 while the corresponding protonoted species — $CH_2NH(CH_3)_2$ has a σ_m figure of 0.40 [11]. Since piperidine is more basic than dimethylamine (pK_a of 11.12 versus 10.73 [12]), the placement of a 1-piperidinylmethyl substituent in an aryl ring should give rise to an even higher percentage of charged species under acidic conditions than utilization of a dimethylaminomethyl group. These considerations led to the decision to prepare series **3**.

Other prototypic molecules were considered, namely **4** and **5a,b**. Certain Mannich bases of conjugated styryl ketones react much more rapidly with thiols than the precursor enones [13]. Hence, the preparation of **4** was suggested, which should be appreciably more cytotoxic than **1f** if thiol alkylation contributes significantly to potency. The dienone **5a** would be predicted to have greater cytotoxicity than **2f**, if the presence of a 1-piperidinylmethyl substituent markedly increases the capacity for alkylation of cellular nucleophiles. Should this be the case, further evidence of this phenomenon would be obtained if the activity of **5a** would be greater than that of the dimethylamino analogue **5b** (*vide supra*).

In summary, the aim of the present investigation was the preparation of a number of prototypic

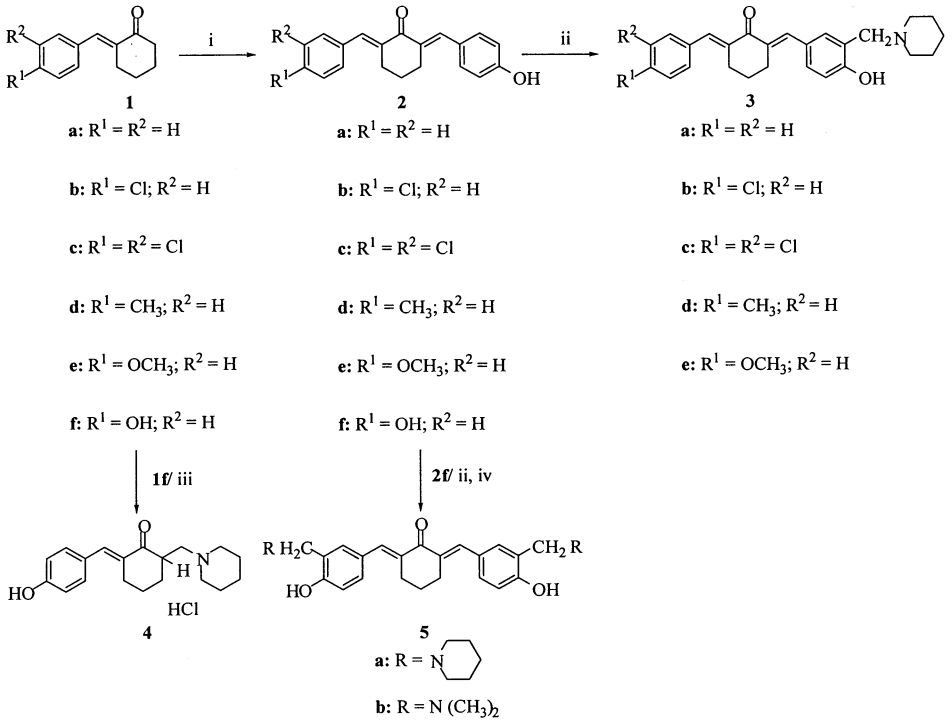

Figure 1. Structures and synthesis of series **1**–**5**. i = 4-hydroxybenzaldehyde/hydrochloric acid; ii = dipiperidinomethane; iii = 1-methylenepiperidinium chloride; iv = *N*,*N*,*N*′,*N*′-tetramethylethylaminediamine.

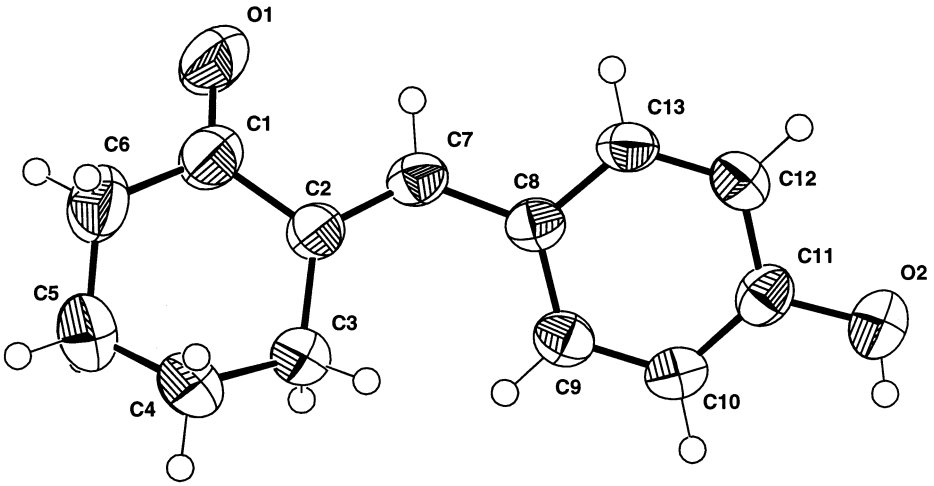

Figure 2. ORTEP diagram of **1f**.

molecules **1**–**5** as candidate cytotoxic agents with a view to determining the important structural features of the molecules that contribute to potency. The data obtained may provide guidance for the ways in which this area of investigation can be expanded on a rational basis.

2. Chemistry

The compounds in series **1** were prepared by a Claisen–Schmidt condensation between cyclohexanone and the appropriate aryl aldehyde. A similar reaction using **1f** in place of cyclohexanone led to series **2**. Condensation of **2a**–**e** with dipiperidinomethane gave rise to the corresponding Mannich bases **3a**–**e**, while **4** was synthesized by reaction of **1f** with the Mannich reagent 1-methylenepiperidinium chloride. The reaction of **2f** with either dipiperidinomethane or *N*,*N*,*N*′,*N*′-tetramethylethylenediamine led to **5a** and **5b**, respectively. These reaction sequences are illustrated in *figure 1*. The X-ray crystallographic data obtained for **1f** are displayed in *figure 2*. Molecular modelling was undertaken on all compounds and the specific torsion angles and charge densities determined are illustrated in *figure 3*, while the details are summarized in *table I*.

3. Cytotoxicity

Compounds **1a**–**g, 2a**–**g, 3a**–**c,e, 4, 5a,b** were evaluated against murine P388 and L1210 leukaemic cells as well as human Molt 4/C8 and CEM T-lymphocytes. These data are summarized in *table II*. In addition, a number of compounds were examined using a panel of approximately 55 human cancer cell lines from a variety of neoplastic diseases. The results are presented in *table III*, together with an indication of whether selective toxicity to colon and/or leukaemic cells was observed. The Mannich bases **3a,d** were also evaluated using human Jurkat leukaemia cells. The IC_{50} for **3a,d** against this cell line were 4.47 and 5.36 μmoles respectively, while the IC_{80} values of 7.15 and 8.57 μmoles, respectively, were used in subsequent biochemical determinations. The apoptotic indices [(number of cells with apoptotic nuclei/number of cells counted) × 100] for **3a,d** were 50.3 and 64.7%, respectively, while **3a** inhibited the syntheses of RNA and protein by 73.2 and 28.9%, respectively.

4. Results and discussion

The synthesis of the compounds in series **1**–**5** was achieved successfully. The evidence from thin-layer

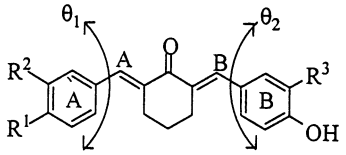

Figure 3. Torsion angles (θ_1, θ_2) and atomic charges (C^A, C^B) of compounds **1**–**5** determined by molecular modelling.

Table I. Torsion angles (θ_1, θ_2) and atomic charges (C^A, C^B) of compounds **1–5** determined by molecular modelling.[a]

Compound	θ_1	θ_2	C^A	C^B
1a	9.07	–	0.055	–
1b	9.02	–	0.053	–
1c	8.78	–	0.047	–
1d	8.90	–	0.058	–
1e	9.18	–	0.062	–
1f	8.87	–	0.063	–
2a	13.27	−13.06	0.044	0.053
2b	13.31	−13.08	0.042	0.055
2c	13.38	−13.10	0.036	0.056
2d	13.23	−13.07	0.047	0.053
2e	13.39	−13.06	0.051	0.052
2f	13.08	−13.08	0.052	0.052
3a	13.17	−14.61	0.044	0.054
3b	13.14	−15.37	0.042	0.055
3c	13.22	−15.40	0.036	0.056
3d	13.07	−15.32	0.047	0.052
3e	12.94	−14.47	0.051	0.053
4	9.44	–	0.064	–
5a	14.52	−15.38	0.052	0.052
5b	13.91	−13.90	0.052	0.052

[a] The torsion angles (°) and atomic charges (esu) in series **1–5** are indicated in *figure 3*. Torsion angles are indicated as positive or negative if the rotation is anticlockwise or clockwise, respectively.

chromatography (TLC) and ^{1}H-NMR spectroscopy indicated that the compounds were isomerically pure. The absorptions of the olefinic protons in the ^{1}H-NMR spectra were located at 7.3–7.8 ppm, which is indicative of compounds possessing the *E* configuration [14]; for the corresponding *Z* isomers, these protons would be predicted to be located at higher field. For example, the olefinic hydrogen atoms of *Z*-2-phenylmethylenecylohexanone and *Z*-2-phenylmethylene-6,6-diphenylcyclohexanone absorb at 6.27 ppm [15] and 6.22 ppm [16], respectively. In addition, X-ray crystallography of a representative compound **1f** revealed that it had the *E* configuration (*figure 2*), which is consistent with similar studies involving a 2-arylidenecyclohexanone [17] and a 2,6-bis-(arylidene)cyclohexanone [18]. The location of the 1-piperidinylmethyl and dimethylaminomethyl substituents in series **3** and **5** *ortho* to the aryl hydroxy group was assigned on the basis of ^{1}H-NMR spectroscopy and literature precedents [19, 20]. ^{1}H-NMR spectroscopy of **4** confirmed that condensation of **1f** with 1-methylenepiperidinium chloride led to substitution on the alicyclic rather than aryl ring.

If the compounds exert their cytotoxicity by electrophilic attack of cellular constituents, two structural

Table II. Cytotoxicity of **1, 2, 3a–c,e, 4** and **5** towards murine P388 and L1210 cells and human Molt 4/C8 and CEM T-lymphocytes.

Compound	IC_{50} (μM)				
	P388 cells	L1210 cells	Molt 4/C8 cells	CEM cells	Av. of four cell lines
1a	19.25 ± 0.72	77.7 ± 14.6	38.1 ± 2.3	36.8 ± 3.0	42.96
1b	6.48 ± 0.28	10.4 ± 0.3	4.21 ± 1.08	3.50 ± 0.17	6.15
1c	3.87 ± 0.54	2.91 ± 0.01	1.06 ± 0.41	3.37 ± 0.07	2.80
1d	30.76 ± 0.27	13.4 ± 0.7	13.4 ± 0.2	14.4 ± 0.3	17.99
1e	20.52 ± 0.96	16.8 ± 0.1	14.8 ± 0.2	15.8 ± 0.1	16.98
1f	16.65 ± 0.23	45.2 ± 3.8	39.2 ± 4.6	26.0 ± 6.8	31.76
2a	3.58 ± 0.21	7.32 ± 0.87	5.17 ± 1.83	5.82 ± 0.83	5.47
2b	3.13 ± 0.43	6.44 ± 1.14	4.90 ± 0.67	2.69 ± 0.39	4.29
2c	2.93 ± 0.21	7.15 ± 0.01	7.71 ± 0.61	4.23 ± 2.05	5.51
2d	5.69 ± 0.37	7.41 ± 0.86	5.22 ± 1.72	4.31 ± 1.16	5.66
2e	3.84 ± 0.07	6.85 ± 1.18	4.18 ± 1.92	5.68 ± 1.65	5.14
2f	5.80 ± 0.68	13.5 ± 2.3	5.52 ± 2.12	6.84 ± 0.14	7.92
3a	2.04 ± 0.11	6.96 ± 0.61	7.14 ± 0.75	4.35 ± 0.96	5.12
3b	2.79 ± 0.24	15.2 ± 0.29	15.5 ± 6.9	8.21 ± 0.09	10.43
3c	2.16 ± 0.07	10.2 ± 0.2	7.82 ± 0.73	8.12 ± 0.59	7.08
3e	2.39 ± 0.07	7.28 ± 0.57	7.21 ± 0.75	5.62 ± 0.88	5.63
4	0.161 ± 0.01	2.60 ± 0.47	5.80 ± 2.25	3.85 ± 2.50	3.10
5a	0.581 ± 0.10	318 ± 26	498 ± 2	316 ± 1	283
5b	1.28 ± 0.04	6.26 ± 0.76	6.98 ± 1.60	5.85 ± 2.49	5.09
Melphalan	0.22 ± 0.01	2.13 ± 0.03	3.24 ± 0.79	2.47 ± 0.30	2.02
5-Fluorouracil	0.49 ± 0.01	0.28 ± 0.14	23 ± 3.0	8.9 ± 0.43	8.17

features of the compounds in series **1**–**5** may determine the relative potencies. First, the orientation of the aryl rings will be expected to influence the alignment of the molecules at critical sites of the biomacromolecules. Thus, the measurement of the torsion angles θ_1 and θ_2 between the aryl rings and the adjacent olefinic groups, as illustrated in *figure 3*, was considered of interest since previous studies have demonstrated that potency is influenced by coplanarity, or lack thereof, between different groups [21]. Second, the atomic charges on carbon atoms C^A and C^B (*figure 3*) will permit a prediction of relative potencies, if thiol alkylation is the principal mode of action of these compounds. For example, a reduction in the electron density on the olefinic carbon atoms would be expected to lead to increased cytotoxicity.

The data generated by molecular modelling are summarized in *table I*. The average θ_1 value in series **1** was 8.97°, while the introduction of a second arylidene group into the alicyclic ring leading to **2** led to an approximately 50% increase in the θ_1 figures. The θ_2 values in series **2** were virtually constant, being slightly lower than the θ_1 figures. The increase in torsion angles in **2** compared to **1** was difficult to rationalize since a lowering of the θ_1 and θ_2 values in **2** (as well as series **3** and **5**) would lead to increased conjugation in these dienones. The presence of the 3-(1-piperidinyl)methyl group in series **3** led to a 13% increase in the θ_2 figures, on average, compared to series **2**. This observation may be attributed to a buttressing effect, i.e. the substituents in the *meta* position forced the adjacent *ortho* protons towards the olefinic bond, thereby increasing steric hindrance to coplanarity of the aryl ring with the adjacent unsaturated linkage. The percentage variation of the θ_1, θ_2 and C^B values within each in the series **1**–**3** was invariably less than 8%. There was no linear correlation between the average potencies of the compounds in series **2** and the θ_2 values ($P > 0.1$). However, a linear relationship between the θ_2 figures of **3a**–**c,e** and average cytotoxicities was observed ($P < 0.1$) whereby potencies increased as the size of the interplanar angles diminished. On the other hand, the atomic charges at C^A within each of these three series varied by 34, 44 and 42, respectively. Thus, within each series of compounds, variation in cytotoxicity is likely to be influenced principally by the charges on the C^A atoms. Linear plots between the atomic charges on the C^A atoms and the Hammett σ values of aryl ring A revealed that correlations occurred within each of the series **1**–**3** ($P < 0.05$) as indicated in *figure 4*.

All of the compounds with the exception of **3d** were evaluated against murine P388 and L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes. The murine cell lines were chosen on the basis of the claim that they are good indicators of a number of clinically useful antineoplastic agents [22], while evaluation

Table III. Evaluation of various compounds against a panel of human tumour cell lines.

Compound	All cell lines MG MID[a] (μM)	Colon cancer cells		Leukaemic cells	
		MG MID[a] (μM)	SI[b]	MG MID[a] (μM)	SI[b]
1c	21.9	22.2	1.0	19.4	1.1
1d	67.6	72.0	0.9	21.6	3.1
1e	97.7	100	1.0	100	1.0
2c	3.16	2.15	1.5	1.53	2.1
3a	4.26	2.07	2.1	2.56	1.7
3b	16.2	10.3	1.6	6.43	2.5
3d	6.64	3.27	2.0	5.03	1.3
3e	6.63	3.76	1.8	3.38	2.0
4	3.98	2.13	1.9	1.46	2.7
5a	6.31	3.49	1.8	3.63	1.7
5b	6.61	3.97	1.7	2.02	3.3
Melphalan	23.5	44.6	0.5	4.65	5.1
5-Fluorouracil	32.6	7.90	4.1	27.6	1.2
Helenalin	1.45	1.42	1.0	0.620	2.3

[a] The letters MG MID refer to the mean graph midpoint values. This term is explained in Section 4.
[b] The letters SI indicate the selectivity index, i.e. the ratio between the MG MID figure for all cell lines and the MG MID value for either the colon cancers or leukaemic cells.

against the T-lymphocytes may reveal whether cytotoxicity to human cancer cells is likely. The data generated are summarized in *table II* along with the relevant figures for the reference drugs melphalan and 5-fluorouracil.

A review of the data in *table II* indicated that most compounds were markedly cytotoxic to the four cell lines except **5a**, which had only very weak activity in the L1210, Molt 4/C8 and CEM screens. The most potent compounds (potencies compared to melphalan and 5-fluorouracil, respectively) in the P388, L1210, Molt 4/C8 and CEM screens were **4** (1.4, 3.0), **4** (0.82, 0.11), **1c** (3.1, 21.7) and **2b** (0.92, 3.3), respectively. Hence, development of analogues of **1c, 2b** and **4** is warranted. If the potencies of compounds **1**–**5** are mediated by interaction with thiols on the olefinic carbon atoms, then series **2** would be expected to be twice as potent as series **1**, having double the number of alkylation sites. However, if the theory of sequential cytotoxicity is validated in this cluster of compounds, then the average IC_{50} value of **2a**–**f** should be substantially less than half of the average IC_{50} figure for **1a**–**f**. The average IC_{50} figures for the four cell lines for **1a**–**f** and **2a**–**f** were 19.77 and 5.67 μM, respectively, i.e. the compounds in series **2** are 3.5 times more potent than the analogues in series **1** suggesting the viability of the theory. Furthermore, the IC_{50} figures for the cell lines for **1a**–**c,e** and **3a**–**c,e** were 17.22 and 7.07 μM, respectively, revealing a 2.4-fold increase in potency for the dienones, which also supports the theory of sequential cytotoxicity. The insertion of a 3-(1-piperidyl)methyl group into an aryl ring in series **3** did not lead to an increase in potency over the precursor α,β-unsaturated ketones in series **2**. Thus, the average IC_{50} figures of the four cell lines for **2a**–**c,e** and **3a**–**c,e** were 5.10 and 7.07 μM, respectively, indicating a nearly 40% reduction in cytotoxicity. Similarly, the average cytotoxicities of **2f** and **5a** were 7.92 and 283 μM, respectively, revealing an even greater decrease in potency. It is conceivable that a bulky substituent in the *meta* position of the aryl rings impedes alignment at a binding site in the cells, or that the increase in the θ_2 figures of **3a**–**c,e** and **5a**, compared to the analogues in series **2**, is detrimental to cytotoxicity. As mentioned previously, Mannich bases of conjugated styryl ketones react

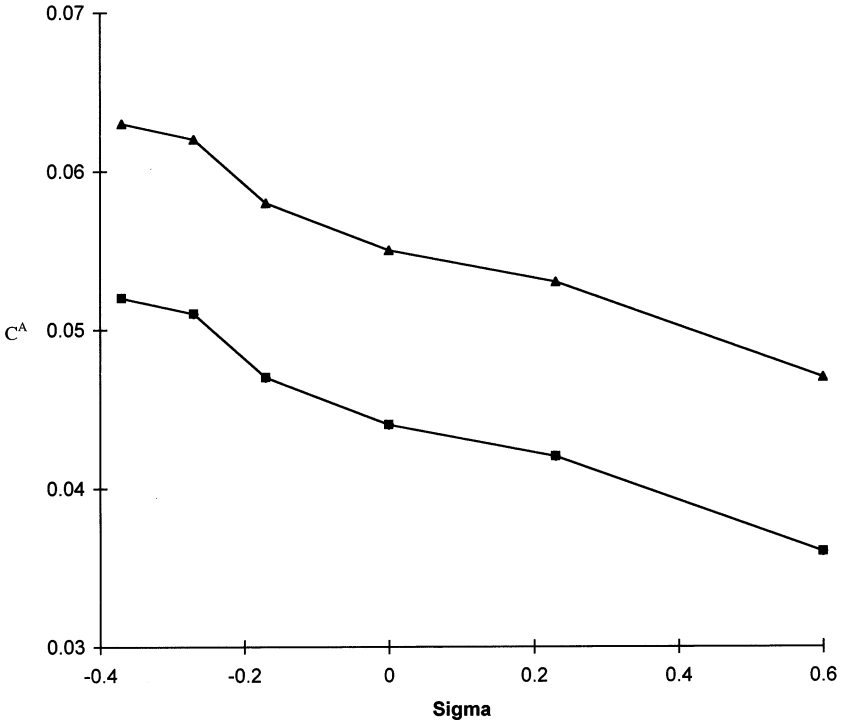

Figure 4. Correlations between the average potencies of **1, 2** and **3a**–**c,e** and the Hammett σ values. The triangles represent the data for series **1** while the results for series **2** and **3a**–**c,e** were superimposable and are presented as squares.

much more rapidly with thiols than the analogous unsaturated ketones [13]. The fact that compound **4** was 10 times more potent than the precursor ketone **1f** supports the view that an important mode of action of these compounds is based upon interference with cellular thiols. In addition, interaction of the piperidyl group of **4** with a binding site may account for its greater potency than **1f**.

In order to seek correlations between the potencies of various groups of compounds and the electronic, hydrophobic and steric properties of the aryl substituents, linear plots were made between the average IC_{50} figures of **1a**–**f, 2a**–**f, 3a**–**c,e** and the Hammett σ, Hansch π and MR constants in each series of compounds. The test for zero correlation [23] was employed. The potencies of **1a**–**f** were correlated with the σ ($P < 0.1$), π ($P < 0.05$) and MR ($P < 0.05$) constants and, in addition, the cytotoxicity of **2a**–**f** was related to the π constants ($P < 0.1$). These correlations were all positive, i.e. potencies increased as the physicochemical constants rose. Thus, development of series **1** and **2** with the aim of increasing cytotoxicity should bear in mind these interrelationships. No other correlations ($P > 0.1$) were noted.

Over half of the compounds were evaluated against a panel of approximately 55 human tumour cell lines from different groups of neoplasms, including leukaemia, melanoma, non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers. The concentrations of compounds used in this assay were normally log 10^{-8} to log 10^{-4} M, and the amounts of compounds required to inhibit the growth of the cells by 50% were noted. Mean graphs were constructed indicating the sensitivities of individual tumours to the compounds relative to the average IC_{50} figures for all cell lines. However, if the IC_{50} values were greater than log 10^{-4} M for one or more tumours, this figure of log 10^{-4} M was used in calculating the average cytotoxicity towards all cell lines. Hence, in some cases, the average IC_{50} figures against all cell lines were not generated and are consequently referred to as mean graph midpoint (MG MID) values [24].

The cytotoxicity of various compounds in series **1**–**5**, as well as three reference compounds, is presented in *table III*. The data reveal that, with the exception of **1c**–**e**, the compounds are, on average, approximately 3.5 and 5 times more potent than melphalan and 5-fluorouracil, respectively. The compounds were less cytotoxic than the antineoplastic agent helenalin, whose mode of action is reported to be by thiol alkylation [25]. The observation that the potencies of the dienones **2c** and **3d,e** are appreciably more than double those of the analogues **1c**–**e** having capacity for monoalkylation supports the hypothesis of sequential cytotoxicity. An examination of the mean graphs [24] revealed that many of the compounds demonstrated greater potencies to colon cancers and leukaemia than towards other cell lines. Thus, a comparison of the average cytotoxicity figures for either colon or leukaemia cell lines with the MG MID values for all tumours gave the selectivity index (SI) for each compound. With the exception of **1c**–**e**, all of the compounds had a SI figure of 1.50 or greater when colon tumours were considered. This criterion for preferential cytotoxicity, i.e. SI = 1.50 or more, was displayed against leukaemia by all the compounds except for **1c,e** and **3d**. Thus, 94% of the compounds in series **2**–**5** that were evaluated in this screen displayed a 50% or more increased toxicity to both colon and leukaemic neoplasms. Hence, these compounds are templates for molecular modification with a view to obtaining analogues with even greater selective toxicity to these important groups of cancers.

A number of anticancer agents act by inducing apoptosis [26] as well as by inhibiting the biosynthesis of RNA and proteins [27]. Work from this laboratory using human Jurkat T cells revealed that cytotoxicity could be explained, at least in part, by these mechanisms of action for various conjugated arylidene ketones [14] and a Mannich base of an unsaturated ketone [28]. In the present investigation, the IC_{80} concentrations of compounds were used in order to cause the death of the majority of the Jurkat T cells. Under these conditions, the apoptotic indices of two representative compounds **3a,d** were 50 and 65%, respectively. A further investigation with **3a** revealed that RNA and protein syntheses were inhibited by 73 and 29%, respectively. The reference compounds melphalan (an antineoplastic alkylating agent), actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (a protein synthesis inhibitor) also caused apoptosis and were inhibitors of RNA and protein syntheses, details of which are recorded in Section 6 of this paper. The conclusion to be drawn is the likelihood that the cytotoxicity of the compounds prepared in this study is based upon induction of apoptosis and interference with the biosyntheses of RNA and proteins.

5. Conclusions

The investigation has revealed that a number of conjugated arylidenecycloalkanones and related Mannich bases have promising cytotoxic properties. In particular, **1c**, **2b** and **4** displayed significant activity towards P388, L1210, Molt 4/C8 and CEM cells. All of the compounds in series **2**–**5** that were evaluated against a panel of human tumour cell lines were more potent than the clinically useful drugs melphalan and 5-fluorouracil. Furthermore, in this screen, selective toxicity to colon cancers and leukaemia was demonstrated; this observation alone mandates that further molecular modification of these compounds is warranted. The modes of action of these compounds likely include the induction of apoptosis and inhibition of RNA and protein syntheses.

6. Experimental protocols

6.1. Chemistry

Melting points are uncorrected and are quoted in degrees Celcius. Elemental analyses were undertaken on **1f**, **2a**–**f** (C,H) and **3a**–**e**, **4** and **5a,b** (C,H,N) by Mr K. Thoms, Department of Chemistry, University of Saskatchewan, and are within 0.4% of the calculated values. TLC was employed using silica gel 60 F_{254}-precoated plastic sheets. ^{1}H-NMR spectroscopy utilized a Bruker AM 300FT instrument (300 MHz). Compounds **1f** and **3c** were obtained with one quarter and one half the molecules of water of crystallization, respectively.

*6.1.1. Synthesis of series **1***

The synthesis of **1a**–**e** has been described previously [29]. The preparation of **1f** was undertaken by a modification of a literature method [30] as follows. A solution of 4-hydroxybenzaldehyde (0.1 mol) in aqueous sodium hydroxide solution (18%, 25 mL) was added slowly to a mixture of cyclohexanone (0.5 mol) and aqueous sodium hydroxide solution (14%, 25 mL). After dilution with water (300 mL), the reaction mixture was stirred at room temperature for 100 h, then acidified with hydrochloric acid (1 M, 300 mL) and extracted with chloroform (3 × 30 mL). The combined organic extracts were washed with water and dried (anhydrous magnesium sulfate). After evaporation of some of the solvent (~ 20 mL), cyclohexane (100 mL) was added and the resultant precipitate was collected, dried and recrystallized from water–methanol to give **1f**, m.p. 172–173 °C (lit. [31], m.p. 172 °C) in 27% yield. TLC using a solvent system of cyclohexane:ether:methanol (5:3:2) revealed it to be a single compound.

*6.1.2. Synthesis of series **2***

Compounds **2a**–**e** were prepared as follows. Hydrogen chloride was passed into a solution of the 2-arylidenecyclohexanone (0.02 mol) and 4-hydroxybenzaldehyde (0.02 mol) in a mixture of ether (100 mL) and methanol (5 mL) for 35–40 min at room temperature. After stirring at room temperature for 1 h, the solvent was removed and the dark red precipitates which formed were collected and washed with aqueous sodium bicarbonate solution (0.1 M, 50 mL). Recrystallization from methanol afforded the following 2,6-bis(arylidene)cyclohexanones [m.p., yield (%)] namely **2a**: 203–208, 78; **2b**: 227–229, 66; **2c**: 164, 38; **2d**: 188, 43 and **2e**: 207–211, 59.

The preparation of **2f** was accomplished by the following procedure. Hydrogen chloride was passed into a solution of cyclohexanone (0.05 mol) and 4-hydroxybenzaldehyde (0.1 mol) in methanol (50 mL) for 1 h at room temperature. After stirring at room temperature for 1 h, evaporation of the solvent led to yellow precipitates which were filtered and washed with sodium bicarbonate solution (0.1 M, 50 mL). Recrystallization from methanol gave **2f**, m.p. 279–282 °C in 82% yield.

TLC using solvent systems of benzene:cyclohexane:methanol (8:1:1) for **2a**–**e** and cyclohexane:ether:methanol (5:3:2) for **2f** revealed the compounds to be homogeneous. The ^{1}H-NMR spectrum of a representative compound **2b** in which the aryl rings containing the chloro and hydroxy groups are referred to as rings A and B, respectively, was as follows: δ ($CDCl_3$): 7.73 (d, 2H, 2,6 aryl A H, $J = 9.94$ Hz), 7.60–7.35 (m, 6H, 3,5 aryl A H, 2,6 aryl B H, olefinic H), 6.87 (d, 2H, 3,5 aryl B H, $J = 8.58$ Hz), 2.90 (m, 4H, 3,5 cyclohexyl H), 1.82 (m, 2H, 4 cyclohexyl H).

*6.1.3. Synthesis of series **3***

A solution of dipiperidinomethane (0.01 mol) in acetonitrile (10 mL) was added to a solution of the 2,6-bis(arylidene)cyclohexanone **2** (0.0075 mol) in acetonitrile (150 mL) at 70–80 °C. The mixture was heated under reflux and the progress of the reaction was monitored by TLC using a solvent system of ethyl acetate:chloroform:methanol (6:3:1). After 24 h, the reaction mixture was concentrated to 25 mL and the resultant yellow precipitates were collected and recrys-

tallized from methanol to give the following Mannich bases [m.p., yield (%)]: **3a**: 97, 42; **3b**: 157, 70; **3c**: 156–157, 40; **3d**: 141, 65 and **3e**: 173, 32. The compounds were homogeneous by TLC using the same solvent system as employed in monitoring the reaction. The ^{1}H-NMR spectrum of a representative compound **3d** was determined in which the aryl rings containing the methyl and hydroxy groups are referred to as rings A and B, respectively. δ ($CDCl_3$): 7.76 (s, 1H, CH = attached to ring A), 7.72 (s, 1H, CH = attached to ring B), 7.36 (def d, 3H, 2,6 aryl A H, 6 aryl B H), 7.20 (d, 2H, 3,5 aryl A H, $J = 8.03$ Hz), 7.13 (s, 1H, 2 aryl B H), 6.83 (d, 1H, 5 aryl B H), 3.70 (s, 2H, aryl B–CH_2–N), 2.91 (t, 4H, 3,5 cyclohexyl H), 2.12–2.78 (broad s, 4H, 2,6 piperidinyl H), 2.37 (s, 3H, CH_3 of aryl A), 1.79 (quintiplet, 2H, 4 cyclohexyl H), 1.64 (def t, 4H, 3,5 piperidinyl H), 1.18–1.57 (broad s, 2H, 4 piperidyl H).

6.1.4. Synthesis of **4**

1-Methylenepiperidinium chloride (0.006 mol), prepared by a literature procedure [32], was added to a solution of **1f** (0.003 mol) in acetonitrile (50 mL) at 40–50 °C and the mixture was heated under reflux. The progress of the reaction was monitored by TLC using a solvent system of ethyl acetate:methanol (7:3). After heating under reflux for 2–3 h, the mixture was concentrated to 15 mL and the colourless crystals were collected and recrystallized from ether–methanol to give 2-(4-hydroxyphenylmethylene)-6-(1-piperidinylmethyl)-cyclohexanone hydrochloride **4**, m.p. 168–170 °C in 64% yield. The compound was homogeneous by TLC using the same solvents as employed in monitoring the reaction. ^{1}H-NMR (DMSO-d_6): δ 10.11 (def s, 1H, OH), 9.91 (br s, 1H, NH), 7.36 (d, 2H, 2,6 aryl H, $J = 8.24$ Hz), 7.32 (s, 1H, olefinic H), 6.86 (d, 2H, 3,5 aryl H, $J = 8.51$ Hz), 3.42–3.50 (m, 3H, cyclohexyl 3H, piperidinyl 2H, 6H), 2.97 (m, 5H, cyclohexyl 3H, piperidinyl 2H, 6H, NCH_2), 2.68–2.70 (m, 1H, cyclohexyl 6H), 2.26 (m, 1H, piperidinyl 4H), 1.64–1.86 (m, 8H, cyclohexyl 4-CH_2, 5-CH_2; piperidinyl 3-CH_2, 5CH_2), 1.41 (m, 1H, piperidinyl 4H).

6.1.5. Synthesis of series **5**

A solution of dipiperidinomethane or *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.01 mol) in acetonitrile (10 mL) was added to a solution of **2f** (0.0025 mol) in acetonitrile (90 mL) at 70–80 °C and the mixture was heated under reflux. The reaction was monitored by TLC using a solvent system of ether:methanol (4:6). After 18 h, the mixture was concentrated to 20 mL and the resultant yellow precipitates were crystallized from acetone–chloroform (**5a**) or methanol (**5g**) to give the following compounds [m.p., yield (%)]: **5a**: 228–232, 82; **5b**: 170–173, 49. TLC revealed that the compounds were homogeneous using the same solvent system as employed in monitoring the reaction. The ^{1}H-NMR spectrum of a representative compound **5a** was determined in which the aryl protons *ortho* to the 1-piperidinylmethyl, hydroxy and olefinic groups are designated as the aryl 2, 5 and 6 protons, respectively. δ ($CDCl_3$): 7.70 (s, 2H, olefinic H), 7.35 (dd, 2H, aryl 5H, $J = 8.75$ Hz), 7.18 (s, 2H, aryl 2H), 6.82 (d, 2H, aryl 6H, $J = 8.37$ Hz), 3.69 (s, 4H, aryl-CH_2-N), 2.90 (t, 4H, 3,5-cyclohexyl H), 2.08–2.80 (br s, 8H, 2,6-piperidinyl H), 1.79 (quintiplet, 2H, 4-cyclohexyl H), 1.22–1.73 (br m, 12H, 3,4,5-piperidyl H).

6.1.6. Charge density calculations

The structures of the compounds **1**–**5** were built and minimized using a HyperChem molecular modelling programme [33]. The molecules were minimized using the MM+ molecular mechanics module with the Polak–Ribiere algorithm. A minimum energy difference between cycles of less than 0.001 kcal/mol was achieved for all compounds except for **1a,c,d,f** and **2b,d**, in which cases the energy differences between conformations were in the range of 0.0015–0.0380 kcal/mol. In order to obtain the atomic charges, the SNDO semi-empirical force field was used to calculate the wave functions of the minimum energy conformers from which the charges listed in *table I* were generated.

6.1.7. Statistical analyses

The σ, π and MR values used in the statistical analyses were taken from the literature [34].

6.1.8. X-ray crystallographic determination of **1f**

Data collection was undertaken using an Enraf-Nonius CAD-4 diffractometer with an ω scan. The structure was solved by direct methods using NRCVAX [35] and refined using SHELXL 97 [36]. *Figure 2* was generated using ORTEP [37] as implemented in XTAL 3.6 [38]. Atomic scattering factors and anomalous dispersion corrections were obtained from the literature [39]. Non-hydrogen atoms were found on the *E* map and refined anisotropically. Hydrogen atoms were placed by geometry and are not refined.

Crystal data were as follows: $C_{13}H_{14}O_2$, $M_r = 202.24$, $0.3 \times 0.3 \times 0.04$ mm^3, orthorhombic, space group =

$P2_1$ a b. $a = 7.4879(10)$ Å, $b = 11.2216(10)$ Å, $c = 12.7460(10)$ Å, $V = 1\,071.00(19)$ Å^3, $Z = 4$, $D_x = 1.254$ mg/m^3, λ (Mo Kα) = 0.70930 Å, $\mu = 0.083$ mm^{-1}, $F(000) = 432$, $T = 288(2)$ K, $\theta_{max} = 25.0°$, $-8 \leq h \leq 1$, $0 \leq k \leq 13$, $-15 \leq l \leq 5$. A total of 1 114 reflections were measured, of which 1 023 [R(int) = 0.0091] were independent. Refinement on F^2, $R1$ and $wR2$ [$F^2 > 2\sigma(F^2)$] = 0.0329 and 0.0721, respectively, $R1$ and $wR2$ (all data) = 0.0954 and 0.0849, respectively. Parameters refined = 137, $w = 1/[\sigma^2(F_o^2) + (0.0380P)^2 + 0.07P]$, where $P = (F_o^2 + 2F_c^2)/3$; final $(\Delta/\sigma)_{max} = 0.000$. $\Delta\rho$ in the final difference map within $+0.114$ and -0.138 eÅ^3. Intermolecular hydrogen bonds produce spirals of molecules.

6.2. Screening

6.2.1. Cytotoxicity evaluations

The examination of the cytotoxicity of various compounds towards murine P388D1 cells was undertaken by a literature procedure [40]. The method employed for determining the bioactivity towards murine L1210 cells and human Molt 4/C8 and CEM T-lymphocytes has been described previously [41].

The procedure for evaluating compounds against a panel of human tumour cell lines utilized a reported procedure [42]. In this latter assay, the following neoplastic cell lines were employed: leukaemia, melanoma, non-small cell lung, colon, central nervous system, ovarian and renal neoplasms. In addition, **1c,d,e** were evaluated against small cell lung tumour cell lines and, in the case of the remaining compounds (including melphalan, 5-fluorouracil and helenalin), prostate and breast cancers were also employed. The highest concentration of compounds was log 10^{-4} M except for one of the determinations of **3e**, whereby the maximum concentration used for most of the cell lines was log 10^{-5} M. In addition, the highest concentrations for melphalan and 5-fluorouracil were log $10^{-3.6}$ and log $10^{-2.6}$ M, respectively. The numbers of cell lines whose growth was inhibited by 50% or more at the maximum concentration of compound used per total number of cell lines were as follows: **1c**: 46/46; **1d**: 27/47; **1e**: 2/47; **2c**: 55/55; **3a**: 55/55, 59/59; **3b**: 56/56; **3d**: 55/55, 58/59; **3e**: 56/56, 59/59, 45/57, 57/57; **4**: 56/56, 59/59; **5a**: 57/57; **5b**: 54/54; melphalan: 55/55, 59/59, 57/57; 5-fluorouracil: 44/50, 54/57, 56/58 and helenalin: 51/51. The ranges of the MG MID figures for all cell lines, colon cancer cells and leukaemic cells, respectively, when the assays were conducted in duplicate (**3a,d** and **4**), triplicate (melphalan and 5-fluorouracil) or quadruplicate (**3e**) were as follows: **3a**: 3.02–5.50, 2.06–2.07, 1.82–3.29; **3d**: 6.03–7.25, 3.01–3.52, 3.67–6.38; **4**: 2.95–5.01, 1.88–2.38, 0.887–2.03; melphalan: 19.1–26.9, 41.7–49.9, 2.82–7.16; 5-fluorouracil: 12.0–56.2, 3.09–14.8, 8.05–52.9; and **3e**: 3.72–12.3, 2.44–5.50, 1.73–4.47.

6.2.2. Measurement of apoptotic indices and inhibition of RNA and protein syntheses in human Jurkat T cells

The cytotoxicity and apoptosis studies of **3a,d**, as well as the reference drugs melphalan, actinomycin D and cycloheximide, were determined by a literature method [28], except that nigrosin dye was used in place of trypan blue in the cytotoxicity experiments and a solution of ethidium bromide (0.1% w/v) and acridine orange (0.1 % w/v) was used in the measurements of apoptosis. Inhibition of RNA and protein syntheses was undertaken by a method which has been reported previously [43]. Determinations were made at the end of 48 h (IC_{50} and IC_{80} experiments), 24 h (apoptosis studies) or 8 h (inhibition of RNA and protein syntheses). The IC_{50} (μmol), IC_{80} (μmol), apoptotic index (%), inhibition of RNA synthesis (%) and inhibition of protein synthesis (%) figures for these compounds were as follows: **3a**: 4.47, 7.15, 50.3, 73.2 ± 4.71, 28.9 ± 8.57; **3d**: 5.36, 8.57, 64.7, –, –; melphalan: 2.20, 3.52, 63.2, 85.1 ± 4.71, 17.3 ± 4.94; actinomycin D: 0.0045, 0.0072, 35.5, 90.5 ± 2.15, 49.2 ± 1.53; and cycloheximide: 0.93, 1.54, 38.3, 93.3 ± 3.56, 71.9 ± 3.52.

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