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Original article

# Cytotoxic 5-aryl-1-(4-nitrophenyl)-3-oxo-1,4-pentadienes mounted on alicyclic scaffolds

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**Abstract**

The 5-aryl-1-(4-nitrophenyl)-3-oxo-1,4-pentadienyl pharmacophore was incorporated into four series of compounds **1**–**4**. Compounds **1a–g** comprised a cluster of 3-arylidene-1-(4-nitrophenylmethylene)-2-oxo-3,4-dihydro-1*H*-naphthalenes while the analogues **2a–g** consisted of a group of 6-arylidene-2-(4-nitrophenylmethylene)cyclohexanones. Three other compounds prepared in this study were 1-(4-nitrophenylmethylene)-3-(3,4,5-trimethoxyphenylmethylene)-2-oxo-2,3-dihydro-1*H*-indene **3a** as well as two 5-arylidene-2-(4-nitrophenylmethylene)cyclopentanones **4a, b**. The compounds were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells. In general, the compounds in series **1** displayed marked cytotoxicity having $IC_{50}$ values in the 1–5 μM range while the related cyclohexyl analogues in series **2** were slightly less potent ($IC_{50}$ figures were mainly 5–10 μM). The relative locations of two aryl rings present in all four series were considered to contribute significantly to bioactivity and may have accounted for the virtual absence of cytotoxic properties in series **3** and **4**. Most of the compounds were administered intraperitoneally to mice using doses up to and including 300 mg/kg. No mortalities were noted. The inhibiting effect of most of the compounds towards *Helicobacter pylori* is noteworthy. The modes of action of representative compounds include the induction of apoptosis while some compounds weakly inhibited tubulin polymerisation and human *N*-myristoyltransferase.


*Keywords:* Conjugated unsaturated ketones; Molecular modelling; Cytotoxicity; Structure–activity relationships; Apoptosis; Rodent toxicity

## 1. Introduction

The principal interest in our laboratory is focussed on studies aimed at producing novel cytotoxic and anticancer agents. Various structural motifs have been investigated and recently different series of compounds have been designed which utilized the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Fig. 1) [1–3]. In particular, 1-(4-nitrophenylmethylene)-3-(3,4,5-trimethoxyphenylmethylene)-2-oxo-3,4-dihydro-1*H*-naphthalene **1a** demonstrated marked cytotoxic potencies having $IC_{50}$ values of 1–2 μM towards human Molt 4C8 and CEM T-lymphocytes as well as murine L1210 cells (Table 1) and 0.59 μM when evaluated against a panel of 56 human tumour cell lines [3].

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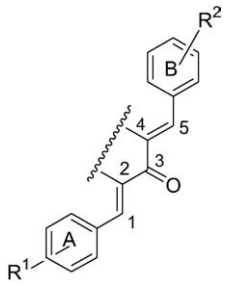



Fig. 1. The 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore.

In order to develop further compounds based on **1a**, the molecular modification of this dienone was planned in two general ways. First, conjugated unsaturated ketones are thiol alkylators [4,5]. By varying the electronic properties of the substituents in rings A and B (Fig. 1), sequential interactions with cellular constituents is likely. Since successive chemical insults may be more detrimental to malignant cells than the corresponding normal cells [6], selective toxicity to neoplasms may occur. Thus the nitro group was retained in ring A while in general electron-donating substituents were placed in ring B. Second, incorporation of the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore onto various alicycles may cause the relative positions of aryl rings A and B to vary. A correlation between the topography of the molecule and cytotoxicity may therefore emerge. These considerations led to the decision to prepare the compounds in series **1**–**4**.

An initial study was directed to determining if the compounds in series **1**–**4** displayed greater toxicity to neoplasms than normal cells and whether reversal of multidrug resistance (MDR) in cancer cells would be demonstrated. The use of HSC-2, HSC-3, HSG and HL-60 human tumour cells as well as the non-malignant human HGF, HPC and HPLF cell lines revealed that a number of the compounds were selectively toxic to the neoplasms while MDR reversal was noted in both human colon cancer Colo 320 and murine lymphoma L-5178 cells in several cases [7]. These encouraging results confirmed that further experimentation on these compounds should be conducted which is described herein. The development included evaluating the compounds in series **1**–**4** against additional cancer cell lines which have been used previously in evaluating candidate cytotoxics, examining the topography of various molecules, probing as to the mode(s) of action of representative compounds and undertaking toxicity assessments in mice.

Table 1
Evaluation of various compounds in series **1**–**4** against human Molt 4/C8 and CEM T-lymphocytes, murine L1210 cells and *Helicobacter pylori*

| Compound | $IC_{50}$(μM)[a] | | | |
|---|---|---|---|---|
| | Molt 4/C8 | CEM | L1210 | *H. pylori* |
| **1a**[b] | 1.56 ± 0.01 | 1.54 ± 0.08 | 1.94 ± 0.12 | 6.17 ± 0.12 |
| **1b** | 12.3 ± 4.1 | 25.5 ± 2.5 | 49.5 ± 0.8 | 23.2 ± 0.08 |
| **1c** | 0.968 ± 0.186 | 0.953 ± 0.377 | 3.20 ± 0.48 | 8.07 ± 0.14 |
| **1d** | 0.992 ± 0.672 | 1.64 ± 0.61 | 2.52 ± 0.01 | 6.63 ± 0.15 |
| **1e** | 1.13 ± 0.64 | 1.62 ± 0.11 | 4.82 ± 2.43 | 8.34 ± 0.12 |
| **1f** | 6.16 ± 1.11 | 7.37 ± 0.14 | 8.67 ± 0.48 | 17.4 ± 0.09 |
| **1g**[b] | 1.72 ± 0.03 | 1.70 ± 0.01 | 4.18 ± 2.16 | – |
| **2a** | 6.42 ± 1.07 | 4.61 ± 3.89 | 6.97 ± 1.80 | 46.6 ± 0.10 |
| **2b** | 300 ± 54 | 250 ± 6 | 240 ± 8 | >100 |
| **2c** | 8.48 ± 0.25 | 8.14 ± 0.01 | 11.2 ± 4.1 | 14.2 ± 0.11 |
| **2d** | 9.49 ± 1.71 | 7.61 ± 0.01 | 9.78 ± 0.87 | 5.80 ± 0.13 |
| **2e** | > 500 | > 500 | > 500 | >100 |
| **2f** | 9.41 ± 0.18 | 7.27 ± 0.93 | 9.95 ± 0.35 | 11.3 ± 0.14 |
| **2g** | 9.12 ± 0.28 | 8.18 ± 0.20 | 9.41 ± 0.97 | 5.91 ± 0.14 |
| **3a** | 442 ± 82 | 320 ± 43 | 216 ± 13 | – |
| **4a** | > 500 | > 500 | > 500 | 19.0 ± 0.09 |
| **4b** | > 500 | > 500 | > 500 | >100 |
| **5**[b] | 34.0 ± 5.9 | 25.5 ± 14.8 | 39.4 ± 0.0 | – |
| **6**[c] | 14.9 ± 0.5 | 14.5 ± 0.4 | 54.5 ± 1.7 | – |
| Melphalan | 3.24 ± 0.79 | 2.47 ± 0.30 | 2.13 ± 0.03 | – |
| Metronidazole | – | – | – | 309 |

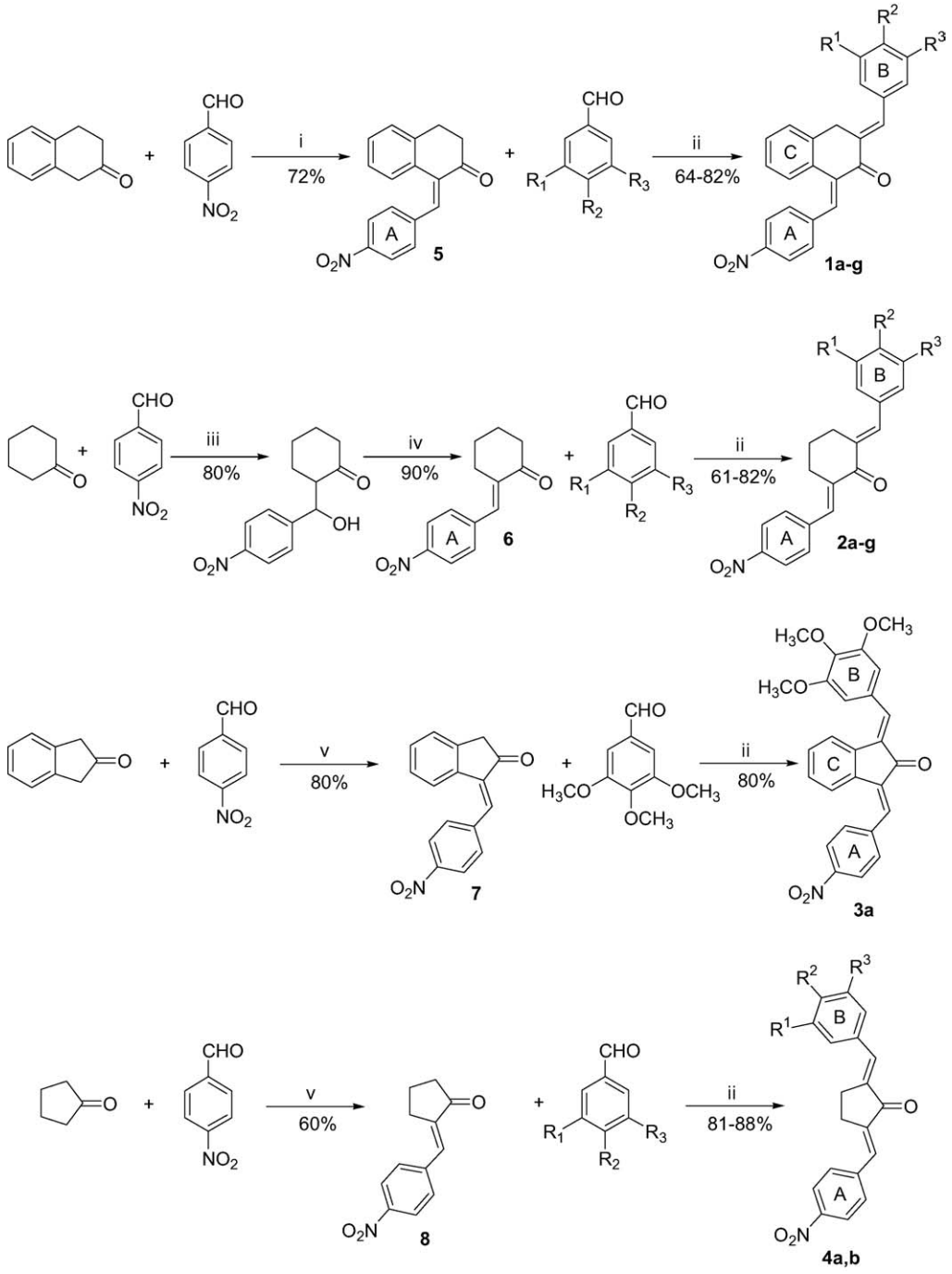
[a] The $IC_{50}$ values are the concentrations required to inhibit the growth of 50% of the neoplastic cells (with standard deviation figures) and *H. pylori* (standard errors are presented).

[b] Data taken from Ref. [3] and reproduced with the permission of the copyright owner.

[c] Data taken from Ref. [38] and reproduced with the permission of the copyright owner (www.tandf.co.uk).

## 2. Chemistry

The synthetic chemical routes employed which led to the preparation of the compounds in series **1**–**4** are presented in Scheme 1. Various strategies were used to prepare the intermediates **5**–**8** which were then condensed with different aryl aldehydes producing the compounds **1**–**4**. The manner in which the monoarylideneketones **5**–**8** were synthesized is as follows. Condensation of 2-oxo-3,4-dihydro-1*H*-naphthalene with 4-nitrobenzaldehyde led to the formation of the enone **5**. Reaction of cyclohexanone with 4-nitrobenzaldehyde under basic conditions led initially to the isolation of the corresponding aldol. Subsequently the reaction was repeated whereby the intermediate aldol formed was dehydrated with acid giving rise to 2-(4-nitrophenylmethylene)cyclohexanone **6**. Reaction of 2-oxo-2,3-dihydro-1*H*-indene with 4-nitrobenzaldehyde in the presence of sodium hydroxide or hydrochloric acid led to the formation of polymers and 1,3-bis(4-nitrophenylmethylene)-2-oxo-2,3-dihydro-1*H*-indene one, respectively. An alternative procedure was employed whereby an enamine from 2-oxo-2,3-dihydro-1*H*-indene was prepared which reacted in good yield with 4-nitrobenzaldehyde to give the desired product **7**. A similar route was undertaken in the conversion of cyclopentanone into 2-(4-nitrophenylmethylene)cyclopentanone **8**. The compounds in series **1**–**4** were shown from 1H NMR spectroscopy to be stereoisomerically pure and exhibited the *E,E* configuration. This observation is in accord with recent X-ray crystallographic determinations of various 1,3-diarylidene-2-oxo-3,4-dihydro-1*H*-naphthalenes [3] and 2,6-bis(arylidene)cyclohexanones [8–10] which revealed that the olefinic double bonds adopted the *E,E* configuration. Molecular models of **1a**, **2a**, **3a** and **4a** were built in order to find the relative positions of different atoms and groups. These determinations are illustrated in Fig. 2 while the data are presented in Table 2.




Scheme 1. The reagents used in the syntheses of the compounds in series **1**–**4** were as follows, namely i = piperidine/acetic acid/toluene; ii = dry HCl/chloroform/ether; iii = $NaOH/H_2O$; iv = HCl/ethanol; v = morpholine/PTSA/benzene. The aryl substituents were as follows: **a**: $R^1 = R^2 = R^3 = OCH_3$; **b**: $R^1 = R^3 = H$, $R^2 = OCH_3$; **c** : $R^1 = R^2 = OCH_3$, $R^3 = H$; **d**: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = H$; **e** : $R^1 = R^2 = OCH_2O$, $R^3 = H$; **f** : $R^1 = R^3 = H$; $R^2 = OH$; **g**: $R^1 = R^3 = H$, $R^2 = Cl$.

## 3. Bioevaluations

The enones **1a–g**, **2a–g**, **3a**, **4a, b, 5** and **6** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells. These data are presented in Table 1. The unsaturated ketones **1b, e, f** were examined towards approximately 54 human tumour cell lines from nine different neoplasias, namely leukemia, melanoma and non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers and the results are summarized in Table 3. Most of the compounds in series **1**–**4** were screened against *Helicobacter pylori* and the $IC_{50}$ values are given in Table 1. HepG2 cells treated with 1 μM of **1e** underwent apoptosis. The $IC_{50}$ values of **1a–f**, **2a–g** and **4a, b** towards urease were greater than 200 μg/ml. The $IC_{50}$ values of **1c**, **2a** and **4a** when examined for their efficacy in inhibiting tubulin polymerisation were in

Table 2
Interatomic distances $d_1$–$d_3$, bond angle $\psi_1$ and torsion angles $\theta_1$ and $\theta_2$ determined by molecular modelling in **1a**, **2a**, **3a** and **4a**

| Compound | $d_1$ (Å) | $d_2$ (Å) | $d_3$ (Å) | $\psi_1$ (°) | $\theta_1$(°) | $\theta_2$(°) |
|---|---|---|---|---|---|---|
| **1a** | 10.07 | 5.643 | 5.625 | 126.8 | 58.3 | 80.3 |
| **2a** | 10.15 | 5.600 | 5.593 | 130.1 | 67.9 | -66.2 |
| **3a** | 9.799 | 5.749 | 5.742 | 117.0 | -89.7 | -90.0 |
| **4a** | 9.685 | 5.780 | 5.772 | 113.9 | -56.6 | -54.9 |

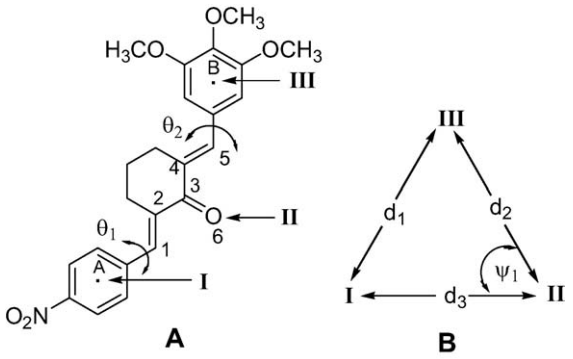



Fig. 2. A. Designations of different parts of the 1,5-diaryl-3-oxo-1,4-pentadienyl group in **2a.** B. The interatomic distances $d_1$-$d_3$ and bond angle $\psi_1$ in **1a**, **2a**, **3a** and **4a**.

Table 3
Evaluation of **1b, e, f** and reference drugs against human tumour cell lines

| Compound | All cell lines | | Leukemic cells | | Colon cancer cells | |
|---|---|---|---|---|---|---|
| | $GI_{50}$ (μM) | SR [a] | $IC_{50}$ (μM) | SI[b] | $IC_{50}$ (μM) | SI[b] |
| **1b** | 19.1 | > 36.3 | 7.76 | 2.46 | 8.32 | 2.30 |
| **1e** | 3.80 | > 191 | 1.35 | 2.82 | 3.39 | 1.12 |
| **1f** | 2.82 | 21.9 | 1.51 | 1.87 | 1.98 | 1.42 |
| Melphalan | 24.5 | 178 | 3.98 | 6.16 | 49.9 | 0.49 |
| 5-Fluorouracil | 29.5 | > 4365 | 52.9 | 0.56 | 5.82 | 5.07 |

[a] The letters SR refer to the selectivity ratio viz the ratio of the $IC_{50}$ values of the least and most sensitive cell lines.

[b] The letters SI indicate the selectivity index viz. the ratio of the $GI_{50}$ figures divided by the average $IC_{50}$ values for leukemic and colon cancer cell lines.

excess of 50 μM (**1c**, **2a**) or 25 μM (**4a**). The enzyme human *N*-myristoyltransferase (hNMT) catalyses the covalent attachment of the myristoyl group to the N-terminal glycine residue of a variety of proteins [11]. A representative compound **1a** was evaluated against hNMT and at the maximum concentration utilized, namely 1 mM, the percentage inhibition of this enzyme was 23.2. Compounds **1b, e, f** were screened against five species of *Aspergillus fumigatus* and four *Candida* species; however the minimal inhibitory concentration (MIC) values were in excess of 32 μg/ml. Doses of 30, 100 and 300 mg/kg of **1a, b, e, f**, **2a–g**, **3a** and **4a, b** were administered intraperitoneally to mice and the animals were examined after 0.5 and 4 hours. No mortalities were noted, while minimal neurotoxicity was noted with **2a** and **3a**. Doses of 30 or 50 mg/kg of **1e, f** and **2g** were administered orally to rats while a further study with **1e** used a dose range of 25–250 mg/kg. A 30 mg/kg dose of **1e** and **2g** was injected intraperitoneally into rats. Over the time span of 0.25–4 hours, neither deaths nor neurotoxicity were observed in any of these experiments with rats.

## 4. Results and discussion

The compounds in series **1**–**4** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells. The results are summarized in Table 1.

The relative potencies between the lead molecule **1a** and related compounds in series **1** revealed a consistent pattern using the Molt 4/C8 and CEM assays. These determinations showed that in comparison to **1a**, **1c** was more potent, **1d, e** were equipotent and **1b, f, g** had higher $IC_{50}$ values than **1a**. This observation indicated that oxygen-containing substituents at positions 3 and 4 in ring B were optimal in terms of potency while a single substituent in the *para* location of this ring in **1b, f, g** lowered cytotoxic potencies significantly. Somewhat different structure–activity relationships were noted in the murine L1210 assay. For example, while **1g** and **1a** were equipotent, other members of series **1** had higher $IC_{50}$ values than **1a**.

The $IC_{50}$ values of **1a–g** in these three cytotoxicity screens were compared with those for melphalan. Greater potencies than the reference drug were displayed by **1a, c–e, g**, **1a, c, e, g** and **1a** in the Molt 4/C8, CEM and L1210 screens, respectively, while **1d, g** and melphalan were equipotent in the CEM and L1210 assays. Thus, in these three screens, over one half of the compounds possessed greater or equal potencies compared to the established alkylating agent melphalan revealing the potential of this cluster of compounds for further development.

A question to be resolved was whether greater cytotoxicity was obtained using the 3,4-dihydro-1*H*-naphthalenyl or cyclohexyl frameworks in series **1** and **2**, respectively. A comparison was therefore made between the $IC_{50}$ values of those compounds bearing identical substituents in rings A and B in each of the screens, e.g. the data for **1a** and **2a** were compared in the Molt 4/C8 screen, then in the CEM test and so forth. With the exception of **1f** and **2f**, which had statistically indistinguishable $IC_{50}$ values in the CEM screen, all the compounds in series **1** had greater cytotoxicity; the largest difference being between the biodata generated in all three screens for **1e** and **2e**.

In order to seek explanations for the variation in cytotoxicity between series **1**–**4**, molecular models of a representative compound in each series were built, namely **1a, 2a, 3a** and **4a**. Overlap of atoms 1–6 (Fig. 2A) were made between pairs of compounds so that each unsaturated ketone was compared with the other analogs in turn. The root mean square (RMS) values generated were invariably less than 0.2 Å. Thus, this group

occupied similar spatial arrangements in all four compounds. Hence, the differences in cytotoxicity between the compounds in series **1**–**4** are unlikely to be due to variations in the shapes of the dienone substructure. However, the overlap of atoms 1–6 revealed that rings A and B occupied significantly different locations. In order to compare the locations of rings A and B in **1a**, **2a**, **3a** and **4a**, measurements were made between the centres of the aryl rings (I and III in Fig. 2A) and secondly, in relation to the ketonic oxygen atom (II in Fig. 2A), taken as the reference point. The distances $d_1$–$d_3$ and angle $\psi_1$, as indicated in Fig. 2B, enabled the relative positions of the aryl rings in **1a**, **2a**, **3a** and **4a** to be found. These data are presented in Table 2. In addition, the literature records a number of correlations between the torsion angles between aryl rings and the adjacent unsaturated groups with bioactivity [12]. Hence the $\theta_1$ and $\theta_2$ angles as indicated in Fig. 2A were measured and are presented in Table 2.

The cytotoxicity data in Table 1 revealed that **1a** and **2a** have significant cytotoxic potencies ($IC_{50}$ figures in the 1–7 μM range) while for **3a** and **4a**, the $IC_{50}$ values are in excess of 200 μM. The results in Table 2 indicate that the $d_1$–$d_3$ and $\psi_1$ figures are similar for **1a** and **2a** while little variation was noted between **3a** and **4a**. Thus, the preferable locations of rings A and B in series **1** and **2** likely contributed to the greater toxicity of these compounds compared to the analogues containing a five-member alicyclic ring. The $\theta_1$ values of **1a**, **2a**, **3a** and **4a** were 58°, 68°, 90° and 123° when considered in a clockwise rotation of the deviation from coplanarity between ring A and the adjacent olefinic linkage. Since the relative potencies of these four compounds in the Molt 4/C8, CEM and L1210 assays were **1a** > **2a** > **3a** > **4a**, an inverse relationship between the size of the $\theta_1$ value and cytotoxic potency was suggested. On the other hand, no correlations between the $IC_{50}$ figures in these cytotoxicity screens with the $\theta_2$ values were discerned.

If cytotoxicity was caused by thiol alkylation, the possibility of chemosensitivity occurring in the tumour cells was addressed by considering the cytotoxicity data in Table 1 for the compounds **1a–g**, **2a–g**, **5** and **6**. By chemosensitivity is meant that initial depletion of thiols in the tumours sensitise these cells to a further chemical insult which could be caused by **1a–g** and **2a–g** but not **5** and **6**. In the absence of chemosensitivity, the potencies of the bis thiol alkylators **1a–g** should be twice that of the monoalkylator **5**; similarly **2a–g** would be predicted to have double the potencies of **6**. However, if sensitisation of the cells occurred, then the second attack on cells which had been made vulnerable by the initial thiol depletion [6] will be demonstrated by the $IC_{50}$ values of **1a–g** and **2a–g** being greater than twice those of **5** and **6**, respectively. The data in Table 1 revealed that **1a–g** were more than twice as potent as **5** in 19 of the 21 comparisons made (91%). On the other hand, **2a–g** possessed more than twice the potency of **6** in only 33% of the cases. Thus, while synergism was observed 62% of the time thus favouring the design of bifunctional alkylators, it is clearly dependent on the general structure of the series of compounds under examination.

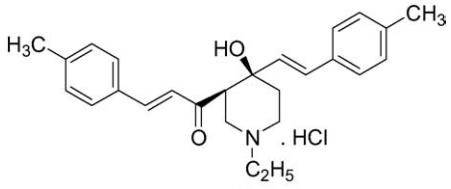
Three compounds in series **1**, namely **1b, e, f**, were evaluated against 54 ± 2 human tumour cell lines from nine different neoplastic diseases. The concentration range employed was $10^{-4}$–$10^{-8}$ M and on rare occasions the growth of the cells at the maximum concentration of $10^{-4}$ M was not inhibited by 50%. Hence, the term $GI_{50}$ was used and not $IC_{50}$. The data are presented in Table 3. The $GI_{50}$ values demonstrated that **1b, e, f** were more cytotoxic than melphalan and, in particular, **1e** and **1f** possessed 6.5 and 8.7 times the potency of the reference drug. A positive feature of a candidate cytotoxin is exhibition of varying potencies towards different cell lines. This property may reveal that the compound in question possesses a selective toxicity for one or more groups of cancers accompanied by a much lower toxicity to the corresponding normal cells. The enones **1b, e, f** displayed varying potencies to the cell lines and **1e** possessed a greater selectivity ratio than melphalan. An examination of the mean graphs [13] of **1b, e, f** revealed that, in general, a preferential toxicity for leukemic and colon cancer cells was observed. The data in Table 3 indicated that towards leukemic cells, **1e, f** were more potent than melphalan, which is effective towards chronic lymphatic leukemia [14]. The average selectivity index (SI) value for **1b, e, f** was 2.4, which is significant although less than the value for melphalan. 5-Fluorouracil is used in treating colon cancers [15]. The enones **1e, f** were more potent than 5-fluorouracil, especially **1f**, which possessed three times the potency of this drug. The average SI value of **1b, e, f** is 1.6, which is less than for 5-fluorouracil. The conclusions that may be drawn from the assay using a panel of human tumour cell lines is that **1e, f** are very useful lead molecules due to their potencies towards malignant cells in general and leukemic and colon cancers in particular.

A major challenge in cancer research is the successful treatment of stomach cancer. This condition arises from various causes, one of which is chronic infections with *Helicobacter pylori* [16]. A useful drug used in treating these infections is the nitroheterocycle metronidazole and thus the evaluation of some of the nitroaromatics prepared in this study as candidate drugs for treating *H. pylori* was considered a worthwhile investigation. A wide range of concentrations was employed viz. approximately 2–5000 μM. In most cases the growth of the microorganism was inhibited at lower concentrations up to 70–200 μM while higher concentrations led to stimulation of the growth of *H. pylori*. The $IC_{50}$ values of **1a–f**, **2a–g** and **4a, b** generated in this screen are presented in Table 1. The following observations were made pertaining to structure–activity relationships. First, in general, the compounds in series **1** had lower $IC_{50}$ values than the analogues in series **2**. Thus **1a–c, e** were more potent than **2a–c, e** and the average $IC_{50}$ figures for **1a–f** and **2a–f** were 11.6 and > 46.3 μM, respectively. Second, the order of potency in series **1** was **1a** > **1d** > **1c** > **1e** > **1f** > **1b** revealing that the structures of the four most potent compounds ($IC_{50}$ values < 10 μM) each possess substituents in both the 3 and 4 positions in ring B. Third, the relationship between the nature of the aryl substituents in ring B of series **2** and antibacterial potencies was different from series **1** insofar as the relative potencies in series **2**

were **2d, g** > **2f** > **2c** > **2a** > **2b, e**. Fourth, **4a,** which contains a cyclopentane ring, possessed more than twice the potency of the cyclohexyl analogue **2a**. Finally, most of the compounds were far more potent than the established drug metronidazole. An important conclusion to be drawn from these experiments is that a number of the compounds prepared in this study are novel prototypic anti-*H. pylori* agents.

With a view to identifying the way in which these compounds exert their cytotoxic activity, experiments were initiated to determine whether these enones would (i) induce apoptosis, (ii) inhibit the sulphydryl enzyme urease, (iii) prevent tubulin formation and (iv) exert any effect on hNMT.

Several years ago, compound **9**, which contains two olefinic groups in different electronic environments, as well as melphalan, were shown to induce apoptosis in human Jurkat T leukemia cells [17]. In fact a number of cytotoxic compounds are apoptotic [18]. The question posed was whether a representative compound prepared in this study would also induce apoptosis in a malignant cell line. Compound **1e** was chosen since the data in Tables 1 and 3 reveal not only its excellent cytotoxicity but also its superior SR value compared to melphalan (Table 3). A concentration of 10 μM of **1e** was lethal to human HepG2 liver cancer cells while the death of some of the cells was noted at lower concentrations of 1 and 0.1 μM. A TUNEL assay was conducted using 1 μM of **1e** which provided unequivocal evidence that **1e** causes apoptosis. The concentrations at which apoptosis is noted is similar to the quantities of compound required to generate the data for **1e** in Tables 1 and 3.




**9**

Second, ureases are sulphydryl enzymes which have a cysteine residue in the active site [19]. In particular, *H. pylori* produces a urease which catalyses the hydrolysis of urea to ammonia which makes possible the inhabitation of this microorganism in the stomach [20]. A number of α,β-unsaturated ketones have demonstrated urease-inhibiting properties [21] but the $IC_{50}$ values of **1a–f**, **2a–g**, **4a, b** towards this enzyme were in excess of 200 μg/ml. Third, representative compounds in series **1**, **2** and **4** were examined for inhibition of tubulin polymerisation. A concentration of 50 μM of **1c** and **2a** caused 16% and 40% inhibition, respectively, while solubility problems were encountered with **4a** at this concentration. Using 25 μM of **4a**, 16% inhibition of tubulin polymerisation was observed. Thus, the compounds interact at one of the tubulin binding sites [22]. Since the average $IC_{50}$ values of **1c**, **2a** and **4a** against the Molt 4/C8, CEM and L1210 cells is 1.71, 6.00 and > 500 μM, respectively, no correlation between inhibition of tubulin polymerisation and cytotoxic potencies was observed. Fourth, one of the differences between certain tumours, such as some colon cancers and the surrounding normal tissues, is the higher activity and expression of hNMT in the malignant cells [23]. The active site of a fungal NMT is believed to contain a mercapto group [24] and hence the thiol alkylators prepared in this study may exert their bioactivity, at least partially, by this mechanism. Accordingly, the effect of the lead molecule **1a** on hNMT was undertaken. At the highest concentration of **1a** utilized, namely 1 mM, the enzyme was inhibited by 23.2%. The $IC_{50}$ figures of **1a** in Table 1 are < 2 μM and the average $IC_{50}$ values of this compound towards human colon and leukemic lines are < 0.4 μM [3]; hence the inhibition of hNMT exerts, at the most, only a minor role in causing the cytotoxicity of **1a**.

The final experimentations were undertaken with the objective of discerning whether the compounds in this study were biocidal in general or whether some specificity for malignant cells could be discerned. Two approaches were adopted to assess this possibility, namely the examination of representative compounds towards certain pathogenic fungi as well as toxicity evaluations in mice and rats.

Compounds **1b, e, f** were examined for antifungal properties using different strains of *A. fumigatus* and four *Candida* species. In each case the MIC values of these compounds were in excess of 32 μg/ml (78–91 μM). In view of the data presented in Tables 1 and 3, **1b, e, f** were clearly far more toxic to malignant cells than to the fungi. No acute mortalities were noted when doses up to and including 300 mg/kg of **1a, b, e, f, 2a–g, 3a, 4a, b** were administered intraperitoneally to mice. Studies with rats involving intraperitoneal injection of **1e, 2g** and oral administration of **1e, f, 2g** did not lead to acute mortalities or neurotoxicity. These data suggested that, in contrast to many cytotoxic and anticancer agents such as melphalan (the $LD_{50}$ in mice is 21.7 mg/kg [25]), the enones were well tolerated in rodents.

## 5. Conclusion

When the 5-aryl-1-(4-nitrophenyl)-3-oxo-1,4-pentadienyl pharmacophore is aligned in a certain fashion as in series **1** and **2**, the compounds exert potent cytotoxicity. These molecules are structurally divergent from the anticancer drugs used currently and may differ from them in their mechanisms of action. Both the absence of rapid onset of toxicity in rodents and the inhibition of the growth of *H. pylori* further enhance the potential of thse compounds for development as therapeutic agents. Further studies should be undertaken in order to find the precise mechanisms whereby apoptosis is induced as well as probing for other ways whereby the compounds exert their cytotoxic effects.

## 6. Experimental protocols

### 6.1. Chemistry

The melting points, which are quoted in °C, were determined using a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses (C, H, N) were undertaken on **1b–f**, **2a–g**, **3a** and **4a, b** by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan and were within 0.4% of the calculated values except for **2e** (calcd. for C: 69.41%. Found: C: 68.96%). 1H NMR spectra were determined on all compounds using a Brucker AM 500 FT NMR machine (500 MHz). The positions of the hydrogen atoms in the aryl rings are designated as unprimed (ring A), single primed (ring B) and, when present, double primed (ring C). The letters br, s, d, t, qu, m refer to broad, singlet, doublet, triplet, quintet and multiplet, respectively. The compounds were recrystallized from chloroform/methanol except for chloroform which was used to purify **2b** and **4b** while **3a** was crystallised from chloroform/acetone.

#### 6.1.1. Synthesis of series 1

The preparation of **1a, g** has been described previously [3]; however **1a** was resynthesised for this project. The intermediate required in the preparation of **1a–g**, namely 1-(4-nitrophenylmethylene)-2-oxo-3,4-dihydro-1*H*-naphthalene **5**, was synthesized by a literature procedure [3] in 72% yield, m.p. 155 (lit. [3] m.p. 154–155). The general method followed in preparing **1a–g** was as follows. A stream of hydrogen chloride gas was passed into a solution of **5** (0.01 mol) and the appropriate aryl aldehyde (0.01 mol) in chloroform (10 ml) and diethyl ether (25 ml) for 10–15 min. For the synthesis of **1f**, 2 ml of methanol was added to the reaction mixture prior to the addition of hydrogen chloride gas. After stirring the reaction mixture at room temperature for 3–4 h, the precipitate was collected and recrystallized. The m.p.s and yields were as follows: **1a**: (185 (lit. [3] 185–186) 72%; **1b** : 212, 82%; **1c**: 176–177, 68%; **1d**: 207–208, 71%; **1e**: 204, 76%; **1f**: 251, 64%. The 1H NMR spectrum of a representative compound **1e** is as follows: δ ($CDCl_3$): 4.15 (s, 2H, $CH_2$), 6.08 (s, 2H, $OCH_2O$), 6.93 (d, $J = 7.99$ Hz, 1H, $C_5'H$), 7.01 (s, 1H, C2'H), 7.05 (m, 2H, C6′ H, C6″H), 7.20 (d, $J = 7.76$ Hz, 1H, $C_5''H$), 7.28 (m, 2H, $C_7''H$, $C_8''H$), 7.57 (s, 1H, olefinic H ring B), 7.62 (d, $J = 8.70$ Hz, 2H, $C_2H$, $C_6H$), 7.80 (s, 1H, olefinic H ring A), 8.15 (d, $J = 8.69$, 2H, $C_3H$, $C_5H$).

#### 6.1.2. Synthesis of series 2

The intermediate required in the synthesis of **2a–g**, namely 2-(4-nitrophenylmethylene) cyclohexanone **6**, was prepared by a previously reported methodology [26] to give **6**, m.p. 119 (lit. [26] m.p. 118–120) in 72% yield with respect to 4-nitrobenzaldehyde.

The condensation of **6** with various aryl aldehydes was accomplished using the same methodology as the reaction of **5** with different aromatic aldehydes vide supra. In the case of **2f**, methanol (2 ml) was added to the reaction mixture prior to the passage of hydrogen chloride gas. The m.p.s and yields for **2a–g** were as follows: **2a**: 164–165, 61%; **2b**: 197–199, 75%; **2c**: 162–164, 80%; **2d**: 191–192, 76%; **2e**: 218–220, 65%; **2f**: 199–200, 66%; **2g**: 145–146, 82%. The 1H NMR spectrum of a representative compound **2e** is as follows: δ ($CDCl_3$): 1.84 (qu, 2H, $CH_2$), 2.91 (t, $J = 5.13$ Hz, $J = 5.26$ Hz, 2H, $CH_2$), 2.96 (t, $J = 5.34$ Hz, $J = 5.22$ Hz, 2H, $CH_2$), 6.04 (s, 2H, $OCH_2O$), 6.88 (d, $J = 8.03$ Hz, 1H, C5′H), 7.03 (m, 2H, C2′H, C6′H), 7.59 (d, $J = 8.69$ Hz, 2H, $C_2H$, $C_6H$), 7.76 (s, 1H, olefinic H ring B), 7.82 (s, 1H, olefinic H ring A), 8.27 (d, $J = 8.74$ Hz, 2H, $C_3H$, $C_5H$).

#### 6.1.3. Synthesis of 3a

The intermediate 1-(4-nitrophenylmethylene)-2-oxo-2,3-dihydro-1*H*-indene **7** was prepared as follows. A solution of 2-oxo-2,3-dihydro-1*H*-indene (0.02 mol), morpholine (0.022 mol), *p*-toluenesulfonic acid (20 mg) and benzene (100 ml) was heated under reflux in a Dean-Stark apparatus for approximately 24 h until the stoichiometric amount of water was collected. 4-Nitrobenzaldehyde (0.02 mol) was added to the reaction mixture and heating under reflux was continued for 22 h. On cooling, hydrochloric acid (37% w/v, 30 ml) was added and the solution was stirred at room temperature for 8 h. Benzene was removed in vacuo and the residue was extracted with chloroform. The organic extract was washed with hydrochloric acid (2 N, 3 × 50 ml), water (3 × 50 ml) and dried over anhydrous sodium sulphate. Removal of the chloroform led to a solid which was recrystallized from chloroform–methanol to give **7**, m.p. 164–167 in 80% yield. The 1H NMR spectrum was as follows: δ ($CDCl_3$): 3.64 (s, 2H, $CH_2$), 7.18 (t, $J = 7.62$ Hz, $J = 7.58$ Hz, 1H, C5″H), 7.34 (s, 1H, C2′H), 7.37 (t, $J = 7.49$ Hz, $J = 7.39$ Hz, 1H, C2″H), 7.43 (d, $J = 7.53$ Hz, 1H, C4″H), 7.49 (s, 1H, olefinic H), 7.53 (d, $J = 7.92$ Hz, 1H, C7″H), 7.77 (d, $J = 8.57$, 2H, $C_2H$, $C_6H$), 8.35 (d, $J = 8.63$ Hz, 2H, $C_3H$, $C_5H$).

Compound **7** reacted with 3,4,5-trimethoxybenzaldehyde using the same procedure as employed for the synthesis of **1a–e, g** to give **3a**, m.p. 220–222, in 80% yield. The 1H NMR spectrum was as follows: δ ($CDCl_3$): 4.02 (s, 6H, 2 x$OCH_3$), 4.04 (s, 3H, $OCH_3$), 7.16 (t, $J = 7.61$ Hz, $J = 7.54$ Hz, 1H, C5″H), 7.34 (s, 1H, $C_2'H$), 7.40 (t, $J = 7.59$ Hz, $J = 7.51$ Hz, 1H, C6″H), 7.46 (d, $J = 7.85$ Hz, C4″H), 7.61 (s, 1H, $C_6'H$), 7.69 (d, $J = 7.83$ Hz, 1H, C7″H), 7.74 (s, 2H, olefinic H), 7.78 (d, $J = 8.53$ Hz, 2H, $C_2H$, $C_6H$), 8.33 (d, $J = 8.65$ Hz, 2H, $C_3H$, $C_4H$).

#### 6.1.4. Synthesis of *4a, b*

The procedure employed in forming **7** was utilized in synthesizing 2-(4-nitrophenylmethylene)cyclopentanone **8**. Recrystallization of the crude product from chloroform-methanol afforded **8**, m.p. 138 (lit. [27] m.p. 145–146) in 60% yield.

Compound **8** was condensed with the appropriate aryl aldehyde using the procedure for preparing **1a–e, g** to give **4a**, m.p. 185–186 and **4b**, m.p. 238–240 in yields of 81% and 88%, respectively. The 1H NMR spectrum of a representative compound **4a** was as follows: δ ($CDCl_3$): 3.18 (s, 4H, 2 x$CH_2$),

3.94 (s, 9H, 3 x$OCH_3$), 6.90 (s, 2H, olefinic H), 7.60 (d, $J = 9.15$ Hz, 2H, C2′H, C6′H), 7.74 (d, $J = 8.72$ Hz, 2H, $C_2$H, $C_6$H), 8.31 (d, $J = 8.73$ Hz, 2H, $C_3$H, $C_5$H).

#### 6.1.5. Molecular modelling

Models of **1a**, **2a**, **3a** and **4a** were built using the Macro-Model 8.0 programme [28] followed by a Monte Carlo search for the conformations with the lowest energies using an Amber force field of 1000 initial conformations. Overlaps were made of atoms 1–6 as indicated in Fig. 2A between the following pairs of compounds (RMS value in parenthesis) viz. **1a**/**2a** (0.1687), **1a**/**3a** (0.1501), **1a**/**4a** (0.1228), **2a**/**3a** (0.1613), **2a**/**4a** (0.1746) and **3a**/**4a** (0.0538).

### 6.2. Bioevaluations

#### 6.2.1. Cytotoxic screens

The evaluations using human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells were undertaken by a previously reported method [29].

Evaluation against the panel of human cell lines was undertaken using a literature procedure [30]. The concentrations of compounds used were $10^{-4}$ to $10^{-8}$ M for **1b**, **1e** and **1f**, $10^{-3.6}$–$10^{-7.6}$ M for melphalan and $10^{-2.6}$–$10^{-6.6}$ M for 5-fluorouracil. The number of cell lines whereby 50% inhibition of the growth of the cells at the highest concentrations was not achieved/total number of cell lines were as follows, namely **1b** (1/54), **1e** (1/56), **1f** (0/52), melphalan (0/55) and 5-fluorouracil (3/57). The data for the leukemic and colon cancer cells are $IC_{50}$ values.

#### 6.2.2. Anti-Helicobacter pylori assay

This determination was undertaken by a literature procedure [31]. In brief, *H. pylori* was inoculated into brain heart infusion broth containing 10% fetal bovine serum (Biofluid, Inc., Rockville, USA) and 0.1% glucose and cultured at 37 °C. After 2 days, the bacterial colonies were collected and diluted with medium to give $10^7$ CFU/ml to which a solution of the compound in dimethylsulphoxide was added. The mixture was incubated at 37 °C for 5 days. Eight concentrations were used in the range of 0.001–2.222 mg/ml. The experiment was performed in triplicate for each compound except in the case of metronidazole which was evaluated twice. The $IC_{50}$ values (± S.E.M.) were obtained using nonlinear regression analysis [32].

#### 6.2.3. Urease assay

The assay was undertaken by a previously reported methodology [33]. In brief, the test compound and Jack bean urease (Sigma-Aldrich, Milwaukee, USA) were preincubated for 3 hours at room temperature after which time a solution of urea and phenol red in phosphate buffer was added. The reaction time was measured by a microplate reader (570 nm).

#### 6.2.4. Apoptosis experimentation

The HepG2 cells line was obtained from the ATCC. These cells were grown in DMEM supplemented with 10% fetal calf serum and a 1% antibiotic-antimycotic solution (GIBCO, Burlington, Canada) which contained penicillin G sodium, streptomycin sulphate and amphotericin B in an environment of humidified air containing 5% carbon dioxide at 37 °C. For the apoptosis assay, the HepG2 cells were cultured in chambered slides in the presence of 1 μM of **1e**. Control experiments were undertaken with the HepG2 cells in the presence and absence of dimethylsulphoxide. After 48 h, the cells were fixed and permeabilized. The apoptotic cells were detected by the TUNEL assay. The apoptosis assay was carried out using the Apoptosis Detection System, Fluorescein (Promega, Madison) and following the protocol of the manufacturer. Apoptotic cells having fluorescently labelled DNA were visualized under a fluorescent microscope. The percentage of cells which are apoptotic was 51.6 ± 5.14.

#### 6.2.5. Tubulin assembly assay

The effect of the compounds on the assembly of porcine brain tubulin was assessed by measuring changes in turbidity as described previously [34]. The $IC_{50}$ for a reference compound podophyllotoxin is 1.9 ± 0.7 μM.

#### 6.2.6. Assay of *1a* versus hNMT

The evaluation of **1a** towards hNMT was undertaken by a previously reported procedure [35]. The percentage inhibition of hNMT when 1 mM of **1a** was used was 23.2 ± 5.23.

#### 6.2.7. Antifungal evaluations

Compounds **1b, e, f** were examined against five strains of *Aspergillus fumigatus*, namely H27023 (ATCC 280997), W73355 (ATCC 280996), F55064 (ATCC 280995), M44251 and F69827. Evaluation was also undertaken using *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 66032), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 13803). The broth microdilution assay was employed [36]. The reference drug was voriconazole which had MIC values of 0.25 μg/ml using the *A. fumigatus* isolates and *C. glabrata* and 0.062 μg/ml towards *C. albicans*, *C. parapsilosis* and *C. tropicalis*.

#### 6.2.8. Rodent toxicity screens

Various compounds were evaluated for toxicity in mice and rats using previously reported protocols [37]. The enones **1a, b, e, f, 2a–g, 3a, 4a, b** were injected intraperitoneally into mice using doses of 30, 100 and 300 mg/kg with the view of determining if lethality and/or neurotoxicity could be demonstrated. The animals were examined after 0.5 and 4 h. In addition, mice receiving the 100 mg/kg dose of **1b, e, 2g** were observed after 2 and 6 h (**1e**, **2g**) and 0.25 and 1 h (**1f**). No deaths were noted, while marginal neurotoxicity was displayed by **2a** in 2/8 animals and **3a** in 1/4 mice using doses of 100 and 300 mg/kg, respectively.

The enones **1e, f, 2g** were administered orally to four rats and observed after 0.25, 0.5, 1, 2 and 4 h using doses of 30 (**1e, 2g**) or 50 (**1f**) mg/kg. In addition, **1e** was given to rats per os using doses of 25, 60 and 120 mg/kg (four animals per dose) and 250 mg/kg (eight animals were employed). Using a dose of 30 mg/kg, **1e** and **2g** were each injected intraperitoneally to four rats and observed after 0.25, 0.5, 1, 2 and 4 h. In all of these experiments, neither deaths nor neurotoxicity were observed.

These bioevaluations were undertaken by the National Institutes of Neurological Disorders and Stroke, USA, under the Anticonvulsant Screening Program. The animals were housed, fed and handled following the procedures documented in the National Research Council publication "Guide for the Care and Use of Laboratory Animals". The mice and rats were euthanised using the policies of the Institute of Laboratory Resources.

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