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Original article

Cytotoxic 1,4-bis(2-oxo-1-cycloalkylmethylene)benzenes and related compounds

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Abstract

A series of 1,4-bis(2-oxo-1-cycloalkylmethylene)benzenes **2a**–**c** and **4** and a related acyclic analogue **6a** were synthesised and converted to the corresponding Mannich bases **3a**–**c**, **5** and **6b**. Evaluation of these compounds against murine P388 and L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes revealed that the Mannich bases were more cytotoxic than the corresponding unsaturated ketones. 1,4-bis(3-Dimethylaminomethyl-2-oxo-1-cyclohexylmethylene)benzene dihydrochloride (**3a**) had lower IC_{50} values than melphalan against the four cell lines and was 15 times more potent than this drug when examined against a panel of human tumours. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: cytotoxicity; α,β-unsaturated ketones; Mannich bases; *N*-myristoyltransferase; X-ray crystallography

1. Introduction

The aim of the present investigation was to prepare a small number of novel prototypic molecules for evaluation as candidate cytotoxic agents. The specific compounds **2**–**6** were designed for the following reasons. First, the compounds chosen are α,β-unsaturated ketones which is a class of molecules having a marked preference or exclusivity for thiols rather than the amino or hydroxy groups found in nucleic acids [1]. The synthesis of the Mannich bases of the unsaturated ketones was proposed on the basis of their more effective thiol alkylating properties compared to the precursor ketones [2]. Second, compounds containing two or more alkylation sites may demonstrate a selective toxicity towards neoplastic cells in contrast to the corresponding normal tissues [3]. This possibility is based upon the observation that various tumours are more susceptible to chemical insult than the corresponding normal cells [4,5], making the neoplasms more vulnerable to a second chemical attack. In fact, various studies have revealed the increased tumour sensitivity to antineoplastic drugs by potentiators [6–8]. Therefore, in the present cluster of compounds, two sites for thiolation were incorporated into the molecules. Third, myristoyl-

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CoA: protein *N*-myristoyltransferase (NMT) catalyzes the co-translational transfer of myristic acid from myristoyl-CoA to the amino terminal glycine residue of various proteins [9]. Its importance as a molecular target in developing cytotoxic agents includes the following considerations. Significantly elevated levels of NMT have been found in colon neoplasias compared to normal colon cells [10]. This study demonstrated that an increase in NMT activity appeared at an early stage in colonic carcinogenesis [10]. Furthermore, immunohistochemical studies also showed increased staining for NMT in colorectal tumours compared to normal mucosa and the staining appeared to be cytoplasmic rather than nuclear [11]. In addition, gallbladder carcinoma showed moderate to strong cytoplasmic positivity for NMT with increased intensity in the invasive component whereas the normal gallbladder mucosa showed weak to negative cytoplasmic staining [12]. In addition, higher concentrations of various proteins that require myristoylation have been found in certain preneoplastic lesions and neoplasms compared to normal tissues [13,14]. Of relevance to the present investigation is the hypothesis of Peseckis and Resh that interaction of NMT with a substrate involves covalent bonding with the cysteine 169 mercapto group of the enzyme [15]. Consequently, the compounds under consideration may be effective inhibitors of NMT.

In addition, the importance of the shapes of the molecules on bioactivity was addressed by incorporating part of the α,β-unsaturated keto group into alicyclic rings (series **2**–**5**). Changes in the sizes of the rings would be expected to alter the relative positions of the alkylating sites which may influence potency considerably. The importance of the shapes of molecules was also considered in the design of the acyclic analogues **6**. It is conceivable that any marked variation in bioactivity could be attributed, at least in part, to differences in the topology of the molecules **2**–**6**.

In summary, this study focussed on the preparation of the compounds in series **2**–**6** with a view to determining those structural features which contribute to cytotoxicity and inhibition of the enzyme NMT.

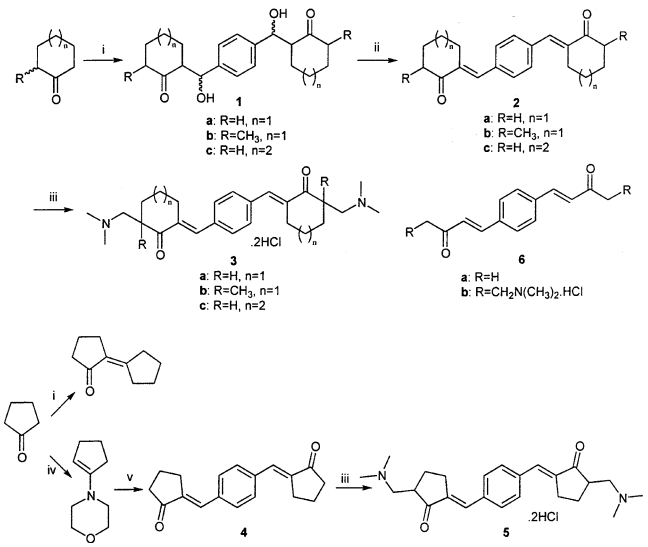

Fig. 1. Synthetic scheme leading to compounds **1**–**5** and structure of **6**. The reagents used in the synthetic route were as follows: (i) terephthaldicarboxaldehyde, aqueous sodium hydroxide solution; (ii) hydrochloric acid; (iii) *N*,*N*-dimethylmethyleneammonium chloride; (iv) 4-toluenesulphonic acid–morpholine; (v) terephthaldicarboxaldehyde.

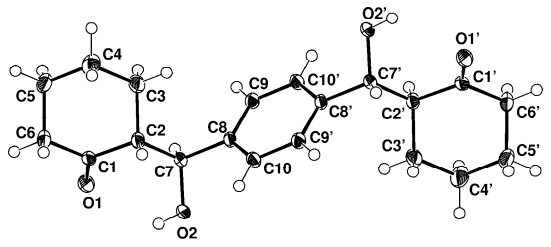

Fig. 2. ORTEP diagram of **1a** (50% of elipsoids).

2. Chemistry

The synthetic routes for preparing the target compounds **2**–**6** are portrayed in Fig. 1. Reaction of terephthaldehyde with either cyclohexanone, 2-methylcyclohexanone or cycloheptanone in the presence of aqeous sodium hydroxide solution led to the aldols **1a**–**c**. X-ray crystallography of **1a** and **1b** not only confirmed the aldol structures but revealed different orientations of the two alicyclic rings relative to the aryl ring. In the case of **1a** for example, the oxygen atoms 1 and 2 were on opposite sides of the centre of symmetry than the O1′ and O2′ atoms (Fig. 2). Treatment of **1a**–**c** with hydrochloric acid led to the formation of the unsaturated ketones **2a**–**c**. X-ray crystallography of a representative compound **2c** also indicated that the O1 and O1′ atoms were on different sides of the centre of symmetry (not shown). It is likely therefore that the molecules described herein have this spatial arrangement, a view that was strengthened by the molecular modelling of compound **4**. In this case, the energies of **4** modelled with the two oxygen atoms on opposite or the same sides of the centre of symmetry were 28.03 and 40.13 kcal mol^{-1}, respectively. The crystal structure of **2c** revealed that the olefinic double bonds adopted the *E* configuration which is consistent with the X-ray crystallographic and ^{1}H-NMR studies of related compounds [16,17]. Reaction of **2a**–**c** with *N*,*N*-dimethylmethyleneammonium chloride led to the formation of the corresponding Mannich bases **3a**–**c**. An attempt was made to prepare the enone **4** by the method of synthesising the compounds in series **2** via

Table 1
Cytotoxicity of **2a–c**, **3a–c**, **4**, **5**, **6a,b** and melphalan against murine P388 and L1210 cells and human Molt 4/C8 and CEM T-lymphocytes.

Compound	IC_{50} (μM)				Potency score [a]
	P388	L1210	Molt 4/C8	CEM	
2a	15.84 ± 1.0	218 ± 12	220 ± 8	153 ± 39	9
2b	> 50 ± 1.2	≥ 500	402 ± 93	265 ± 4	4
2c	15.90 ± 1.4	56.4 ± 14.6	96.9 ± 51.0	87.2 ± 57.7	12
3a	0.04 ± 0.01	1.08 ± 0.11	1.72 ± 0.11	1.48 ± 0.13	39
3b	1.23 ± 0.04	6.14 ± 1.41	1.65 ± 0.13	1.93 ± 0.28	31
3c	1.06 ± 0.02	1.56 ± 0.22	3.40 ± 2.54	2.23 ± 0.68	30
4	9.05 ± 0.7	59.5 ± 4.7	53.1 ± 6.8	35.3 ± 0.14	15
5	0.051 ± 0.01	7.65 ± 0.83	8.69 ± 0.55	7.76 ± 1.15	25
6a	3.27 ± 0.07	10.3 ± 0.5	7.44 ± 0.91	7.30 ± 0.54	22
6b	0.21 ± 0.01	1.78 ± 0.21	1.84 ± 0.37	1.80 ± 0.17	33
Melphalan [b]	0.22 ± 0.01	2.13 ± 0.03	3.24 ± 0.79	2.47 ± 0.30	–

[a] Potency score reflects the relative cytotoxicities towards all four cell lines (see Section 4 for details).
[b] These data are taken from Eur. J. Med. Chem. 35 (2000) 970 and are reproduced with the permission of the copyright owner.

the aldols **1** but the isolated product was shown to be 2-cyclopentylidenecyclopentanone. Thus an alternate strategy was undertaken using an enamine intermediate leading to the desired product **4** which was converted in to the β-aminoketone **5**. Condensation between terephthalaldehyde and acetone afforded **6a** which was successfully transformed into the Mannich base **6b**.

The log *P* values, surface areas and volumes of **2a–c**, **3a–c**, **4**, **5**, **6a** and **6b** were obtained by computational means; these results are portrayed in Table 2.

3. Biological evaluations

All of the compounds in series **2–6** were evaluated against murine P388 and L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes. Bioevaluation was undertaken using a maximum concentration of 50 μM of the compounds in the case of the most sensitive cell line, i.e. the P388 cells. The maximum concentration employed for the three other cell lines was 500 μM. These data are summarised in Table 1. Compounds **2c** and **3a** were examined against a panel of human tumours and the results are given in Table 3. Compounds **3b**,**c**, **5** and **6b** inhibited NMT activity in the presence of cAMP-dependent protein kinase derived peptide, while all four compounds stimulated enzyme activity when $pp60^{src}$ was used as the substrate. Four representative compounds, namely **2b**,**c** and **3b**,**c**, had minimum inhibitory concentration (MIC) figures in excess of 25 μg mL^{-1} when evaluated against different strains of *Aspergillus fumigatus* and *Candida albicans*. Administration of **2b**,**c**, **3b**,**c**, **4**, **5**, **6a** and **6b** to mice revealed that anticonvulsant activity was displayed in the cases of **3b** and **5** and the Mannich bases **3b**,**c**, **5** and **6b** were lethal in the 30–300 mg kg^{-1} dose range.

4. Results and discussion

Initially the cytotoxicity of the compounds in series **2–6** against P388, L1210, Molt 4/C8 and CEM cells (Table 1) will be discussed. The choice of cell lines was determined on the basis that the murine cell lines have been claimed to be good predictors of clinically useful anticancer agents [18] while activity towards the T-lymphocytes would indicate a capacity to inhibit the growth of human tumours. Since melphalan is a clinically useful antineoplastic agent which acts by alkylation, the potencies of the compounds listed in Table 1 were compared with this reference drug. The Mannich bases were either more potent than (**3a–c**, **5** and **6b**) or equipotent with (**3b**,**c** and **6b**) melphalan against some or all of the cell lines. Of particular note were **3a** and **6b** which were more active than melphalan in all screens with the exception of the equiactivity of **6b** against P388 cells. These two compounds are clearly important lead molecules for further development.

In order to compare the bioactivities of the compounds in series **2–6**, the following approach was adopted. A comparison was made in each cell line of all 10 compounds and a score of 10 was assigned to the most potent compound, 9 to the analogue possessing the next highest activity and so on while the least active compound was assigned a rating of 1. The scores for each of the four cell lines were combined and the results are presented in Table 1. The order of cytotoxicity was found to be **3a** > **6b** > **3b** > **3c** > **5** > **6a** > **4** > **2c** > **2a** > **2b**, indicating that the Mannich bases were more cytotoxic than the corresponding unsaturated ketones. The similar cytotoxicity of **3a** and **3b** suggests that bioactivity is due to the Mannich bases per se and not to any deamination process which is blocked in the case of **3b**.

In an attempt to discern the physicochemical properties of these molecules that influence cytotoxicity, the

Table 2
Log *P* values, surface areas and volumes of **2a–c**, **3a–c**, **4**, **5** and **6a**,**b**.

Compound	Log *P*	Surface areas (Å^2)	Volume (Å^3)
2a	3.87	544	908
2b	5.00	590	993
2c	4.67	586	991
3a (base)	3.84	736	1289
3a (salt)	2.39	740	1298
3b (base)	5.17	753	1349
3b (salt)	3.73	755	1356
3c (base)	4.63	781	1375
3c (salt)	3.19	781	1329
4	3.08	509	826
5 (base)	3.05	710	1210
5 (salt)	1.93	684	1175
6a	2.28	445	708
6b (base)	2.37	667	1111
6b (salt)	0.93	678	1124

log *P* values, surface areas and volumes of **2a–c**, **3a–c**, **4**, **5**, **6a** and **6b** were determined computationally. The pK_a of a Mannich base of a conjugated styryl ketone, namely 5-diethylamino-4,4-dimethyl-1-phenyl-1-penten-3-one hydrobromide is 7.19 [19] and if the basicities of the Mannich bases described in this study are comparable, then the ratio of free bases to ionised species will be ca. 3:2 at pH 7.4 [20]. Thus the physical properties of both molecular species were obtained and these results are presented in Table 2. Both linear and logarithmic plots were made between the individual physicochemical constants and the IC_{50} values in each of the four cell lines with firstly the enones, **2a–c**, **4** and **6a**, secondly the Mannich bases, **3a–c**, **5** and **6b** (free bases) and, finally, with these five Mannich bases as the protonated species. The *P* values listed as either < 0.05 or < 0.1 are given in the Section 6 of this report. A positive correlation was noted between the cytotoxicity of the unsaturated ketones **2a–c**, **4** and **6a** in the P388, Molt 4/C8 and CEM screens and their log *P* values, surface areas and volumes. Thus the insertion of large lipophilic groups into this series of compounds may lead to increased cytotoxicity. In contrast to the enones, the only relationship noted with **3a–c**, **5** and **6b** was a positive correlation using linear but not logarithmic plots between the log *P* values of the Mannich bases (both unprotonated and protonated species) and potency in the P388 screen. These data indicated that the significant activity of the Mannich bases is not correlated with their surface areas and volumes.

Several compounds were considered for inclusion in a screen utilizing a panel of human tumours. In this process, a prescreen is conducted initially using NCI-H460, MCF7 and SF-628 cells which are lung, breast and central nervous system tumours, respectively. The criterion for activity in this prescreen is that the growth of at least one cell line should be inhibited by 68% at a concentration of 10^{-4} M. Compounds **2a**,**c** and **3a** were examined in the prescreen and the specific figures for the inhibition, or lack thereof, of growth of the three cell lines are summarised in Section 6. The prescreen revealed that maximum activity was displayed by **3a** which inhibited completely the growth of all three cell lines. The enone **2c** and the Mannich base **3a** met the criterion of the prescreen and were evaluated in vitro against ca. Fifty six human tumours from nine different neoplastic diseases, namely leukemia, melanoma, non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers. The results of the evaluation of **2c** and **3a** as well as three reference compounds in the human tumour panel are presented in Table 3. In this screen concentrations of 10^{-8}–10^{-4} M were used and the amount of compound required to inhibit the growth of the cells by 50% was noted. However, when the maximum concentration of compound, namely 10^{-4} M, did not inhibit the growth of a cell line by 50%, the figure of 10^{-4} M was still incorporated into the calculation of average cytotoxicity. Hence the designation of mean graph midpoint (MG MID) rather than IC_{50} values were used in presenting these data.

Table 3
Evaluation of **2c**, **3a** and reference compounds against various human tumour cell lines.

Compound	All cell lines	Colon cancer cells		Leukemic cells	
	MG MID [a] (μM)	MG MID [a] (μM)	SI [b]	MG MID [a] (μM)	SI [b]
2c	63.1	53.2	1.2	48.1	1.3
3a	1.55	0.683	2.3	0.447	3.5
Melphalan [c]	23.5	44.6	0.5	4.65	5.1
5-Fluorouracil [c]	32.6	7.90	4.1	27.6	1.2
Helenalin [c]	1.45	1.42	1.0	0.620	2.3

[a] The letters MG MID indicate the mean graph midpoint which is explained in the text.
[b] The letters SI refer to the selectivity index which is calculated by dividing the MG MID figures for all cell lines by either the MG MID values of the colon cancer or leukemic cells.
[c] The data are taken from Eur. J. Med. Chem. 35 (2000) 971 and are reproduced with the permission of the copyright owner.

The data presented in Table 3 revealed that **3a** was 15 times more potent than melphalan when cytotoxicity to all cell lines was considered. In view of the interest of this laboratory in discovering compounds which are effective towards colon cancers [19] and leukemic cells [21], the MG MID values against these specific tumours were obtained and compared to the average figures against all cell lines in order to produce selectivity index (SI) figures. A SI value of 1.5 was arbitrarily chosen as an indicator of selectivity, i.e. the colon cancers or the leukemic cells were 50% more vulnerable to the compound than the average cytotoxicity towards all cell lines. The SI figures are also presented for 5-fluorouracil and melphalan which are useful drugs in treating colon neoplasms [22] and leukemia [23], respectively. In addition, the figures are also given for helenalin which is a cytotoxic thiol alkylator [24]. Compound **3a** demonstrated selectivity for colon cancer and leukemic cells. In particular, the SI figures for **3a** were 56 and 69% of the established drugs, 5-fluorouracil and melphalan, respectively. Helenalin was markedly cytotoxic and demonstrated selectivity to leukemic cells although its SI figure was lower than those obtained by **3a**. The results of evaluation of selected compounds against a panel of human tumours revealed the emergence of a significant lead compound **3a** which can be considered a prototypic molecule for subsequent development.

An attempt was made to determine whether representative compounds in series **2**–**6** inhibited NMT. The amino terminal glycine residue of various proteins is myristoylated as noted for the catalytic subunit of cAMP-dependent protein kinase [9,25] and various tyrosine kinases including $pp60^{src}$ [26]. In the concentration range of 15–40 mM, the unsaturated ketones **2a**–**c**, **4** and **6a** were virtually insoluble in both water and dimethylsulphoxide or only sparingly soluble in dimethylsulphoxide. Hence the efficacy of these compounds to inhibit NMT was not determined. The Mannich bases **3b**,**c**, **5** and **6b** however were water soluble and a study of their effect on NMT using both substrates was determined. In the presence of cAMP-dependent protein kinase derived peptide, the IC_{50} figures of **3b**,**c**, **5** and **6b** towards NMT were 1.5, 25.0, 4.0 and 10.0 mM, respectively. Inhibition therefore was favoured by the compounds with five and six membered alicyclic rings (**5** and **3b**) or **6b** which has no aliphatic ring. This effect may have been due to **3b**, **5** and **6b** causing less steric impedance to interaction with the enzyme than **3c**. The potency scores listed in Table 1 were in the order $\mathbf{6b} > \mathbf{3b} > \mathbf{3c} > \mathbf{5}$ while the relative NMT-inhibiting cytotoxicity properties were $\mathbf{3b} > \mathbf{5} > \mathbf{6b} > \mathbf{3c}$ indicating that there was no correlation between cytotoxicity and inhibition of this enzyme. While some effect on NMT occurs, the concentrations used are considerably higher than those causing cytotoxicity to P388, L1210, Molt 4/C8 and CEM cells. The average of IC_{50} values of **3b**,**c**, **5** and **6b** against these four cell lines were 2.74, 2.06, 6.04 and 1.41 μM, respectively. Thus the IC_{50} values of these Mannich bases towards NMT were 548, 12 136, 662 and 7092 times, respectively, higher than those required to generate IC_{50} figures against malignant cells.

In contrast, when the $pp60^{src}$ substrate was employed, no inhibition was noted using concentrations up to and including 40 mM except **6b** when 75% of the activity of the enzyme was inhibited using 20 mM of the Mannich base. However, all of the compounds caused stimulation of the enzyme, with a 50% increase in enzyme activity was noted for **3b**,**c**, **5** and **6b** at concentrations of 2, 10, 0.3 and 1.5 mM, respectively. Similar to the enzyme-inhibiting properties of these compounds, the greatest potencies were noted with those molecules possessing small alicyclic rings (**3b** and **5**) and the acylic Mannich base **6b**. As the concentrations of compounds were elevated, stimulation of enzyme activity increased; the fold increases in stimulation for **3b**,**c**, **5** and **6b** (concentrations in mM) were 2.8 (5), 5 (20), 2.5 (20) and 3.3 (10), respectively. Recent results from this laboratory have demonstrated stimulation of NMT activity by dimethylsulphoxide and related solvents in the presence of different substrates.[1] The postulate was made that hydrogen bonding between the oxygen atoms of the solvents and protons of the substrate, particularly the hydroxy hydrogen atoms of serine and threonine residues, accounts for this phenomenon. In the current investigation, support for this hypothesis was obtained since there was no stimulation noted using cAMP-dependent protein kinase derived peptide, which contains no serine or threonine residues. However, stimulation was found using $pp60^{src}$ which is a nonamer with three serine residues. The stimulation noted is likely due to intermolecular bonding between the oxygen atoms of the Mannich bases and the hydrogen atom of the serine hydroxyl groups.

The conclusions that may be drawn from the NMT studies are that it is unlikely that the Mannich bases interact with the sulfhydryl group of NMT to an appreciable extent, the inhibition-stimulation phenomena do not contribute significantly to cytotoxicity and there must be other molecular targets that are primarily responsible for the marked cytotoxicities of these compounds.

A number of conjugated styryl ketones and related Mannich bases possess antifungal activity [27,28]. The two most common fungal pathogens are *A. fumigatus* and *C. albicans*. Consequently four representative compounds, namely **2b**,**c** and **3b**,**c**, were evaluated against three strains of both of these fungi. The MIC figures of

[1] Pasha, M.K., Dimmock, J.R., Hollenberg, M.D., Sharma, R.K., unpublished results.

all four compounds were in excess of 25 μg mL^{-1} in contrast to the reference drugs amphotericin B and itraconazole, which have MIC values of less than 1 μg mL^{-1}. This observation is of interest because **3b** and **3c**, in particular, demonstrated marked cytotoxicity to neoplastic cells, so that their lack of appreciable antifungal activity may indicate that the compounds are not general biocidal agents but have preferential toxicity to neoplastic tissue although further work in this regard is required.

Finally, an estimate of the behaviour of representative compounds in vivo was made. A useful method is to undertake anticonvulsant and toxicity screens which afford not only evidence of the tolerance of mice to the compounds but also provides information as to whether penetration of the CNS takes place. Activity in the anticonvulsant screen may be beneficial in revealing compounds of potential use in treating CNS tumours; on the other hand such properties may be dystherapeutic by causing neurotoxicity (NT). Compounds **2b**,**c**, **3b**,**c**, **4**, **5**, **6a** and **6b** were administered to mice and examined for protection in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (scPTZ) screens as well as for neurological deficit [29]. In general, doses of 30, 100 and 300 mg kg^{-1} were employed in these tests. The α,β-unsaturated ketones **2b**,**c**, **4** and **6a** displayed no anticonvulsant activity. Neither NT nor other signs of impairment were noted with these compounds with the exception of minimal NT displayed by **4**. In the case of the Mannich bases **3b**,**c**, **5** and **6b**, marginal protection was afforded by **3b** in the MES screen and activity was demonstrated by **5** in the scPTZ test. However, in contrast to the enones, deaths due to respiratory depression were noted for all of the Mannich bases. Specific details of these and other pathological symptoms are given within the Section 6 of this report.

5. Conclusions

This study has revealed a synthetic chemical route to various novel enones and the corresponding Mannich bases. The latter group of compounds demonstrated marked cytotoxicity. In particular **3a** is a prototypic molecule having considerably greater potency than melphalan towards a number of human tumour cell lines. Selective toxicity was displayed by **3a** towards human colon and leukemic cells. While some effects on NMT were demonstrated by four of the Mannich bases, it is unlikely that their mode of action is mediated via this enzyme. Molecular modification will need to be conducted in conjunction with murine toxicity studies with a view to enhancing the differential between selective cytotoxicity and mammalian general toxicities.

6. Experimental

6.1. Chemistry

Melting points are in Celsius degrees and are uncorrected. Elemental analyses were undertaken by K. Thoms, Department of Chemistry, University of Saskatchewan, on the compounds **1a**–**c**, **2a**–**c**, **3a**–**c**, **4**, **5**, **6a** and **6b**, and were within 0.4% of the calculated values. The following compounds were obtained in hydrated forms (moles of water of crystallisation), namely **1a** (0.25), **2a** (0.33), **2c** (0.2), **3a** (0.5) and **3b** (0.5). ^{1}H-NMR spectra were recorded using a Bruker AM 500 FT-NMR machine (500 MHz). Some of the protons on the alicyclic rings are designated by the subscripts a and b since they were not unequivocally assigned to the equatorial and axial positions. Silica gel (60–120 mesh) was used in column chromatography. TLC utilised silica gel 60 F_{254}-precoated plastic sheets.

6.1.1. Synthesis of series *1*

The preparation of **1a** was accomplished as follows. A solution of NaOH (0.1 mol) in water (50 mL) was added slowly to an ice-cold mixture of cyclohexanone (0.4 mol), terephthaldicarboxaldehyde (0.04 mol) and water (100 mL). The reaction mixture was stirred with ice cooling for 6 h. The resultant precipitate was collected, washed with HCl (3.75% w/v, 3 × 50 mL), dried and recrystallised from Et_2O–$CHCl_3$ to give **1a**, m.p. 198–202 °C in 42% yield.

Compound **1b** was prepared in a similar fashion as **1a** except the molar concentrations of NaOH, 2-methylcyclohexanone and terephthaldicarboxaldehyde were 0.05, 0.135 and 0.015, respectively. In addition, the reaction mixture was stirred at room temperature (r.t.) for 3 days. After acidification, an oil formed which was separated and extracted with $CHCl_3$. The organic extract was dried and the solution was concentrated to 15 mL. After standing at r.t. for 1 h, a colourless precipitate was collected and washed with MeOH (3 × 20 mL). Recrystallisation from Et_2O–$CHCl_3$ gave **1b**, m.p. 184–188 °C in 34% yield.

The aldol **1c** was synthesised in the same manner as **1b** except the molar concentrations of cycloheptanone and terephthaldicarboxyaldehyde were 0.1 and 0.02, respectively, and the time of stirring at r.t. was 24 h. The crude product was chromatographed on silica gel and eluted with a mixture of EtOAc and C_6H_{14} (5:95) to yield material which was recrystallised from $CHCl_3$–MeOH to give **1c**, m.p. 139–144 °C in 22% yield.

Compounds **1a**–**c** were obtained as a mixture of diastereoisomers as revealed by TLC using a solvent system of C_6H_{14}–EtOAc (4:1) and ^{1}H-NMR spectroscopy. The assignments made, where possible, of a representative compound **1c** were as follows. δ ($CDCl_3$): 1.02–1.03 (6H, dd, $J = 3.0$ Hz, 2 × CH_3),

1.25–1.40 (4H, m, 2 × 4-CH_2), 1.51–1.80 (8H, m, 2 × 3-CH_2, 2 × 5-CH_2), 2.05–2.11 (2H, m), 2.40–2.52 (2H, m, 2 × 6-CH), 2.53–2.61 (2H, m, 2 × 2-CH), 4.77 (1H, d, $J = 9.0$ Hz, C*H*OH), 5.34 (1H, d, $J = 2$ Hz, C*H*OH), 7.23–7.28 (4H, m, aryl H).

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

Hydrochloric acid (37% w/v, 25 mL) was added dropwise over a period of 10 min to a stirred mixture of the hydroxyketone **1** (0.02 mol) and MeOH (50 mL) at r.t. After heating at 80 °C for 3 h, the mixture was cooled and the precipitates were collected. Compound **2a**, m.p. 168–173 °C (dec.), was obtained in 62% yield upon recrystallisation from Et_2O–$CHCl_3$ while **2b**, m.p. 193–196 °C, was isolated in 72% yield by recrystallisation from EtOAc–$CHCl_3$. The unsaturated ketone **2c**, m.p. 129 °C, was purified by chromatography using a column of silica gel and elution was accomplished using a gradient solvent of C_6H_{14} and EtOAc. Finally **2c** was recrystallised from EtOAc–$CHCl_3$ to give the ketone in 51% yield. The ^{1}H-NMR spectrum of a representative compound **2b** was as follows: δ ($CDCl_3$): 1.06 (6H, d, $J = 6.8$ Hz, 2 × CH_3), 1.42–1.61 (4H, m, 2 × C5–$H_{a,b}$), 1.72–1.80 (2H, m, 2 × C4–H_a), 1.91–1.98 (2H, m, 2 × C4–H_b), 2.28–2.36 (2H, m, 2 × C3–H_a), 2.50–2.58 (2H, m, 2 × C3–H_b), 2.82–2.90 (2H, m, 2 × C6–H), 7.24 (6H, s, 4 aryl H, 2 vinylic H).

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

A mixture of *N*,*N*-dimethylmethyleneammonium chloride (0.009 mol), which had been freshly prepared by a literature procedure [30], the appropriate unsaturated ketone **2** (0.003 mol) and dry MeCN (100 mL) was heated at 80 °C for 9 h. The progress of the reaction was monitored by TLC using a solvent system of MeOH–$CHCl_3$ (1:4). Approximately one third of the solvent was removed in vacuo and the precipitate was collected, washed with dry MeCN and dried. Compound **3a**, m.p. > 285 °C, was obtained in 22% yield by recrystallisation from Et_2O–MeOH. The other two Mannich bases were digested in dry MeCN (20 mL) at 60° for 3 h to give **3b**, m.p. 227 °C and **3c**, m.p. 213 °C in yields of 79 and 76%, respectively.

The ^{1}H-NMR spectrum of a representative compound **3b** was as follows: δ (DMSO-d_6): 1.14 (6H, s, 2 × CH_3), 1.74–1.84 (4H, m, 2 × C4–$H_{a,b}$), 2.24–2.36 (2H, m, 2 × C5–H_a), 2.72–2.84 (16H, m, 4 × NCH_3, 2 × NCH_2), 2.96–3.20 (2H, m, 2 × C5–H_b), 3.12–3.18 (2H, m, 2 × C3–H_a), 3.56–3.60 (2H, m, 2 × C3–H_b), 7.32 (2H, s, vinylic H), 7.53 (4H, s, aryl H), 10.25 (2H, bs, $\overset{(+)}{N}H$).

*6.1.4. Synthesis of **4** and **5***

A solution of cyclopentanone (0.1 mol), morpholine (0.11 mol), 4-toluenesulfonic acid (20 mg) and C_6H_6 (100 mL) was heated under reflux in a Dean–Stark apparatus for 9 h. Terephthaldicarboxaldehyde (0.05 mol) was added and the reaction mixture was heated under reflux for a further 12 h. On cooling to r.t., HCl (37% w/v, 20 mL) was added and the mixture stirred vigorously for 3 h at r.t. The product was extracted with $CHCl_3$, the organic extracts dried and removal of the solvents led to the isolation of a brown semisolid which was chromatographed using silica gel and an eluting solvent of EtOAc–C_6H_{14} (1:4) to give **4**, m.p. 185 °C in 39% yield. ^{1}H-NMR ($CDCl_3$): δ 2.04 (4H, m, 2 × C4–H_2), 2.40 (4H, t, $J = 7.9$ Hz, 2 × C5–H_2), 2.96–3.00 (4H, m, 2 × C3–H_2), 7.35 (2H, t, $J = 2.57$ Hz, 2 × vinylic), 7.56 (4H, s, aryl H).

An initial attempt to prepare **4** by using the method employed for synthesising series **2** via the intermediates **1** led to the isolation of a product which was purified by column chromatography using silica gel and an eluting solvent of a mixture of EtOAc and C_6H_{14} (1:9) to give 2-cyclopentylidenecyclopentanone [31] as a colourless oil in 37% yield. ^{1}H-NMR ($CDCl_3$): δ 1.16–1.71 (4H, m, 8,9-CH_2), 1.84–1.90 (2H, m, 4-CH_2), 2.26 (4H, m, 7,10-CH_2, $J = 8.0$ Hz), 2.48–2.51 (2H, m, 3-CH_2), 2.60–2.85 (2H, m, 5-CH_2). The carbon atoms in the cyclopentanone ring were designated 1–5 and the cyclopentylidene carbon atoms were assigned numbers 6–10. In this numbering sequence, carbon atom 2 was attached to carbon atom 6.

Compound **4** was converted into the corresponding Mannich bases **5** by the same procedure used for the preparation of the compounds in series **3**. It was purified by digestion in MeOH in the same manner as **3b** to give the desired product m.p. 200 °C (dec.) in 71% yield. ^{1}H-NMR (D_2O): δ 1.56–1.62 (2H, m, 2 × 4-CH_a), 2.37–2.43 (2H, m, 2 × 4-CH_b), 2.75–2.92 (18H, m, 4 × NCH_3), 2 × CH_2N, 2 × 5-CH), 3.14–3.18 (2H, m, 3-CH_a), 3.40–3.44 (2H, m, 3-CH_b), 7.17 (2H, s, 2 × vinylic H), 7.48 (4H, s, 4 × aryl H). In this compound, the olefinic and dimethylaminomethyl substituents were assigned positions 2 and 5, respectively, on the alicyclic ring.

*6.1.5. Synthesis of series **6***

A literature procedure [32] was employed for the synthesis of **6a**. Purification was accomplished by chromatography on silica gel and using C_6H_{14}–EtOAc as the gradient solvent led to the isolation of the desired compound m.p. 159 °C (lit. [33] m.p. 156 °C) in 42% yield.

Conversion of **6a** into the Mannich base **6b** was undertaken using the general procedure for preparing the analogues **3a**–**c**. The crude product was purified by recrystallisation from MeOH to give **6b**, m.p. 234–235 °C (dec.) in 49% yield. ^{1}H-NMR (D_2O): δ 2.83 (12H, s, 4 × NCH_3), 3.24 (4H, t, 2 × $COCH_2$, $J = 6.3$ Hz), 3.38 (4H, t, 2 × NCH_2, $J = 6.3$ Hz), 6.78 (2H, d, 2 × olefinic H, $J = 16.4$ Hz), 7.58–7.62 (6H, m, 4 × aryl H, 2 × olefinic H).

*6.1.6. X-ray crystallographic determination of **1a**,**b** and **2c***

The data were collected using an Enraf–Nonius CAD-4 diffractometer with an ω scan. The structure was solved by NRCVAX [34] and refined using SHELXL [35]. Fig. 1 was generated by ORTEP [36] as described in XTAL 3.6 [37]. Atomic scattering factors and anomolous dispersion corrections were taken from the literature [38]. Non-hydrogen atoms were found on the *E* map and were refined anisotropically. Hydrogen atoms were not refined and were placed on atoms by geometry. Specific details of the X-ray crystallography of **1b**,**c** and **2c** are available from the authors on request.

6.1.7. Molecular modelling and statistical analyses

The structures of the compounds in series **2**–**6** were built and minimised using the HyperChem molecular modelling programme [39] which also generated the log *P* values, surface areas and volumes of these molecules. The linear and logarithmic plots between the IC_{50} values of various compounds in the P388, L1210, Molt 4/C8 and CEM screens and the log *P* figures, surface areas and volumes were generated using a commercial software package [40]. The cytotoxicity of **2a**–**c**, **4** and **6a** was positively correlated with the log *P* values, surface areas and volumes using linear (lin) or logarithmic (log) plots in the following cases (*P* values in parentheses): $P388_{lin}$: log *P* (<0.1), surface area (<0.05), volume (<0.05); $P388_{log}$: log *P* (<0.05), surface area (<0.05), volume (<0.05); Molt $4/C8_{log}$: log *P* (<0.05), surface area (<0.05), volume (<0.05); CEM_{lin}: log *P* (<0.1) and CEM_{log}: log *P* (<0.05), surface area (<0.05), volume (<0.05). Linear plots between the IC_{50} values of the Mannich bases **3a**–**c**, **5** and **6b** (both free bases and dihydrochloride salts) in the P388 screen were positively correlated with the log *P* figures ($P<0.1$). In all other cases the *P* values were >0.1.

6.2. Biological evaluations

6.2.1. Cytotoxicity screening

Evaluation of the compounds in the P388D1 screen was carried out using a literature procedure [41], while the L1210, Molt 4/C8 and CEM tests were undertaken by a method described previously [42].

In the case of the human tumour panel assay, compounds **2a**,**c**, and **3a** were examined against the NCI-H460, MCF7 and SF-268 cell lines at a concentration of 10^{-4} M. After incubation for 48 h, the viability of the cells was determined using the protein-binding dye sulforhodamine B. The percentage inhibition against these three cell lines was as follows namely **2a**: 18, 64, 37; **2c**: 71, 56, 43; and **3a**: 100, 100, 100. The Mannich bases **2c** and **3a** were subsequently evaluated using ca. 56 cell lines from different neoplastic diseases by a literature method [43]. Some of these data are presented in Table 3.

6.2.2. Evaluation using NMT

Escherichia coli DH5α with recombinant pTrcHisC.hNMT was grown to stationery phase in LB medium at 37 °C containing ampicillin (50 mg mL^{-1}) and isopropyl-β-D-thiogalactopyranoside (1 mM) for 2 h. The cells were harvested by centrifugation at $10{,}000 \times g$ for 30 min and suspended in tris(hydroxymethyl)aminomethane hydrochloride (50 mM, pH 8.0) containing ethylenediaminetetracetic acid (2 mM), phenylmethanesulphonyl fluoride (1 mM), leupeptin (0.5 μg mL^{-1}) and polyoxyethylene (10) isooctylphenyl ether (0.1%). The suspension was sonicated on ice for 10 s. After centrifugation of $15{,}000 \times g$ for 20 min, the supernatent was removed for the purification of the expressed protein. The recombinant hNMT was purified by a literature procedure [44]. The assay using NMT has been described previously [45,46].

6.2.3. Antifungal evaluation

The antifungal evaluations of **2b**,**c**, **3b**,**c** amphotericin B and itraconazole was accomplished using a broth dilution method [47] and three isolates of *A. fumigatus* (ATCC 208995, ATCC 208996 and ATCC 208997) and *C. albicans* (ATCC 90028, ATCC 32354 and ATCC 28367). The highest concentrations (in μM) used were as follows: **2b**: 77.5; **2c**: 76.7; **3b**: 48.2; and **3c**: 49.1. The MIC values of amphotericin B and itraconazole against the six organisms were 0.101–0.812 and 0.19–0.39 μM, respectively.

6.2.4. Anticonvulsant and NT screens

The compounds were evaluated in the murine MES, scPTZ and NT screens using a literature procedure [29]. The doses employed were 30, 100 and 300 mg kg^{-1} except in the case of **3b** and **6b**, 30 and 100 mg kg^{-1} doses only were used in the MES and scPTZ tests. The animals were observed 0.5 and 4 h after administration of the compounds. No results were available in the following screens (test, dose in mg kg^{-1}, time in hours): **3c**: (scPTZ, 300, 0.5); **5**: (MES, 300, 4); **5**: (scPTZ, 100 and 300, 4); **6b**: (MES, 100, 4); **6b** (scPTZ, 100, 4); and **6b**: (NT, 300, 4). Protection was noted in the MES screen with **3b** (1/3 mice, 100 mg kg^{-1}, 4 h) and in the scPTZ test (1/1 mouse, 300 mg kg^{-1}, 0.5 h).

At the end of 0.5 h, NT was demonstrated in the following cases (dose in mg kg^{-1}, toxicity/number of animals used): **3b**: (30, 1/4), (100, 3/8), (300, 4/4); **3c**: (100, 2/8), (300, 3/4); **4**: (100, 1/8); **5**: (30, 2/4), (100, 6/8), (300, 4/4); and **6b**: (30, 1/4), (100, 5/8), (300, 4/4). After 4 h, the following NT was observed: **3c**: (100, 1/4); **5**: (30, 1/2), (100, 4/4), (300, 2/2); and **6b**: (30, 2/2), (100, 1/1).

The following compounds caused death and/or other pathological symptoms (dose in mg kg^{-1}, time in hours and observation). In the scPTZ screen, the adverse effects were as follows: **3b**: (30, 0.5 and 4, death following tonic extension); **3b**: (100, 4, tonic extension); **5**: (300, 0.5, myoclonic jerks); and **6b**: (100, 0.5, death following clonic seizure). In the NT test, deaths were caused by the following compounds (dose in mg kg^{-1}, time in hours, mortality/number of animals used): **3b**: (300, 0.5, 4/4); **3c**: (300, 0.5, 2/4); **5**: (100, 4, 4/4); and **6b**: (100, 0.5, 5/8), (300, 0.5, 4/4), (100, 4, 1/1).

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