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Articles

Cytotoxic Activities of Mannich Bases of Chalcones and Related Compounds

Jonathan R. Dimmock,*,† N. Murthi Kandepu,† Mark Hetherington,† J. Wilson Quail,‡ Uma Pugazhenthi,‡ Athena M. Sudom,‡ Mahmood Chamankhah,§ Patricia Rose,|| Eric Pass,|| Theresa M. Allen,⊥ Sarah Halleran,⊥ Jen Szydlowski,⊥ Bulent Mutus,# Marie Tannous,# Elias K. Manavathu,▽ Timothy G. Myers,@ Erik De Clercq,○ and Jan Balzarini○

College of Pharmacy and Nutrition, Department of Chemistry and Department of Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C9, Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan S7N 0W9, Canada, Department of Pharmacology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario N9B 3P4, Canada, Department of Internal Medicine, Wayne State University, Detroit, Michigan 48201, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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Various Mannich bases of chalcones and related compounds displayed significant cytotoxicity toward murine P388 and L1210 leukemia cells as well as a number of human tumor cell lines. The most promising lead molecule was **21** that had the highest activity toward L1210 and human tumor cells. In addition, **21** exerted preferential toxicity to human tumor lines compared to transformed human T-lymphocytes. Other compounds of interest were **38**, with a huge differential in cytotoxicity between P388 and L1210 cells, and **42**, with a high therapeutic index when cytotoxicity to P388 cells and Molt 4/C8 T-lymphocytes were compared. In general, the Mannich bases were more cytotoxic than the corresponding chalcones toward L1210 but not P388 cells. A ClusCor analysis of the data obtained from the *in vitro* human tumor screen revealed that the mode of action of certain groups of compounds was similar. For some groups of compounds, cytotoxicity was correlated with the σ, π, or molar refractivity constants in the aryl ring attached to the olefinic group. In addition, the IC_{50} values in all three screens correlated with the redox potentials of a number of Mannich bases. X-ray crystallography and molecular modeling of representative compounds revealed various structural features which were considered to contribute to cytotoxicity. While a representative compound **15** was stable and unreactive toward glutathione (GSH) in buffer, the Mannich bases **15**, **18**, and **21** reacted with GSH in the presence of the π isozyme of glutathione S-transferase, suggesting that thiol alkylation may be one mechanism by which cytotoxicity was exerted in vitro. Representative compounds were shown to be nonmutagenic in an intrachromosomal recombination assay in yeast, devoid of antimicrobial properties and possessing anticonvulsant and neurotoxic properties. Thus Mannich bases of chalcones represent a new group of cytotoxic agents of which **21** in particular serves as an useful prototypic molecule.

Introduction

A number of chalcones (1,3-diaryl-2-propen-1-ones) have demonstrated cytotoxic[1,2] and anticancer properties.[3,4] Results from this laboratory have revealed that conversion of various acyclic conjugated styryl ketones into the corresponding Mannich bases was often accompanied by increased bioactivity both in vitro and in vivo.[5] However the synthesis of Mannich bases of chalcones for cytotoxic and anticancer properties appears to be an unexplored field. The aim of the present investigation therefore was to prepare a number of such prototypic molecules and related analogues in order to evaluate their cytotoxic activity. Furthermore, representative compounds would be examined in order to discern structure−activity relationships and the shapes of such molecules, their stabilities, and reactivities as well as possible toxicities. From the information generated, one or more lead compounds may be identified for subsequent development. The specific aims of the project are given below.

First, the preparation of compounds **1**−**44** was suggested. The general structures of most of these compounds are given in Scheme 1 while the nature of the aryl substituents is presented in Table 1. The reasons for their proposed synthesis and cytotoxic evaluation

* To whom inquiries should be directed.
† College of Pharmacy and Nutrition, Saskatoon.
‡ Department of Chemistry, Saskatoon.
§ Department of Microbiology, Saskatoon.
|| Plant Biotechnology Institute, Saskatoon.
⊥ Department of Pharmacology, Edmonton.
Department of Chemistry and Biochemistry, Windsor.
▽ Department of Internal Medicine, Detroit.
@ National Cancer Institute, Bethesda.
○ Rega Institute for Medical Research, Leuven.

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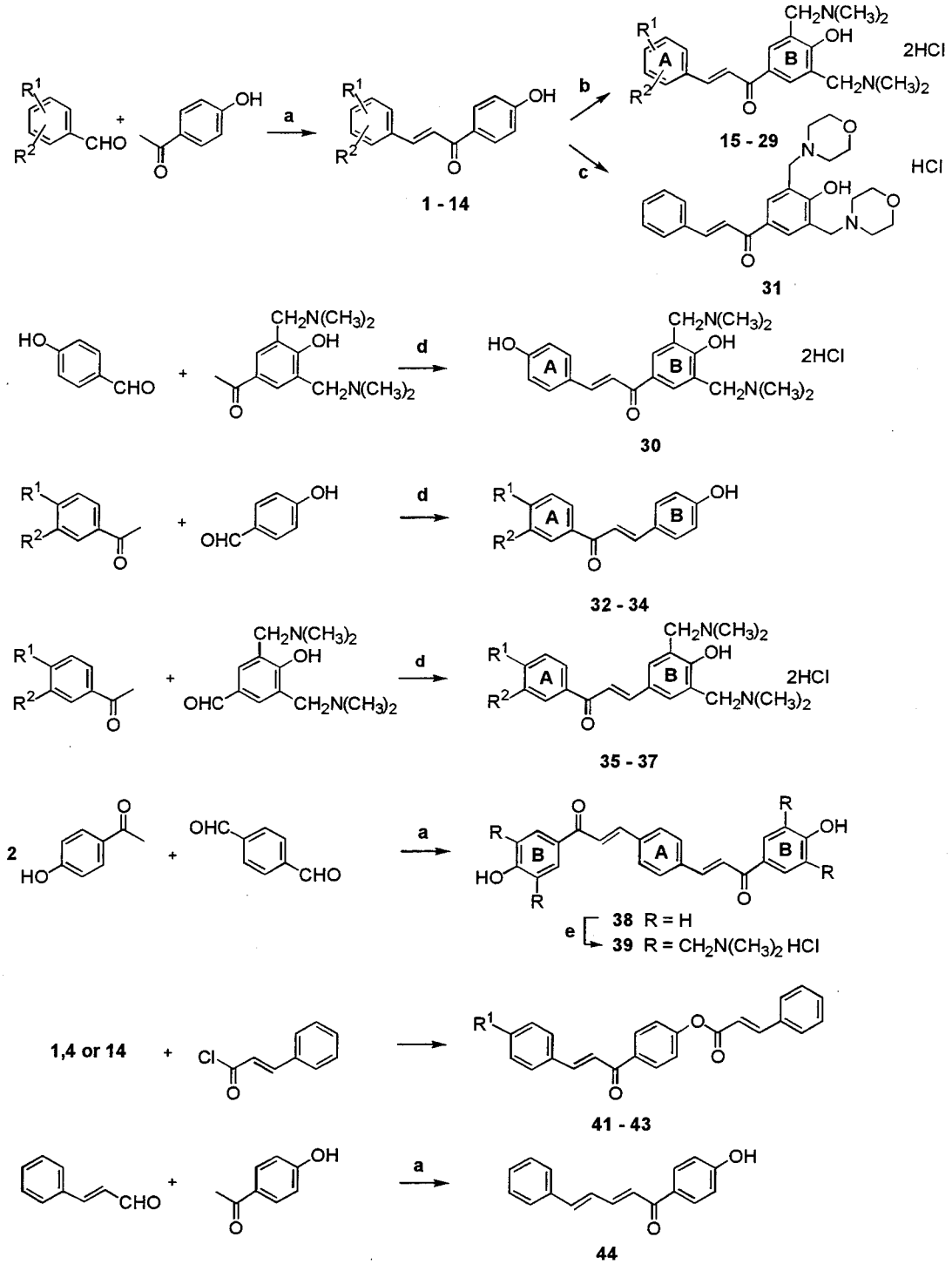
Scheme 1. Preparation of the Mannich Bases of Some Chalcones and Related Compounds **1**−**44**[a]



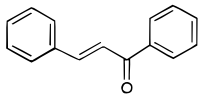
[a] (a) Aqueous sodium hydroxide solution; (b) *N,N*-dimethyleneammonium chloride; (c) (i) formaldehyde and morpholine, (ii) hydrogen chloride; (d) hydrogen chloride; (e) (i) formaldehyde and dimethylamine, (ii) hydrogen chloride.

were as follows. A number of α,β-unsaturated ketones have demonstrated preferential reactivity toward thiols in contrast to amino and hydroxy groups,[6,7] and hence these compounds may be free from the problems of mutagenicity and carcinogenicity which are associated with a number of alkylating agents used in cancer chemotherapy.[8] In addition, the theory of sequential cytotoxicity proposed by one of the authors states that the successive release of two or more cytotoxic compounds may cause greater toxicity to malignant tissue than normal cells.[9] This concept is illustrated in Scheme 2. Thus alkylation with a cellular thiol such as glutathione (GSH) may occur with the chalcones **1**−**14** leading to the adducts A. On the other hand, after formation of the initial thiol adducts B from the Mannich bases **15**−**30**, deamination leading to the formation of C could take place. This reactive intermediate may lead to the formation of the bisadduct D. Furthermore extrusion of dimethylamine from D would enable further alkylation to occur by the B → D sequence (not shown in Scheme 1). If such a process occurs, then the Mannich bases **15**−**30** should be markedly more potent

Table 1. Physical Data and Cytotoxicity Evaluation of Compounds **1**−**44** Against Murine P388 and L1210 Cells and Human T-lymphocytes

					IC_{50} (μM)			
compd	R^1	R^2	mp (°C)	yield (%)	P388 cells	L1210 cells	Molt 4/C8 cells	CEM cells
1	H	H	172−173	85	3.12	23.5 ± 12.9	13.1 ± 4.2	14.7 ± 0.45
2	2-Cl	H	168−169	80	6.85	24.5 ± 6.7	18.2 ± 6.33	13.9 ± 1.5
3	3-Cl	H	176−178	84	2.82	21.2 ± 5.9	6.80 ± 0.39	5.4 ± 0
4	4-Cl	H	187−189	90	3.67	9.07 ± 0.39	6.91 ± 0.04	3.47 ± 1.16
5	2-Cl	4-Cl	171−173	82	7.89	25.2 ± 1.7	13.9 ± 9.3	10.2 ± 0.3
6	2-Cl	6-Cl	190−192	85	3.17	5.15 ± 0.58	5.15 ± 0.41	1.36 ± 0.07
7	3-Cl	4-Cl	186−188	87	11.70	13.1 ± 4.4	5.66 ± 0.03	1.71 ± 0.34
8	4-F	H	185−187	86	8.55	15.7 ± 6.4	16.1 ± 10.7	3.30 ± 0.12
9	2-F	5-F	186−188	75	0.864	12.2 ± 4.0	7.88 ± 1.50	3.57 ± 0.19
10	4-Br	H	199−201	84	16.65	13.2 ± 8.4	6.07 ± 1.32	1.98 ± 0.66
11	4-CF_3	H	181−183	86	2.49	13.0 ± 4.6	6.33 ± 0.92	1.88 ± 0.17
12	4-NO_2	H	247−249	84	13.14	7.32 ± 1.26	5.94 ± 0.11	2.23 ± 0.11
13	4-CH_3	H	190−192	86	5.51	24.7 ± 7.6	25.4 ± 6.8	11.3 ± 1.7
14	4-OCH_3	H	180−182	86	8.70	32.2 ± 5.5	27.9 ± 2.5	5.90 ± 0.39
15	H	H	218−220	72	29.6	17.7 ± 7.5	20.7 ± 17.0	19.6 ± 14.9
16	2-Cl	H	228−229	74	13.2	6.84 ± 0.36	7.13 ± 1.95	8.08 ± 1.79
17	3-Cl	H	223−224	73	8.66	5.96 ± 0.25	5.75 ± 1.05	2.68 ± 0.20
18	4-Cl	H	227−229	75	13.7	8.33 ± 0.85	8.41 ± 1.55	6.48 ± 3.28
19	2-Cl	4-Cl	221−223	78	5.44	5.28 ± 2.01	5.74 ± 3.66	4.87 ± 3.21
20	2-Cl	6-Cl	217−219	76	5.92	3.83 ± 1.47	5.38 ± 4.10	4.33 ± 3.00
21	3-Cl	4-Cl	253−254	76	5.09	2.49 ± 0.42	6.99 ± 2.42	2.48 ± 1.08
22	4-F	H	238−240	75	26.9	16.9 ± 6.4	17.2 ± 11.2	13.8 ± 8.1
23	2-F	5-F	232−233	74	8.86	15.9 ± 2.7	15.4 ± 2.2	2.91 ± 0.31
24	4-Br	H	226−227	76	10.9	7.09 ± 0.52	8.42 ± 0.52	4.78 ± 3.83
25	4-CF_3	H	228−230	75	3.67	8.14 ± 3.90	8.30 ± 2.96	1.56 ± 0.03
26	4-NO_2	H	219−220	45	17.7	8.73 ± 5.99	4.82 ± 0.77	1.75 ± 0.17
27	4-CH_3	H	227−229	78	30.8	40.1 ± 1.7	40.2 ± 5.9	35.2 ± 13.8
28	4-OCH_3	H	238−240	76	44.1	25.8 ± 9.7	25.3 ± 22.0	14.8 ± 8.8
29	2-Cl	6-F	213−214	75		8.29 ± 1.60	7.92 ± 0.11	7.74 ± 0.40
30	4-OH	H	238−240	80	>50	54.0 ± 8.3	44.3 ± 1.8	39.1 ± 1.4
31			236−238	78	10.3	15.7 ± 3.9	17.2 ± 0.5	6.11 ± 0.44
32	H	H	180−181	85	14.3	37.9 ± 1.0	8.58 ± 0.78	9.31 ± 1.96
33	Cl	Cl	199−201	86	8.53	33.8 ± 1.9	10.8 ± 0.1	20.0 ± 8.5
34	Br	H	164−166	85	12.9	29.4 ± 4.7	9.38 ± 2.00	12.8 ± 5.2
35	H	H	214−215	78	>50	108 ± 4	98.0 ± 5.6	22.1 ± 6.6
36	Cl	Cl	227−229	77	5.45	9.32 ± 1.24	12.4 ± 0.8	10.4 ± 0.5
37	Br	H	232−234	78	15.1	32.3 ± 4.2	39.2 ± 2.3	21.9 ± 3.4
38			>300	85	0.564	395 ± 149	30.3 ± 2.6	78.6 ± 1.2
39			275−285	40				
40	H				9.63	41.4 ± 4.9	12.7 ± 4.5	12.5 ± 3.4
41	H		158.5	50	4.62	46.5 ± 1.4	39.2 ± 3.1	13.2 ± 6.4
42	Cl		185	94	3.82	265 ± 47	220 ± 8	45.3 ± 10.1
43	OCH_3		151.4	76	10.2	39.5 ± 3.5	27.9 ± 16.8	10.8 ± 3.0
44			133−135	60	8.83	33.8 ± 4.0	30.8 ± 2.64	10.0 ± 3.2
melphalan					0.22	2.13 ± 0.02	3.24 ± 0.56	2.47 ± 0.21

than the analogous chalcones **1**−**14,** not only due to the greater number of sites in the molecules for electrophilic attack with cellular constituents but also due to the cascade effect whereby preferential chemosensitization to chemical insult of the malignant cells, rather than normal cells, may occur.[9] Since the rate of deamination is inversely proportional to the basicity of the liberated amine, the weaker basic group in **31** may lead to a more rapid formation of a cyclohexadienone than in the case of **15**.



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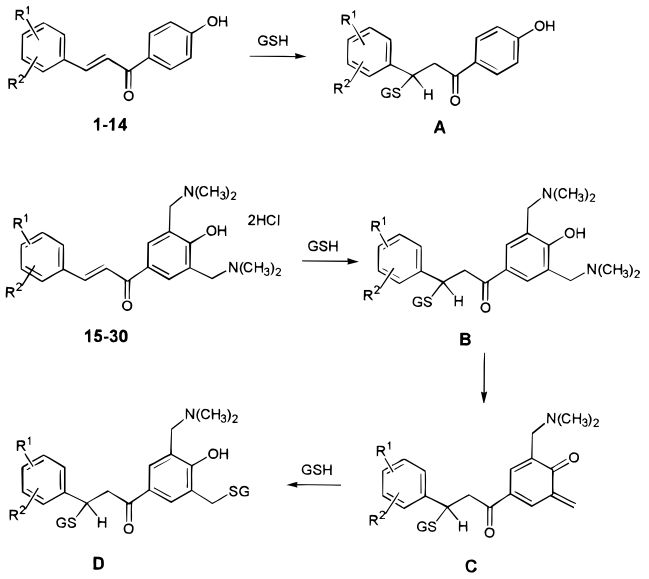
The choice of substituents in the aryl ring A of **15**−**30** was made for the following reasons. First, groups with widely divergent Hammett σ and Hansch π values were chosen; in fact substituents from three of the four quadrants of a Craig plot[10] were used. In this manner, the contributions of chemical reactivity and lipophilicity to cytotoxicity may be discerned. Second, the aryl substitution pattern would enable a Topliss analysis[11] to be undertaken. Third, compounds containing ortho and meta substituents may force the aryl ring A out of coplanarity. The adjacent olefinic linkage enables an interplanar angle θ to be calculated by electronic absorption spectroscopy and other physicochemical techniques, allowing study of any correlation between θ values and cytotoxicity.

The synthesis and cytotoxic evaluation of compounds **32**−**44** were suggested due to the following considerations. The cluster of compounds **32**−**37** may be referred to as "reversed chalcones" whereby the carbonyl and ethylenic groups present in the compounds discussed previously are interchanged. Their cytotoxic evaluation may shed light on the importance of the structure of the spacer link between the two aryl rings. Compounds **38**, **39**, and **41**−**44** contain two olefinic double bonds present in the "bischalcones" **38**, **39**, the *O*-cinnamoyl esters **41**−**43** (whose cytotoxicity may be compared to the parent compound **40**), and the diolefinic ketone **44**. If the activity of the chalcones is due to thiol

Scheme 2



alkylation, then compounds containing two double bonds should be more cytotoxic than analogues containing only one nucleophilic center.

In addition to the syntheses and cytotoxicity evaluations, a number of physicochemical, biochemical, and toxicological determinations on representative compounds were planned with a view to determining correlations between the structures of these molecules and bioactivities.

Results

The methods employed for the preparation of compounds **1**−**39**, and **41**−**44** are outlined in Scheme 1. With very few exceptions, they were all evaluated against murine P388 and L1210 leukemic cells as well as transformed human T-lymphocytes. These data are summarized in Table 1. Various Mannich bases were examined against a panel of approximately 55−60 human tumor cell lines from the following neoplastic diseases, namely leukemia, melanoma, and non small cell lung, colon, central nervous system, ovarian, renal, prostate, and breast cancers.[12] These results, along with the figures for **38** and **40**, are presented in Table 2.

A number of physicochemical experiments were undertaken with a view to understanding those factors which contributed to cytotoxicity. These measurements involved the determination of the torsion angles θ using electronic absorption spectroscopy and redox potentials of various Mannich bases (Table 2) as well as examining data provided by X-ray crystallography (Table 3) and molecular modeling (Table 4).

Compounds **1, 9**, **15**, and **23** were examined for mutagenic properties using deletion and intrachromosomal recombination tests in *Saccharomyces cerevisiae*.[13] No mutagenicity was observed.

The Mannich bases **15**, **16**, **18**−**22**, **24**, **27**, and **28** were evaluated against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus fumigatus*. The concentration of compound required to inhibit the growth of the microorganisms by 100% was greater than 1000 μM in each case.

Table 2. Cytotoxicity to Human Tumor Cell Lines, Electronic Absorption Spectroscopy, and Redox Potentials of Various Mannich Bases and Related Compounds

compd	IC_{50} (μM)	electronic absorption spectroscopy λ_{max}	ϵ	θ	redox potential E_{pk} (mv)
15	21.9	374.6	24,350	0	−773.1
16	9.55	377.1	21,912	19.7	−742.9
17	4.68	376.9	23,628	11.9	−742.9
18	10.0	376.2	24,668	0	−755.2
19	3.09	379.3	21,709	21.5	−681.2
20	5.88	373.0	18,577	30.9	−694.5
21	2.35	379.2	22,454	18.8	−753.8
22	20.0	373.6	25,891	0	−784.1
23		378.3	22,989	25.3	−694.5
24	6.31	378.2	27,368	0	−739.3
25		379.6	22,787	0	−742.9
26	8.91				
27	17.8	375.9	26,621	0	−834.8
28	29.5	382.3	31,939	0	−854.2
29	9.12				
30	39.8	383.7	33,398	0	−851.7
31	14.5				
35		405.5	22,811	0	−820.4
38	93.3				
39	26.9				
40	8.32				
melphalan	26.3				

Compounds **15**, **18**, **27**, and **40** were examined for anticonvulsant properties and neurotoxicity in rodents using a literature protocol.[14] The Mannich bases **15**, **18**, and **27** were active in the maximal electroshock (MES) screen at doses of 30 (**15**) and 100 (**18**, **27**) mg/kg when administered intraperitoneally to mice while **40** was inactive at the highest dose of 300 mg/kg. No activity was displayed in the subcutaneous pentylenetetrazole test except for **40** at the maximum dose. Neurotoxicity (NT) was displayed by all four compounds at a dose of 300 mg/kg. Oral administration of 30 mg/kg of **15**, **18**, and **27** to rats afforded protection in 0, 0 and 1 of 4 rats, respectively, in the MES screen while no neurological deficit was noted.

Discussion

The chalcones **1**−**14** and related unsaturated ketones **32**−**34**, **38**, and **44** were prepared in high yields. The corresponding Mannich bases **15**−**31**, **35**−**37**, and **39** were obtained in lower yields, and considerable difficulty was experienced in synthesizing the tetrabasic compound **39**. Both ^{1}H NMR spectroscopy and X-ray crystallography, when utilized, revealed that the olefinic bond in these compounds adopted the *E* configuration. Aminomethylation of phenols occurs preferentially at positions vicinal to the hydroxy group; on occasions reaction at the para position also takes place.[15] For the Mannich bases described in this study, the para position was blocked, and hence the assumption was made that reaction occurred at the positions ortho to the hydroxyl group. The ORTEP diagrams of **21**, **26**, and **31** supported this assignment. Attempts to quaternize the free base of **31** with methyl iodide led to the formation of the corresponding monohydroiodide **45**.

The initial cytotoxicity screens used murine P388 and L1210 leukemic cells due to their being good predictors of clinically useful drugs;[16] these data are summarized in Table 1. The structure−activity relationships (SAR) that were discerned in the P388 screen were as follows.

Table 3. Physical Data Obtained by X-ray Crystallography of **13**, **21**, **26**, and **31**[a]

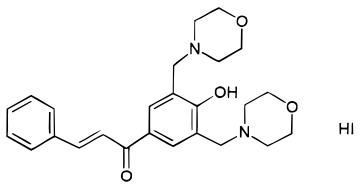
									$IC_{50}(\mu M)$	
compd	θ_A[b]	θ_B[b]	d_1 (Å)	d_2 (Å)	d_3 (Å)	d_4 (Å)[c]	d_5 (Å)[d]	d_6 (Å)[d]	P388 cells	L1210 cells	human tumors
13	−1.6	14.5							5.51	24.7	
21	−6.4	−9.8	2.491	2.499	6.422	10.304	1.315	1.279	5.09	2.49	2.35
26	−3.3	3.8	2.499	2.507	6.014	9.774	−1.445	−1.146	17.7	8.73	8.91
31	13.8	−25.9	2.510	2.471	6.088	10.764	−1.281	0.667	10.3	15.7	14.5

[a] The meaning of the symbols θ_A, θ_B, etc. are explained in the Discussion. [b] The torsion angles are designated as + or − if the rotations about the bonds are clockwise or anticlockwise, respectively. [c] The distances measured were between the following atoms namely H17a−H21a (**21**), H17c−H21b (**26**), and H18b−H24b (**31**). [d] Atoms located above and below the plan of ring B are designated as + and −, respectively.

Table 4. Torsional Angles of **1**, **6**, **15**, and **20** Obtained by Molecular Modeling[a]

	constrained molecule				minimized molecule			
compd	Ψ_1	Ψ_2	Ψ_3	energy (kcal/mol)	Ψ_1	Ψ_2	Ψ_3	energy (kcal/mol)
1	40	−20	−20	6.99	26.4	−23.3	−13.4	6.51
	20	−20	−20	7.18	25.1	−23.0	−14.8	6.51
average				7.09	25.8	−23.2	−14.1	6.51
6	60	−20	−20	7.20	47.8	−22.8	−15.0	6.71
	40	−20	−20	6.99	47.8	−22.1	−15.9	6.71
average				7.10	47.8	−22.5	−15.5	6.71
15	40	−20	−20	36.89	25.9	−24.7	−13.3	36.23
	20	−20	−20	36.43	25.1	−24.5	−14.7	36.24
average				36.66	25.5	−24.6	−14.0	36.24
20	60	−20	−20	37.08	48.6	−23.1	−17.4	36.01
	40	−20	−20	36.82	47.7	−23.4	−19.2	36.01
average				36.95	48.2	−23.3	−18.3	36.01

[a] The Ψ angles were arbitrarily assigned positive numbers, while the Ψ_2 and Ψ_3 angles, having opposite rotations, were described as negative figures.



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First, conversion of the chalcones **1**−**14** into the corresponding Mannich bases **15**−**28** led, on average, to a 2.4-fold reduction in potency; the average IC_{50} figures of the chalcones and corresponding Mannich bases were 6.80 and 16.04 μM, respectively. The compound displaying the greatest cytotoxicity was **9**, which possessed one-quarter of the activity of the reference drug melphalan. Second, replacement of the dimethylamino group of **15** by a morpholino function led to **31**, which had a 3-fold increase in potency. Third, a comparison was made of the cytotoxicity of the reversed chalcones **32**−**37** with the corresponding "normal" chalcones **1**, **7**, **10**, **15**, **21**, and **24**, respectively. With the exception of **33** and **34**, which had greater cytotoxicity than **7** and **10**, respectively, the remaining reversed chalcones had higher IC_{50} values than the related chalcones. The 4-hydroxy group in these molecules appears to contribute to cytotoxicity since **1** has more than 3 times the potency of **40** (the related analogue with no 4-hydroxy substituent in the aryl ring). Fourth, the effect of introducing a second site of alkylation was as follows. A comparison between **1** and the bischalcone **38** led to an approximately 6-fold increase in potency; in fact **38** had nearly 40% of the activity of melphalan. On the other hand, the IC_{50} figures of **1**, **4**, and **14** were lower than the corresponding *O*-cinnamoyl esters **41**−**43**, respectively, and **44** (the chalcone containing a pentadienoyl chain) had one-third of the cytotoxicity of **1**. One may summarize the results of evaluating these compounds in the P388 screen by stating that, in general, the chalcones displayed greater activity than the corresponding Mannich bases and reversed chalcones and also that the introduction of a second olefinic linkage was disadvantageous. However further development of analogues of **38** may prove to be a profitable venture.

The SAR observed in the L1210 screen showed both differences and similarities to the trends observed using P388 cells. Thus in contrast to their evaluation in the P388 screen, the Mannich bases **15**−**28** were 1.4 times more cytotoxic than the corresponding chalcones **1**−**14** in general; the average IC_{50} figures for these two series of compounds were 12.4 and 17.2 μM, respectively. The most active compound was the dichlorinated chalcone **21** which was equipotent with melphalan. In addition, the dimethylamino and morpholino Mannich bases **15** and **31** displayed similar cytotoxicity toward L1210 cells. However in the remaining cases, the SAR in the L1210 and P388 screens were similar. Thus the reversed chalcones **32**−**37** had higher IC_{50} values than the related analogues **1**, **7**, **10**, **15**, **21** and **24**, respectively. The greater activity of **1** compared to that of **40** indicated the contribution of the 4-hydroxy group to cytotoxicity. In addition, compounds **41**−**44** which possessed a second olefinic linkage had higher IC_{50} figures than the related chalcones **1**, **4**, **14**, and **1**, respectively. Thus the most promising compounds emerging from evaluation using L1210 cells were the Mannich bases **15**−**28**, and in particular **21** may be regarded as a useful lead molecule.

An important characteristic of prototypic anticancer molecules is displaying selective toxicity toward neoplastic rather than normal tissues. However the passaging of normal lymphocytes is both difficult and unpredictable; consequently in practice transformed lymphocytes may be employed. Evaluation was under-

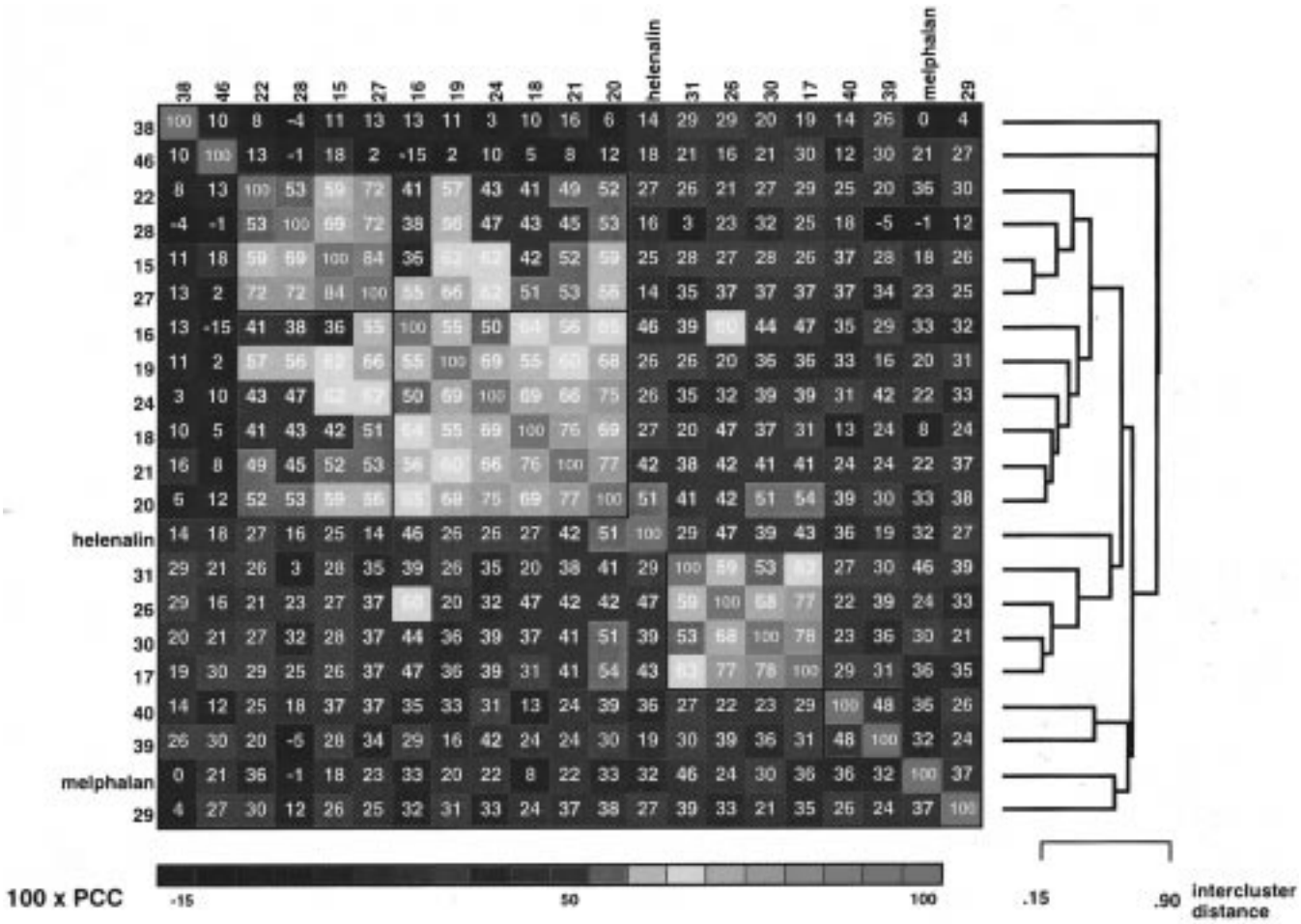


Figure 1. ClusCor analysis showing the degree of similarity between compounds tested against human tumor cell lines. Pairwise Pearson correlation coefficient (PCC) figures were calculated for each compound and all other compounds in the set using GI_{50} values. The figures of 100 on the diagonal occur where compounds are compared to themselves. Cluster analysis was used to order the rows and the columns of the matrix as reflected by the dendogram on the right of the matrix. The similarity values were color-coded in order to aid visualization of clusters according to the scale at the bottom of the figure.

taken using Molt 4/C8 and CEM human T-lymphocytes, and the results are portrayed in Table 1. A comparison between the IC_{50} values of these enones in both the P388 and L1210 assays with their cytotoxicity toward each of the T-lymphocytes gave a therapeutic index (TI). A TI value of 4 or greater was arbitrarily chosen as useful chemosensitivity toward tumorous tissue. A comparison of the IC_{50} figures using P388 and Molt 4/C8 cells indicated that the following compounds had selective toxicity for the leukemic cells (TI values in parentheses) viz **1** (4.20), **9** (9.12), **13** (4.61), **38** (53.7), **41** (8.49), **42** (57.6), and melphalan (14.7). The data for the P388 and CEM cells revealed that **1** (4.71), **9** (4.13), **38** (139), **42** (11.9), and melphalan (11.2) demonstrated selectivity. TI values of 4 or greater were not observed when the cytotoxicity data of the Mannich bases and related compounds as well as melphalan toward L1210 cells was compared to both the Molt 4/C8 and CEM lymphocytes. The very high selectivity demonstrated by the bischalcone **38** and the cinnamoyl ester **42**, which had superior TI figures than melphalan, is noteworthy, and they are clearly useful lead molecules for further development.

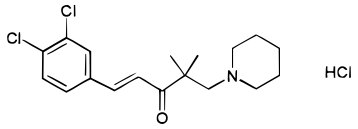
The cytotoxicity of most of the Mannich bases prepared in this study along with **38** and **40** were examined against various human tumor cell lines, and the data are presented in Table 2. The Mannich bases **15**−**22**, **24**, **26**, **27**, **29**, and **31** and the unsubstituted ketone **40** had lower IC_{50} values than melphalan and in particular **21** (which was the most active compound in the L1210 screen) possessed 11 times the activity of melphalan and is a useful lead molecule. None of the compounds listed in Table 2 displayed selective toxicity toward one or more of the neoplastic diseases used in this screen.

Use of the ClusCor program[17] was undertaken in order to identify compounds having similar patterns of cytotoxicity toward the various human tumor cell lines employed in this study. This program measures the similarity between two cytotoxicity patterns using the Pearson correlation coefficient (PCC). Compounds identified in this manner often include those with high structural similarity, which attests to the reproducibility of the screening pattern. In addition, in many cases, structurally dissimilar molecules were shown to share growth inhibition mechanisms which were predicted by high pattern similarity.[18] Artificial neural networks[19] and discriminant analysis[20] have been used to demonstrate that the screen patterns are predictive of mechanisms of action.

The ClusCor output is presented in Figure 1. It is constructed by using a matrix of similarity values (PCC $\times$ 100) between all members of the tested set. The same order was used for the rows as for the columns which results in values of 100 on the diagonal whereby each compound was compared to itself. The order was

obtained by cluster analysis of the screen patterns. The resultant cluster tree is shown on the right of the matrix.

In general, there was a high degree of similarity among the screen patterns for most of the Mannich bases of the chalcones, and in particular three clusters were distinguishable. First, compounds **15** and **27** had the highest similarity ($r = 0.84$) and were grouped with **22** and **28**. Second, the patterns of compounds **16**, **19**, **24**, **18**, **21**, and **20** were similar, and the third group comprised the Mannich bases **31**, **26**, **30**, and **17**. Three reference compounds were also included in this study namely **46**, helenalin, and melphalan. Compound **46**



46

is an acyclic Mannich base possessing marked cytotoxicity toward murine P388 and L1210 leukemic cells as well as to the panel of human tumors.[21] Tumor cells which were resistant to melphalan and related bifunctional alkylators were sensitive to **46** and related Mannich bases, revealing an absence to cross-resistance to melphalan-resistant cells.[21] Compound **46** was designed as a thiol alkylator. The data in Figure 1 revealed that **46** did not cluster with either helenalin, which is an established thiol alkylator,[22] or melphalan, suggesting that while its mode of action is different from melphalan, its cytotoxicity is principally by mechanisms other than interacting with cellular thiols. Helenalin showed marginal similarity to **20** but the impression was gleaned from Figure 1 that the chalcones act by other pathways than those postulated for this sesquiterpenoid lactone. The pattern for melphalan was different than the other compounds as were **29**, **38**−**40**. One may conclude that in general, while one or more physicochemical properties play a common role in eliciting cytotoxicity in these compounds, subsets of these Mannich bases were discovered which act by other modes of action.

A Topliss analysis[11] was undertaken using the IC_{50} values in the P388 and L1210 screens for the chalcones **1**, **4**, **7**, **13**, and **14** and the corresponding Mannich bases **15**, **18**, **21**, **27**, and **28**. In addition, the latter compounds were also employed in this analysis using the IC_{50} data for the human tumor cell lines. The cytotoxic activity of the chalcones in the L1210 screen was closest to a positive correlation with the aryl σ constants, while no parameter dependencies were noted in the P388 test. The bioactivity of the Mannich bases was positively correlated with the σ values of the aryl substituents in the P388 and human tumor screens. However cytotoxicity was not directly related to the π or σ constants in the L1210 test. Thus in certain cases, this analysis reveals that cytotoxicity was elevated as the electron-accepting properties of the aryl substituents were increased.

A further investigation to discern SAR was undertaken by making linear and semilogarithmic plots between the IC_{50} figures and various physicochemical constants which reflected the electronic (σ and/or σ^*; for clarity referred hereafter to σ) hydrophobic (π) and steric (MR i.e., molar refractivity) properties of the aryl substituents in cases where there was at least three compounds in the series. Plots were made using the following clusters of compounds namely the chalcones **1**−**14** (P388, L1210 screens) and the Mannich bases **15**−**28** (P388 screen), **15**−**30** (L1210 test), and **15**−**22, 24, 26**−**30** (human tumor screen). The p values for all these determinations are recorded in the Experimental Section, and the following correlations ($p < 0.1$) were noted. In the case of the chalcones **1**−**14**, semilogarithmic plots revealed correlations between the σ and MR values in the L1210 and P388 screens, respectively. Both linear and semilogarithmic plots revealed correlations between (i) the σ and π values of **15**−**28** in the P388 screen (ii) the σ, π, and MR figures of **15**−**30** in the L1210 test, and (iii) the σ, π, and MR figures of the aryl substituents in **15**−**22, 24, 26**−**30** in both the L1210 and human tumor screens. All correlations were positive, i.e., cytotoxicity increased as the magnititude of the physicochemical constants were elevated except for a negative relationship between the MR values of the chalcones **1**−**14** and the IC_{50} figures in the P388 screen. These results indicated that, in general, cytotoxicity was increased by elevation of the magnititude of the σ values, as suggested by the Topliss analysis. In addition, activity was raised by increasing the lipophilicity of the molecules.

With a view to seeking correlations between the shapes of various Mannich bases of chalcones and related compounds with cytotoxicity, use was made of electronic absorption spectroscopy, X-ray crystallography, and molecular modeling techniques applied to representative compounds.

The interplanar angle θ between ring A of a number of Mannich bases and the adjacent olefinic linkage was measured by electronic absorption spectroscopy and application of eq 1 developed by Braude and co-workers.[23,24] The question posed was whether a correlation existed between cytotoxicity and the θ angles. The data in Table 2 indicated that a lack of coplanarity between aryl ring A and the attached unsaturated group was displayed by **16**, **17**, **19**−**21**, and **23**. The average IC_{50} values of these compounds in the P388, L1210, and human tumor screens were approximately 3 or 4 times lower than the figures for the analogues **15**, **18**, **22**, **24**, **25**, **27**, **28**, and **30**. Hence a lack of coplanarity favors bioactivity although constructing linear and semilogarithmic plots between compounds with θ values greater than 0 and cytotoxicity revealed no correlations.

$$\cos^2 \theta = \frac{\epsilon}{\epsilon_o} \qquad (1)$$

The second physicochemical technique used to examine the shapes of representative molecules was X-ray crystallography. The ORTEP diagrams of **13**, **21**, **26**, and **31** are portrayed in Figures 2−5. The results confirm that the olefinic double bond in all four compounds had the *E* configuration and the bis(dimethylamino)methyl or 4-morpholinylmethyl groups in ring B of **21**, **26**, and **31** were located ortho to the aryl hydroxy function. Two torsion angles θ_A and θ_B and six interatomic distances d_1−d_6 were obtained from the X-ray crystallographic data with a view to seeking

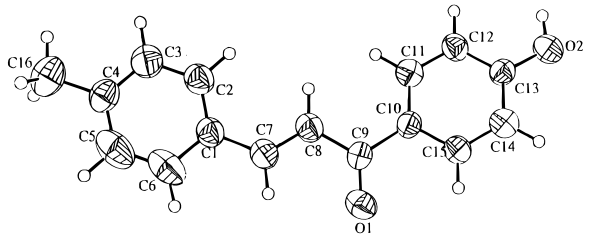


Figure 2. ORTEP diagram of **13**.

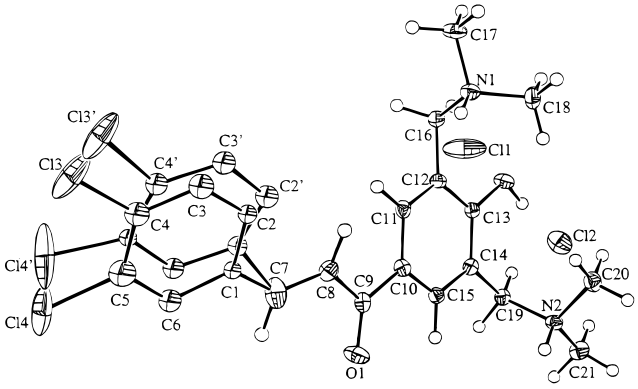


Figure 3. ORTEP diagram of **21**.

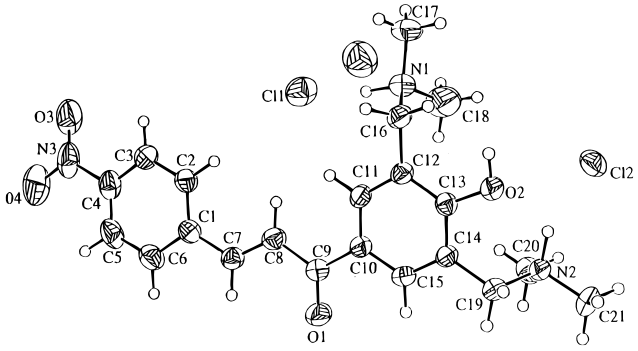


Figure 4. ORTEP diagram of **26**.

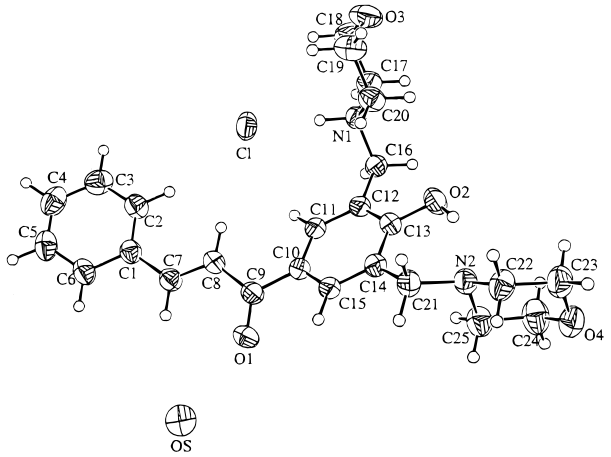


Figure 5. ORTEP diagram of **31**.

correlations between these results and cytotoxicity. These measurements are illustrated for a representative compound **21** in Figure 6, while the results are summarized in Table 3; the relevant cytotoxicity data are also enclosed for the sake of clarity in this table. Linear and semilogarithmic plots between the torsion angles and IC_{50} values revealed no correlation ($p > 0.10$). The

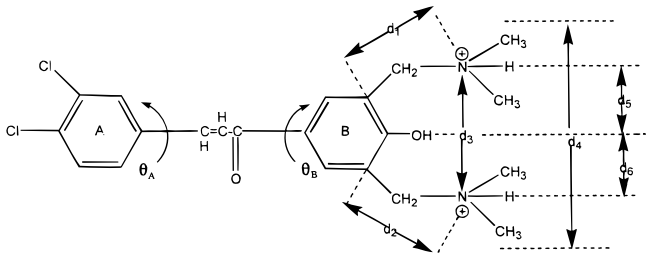


Figure 6. Torsion angles and interatomic distances of **21** measured by X-ray crystallography.

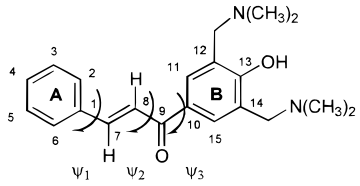


Figure 7. Numbering used in the molecular modeling studies.

distances d_1 and d_2 were similar and therefore did not influence cytotoxicity. On the other hand, **21** had the largest d_3 figure, and its cytotoxicity was approximately 4 times that of **26** and **31**. The distance d_4 in **21** was intermediate between the figures for **26** and **31**. The measurements d_5 and d_6 are the distances either above or below the plane of ring B. Both nitrogen atoms of **21** were above the plane of the aryl ring whereas the least active compound **31** had one nitrogen atom above and the other below this plane. One may conclude that the design of future analogues should aim at increasing the distance d_3 while at the same time the width of the groups attached to the nitrogen atoms in **21** should be retained. Both nitrogen atoms should be at least 1.3 Å above or below the plane of ring B.

Molecular modeling was undertaken on the Mannich bases **15** and **20** as well as the corresponding chalcones **1** and **6** with a view to seeking correlations between various torsion angles and cytotoxicity. The search was carried out by systematically rotating three bonds designated Ψ_1, Ψ_2, and Ψ_3 (indicated in Figure 7) and calculating the energy of each conformation.[25] Using three-dimensional isocontour maps, four regions of lower energy conformations were revealed, indicating Ψ_1 and Ψ_2 angles of 20−40°. Conformations within these areas were minimized further using the Maxmin 2 program with the Tripos force field to produce the global minima. These results are summarized in Table 4.

The presence of two ortho chloro atoms in ring A of compounds **6** and **20** led to a marked inhibition of coplanarity of this group with the adjacent unsaturated linkage as revealed by the Ψ_1 angles of 48°. On the other hand, the variations in either the Ψ_2 and Ψ_3 angles among all four compounds were minimal. The substantially larger Ψ_1 angles displayed by **6** and **20** compared to the unsubstituted analogues **1** and **15** led, in general, to a 5-fold increase in cytotoxicity. Thus the results from molecular modeling suggested that bioactivity was favored in compounds with the highest Ψ_1 figures.

Correlations between cytotoxic and anticancer activities with redox potential have been observed previously,[26] and hence the bioactivity of the Mannich bases of chalcones may be significantly influenced by their

redox potentials. The E_{pk} values of 15 compounds are listed in Table 2. Linear and semilogarithmic plots were made between the E_{pk} figures of **15**−**25**, **27**, **28**, and **30** and the IC_{50} data in the P388, L1210, and human tumor assays where such figures were available. Highly significant positive correlations ($p < 0.03$) were obtained in all cases, i.e., cytotoxicity increased as the E_{pk} values were less negative, revealing the important contributions the redox potentials of the Mannich bases made to cytotoxicity. The lower reducing properties of **15** versus **35**, which may have been due to a greater hindrance for reductions of the carbonyl group, could have contributed to the increased cytotoxicity of **15** in the P388 and L1210 screens.

To glean some insight into the mechanism whereby the Mannich bases exerted their cytotoxic effect, the stability and reactivity toward GSH of representative compounds was examined. Compound **15** was stable in buffered solution and did not react with excess of GSH. However compound **15** and the analogues **18** and **21** reacted with GSH in the presence of the π isozyme of GST.[27] Thus under biological conditions it is likely that the compounds described in this study are thiol alkylators, although additional mechanisms of action are also possible.

The final three lines of investigation related to whether these compounds were carcinogenic, possessed antimicrobial properties, or penetrated the central nervous system. A number of chalcones are mutagenic.[28] However in the present investigation, the representative Mannich bases **15** and **23** as well as the analogous chalcones **1** and **9** were nonmutagenic when concentrations 50 times that of the IC_{50} figures in the P388 screen were employed. An impaired immune system in certain cancer patients may lead to infections. Antimicrobial properties have been reported for various chalcones[29,30] and Mannich bases,[31] and hence compounds described in the present investigation having antibacterial and antifungal activities may enhance their utility. Ten Mannich bases prepared in this study did not inhibit the growth of *E. coli*, *S. typhimurium*, *S. aureus*, *C. albicans*, and *A. fumigatus* completely at the maximum concentration utilized. It is conceivable that the presumed absence of a deamination process in these compounds may be responsible for their lack of antimicrobial properties.

In addition, anticonvulsant activity has been reported for both chalcones[32] and Mannich bases,[33] and hence the evaluation of representative compounds for this property was considered of interest. Anticonvulsant activity could be advantageous insofar as the compounds would have the potential for treating central nervous system (CNS) tumors, or alternatively, penetration of the CNS could lead to such unwanted side effects as neurological deficit. Three Mannich bases **15**, **18**, and **27** as well as the chalcone **40** possessed anticonvulsant and neurotoxic properties in mice. Quantitation of the most potent compound **15** revealed ED_{50} and TD_{50} figures in the MES and NT screens of 55.4 and 235 mg/kg, respectively, leading to the protection index (PI, i.e., TD_{50}/ED_{50}) of 4.24. The comparable figures for a reference drug phenytoin were 6.32 and 41.2 mg/kg, respectively, and a PI value of 6.52. Hence while **15** is less potent than phenytoin, it has two-thirds of the PI figure of this drug. The data reveal that penetration of the CNS occurs with these Mannich bases. Thus future development of these compounds may lead to drugs capable of treating CNS tumors; however at the same time monitoring for neurotoxic symptoms should take place.

Conclusions

This study has revealed that Mannich bases of chalcones are novel cytotoxic agents whose activity is influenced by a number of physicochemical parameters including the π, σ, and MR constants of the aryl rings and redox potentials along with other structural features revealed by X-ray crystallography and molecular modeling. Several lead molecules have been found, including **21**, with high potency toward L1210 cells and human tumor cell lines, **38**, with marked lethality to P388 cells, and **42**, with a good therapeutic index when the IC_{50} values of P388 and Molt 4/C8 cells were compared. The absence of mutagenic properties is a further pointer that development of these compounds may lead to useful therapeutic agents.

Experimental Section

A. Chemistry. Melting points are uncorrected. Elemental analyses (C, H for **1**−**11**, **13**, **14**, **32**−**34**, **38**, and **41**−**44** and C, H, N for **12**, **15**−**31**, **35**−**37**, **39**, and **45**) were undertaken by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan, and were within 0.4% of the calculated values. ^{1}H NMR spectra were recorded using Varian T-60 (60 MHz), Bruker AM 300 FT (300 MHz) or Bruker AMX 500 FT (500 MHz) instruments. The designation of protons in the 500 MHz ^{1}H NMR spectra of representative compounds was as follows. For **1** and **18**, the numbering used was the same as displayed in Figures 2 and 3. In the case of **36**, the protons at positions 2, 5, and 6 in ring A were designated H-A2, H-A5, and H-A6, respectively, while the ortho protons in ring B were described as H-B2 and H-B6. The numbering of the atoms in **42** was the same as in Figure 2 while the protons in the phenyl ring of the ester moiety were designated C2−C6. The electronic absorption spectra were obtained using a Gilford UV/vis spectrophotometer. An EG and G Princeton Applied Research Static HMDE Model 303A with silver/silver chloride reference and platinum counter electrodes interfaced to a 486 33DX PC system control was employed for the measurements of the redox peak potentials. The compounds were homogeneous as revealed by thin-layer chromatography using silica gel sheets with a fluorescent indicator. The eluting solvents were chloroform:methanol (4:1) for **1**−**14**, **32**−**34**, **38,** and **41**−**44** and chloroform:methanol (3:2) containing 1% ammonium hydroxide for **15**−**31**, **35**−**37**, **39**, and **45**. The unsaturated ketones **1**−**14**, **32**−**34**, **41**−**43**, and **44** were recrystallized from water−ethanol and **38** from dimethyl sulfoxide while the Mannich bases **15**−**31**, **35**−**37**, **39**, and **45** were recrystallized from ether−methanol. Compounds **26**, **29**, and **39** were obtained as the hemihydrates.

Synthesis of Compounds 1−14, 38, 40, and 44. The general procedure for preparing **1**−**14** was as follows. An aqueous solution of sodium hydroxide (10% w/v, 10 mL) was added to a solution of the appropriate aryl aldehyde (0.02 mol) and 4-hydroxyacetophenone (0.02 mol) in ethanol (6 mL). The reaction mixture was stirred at room temperature overnight and poured into water (100 mL). After neutralization with hydrochloric acid (10% w/v), a yellow solid or a yellow oil (which solidified on cooling) was obtained which was recrystallized. 3-(2-Chloro-6-fluorophenyl)-1-(4′-hydroxyphenyl)-2-propen-1-one required in the synthesis of **29** was prepared in the same manner in 85% yield and recrystallized from water−ethanol, mp 145 °C.

The chalcone **38** was prepared by the same procedure using terephthaldehyde (0.005 mol), 4-hydroxyacetophenone (0.01 mol), aqueous solution of sodium hydroxide (10% w/v, 10 mL),

and ethanol (25 mL). Compound **40** was prepared previously in these laboratories,[34] and **44** was synthesized by the method described above except cinnamaldehyde was used in place of an aryl aldehyde. The ^{1}H NMR spectrum ($CDCl_3$) of a representative compound **1** was as follows: δ 5.46 (1H, s, OH), 6.91 (2H, d, H-12, H-14, J = 8.7 Hz), 7.37−7.43 (3H, m, H-3, H-4, H-5), 7.52 (1H, d, H-8, J = 15.6 Hz), 7.62−7.64 (2H, m, H-2, H-6), 7.79 (1H, d, H-7, J = 15.6 Hz), 7.99 (2H, d, H-11, H-15, J = 8.7 Hz).

Synthesis of Compounds 15−30. The Mannich reagent *N*,*N*-dimethyleneammonium chloride was prepared by a literature method.[35] A mixture of the appropriate chalcone (0.005 mol) and *N*,*N*-dimethyleneammonium chloride (0.012 mol) in dry acetonitrile (50 mL) was heated under reflux for 36−48 h. On cooling, the precipitate was collected, washed with acetonitrile and subsequently with ether, and recrystallized to give **15−29** as pale yellow crystals. The ^{1}H NMR spectrum (DMSO-d_6) of a representative Mannich base **18** was as follows: δ 2.77 [12H, s, 4 × N(CH_3)$_2$], 4.48 (4H, s, 2 × CH_2), 7.52 (2H, d, H-3, H-5, J = 8.5 Hz), 7.74 (1H, d, H-8, J = 15.6 Hz), 7.98 (2H, d, H-2, H-6, J = 8.5 Hz), 8.10 (1H, d, H-7, J = 15.7 Hz), 8.45 (2H, s, H-11, H-15).

The synthesis of **30** was accomplished as follows. 3,5-Bis-(dimethylaminomethyl)-4-hydroxyacetophenone was prepared by a literature method[36] except that the molar ratio of both formaldehyde and dimethylamine was twice that of acetophenone. Hydrogen chloride was passed into a solution of the free base in anhydrous ether to produce a colorless precipitate which on recrystallization from ether−methanol produced the dihydrochloride salt in 85% yield, mp 200−202 °C. A mixture of 3,5-bis(dimethylaminomethyl)-4-hydroxyacetophenone dihydrochloride (0.01 mol) and 4-hydroxybenzaldehyde (0.012 mol) in ethanol that was saturated with hydrogen chloride (50 mL) was stirred at room temperature overnight. The resultant precipitate was collected, washed with ether, and recrystallized to give **30** as green crystals.

Synthesis of Compounds 31 and 45. A mixture of **1** (0.005 mol), formaldehyde (36−38% w/v, 0.01 mol), and morpholine (0.01 mol) in ethanol (40 mL) was heated under reflux for 40 h. On cooling, the solvent was removed in vacuo, yielding an oil which was dissolved in anhydrous ether (50 mL). The solution was divided into two equal portions. Hydrogen chloride gas was passed into one-half of the solution until a yellow precipitate was obtained which was recrystallized to give **31**. To the remainder of the ethereal solution was added methyl iodide (0.02 mol), and the mixture was stirred at room temperature overnight. The resultant yellow precipitate was collected and recrystallized to give **45** whose structure was confirmed by X-ray crystallography, details of which will be published elsewhere.

Synthesis of Compounds 32−34. These compounds were prepared as follows using a modification of a literature method.[37] A solution of the appropriate 1-aryl-1-ethanone (0.01 mol) and 4-hydroxybenzaldehyde (0.01 mol) in ethanol (100 mL) was saturated with hydrogen chloride. The reaction mixture was stirred at room temperature overnight and poured into water (250 mL). After neutralization with potassium carbonate solution (10% w/v), yellow (**32**, **33**) or pale green (**34**) precipitates were obtained and recrystallized.

Synthesis of Compounds 35−37. The intermediate aldehyde 3,5-bis(dimethylaminomethyl)-4-hydroxybenzaldehyde dihydrochloride was prepared from *N*,*N*-dimethylmethyleneammonium chloride by the method used for preparing compounds **15−29**. It was obtained in 70% yield, mp 234−235 °C, after recrystallization from ether−methanol.

A mixture of the appropriate 1-aryl-1-ethanone (0.01 mol) and 3,5-bis(dimethylaminomethyl)-4-hydroxybenzaldehyde dihydrochloride (0.01 mol) in ethanol which was saturated with hydrogen chloride (50 mL) was stirred at room temperature overnight. The resultant pale pink precipitate was collected and recrystallization afforded **35−37** as pale yellow amorphous powders. The ^{1}H NMR spectrum (DMSO-d_6) of a representative compound **36** was as follows; δ 2.75 [12H, s, 4 × N(CH_3)$_2$], 4.39 (4H, s, 2 × CH_2), 7.69 (1H, d, COC*H*═CH, J = 15.4 Hz), 7.85 (1H, d, H-A5, $J_{H-A5,H-A6}$ = 8.3 Hz), 7.86 (1H, d, COCH═C*H*, J = 15.7 Hz), 8.10 (1H, dd, H-A6, $J_{H-A2,H-A6}$ = 1.9 Hz, $J_{H-A5,H-A6}$ = 8.4 Hz), 8.15 (2H, s, H-B2, H-B6), 8.34 (1H, d, H-A2, $J_{H-A2,H-A6}$ = 1.9 Hz).

Synthesis of Compound 39. To a solution of **38** (0.001 mol) in ethanol (50 mL) were added aqueous solutions of formaldehyde (36−38% w/v, 0.005 mol) and dimethylamine (25−30% w/v, 0.005 mol). The mixture was heated under reflux for 84 h. On cooling, the precipitate was collected, recrystallized from ether−chloroform, and dissolved in chloroform into which excess hydrogen chloride was passed. The resultant pale yellow precipitate was recrystallized to give **39**.

Synthesis of Compounds 41−43. Cinnamoyl chloride (0.025 mol) was added to a solution of **1**, **4**, or **14** (0.025 mol) in pyridine (3 mL), and the mixture was heated under reflux for 3−4 h. On cooling, the reaction mixture was poured into dilute aqueous hydrochloric acid (10% w/v), and the colorless precipitate was collected, washed with methanol, dried, and recrystallized to give **41−43**. The ^{1}H NMR spectrum ($CDCl_3$) of a representative compound **42** was as follows: δ 6.63 (1H, d, OCOC*H*═C*H*, J = 16.0 Hz), 7.32 (2H, d, H-12, H-14, J = 8.7 Hz), 7.39 (2H, d, H-3, H-5, J = 8.4 Hz), 7.41−7.45 (3H, m, H-C3, H-C4, H-C5), 7.48 (1H, d, H-8, J = 15.6 Hz), 7.57 (2H, d, H-2, H-6, J = 8.5 Hz), 7.58−7.61 (2H, m, H-C2, H-C6), 7.76 (1H, H-7, J = 15.7 Hz), 7.89 (1H, d, O COCH═C*H*, J = 16.0 Hz), 8.07 (2H, d, H-11, H-15, J = 8.6 Hz).

Electronic Absorption Spectroscopy. Solutions of the compounds were prepared in water except, in order to achieve dissolution of **21** and **31**, the solvent contained approximately 10% v/v methanol. The concentrations of the solutions were chosen so that the absorption maxima were between 0.5 and 1. All determinations were carried out in duplicate. The error limits of the ϵ values were approximately 2%.

Reduction Peak Potential Determinations. Measurement of the reduction peak potentials was achieved by cyclic voltammetry using solutions of the compounds (1×10^{-4} M) in 1 M potassium chloride except that for **21** and **31** a mixture of methanol and 1 M potassium chloride (6:4) was employed. Five cycles were averaged at a scan rate of 0.5 mV/s for all samples. Data collection was made using the appropriate software.[38]

Stability Studies and Incubation with GSH. There was no change in the electronic absorption spectrum of a solution of **15** in PBS pH 7.4 after incubation at 37 °C for 48 h. The experiment was repeated in the presence of GSH using thiol: Mannich base ratios of 2:1 and 10:1, and no reaction was observed. The rates of reaction of **15**, **18**, **21**, and 1-chloro-2,4-dinitrobenzene with GSH in the presence of the π isozyme of GST was undertaken by a literature procedure.[27] The V_{max}/K_m (μmol/μM) values for the reaction of **15**, **18**, **21**, and a reference compound 1-chloro-2,4-dinitrobenzene with GSH in the presence of the π isozyme of GST were 0.000 34, 0.000 802, 0.000 375, and 0.000 864, respectively.

Statistical Analyses. The σ, π, and MR values were taken from the literature[39] and the σ* figures from a reference source.[40] Since the MR value of hydrogen is 1.03, this figure was subtracted from the values for various substituents. For example, the MR value of fluorine is 0.92 and hence the MR figure for the 2,5-difluoro analogue was taken as −0.22, i.e., (2 × 0.92) − (2 × 1.03).

The p values for the groups of compounds in different screens are given below. The figures in brackets refer to the plots of cytotoxicity versus the σ, π, and MR constants, respectively, and the subscripts l and sl refer to linear and semilogarithmic, respectively. **1−14**: $P388_l$ (0.84, 0.93, 0.11), $P388_{sl}$ (0.45, 0.94, 0.06), $L1210_l$ (0.10, 0.52, 0.80), $L1210_{sl}$ (0.012, 0.54, 0.53). **15−28**: $P388_l$ (0.0002, 0.005, 0.24), $P388_{sl}$ (0.0009, 0.001, 0.13). **15−30**: $L1210_l$ (0.0007, 0.003, 0.073), $L1210_{sl}$ (0.0006, 0.0002, 0.006). **15−22**, **24**, **26−30**: human $tumors_l$ (0.0002, 0.0003, 0.014), human $tumors_{sl}$ (0.0008, 0.0002, 0.002). The p values of the plots of the θ angles obtained by electronic absorption spectroscopy of **16**, **17**, **19−21**, and **23** against cytotoxicity were as follows: $P388_l$ (0.64), $P388_{sl}$ (0.63), $L1210_l$ (0.75), $L1210_{sl}$ (0.88), human $tumors_l$

(0.82), human tumors$_{sl}$ (0.78); in the evaluation using human tumors, the datum for **23** was unavailable. The *p* values of the plots of the E_{pk} data for **15**−**22**, **24**, and **26**−**30** against cytotoxicity were as follows: P388$_l$ (0.0001), P388$_{sl}$ (0.001), L1210$_l$ (0.0006), L1210$_{sl}$ (0.002), human tumors$_l$ (0.0004), human tumors$_{sl}$ (0.0012).

The *p* values obtained by plotting data obtained by X-ray crystallography of **13**, **21**, **26**, and **31** with cytotoxicity were as follows. θ_A: P388$_l$ (0.89), P388$_{sl}$ (0.73), L1210$_l$ (0.61), L1210$_{sl}$ (0.47), human tumors$_l$ (0.27), human tumors$_{sl}$ (0.41). θ_B: P388$_l$ (1.00), P388$_{sl}$ (0.89), L1210$_l$ (0.63), L1210$_{sl}$ (0.74), human tumors$_l$ (0.66), human tumors$_{sl}$ (0.80).

Use of ClusCor Program. The logarithim of the molar concentration of the test compound required to lead to a 50% recovery of intact cells at the end of the 48 h assay relative to vehicle control was used in this experiment. These figures are referred to hereafter as GI_{50} values. The DISCOVERY program was used to generate delta patterns, correlation matrixes, and cluster analyses using scripts written for SAS (SAS institute, Cary, NC) and Microsoft Excel/Visual Basic (Redmond, WA). A matrix of pairwise Pearson correlation coefficients (PCC) between GI_{50} patterns was created. This similarity matrix was converted to a distance matrix by subtracting each PCC from unity. Agglomerative clustering using the group average method[41] was performed using the distance matrix as input. The linear order of the resultant cluster tree was used to sort the rows and columns of the original similarity matrix. Values within the matrix were color-coded to aid in the visual interpretation of cluster groups.

X-ray Crystallography of 13, 21, 26, and 31. The compounds were crystallized from methanol by slow evaporation (**13**, **21**, and **26**) and from 2-propanol−methanol by vapor diffusion (**31**). A Nonius CAD-4 diffractometer with a ω scan was used for data collection and the structure was solved by direct methods using NRCVAX[42] and refined using SHELXL-93.[43] Atomic scattering factors were taken from the literature.[44] All non-hydrogen atoms were found on the E-map and refined anisotropically except for **21** in which case six atoms (C1−C6) were refined isotropically. Atoms C1−C6, Cl3, and Cl4 are disordered and occupied two different positions with occupancies of 0.63 (C1−C6, Cl3, and Cl4) and 0.37 (Cl^1, C1^6, Cl3', and Cl4'). Hydrogen atom positions were calculated and not refined.

The data for **13** were as follows: $C_{16}H_{14}O_2$, M_r = 238.29, colorless blocks 0.45 × 0.40 × 0.10 mm, a = 9.4706(4) Å, b = 11.3523(7) Å, c = 23.9731(11) Å, Z = 8, space group = *Pcab*, orthorhombic, D_x = 1.228 g cm^{-3}, λ(Mo Kα) = 0.7093 Å, μ = 0.08 mm^{-1}, F(000) = 1008, T = 287 K. Merging R is based on intensities 0.0013 for 138 replicate reflections. Refinement on F^2; $R[F^2 > 2\sigma(F^2)] = 0.0385$, $R_w(F^2) = 0.1269$, $S = 1.147$. A total of 2405 reflections were measured of which 2267 were independent. The refinement of the structure used 1575 observed reflections with $I > 2\sigma(I)$. Parameters refined = 164, $[w = 1/[\sigma^2(F_o)^2 + (0.0698P)^2 + 0.1288P]$ where $P = (F_o^2 + 2F_c^2)/3$. $\Delta\rho$ in the final difference map was within +0.161 and −0.148 e Å^{-3}.

The data for **21** were as follows: $C_{21}H_{26}Cl_4N_2O_2$, M_r = 480.26, colorless plates 0.4 × 0.15 × 0.125 mm, a = 11.476(2) Å, b = 10.5414(10) Å, c = 18.713(2) Å, β = 96.79(2), Z = 4, space group = $P2_1/a$, monoclinic, D_x = 1.419 g cm^{-3}, λ(Mo Kα) = 0.7093 Å, μ = 0.55 mm^{-1}, F(000) = 1002, T = 123 K. Merging R is based on intensities 0.013 for 191 replicate reflections. Refinement on F^2; $R[F^2 > 2\sigma(F^2)] = 0.0405$, $R_w(F^2) = 0.1159$, $S = 1.098$. A total of 3644 reflections were measured of which 3453 were independent and used in the refinement. Parameters refined = 274, $[w = 1/[\sigma^2(F_o)^2 + (0.0637P)^2 + 1.1188P]$ where $P = (F_o^2 + 2F_c^3)/3$. $\Delta\rho$ in the final difference map within +0.542 and −0.477 e Å^{-3}.

The data for **26** were as follows: $C_{21}H_{29}Cl_2N_3O_5$, M_r = 474.37, colorless plates 0.55 × 0.2 × 0.1 mm, a = 8.9155(6) Å, b = 11.3197(12) Å, c = 12.9401 (9) Å, α = 111.7500(10), β = 95.2400(10), γ = 101.4500(10), Z = 2, space group = $P1$, triclinic, D_x = 1.348 g cm^{-3}, λ(Mo Kα) = 0.7093 Å, μ = 0.31 mm^{-1}, F(000) = 501, T = 287 K. Merging R is based on intensities 0.009 for 912 replicate reflections. Refinement on F^2; $R[F^2 > 2\sigma(F^2)] = 0.0396$, $R_w(F^2) = 0.1298$, $S = 1.140$. A total of 4332 reflections were measured of which 3420 were independent and used in the refinement. Parameters refined = 280, $[w = 1/[\sigma^2(F_o)^2 + (0.0781P)^2 + 0.2152P]$ where $P = (F_o^2 + 2F_c^2)/3$. $\Delta\rho$ in the final difference map within +0.369 and −0.290 e Å^{-3}. There is a molecule of water present as solvent in this structure.

The data for **31** were as follows: $C_{25}H_{31}ClN_2O_4 \cdot H_2O$, M_r = 474.98, colorless prisms 0.5 × 0.38 × 0.2 mm, a = 9.8680(6) Å, b = 10.7762(7) Å, c = 13.7339(8) Å, α = 66.576(5), β = 68.746(5), γ = 74.194(6), Z = 2, space group = $P1$, triclinic, D_x = 1.277 g cm^{-3}, λ(Mo Kα) = 0.7093, μ = 0.19 mm^{-1}, F(000) = 504, T = 287 K. Merging R is based on intensities 0.005 for 197 replicate reflections. Refinement on F^2; $R[F^2 > 2\sigma(F^2)] = 0.0369$, $R_w(F^2) = 0.1141$ and $S = 1.114$. A total of 4532 reflections were measured, of which 4335 were independent and used in the refinement. Parameters refined = 298 $[w = 1/[\sigma^2(F_o)^2 + (0.0648P)^2 + 0.1921P]$, where $P = (F_o^2 + 2F_c^2)/3$. $\Delta\rho$ in the final difference map within +0.313 and −0.234 e Å^{-3}. There is a molecule of water present as solvent in this structure.

Molecular Modeling of 1, 6, 15, and 20. The GRIDSEARCH and MAXIMIN2 programs in the Tripos Sybyl software (version 6.1a) were run on a Silicon Graphics Indigo 2 workstation using Irix 5.2 as the operating system. Compounds **15** and **20** were examined as the free bases.

B. Bioevaluations. Examination of Compounds for Cytotoxic Activity. Evaluation of compounds against murine P388 D1 cells was undertaken by a reported procedure.[45] The cytotoxicity of various compounds toward murine L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes was performed by a reported method.[46] The examination of different compounds against a variety of human tumor cell lines has been described previously.[47] The compounds are generally screened against 55 cell lines approximately using concentrations of 10^{-8} to 10^{-4} M. Compounds which do not inhibit the growth of the cells by 50% at 10^{-4} M are still employed in the calculation of cytotoxicity. Hence the term meangraph midpoint (MG MID) is used. In the present case, virtually all of the compounds caused 50% inhibition of the growth of cells at 10^{-4} M or lower concentrations with the exception of **38** whereby the growth of 51 of the 55 cell lines was not inhibited by 50% at 10^{-4} M. The IC_{50} figures in Table 2 were taken directly from the MG MID values.

Evaluation of 1, 9, 15, and 23 for Mutagenic Properties. A literature method was followed.[13] Data from less than five colonies were not counted. Each experiment was repeated three times, and two plates were used for each determination and each concentration. Data were generated for a reference compound methyl methanesulfonate (MMS). Using different concentrations of compounds, the percentage of survivors (S) of *S. cerevisiae* was determined as well as the number of histidine$^+$ revertants × 10^4, referred to as the deletion (DEL) assays. The intrachromosomal recombination (ICR) figures obtained were the number of adenine$^+$ revertants × 10^5 found. The concentrations in μg/mL for MMS (S, DEL, ICR figures are in parentheses) were as follows: 0 (100 ± 8; 2.19 ± 0.4; 0.42 ± 0.2), 10 (100 ± 5; 5.73 ± 0.5; 1.55 ± 0.4), 50 (93 ± 6; 25.0 ± 0.3; 12.2 ± 0.7), and 250 (33 ± 3; 187.7 ± 1.5; 69.4 ± 0.9).

Examination of Compounds for Antimicrobial Activity. The microorganisms used were *E. coli*,[48] *S. typhimurium* ATCC 19585, *S. aureus*,[49] *C. albicans* B311, ATCC 32354, and *A. fumigatus* W73355.[50] Literature methods were employed for the screening of the compounds against *E. coli* and *S. typhimurium*,[51] *S aureus*,[52] *C. albicans*,[53] and *A. fumigatus*.[50]

Evaluation of 15, 18, 27, and 40 for Anticonvulsant and Neurotoxic Properties. Mice were administered doses of 30, 100, and 300 mg/kg intraperitoneally and examined at the end of 0.5 and 4 h. In the MES screen, the lowest doses (mg/kg) affording protection in half or more of the animals and the times of observation were as follows: **15**, 30, 0.5 h; 300, 4 h; **18**, 100, 0.5, and 4 h; and **27**, 100, 0.5, and 4 h. For the

scPTZ test, the protection afforded was as follows: **40**, 300, 0.5 h. Continuous seizure activity (CSA) was noted in the scPTZ screen as follows: **15**, 100 and 300, 0.5 h; **18**, 300, 0.5 h; and **27**, 300, 0.5 h. Neurotoxicity (NT) was noted in the following cases: **15**, 300, 0.5 h; **18**, 300, 0.5 and 4 h; **27**, 300, 0.5 h; and **40**, 300, 4 h. One of two mice receiving 300 mg/kg of **18** was dead at the end of 4 h, and after 0.5 h, mice receiving 300 mg/kg of **27** were unable to grasp the rotorod. Quantitation of **15** in mice using the MES and NT screens revealed the following data (ED_{50} or TD_{50} in mg/kg, 95% confidence intervals, slope and SE, time of determination in hours). MES: 55.4, 40.8−72.2, 6.01, 2.08, 1. NT: 235, 190−281, 8.54, 2.51, 0.5. The comparable figures for phenytoin were as follows. MES: 6.32, 5.44−7.23, 11.2, 3.52, 1. NT: 41.2, 36.9−46.1, 14.4, 4.82, 0.5. A dose of 380 mg/kg of **15** in the scPTZ screen afforded no protection, and CSA followed by death was observed.

Rats were dosed orally with 30 mg/kg of **15**, **18**, and **27** and observed at the end of 0.25, 0.5, 1, 2, and 4 h in the MES and NT screens. No activity or toxicity was noted except in the case of **27** when one of four rats was protected in the MES screen after 2 h.

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Supporting Information Available: Atomic anisotropic displacement parameters, hydrogen positional and isotropic displacement parameters, atomic positional and equivalent isotropic displacement parameters, and bond distances and angles for **13**, **21**, **26**, and **31** (21 pages). Ordering information is given on any current masthead page.

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