4-(β-Arylvinyl)-3-(β-arylvinylketo)-1-ethyl-4-piperidinols and Related Compounds: A Novel Class of Cytotoxic and Anticancer Agents

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The syntheses of a series of 1-aryl-5-diethylamino-1-penten-3-one hydrochlorides **1** and 1-aryl-3-diethylamino-1-propanone hydrochlorides **4** were accomplished. Attempts to prepare the corresponding bis(5-aryl-3-oxo-4-pentenyl)ethylamine hydrochlorides **2** and bis(3-aryl-3-oxopropyl)ethylamine hydrochlorides **5** led to the formation of a series of 4-(β-arylvinyl)-3-(β-arylvinylketo)-1-ethyl-4-piperidinol hydrochlorides **9** and 4-aryl-3-arylketo-1-ethyl-4-piperidinol hydrochlorides **11**, most of which were converted subsequently into the corresponding quaternary ammonium salts **10** and **12**, respectively. The structures of these compounds were determined by ^{1}H NMR spectroscopy and confirmed by X-ray crystallography of representative molecules. Most compounds displayed significant cytotoxicity toward murine P388 and L1210 cells as well as human tumors. In general, Mannich bases containing olefinic bonds were more cytotoxic than the analogues without this functional group, while the piperidines **9** and **11** were more potent than the acyclic analogues **1** and **4**, respectively. Correlations were noted between various physicochemical constants in the aryl rings and cytotoxicity. Compound **9d** displayed promising in vivo activity against colon cancers. This study has revealed that the piperidines **9** and **11** constitute new classses of cytotoxic agents.

Introduction

A number of Mannich bases derived from arylidene alkyl ketones and related compounds possess significant cytotoxic and anticancer properties.[1] These compounds were developed as thiol alkylators since α,β-unsaturated ketones have a markedly greater affinity for thiols over amino and hydroxy nucleophiles.[2,3] Hence interactions with nucleic acids may be avoided, and the disadvantages of certain alkylating agents such as mutagenicity[4] and carcinogenicity[5] may be absent. Support for the contention that these compounds have a different mode of action than the widely used alkylating agent melphalan was provided by noting that they displayed similar cytotoxicity toward melphalan-resistant and melphalan-sensitive neoplastic cells, i.e., the melphalan-resistant cell lines were free from cross-resistance to these Mannich bases.[6] In addition, several series of Mannich bases have been prepared recently which were designed using the concept of sequential cytoxicity.[7,8] This theory may be defined as the successive chemical attack by two or more cytotoxic agents whereby greater toxicity to malignant rather than normal cells will be displayed. The reasons for proposing this theory, as well as the relevant literature, have been presented at length.[7]

The original objectives of the present study included the synthesis of series **1** (Scheme 1) for cytotoxic evaluation. In addition, to evaluate the theory of sequential cytotoxicity further, the bis Mannich bases **2** were considered for bioevaluation. Initial thiol attack could occur at one of the olefinic double bonds to be followed by a second thiol interaction which could be more damaging to neoplastic cells than normal tissues. This assumption depends on nonequivalent charges at the olefinic bonds in order to avoid synchronous attack at both olefinic carbon atoms. The pK_a values of the acyclic Mannich base **7** and related analogues were correlated with the magnitude of the Hammett σ values in the aryl ring.[9] Hence basicity and electron densities of the β-arylvinyl group are interrelated. When the nitrogen atoms are ionized, the electron densities at the olefinic bonds will be reduced compared to the corresponding free bases, and thus nucleophilic attack by thiols will be enhanced. Hence the related quaternary ammonium compounds **3** were predicted to be more cytotoxic than **2** since the Mannich bases will exist as a mixture of the free bases and protonated hydrochloride salts. The use of a null hypothesis[10] suggested the preparation of **4**−**6** which lacked olefinic double bonds. These compounds would be predicted to be less cytotoxic than the analogues **1**−**3**; should any bioactivity be displayed by these molecules, then one may conclude that the structural features in **1**−**3** other than the olefinic double bonds contributed to bioactivity. The aryl substitution pattern in series **1**−**6**, which has been employed in a Topliss analysis,[11] was chosen so that atoms and groups with divergent electronic and hydrophobic properties were used. In fact, the chloro, methyl, and methoxy substituents are found in three different quadrants of a two-dimensional Craig plot.[12]

Three related analogues were also considered for cytotoxic evaluation. First, **7** (as the hydrobromide salt)

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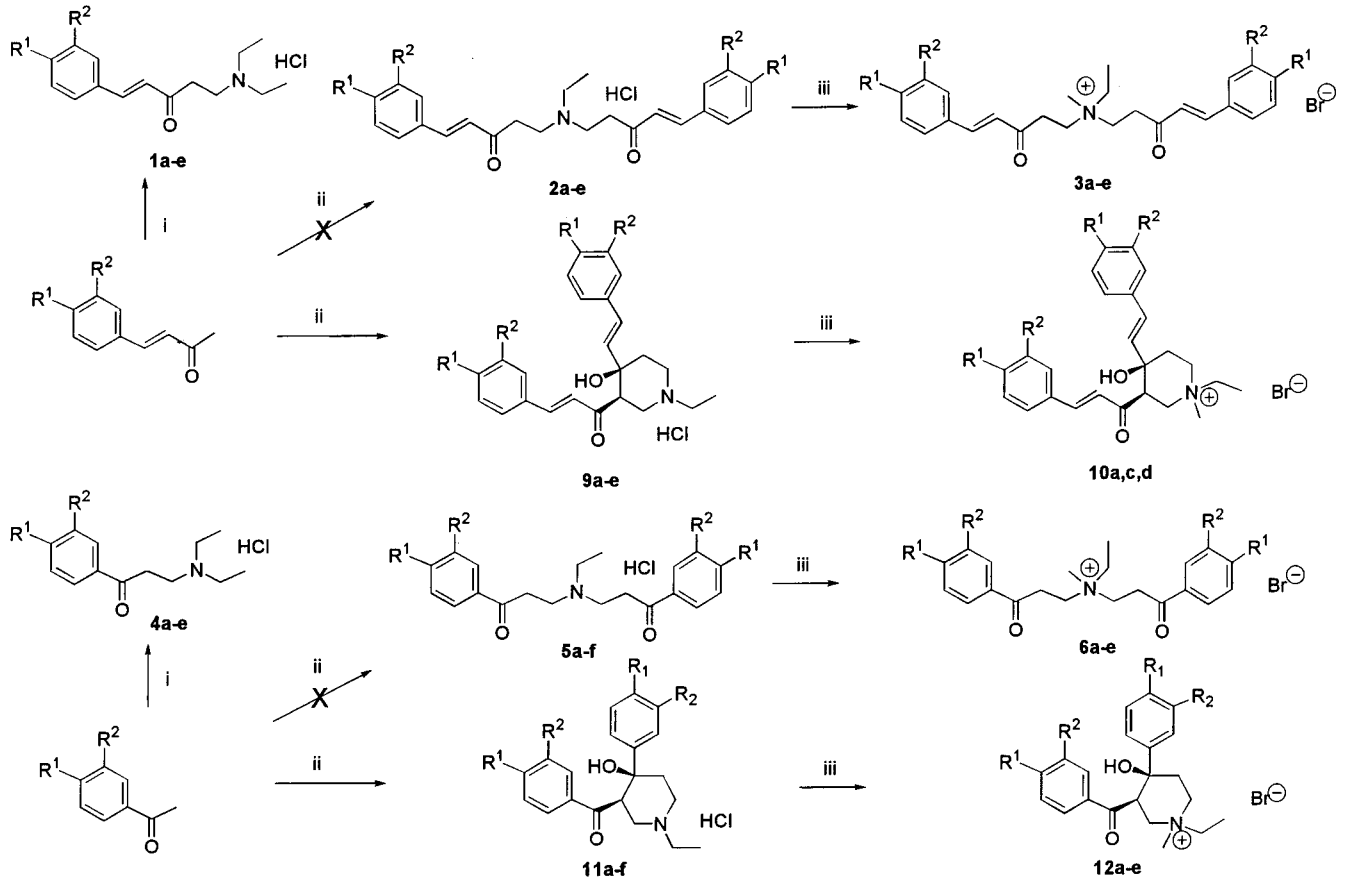
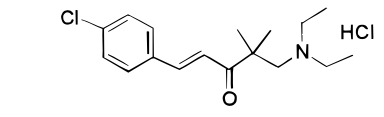
Scheme 1[a]

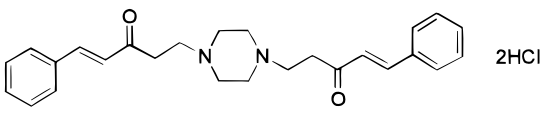


[a] Reagents: (i) HCHO, $HN(C_2H_5)_2HCl$; (ii) HCHO, $H_2NC_2H_5HCl$; (iii) CH_3Br. The letters **a**−**f** reflect the aryl substitution pattern: **a**, $R^1 = R^2 = H$; **b**, $R^1 = Cl$, $R^2 = H$; **c**, $R^1 = R^2 = Cl$; **d**, $R^1 = CH_3$, $R^2 = H$; **e**, $R^1 = OCH_3$, $R^2 = H$; **f**, $R^1 = OH$, $R^2 = H$.

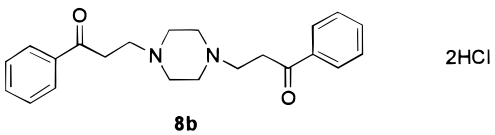
has 1.3 times the activity of 5-fluorouracil against the human WiDr colon cancer in vitro,[9] and a comparison of its cytotoxicity with **1b** using the screens employed in the present study was of interest. Second, **8a**,**b** were suggested in order to determine the effect of pK_a on cytotoxicity. Thus the pK_a values of the nitrogen atoms of piperazine are 5.33 and 9.73, while the figure for triethylamine is 10.75.[13] Hence under biological conditions, **1a** should present a higher percentage of ionized species than **8a** and thus display greater toxicity. A comparison of the screening data of **8a** with **8b** would indicate the importance of olefinic bonds in this group of molecules.



7



8a



8b

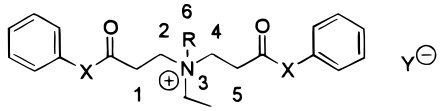


Figure 1. Numbering used in atoms **1a**, **2a**, **3a**, **4a**, **5a**, and **6a** for which electrostatic charges were computed.

In summary therefore the aim of the present investigation was the preparation of the acyclic molecules **1**−**8** as candidate cytotoxic and anticancer agents. The results generated would enable evaluation of the predictions of relative bioactivity to be made and in particular to examine the theory of sequential cytotoxicity.

Results

To give theoretical support to these proposals, the electrostatic charges on several of the atoms of the unsubstituted compounds in series **1**−**6** were computed. The atoms chosen and the results generated are indicated in Figure 1 and Table 1, respectively.

Compounds **1a**−**e**, **7**, and **8a** were prepared from the appropriate β-arylvinyl alkyl ketone, formaldehyde, and secondary amine hydrochloride. The Mannich bases **4a**−**e** and **8b** were prepared in a similar fashion from the appropriate aryl methyl ketone. Attempts to prepare the quaternary ammonium salts from the tertiary amines **1** and **4** led to the isolation of impure products only; these synthetic difficulties have also been noted by other laboratories.[14] However reaction of ethylamine hydrochloride with excess of either β-arylvinyl methyl

Table 1. Electrostatic Charges on Certain Atoms of **1a**, **2a**, **3a**, **4a**, **5a**, and **6a**

	electrostatic charges of atoms[a]					
compd	C1	C2	N3	C4	C5	R6[b]
1a	−0.296	0.017	−0.191			0.332
2a	−0.252	−0.151	−0.074	−0.173	−0.209	0.368
3a	−0.280	−0.110	0.364	−0.184	−0.202	0.108
4a	−0.112	−0.143	−0.112			0.368
5a	−0.136	−0.040	−0.163	−0.087	−0.107	0.382
6a	0.032	−0.192	0.269	−0.202	0.080	0.119

[a] The designation of atoms is given in Figure 1. [b] R = H (**1a**, **2a**, **4a**, **5a**) or CH_3 (**3a**, **6a**).

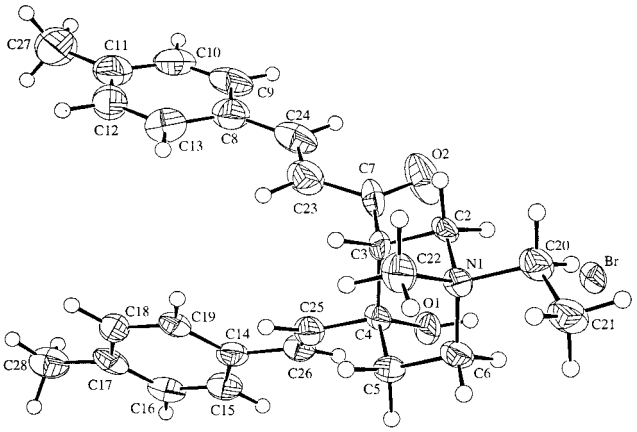
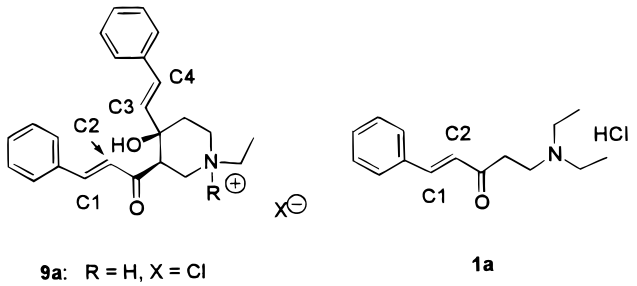


Figure 2. ORTEP diagram of **10d**.



9a: R = H, X = Cl

10a: R = CH_3, X = Br

14: R = X = ----

1a

Figure 3. Designations of atoms in **1a**, **9a**, **10a**, and **14** for which electrostatic charges were computed.

ketones or aryl methyl ketones and formaldehyde led to **9** and **11**, respectively. The significant in vitro and in vivo activity of **9d** vide infra suggested the preparation of the analogues **13a**,**b** which were synthesized by the same route. Reaction of the free bases of **9a**,**c**,**d** with methyl bromide gave rise to **10a**,**c**,**d**, respectively while the quaternary ammonium salts **12** were prepared from the free bases of **11**. The structures of the compounds were confirmed by ^{1}H NMR spectroscopy and elemental analyses. X-ray crystallography was undertaken for **8b**, **10d**, **12d** (as the iodide salt), and **13a**,**b**. The ORTEP diagram of a representative compound, namely, **10d**, is displayed in Figure 2. The electrostatic charges on the olefinic carbon atoms in **9a**, **10a**, the free base of **9a** referred to hereafter as **14**, and **1a** as indicated in Figure 3 were calculated, and the results are given in Table 2.

The cytotoxic properties of the compounds in series **1**, **4**, and **7**−**13** as well as the established anticancer drug melphalan are given in Table 3. The promising in vitro activity of **9d** and **10a** suggested their examination against several human tumor xerografts, and these data are presented in Table 5.

Table 2. Electrostatic Charges on Some Carbon Atoms and Cytotoxicity of **1a**, **9a**, **10a**, and **14**[a]

					IC_{50} (μM)		
compd	C1	C2	C3	C4	P388 screen	L1210 screen	human tumor screen
1a	0.025	−0.576			1.7	25.7	9.8
9a	0.057	−0.552	−0.403	−0.194	1.4	5.3	2.8
10a	−0.001	−0.505	−0.370	−0.253	2.3	NA[b]	8.3
14	−0.055	−0.489	−0.354	−0.273			

[a] The designation of atoms is given in Figure 3. [b] Result not available.

Table 3. Cytotoxicity Data of Various Mannich Bases and Related Compounds

	IC_{50} (μM)		
compd[a]	P388 cells	L1210 cells	human tumors
1a	1.7	25.69 ± 11.09	9.77
1b	0.3	41.03 ± 2.32	
1c	0.39	61.18 ± 20.49	3.80
1d	1.1	52.87 ± 26.61	
1e	0.67	44.32 ± 28.21	
4a	2.6	20.31 ± 3.10	25.12
4b	3.2	36.93 ± 5.79	
4c	0.31	36.38 ± 3.86	32.36
4d	2.2	21.54 ± 2.66	26.30
4e	1.6	43.79 ± 27.96	
7	2.2	6.39 ± 1.76	
8a	0.62	5.78 ± 1.94	12.02
8b	2.84	9.71 ± 2.22	
9a	1.4	5.30 ± 2.44	2.75
9b	1.1	6.96 ± 0.26	25.72
9c	0.4		0.38
9d	2.1	6.08 ± 2.47	5.01
9e	5.0	25.55 ± 14.85	8.31
10a	2.27		8.31
10c	0.64	21.44 ± 1.55	2.19
10d	0.62	13.79 ± 6.89	
11a	1.07	20.07 ± 8.91	30.19
11b	5.14	15.67 ± 8.56	83.18
11c	3.11		16.59
11d	8.6	38.24 ± 12.91	25.12
11e	4.17	13.5 ± 5.19	19.49
11f	1.4	9.75 ± 1.14	20.89
12a	1.3		12.88
12b	1.5		12.88
12c	2.3	33.57 ± 6.17	9.54
12d	2.1		50.11
12e	0.98		10.23
13a	1.89	12.6 ± 10.4	2.46
13b	2.59	20.9 ± 0.3	
melphalan	0.22	2.13 ± 0.02	26.30

[a] Hydrobromide salts obtained from the free bases of **4a** and **11a**−**c**,**e** were used in the human tumor screen. The hydrobromide salt from the free base of **11d** was employed in all screens.

Discussion

The data provided in Table 1 supported the rationale for the synthesis and bioevaluation of the compounds in series **1**−**6**. First, variation in the charges on carbon atoms 1 and 5 in each of the bis compounds **2a** and **3a** meant that in each molecule, a different electronic effect will be exerted on the adjacent unsaturated keto group. Thus initial nucleophilic attack by a cellular constituent at one carbon atom would be followed by a subsequent thiol alkylation as the theory of sequential cytotoxicity requires. Second, in addition to thiol alkylation at the olefinic bonds, Mannich bases can react by amino group

Table 4. Correlations between the σ, π, and MR Constants in the P388, L1210, and Human Tumor Screens

screen	series	aryl substituent constant	plot[a]	*p* value[b]	correlation[c]
P388	**1**	MR	lin, log	<0.05, <0.1	+
	4	MR	lin, log	<0.1, <0.05	+
	9	σ	lin, log	<0.1, <0.005	+
	9	π	lin, log	<0.1, <0.05	+
L1210	**1**	π	lin, log	<0.1, <0.1	−
	1	MR	lin, log	<0.025, <0.025	−
	4	MR	log	<0.1	−
human tumors	**4**	σ	log	<0.1	−
	4	π	lin, log	<0.1, <0.1	−
	4	MR	lin, log	<0.1, <0.1	−
	9	π	log	<0.1	+

[a] Both linear (lin) and semilogarithmic (log) plots were made. [b] When two values are quoted, they refer to correlations obtained from the linear and logarithmic plots, respectively. [c] Positive (+) correlations indicate that cytotoxicity rose as the σ, π, and MR values are increased, while negative (−) correlations reveal that increased bioactivity occurred with diminishing σ, π, and MR figures.

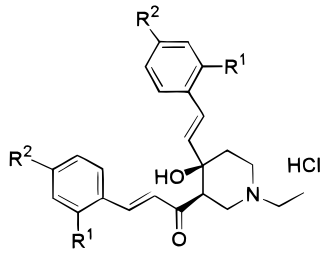
Table 5. Effect of **9d** and **10a** on Various Human Tumor Xenografts Passaged in Athymic Mice

compd	tumor	classification	% T/C[a] (dose in mg/kg)	% ILS[b] (dose in mg/kg)
9d	COLO 205	colon	57 (200)	28 (200)
	SW-620	colon	32 (80)	9 (80)
	NCI-H522	non-small-cell lung	23 (80)	−2 (80)
	LOX IMVI	melanoma	35 (80)	12 (80)
10a	COLO 205	colon	20 (16.8)	14 (16.8)
	KM12	colon	45 (16.8)	5 (25)
	CAKI-1	renal	43 (16.8)	6 (16.8)

[a] % T/C indicates the optimal value of the percentage reduction of the median treated tumor weight compared to the median control tumor weight. [b] % ILS refers to the percentage increase in the median time in days for the treated tumor to reach a certain size compared to controls.

replacement by thiols at the 2 (and 4) atom(s). This reaction may be an elimination−addition process or by nucleophilic attack.[15] The rate-determining step in an elimination reaction is the loss of the proton adjacent to the carbonyl group.[16] Hence, if the elimination−addition mechanism operates at the cellular level, compounds **1**−**3** should be more active than the analogues **4**−**6** since the negative charges on carbon atom 1 (and 5) are greater in **1a**, **2a**, and **3a**, and hence the proton is more acidic than in the analogues **4a**, **5a**, and **6a**. Hence in comparing the olefinic versus the non-olefinic analogues, the potency orders would be **1a** > **4a**, **2a** > **5a**, and **3a** > **6a**.

However while the acyclic mono Mannich bases **1** and **4** were prepared, only the 1,3,4-trisubstituted piperidines **9**−**12** were isolated in the attempts to synthesize **2**, **3**, **5**, and **6**. The structures of **9**−**12** were proved by ^{1}H NMR spectroscopy and confirmed by X-ray crystallography of four representative compounds, namely, **10d** (Figure 2), **12d**, and **13a**,**b**. The data from these two physicochemical determinations revealed the trans arrangement of the bulky groups at positions 3 and 4 and olefinic double bonds displaying the *E* configuration. In addition, the X-ray crystallographic data showed that the piperidines adopted the chair conformations and the larger groups at postions 3 and 4 were in the equatorial



13a: $R^1 = CH_3$; $R^2 = H$
13b: $R^1 = R^2 = CH_3$

conformations. The piperazine analogues **8a**,**b** did not undergo intramolecular cyclizations, the X-ray crystallography of **8b** confirming this conclusion. In these cases, a cyclization process would require the formation of a nine-membered ring system. Finally, a survey of previous investigations revealed reports of this type of intramolecular cyclization[17−21] which probably resulted from an acid-catalyzed aldol reaction of the bis Mannich bases **2** or **5**. In this process, the enol group of one carbonyl function would attack the carbon atom of the second (protonated) carbonyl group.

Predictions were made regarding the way in which compounds containing olefinic groups could interact with cellular thiols. Electrostatic charges of the olefinic groups in **1a**, **9a**, **10a**, and **14** (the free base of **9a**) as indicated in Figure 3 were made. The data in Table 2 revealed that thiol alkylation would be predicted to occur at carbon atoms C1 and C4 since they are more electropositive than the adjacent atoms C2 and C3, respectively. In the case of **9a**, **10a**, and **14**, the C1 atoms are more electropositive than the C4 atoms. Thus the data suggest that initial thiol alkylation will occur at the C1 carbon atom and a subsequent thiolation will take place at the C4 center thus exemplifying the sequential cytotoxicity concept. Based on their electrostatic charges, the rates of thiol attack at the C1 and C4 positions would be predicted to be **9a** > **10a** > **14**. The electrostatic charges for **1a** revealed that nucleophilic attack will occur at the C1 position. If the initial rate of thiol attack is the limiting factor in bioactivity, then the order of cytotoxicity would be predicted to be **9a** > **1a** > **10a**.

Figure 4 indicates three structural features of the compounds in series **9** which could contribute to cytotoxicity. These piperidines are Mannich bases containing many of the structural features found in series **1** as well as possessing an isolated β-arylvinyl group. In addition, loss of the allylic hydroxy group could give rise to a reactive carbonium ion stabilized by the presence of the adjacent β-arylvinyl group. These features should permit interaction with cellular thiols to occur which, if a major contributor to bioactivity, permits the following predictions pertaining to structure and cytotoxicity to be made, namely, **1** > **4**, **9** > **11**, and **10a**,**c**,**d**, > **12a**,**c**,**d**. If quaternization increased chemical reactivity of the olefinic centers, then **10a**,**c**,**d** > **9a**,**c**,**d**. Furthermore, addition of thiols to the β-arylvinyl double bond has been shown to occur at a far greater rate with Mannich bases of conjugated β-arylvinyl ketones than the corresponding α,β-unsaturated ketones.[22] Hence thiol alkylation should occur at carbon atom C1 much

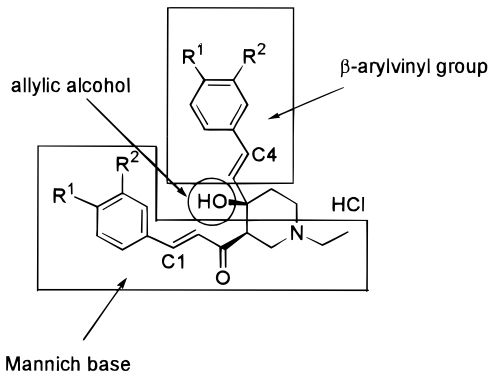


Figure 4. Structural features of series **9** which may confer antineoplastic activity.

more rapidly than at C4 (Figure 4), and the potential of sequential cytotoxicity exists.

To examine the viability of the predictions and hypotheses discussed earlier, three cytotoxicity assays were chosen for the following reasons. First, use of murine P388 and L1210 leukemic cells was employed since these tumors have been claimed to be good predictors of clinically useful anticancer drugs.[23] Second, the human tumor assay employed approximately 55 tumor cell lines from different neoplastic diseases: principally leukemia, melanoma, non-small-cell lung, colon, central nervous system, ovarian, renal, prostate, and breast cancers.[24] If a 50% decrease in the growth of cells was not achieved at the highest concentration, i.e., 10^{-4} M, this figure of 10^{-4} M was still included in the calculation of the average IC_{50} values. Hence the figures are mean graph midpoint values rather than true mean figures. Compounds which have a higher toxicity to one or more of these neoplastic diseases may display a greater activity toward these cancers than normal tissues.

An overview of the biodata generated will be made, followed by an evaluation of the predictions made earlier. In the case of the P388 screen, all of the compounds had IC_{50} figures of less than 10 μM, and for 27% of the compounds, these values were less than 1 μM. Four compounds, namely, **1b**,**c**, **4c**, and **9c**, gave IC_{50} values less than twice that of melphalan, and the significant antileukemic activity of the compounds in series **1** is noteworthy. The activity in the L1210 test ranged from 5.3 (**9a**) to 61.2 (**1c**) μM. In all cases the compounds were less active toward the more rapidly growing L1210 cells than the slower growing P388 leukemia cell line. In contrast to the results from P388 cells, greater cytotoxicity was found with the piperidines **9** and **11** than the analogous Mannich bases **1** and **4**.

Most of the compounds prepared in this study were assessed against a panel of human tumor cell lines. A noteworthy feature was the fact that approximately 80% of the compounds evaluated were more potent than melphalan, and in particular **9c** possessed 69 times the potency of this widely used drug. Selective toxicity toward leukemia was observed for four of the seven quaternary ammonium salts examined in this screen, namely, **10a** and **12a**,**b**,**e**; this property was also noted with **1a** and **9e**. In addition, **9d** had preferential cytotoxicity toward human colon cells.

In view of the in vivo activity of **9d** toward colon tumors vide infra, the impressive cytotoxicity of this piperidinol in the colon subpanel of six human tumor cell lines will be discussed. In addition, comparisons will be made between **9d** and 5-fluorouracil (5-FU) since the latter compound is an established drug used clinically against cancer of the colon. The six tumors (IC_{50} figures in μM of **9d** and 5-FU in parentheses) were COLO 205 (1.7, 7.4), HCC-2998 (2.5, 0.9), HCT-15 (3.7, 12.0), HT29 (1.9, 9.8), KM12 (1.8, 12.0), and SW-620 (2.0, 25.7) indicating that, with the exception of the HCC-2998 neoplasm, **9d** was more cytotoxic than 5-FU to these tumors. In fact, the average IC_{50} figures for **9d** and 5-FU were 2.3 and 11.3 μM, respectively, revealing a 5-fold greater potency for **9d**. Since the average IC_{50} figures for all cell lines for **9d** and 5-FU were 5.0 and 24.6 μM, respectively, an approximately 2.2-fold selectivity for both **9d** and 5-FU for colon cancers was demonstrated.

A comparison of the murine cytotoxicity data for **1b** and **7** was ambiguous pertaining to whether the replacement of the 4-methylene protons of **1b** by two methyl groups, as in **7**, increased cytotoxicity or not. The acyclic Mannich base **8a** may be considered to be a bis analogue of **1a**. Hence if the theory of sequential cytotoxicity is valid, **8a** should possess more than twice the activity of **1a**. This speculation is valid when the IC_{50} figures for the P388 and L1210 screens are considered but not for the human tumor test. As predicted, **8b** lacking olefinic bonds was less cytotoxic than **8a**.

The hypotheses outlined previously predicted that the relative cytotoxicities of different groups of compounds would be as follows: **1** > **4**, **9** > **11**, **10a**,**c**,**d** > **9a**,**c**,**d**, and **10a**,**c**,**d** > **12a**,**c**,**d.** To examine the viability of these postulates, comparisons were made between the cytotoxicities of pairs of compounds having the same aryl substituents. For example, in contrasting the compounds in series **1** and **4**, **1a** was compared with **4a**, **1b** with **4b**, and so forth. Comparisons were made if data were available for at least three pairs of compounds. A correlation was recorded if more than half of the comparisons supported the theory. In the case of the P388 screen, **1** > **4**, **9** > **11**, **10** < **9**, and **10** > **12** indicating that three of the four predictions made were established. The results in the L1210 test showed that **1** < **4** and **9** > **11** revealing that only the latter relationship fulfilled the predictions made. The compounds in series **9** were more cytotoxic than the analogues in series **11** as predicted. In the remaining cases there were insufficient data to make comparisons. Thus in general the results support both the sequential cytotoxicity concept and the importance of the presence of olefinic bonds in these molecules. Hence design of future analogues should incorporate unsaturated centers into their structures permitting alkylation of cellular nuclephiles to occur.

To seek correlations between the cytotoxicity data and the electronic, hydrophobic, and steric properties of the aryl substituents, linear and semilogarithmic plots between the IC_{50} values and the Hammett σ, Hansch π, and molar refractivity (MR) constants in each of the series **1**, **4**, and **9**−**12** were made, providing that screening results were available for at least three members of a particular series. The test for zero correlation[25] was applied at the 95% and 90% significance levels. In cases where good correlations were

noted, the data was further evaluated revealing p values of less than 0.05. The significant relationships which were obtained are summarized in Table 4.

The data in Table 4 revealed that 11 correlations between cytotoxicity and the σ, π, and MR constants were noted in both series of acyclic Mannich bases **1** and **4** as well as the piperidines of series **9**. No correlations were discerned in the other three series of compounds (**10**−**12**). The relationships between cytotoxicity and the MR, π, and σ values of the aryl substituents were noted in five, four, and two cases, respectively. Thus, where correlations were detected, differences in the sizes and hydrophobic properties of the aryl groups influenced activity more than their chemical reactivity.

For the purpose of subsequent drug design, development of each of the series **1**, **4**, and **9** will use these correlations. However the test system being utilized needs to be considered since, as Table 4 indicates, positive correlations were noted with the P388 screen, negative relationships were found in the L1210 test, and both positive and negative correlations were obtained using the human tumor assay. For example, for future expansion of series **4**, an increase in the size of the aryl substituent would be predicted to increase cytotoxicity in the P388 screen. On the other hand, a reduction in the MR value of the aryl group is expected to increase activity in the L1210 and human tumor assays. Similarly for series **1**, while increases in the size of the aryl substituents would be expected to increase activity in the P388 screen, compounds containing aryl substituents with smaller MR values would be predicted to display increased cytotoxicity in the L1210 test.

As indicated previously, several compounds such as **9d** and **10a** displayed selective toxicity in the human tumor screen toward colon and leukemia cells, respectively. To evaluate whether these promising results could be translated into in vivo activity, both compounds were examined in the murine P388 screen and against certain human tumors in athymic mice. Evaluation in the P388 screen revealed that **9d** was inactive and **10a** displayed marginal potency, whereby an increase in the life span of the mice by 20% was noted. The activity of these two compounds toward several xenografts is summarized in Table 5. Reductions in the sizes of the tumors were observed with both compounds, and the potency of **9d** against the COLO 205 tumor is of particular interest. A very recent mode of action study with this lead molecule revealed that **9d** caused apoptosis in human Jurkat leukemia cells.[26]

The promising in vitro and in vivo activity of **9d** suggested that analogues containing one or two aromatic methyl groups may also display selective toxicity to malignant cells. The data in Table 3 revealed that **13a**,**b** had comparable cytotoxicity to **9d**, although neither **13a**,**b** displayed selective toxicity for colon cancers (or any other neoplastic disease) in the human tumor screen.

Conclusions

This study outlined the preparation of various acyclic Mannich bases and the formation of some 1,3,4-trisubstituted piperidines and related quaternary ammonium salts. In general, compounds containing olefinic bonds had greater cytotoxicity than analogues bereft of this functional group; however, these latter compounds displayed cytotoxicity, and therefore structural features other than the presence of chemically reactive double bonds contributed to bioactivity. A number of prototypic molecules emerged from this study, and in addition, the promising in vivo activity of **9d** toward colon cancers was noteworthy. Thus development of this series of compounds is warranted with a view to discovering novel clinically useful antineoplastic drugs.

Experimental Section

A. Chemistry. Melting points are uncorrected. Compounds **1a**,**d**,**e**, **4a**−**e**, and **11a** have been reported previously and, in general, had melting points similar to those recorded in the literature.[27−33] Elemental analyses (C,H,N) were undertaken on **1a**−**e**, **4a**−**e**, **7**, **8a**,**b**, **9a**−**e**, **10a**,**c**,**d**, **11a**−**c**,**e**,**f**, **12a**−**e**, and **13a**,**b**, as well as the hydrobromide salts of the free bases obtained from **4a** and **11a**−**e**, by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan, and were within 0.4% of the calculated values. ^{1}H NMR spectra were determined using a Bruker AM 500 FT NMR machine (500 MHz), while a Varian T-60 (60 MHz) instrument was used to confirm the structures of intermediate α,β-unsaturated ketones. A Nonius CAD-4 diffractometer was used for the collection of X-ray crystallographic data. TLC was undertaken using silica gel plastic-backed sheets. All compounds were homogeneous using solvent systems of hexane−methanol (7:3) for the intermediate α,β-unsaturated ketones, chloroform−methanol (7:3) for the Mannich bases, and chloroform−methanol−ammonium hydroxide (7:3:0.08) for the quaternary ammonium salts. Compounds **8b**, **11f**, and **12c**,**d** were obtained as the hemihydrates and **12a** as the monohydrate. The percentage yields of the Mannich bases were calculated on the basis of the lowest molar fraction of the reactants used. For example, in the case of **4a** the molar ratios of diethylamine hydrochloride, acetophenone, and paraformaldehyde were 0.01, 0.04, and 0.03, respectively, and the 55% yield recorded was based on the premise that a maximum yield would be 0.01 mol of pure product.

Synthesis of Intermediate α,β-Unsaturated Ketones Required in the Preparation of 1, 7, 8a, 9, 10, and 13. 4-Phenyl-3-buten-2-one was obtained from the Aldrich Chemical Co. The remaining arylvinyl ketones were prepared by a literature method[14] and purified by recrystallization or distillation. The products had melting points or boiling points consistent with literature values. The structures were confirmed by ^{1}H NMR spectroscopy (60 MHz, $CDCl_3$), and the spectrum of a representative compound, 4-(4-methoxyphenyl)-3-buten-2-one, was as follows. δ: 2.30 (s, 3H, $COCH_3$), 3.80 (s, 3H, OCH_3), 6.40−6.60 (d, 1H, CH=C*H*CO, J = 18 Hz), 6.70−7.50 (m, 5H, aryl H, C*H*=CHCO).

Synthesis of Series 1 and 4 and Compound 7. A mixture of the appropriate 4-aryl-3-buten-2-one, 1-aryl-1-ethanone, or 1-aryl-4-methyl-1-penten-3-one, paraformaldehyde, diethylamine hydrochloride, trifluoroacetic acid (0.04 mL; **1a**−**e**, **7**), or hydrochloric acid (37% w/v, 0.04 mL; **4a**−**e**), and acetonitrile (100 mL; **1a**−**e**, **4a**,**c**, **7**) or 2-propanol (100 mL; **4b**,**d**,**e**) was heated under reflux for different periods of time. After removal of the solvent in vacuo, the resultant oil was triturated with anhydrous ether and subsequently with acetone. The solid obtained was washed with ether and recrystallized from ether−methanol **(1c**−**e**, **4a**,**d**, **7**), acetone **(1a**, **4b**), acetonitrile (**4c**), ethanol−acetone (**4e**), or methanol **(1b**). A constant quantity of diethylamine hydrochloride was used throughout, namely, 0.01 mol. The molar ratios of ketone and paraformaldehyde, times of heating under reflux (h), yields (%), and melting points (°C) were as follows. **1a**: 0.03:0.03, 36, 61, 130−132. **1b**: 0.03:0.03, 24, 68, 150−152. **1c**: 0.03:0.03, 24, 54, 158−160. **1d**: 0.03:0.03, 48, 48, 156−158. **1e**: 0.03:0.03, 48, 89, 148−150. **4a**: 0.04:0.03, 30, 55, 109−111. **4b:** 0.025:0.03, 17, 61, 138−140. **4c**: 0.03:0.025, 24, 67, 131−

133. **4d**: 0.04:0.03, 42, 53, 118−120. **4e**: 0.04:0.03, 42, 63, 119−121. **7**: 0.05:0.05, 48, 61, 160−162. The ^{1}H NMR (500 MHz) spectra of representative compounds were as follows. **1e**: δ($CDCl_3$) 7.64 (1H, d, C*H*=CHCO, J = 16.2 Hz), 7.51 (2H, d, 2,6 aryl H, J = 8.8 Hz), 6.90 (2H, d, 3,5 aryl H, J = 8.8 Hz), 6.62 (1H, d, CH=C*H*CO, J = 16.1 Hz), 3.83 (3H, s, OCH_3), 3.43 (2H, def t, $COCH_2$), 3.35 [2H, def t, $CH_2N(C_2H_5)_2$], 3.10 [def q, 4H, $CH_2N(CH_2CH_3)_2$, J = 7.1 Hz], 1.39 [6H, t, $N(CH_2CH_3)_2$, J = 7.4 Hz]. **4e**: δ($CDCl_3$) 7.98 (2H, d, 2,6 aryl H, J = 8.9 Hz), 6.92 (2H, d, 3,5 aryl H, J = 8.9 Hz), 3.86 (3H, s, OCH_3), 3.70 (2H, t, $COCH_2$, J = 7.3 Hz), 3.42 [2H, t, $CH_2N(C_2H_5)_2$, J = 7.3 Hz], 3.12 [4H, def q, $N(CH_2,CH_3)_2$, J = 7.1], 1.40 (6H, t, 2 CH_3, J = 7.3 Hz).

Utilization of a literature procedure for preparing a series of 3-dimethylamino-1-aryl-1-propanone hydrobromides[3] led to the synthesis of the hydrobromide salt of the free base of **4a** in 15% yield. It was recrystallized from acetone−methanol, mp 104−107 °C.

Synthesis of Series 8, 9, 11, and 13. A mixture of the 4-aryl-3-buten-2-one or 1-aryl-1-ethanone, paraformaldehyde, piperazine dihydrochloride (**8a**,**b**), ethylamine hydrochloride (**9a**−**e**, **11a**−**c**,**e**,**f**, **13a**,**b**), or ethylamine hydrobromide **(11d)**, hydrochloric acid (37% w/v, 0.04 mL; **8b**, **9a**−**e**, **11b**,**c**, **13a**,**b**) or trifluoroacetic acid (0.04 mL; **11a**,**d**,**e**) (3 mL; **11f**) in acetonitrile (100 mL; **8a**, **11a**,**d**,**e**,), and ethanol (95% v/v, 100 mL; **8b**, **9a**−**e**, **13a**,**b**) or 2-propanol (100 mL; **11b**,**c**,**f**) was heated under reflux for varying lengths of time. In the case of **11b**, the product which deposited from the reaction mixture was collected and washed with 2-propanol. For the other compounds, the solvent was removed in vacuo leading to oils which were washed with ether and dissolved in ethanol (10 mL) to which ether was added to induce precipitation. After refrigerating the solution at 4 °C for 2−3 days, the deposited solids were collected and recrystallized from 60% ethanol (**8a**), 70% ethanol (**8b**), 95% ethanol (**9a**,**c**, **11a**,**b**), ether−methanol (**9b**,**d**,**e**, **11d**,**e**, **13b**), or methanol (**11c**,**f**, **13a**). A constant quantity of amine hydrohalide was used (0.01 mol). The molar ratios of ketone to paraformaldehyde, times of heating (h), yields (%), and melting points (°C) were as follows. **8a**: 0.04: 0.03, 4, 61, 234 dec. **8b**: 0.06:0.06, 17, 41, 198 dec. **9a:** 0.06: 0.04, 36, 57, 194−196. **9b**: 0.06:0.04, 30, 24, 210−212. **9c**: 0.08:0.08, 24, 63, 192−194. **9d**: 0.06:0.04, 42, 21, 190−192. **9e**: 0.05:0.05, 45, 25, 180−182. **11a**: 0.04:0.04, 20, 47, 208−210. **11b**: 0.03:0.025, 48, 71, 202−203. **11c**: 0.03:0.025, 24, 70, 185−187. **11d**: 0.04:0.04, 20, 50, 198−200. **11e**: 0.04: 0.04, 48, 56, 178−179. **11f**: 0.05:0.05, 48, 51, 178−180. **13a**: 0.06:0.04, 36, 30, 164−166. **13b**: 0.06:0.04, 72, 28, 172−174. The ^{1}H NMR (500 MHz) spectra of two representative compounds (**9e** and **11e**) are given below. In the case of **9e**, the aryl rings adjacent to carbon atoms C1 and C4 (Figure 4) are referred to as aryl A and aryl B, respectively. **9e**: δ($CDCl_3$) 8.05 (1H, d, C*H*=CHCO, J = 16.3 Hz), 7.59 (2H, d, 2,6 aryl A H, J = 8.8 Hz), 7.21 (2H, d, 2,6 aryl B H, J = 8.8 Hz), 6.89 (2H, d, 3,5 aryl A H, J = 8.8 Hz), 6.77 (2H, d, 3,5 aryl B H, J = 8.8 Hz), 6.63 (1H, d, CH=C*H* aryl B, J = 15.9 Hz), 6.53 (1H, d, CH=C*H*CO, J = 16.3 Hz), 6.07 (1H, d, C*H*=CH aryl B, J = 15.9 Hz), 4.67 (1H, br d, OH, J = 1.5 Hz), 4.51 (1H, br d, $C3H_a$, J = 8.3 Hz), 3.83 (3H, s, aryl A OCH_3), 3.75 (3H, s, aryl A OCH_3), 3.34−3.41 (2H, m, $C2H_e$, $C6H_e$), 3.00−3.25 (4H, m, $C2H_a$, $C6H_a$, N−CH_2CH_3), 2.43−2.53 (1H, m, $C5H_a$), 1.86 (1H, d, $C5H_e$, J = 14.7 Hz), 1.48 (3H, t, CH_2CH_3, J = 7.3 Hz). **11e**: δ($CDCl_3$) 8.12 (2H, d, 2,6 aroyl H, J = 8.9 Hz), 7.42 (2H, def d, 2,6 aryl H, J = 8.8 Hz), 6.91 (2H, def d, 3,5 aroyl H, J = 9.0 Hz), 6.74 (2H, def d, 3,5 aryl H, J = 8.8 Hz), 5.50 (1H, dd, $C3H_a$, $J_{3a/2a}$ = 12.1 Hz, $J_{3a/2e}$ = 3.6 Hz), 5.24 (1H, d, OH, J = 2.6 Hz), 3.83 (3H, s, aroyl OCH_3), 3.68 (3H, s, aryl OCH_3), 3.41−3.48 (2H, m, $C2H_e$, $C6H_e$), 3.29−3.39 (2H, m, $C2H_a$, $C6H_a$), 3.04−3.18 (2H, m, NCH_2CH_3), 2.74−2.83 (1H, m, $C5H_a$), 1.92 (1H, br d, $C5H_e$, J = 14.9 Hz), 1.50 (3H, t, NCH_2CH_3, J = 7.3 Hz).

The hydrobromide salts of the free bases from **11a**,**c**,**e** were prepared as follows. A solution of **11a**,**c**,**e** (0.01 mol) in water (50 mL) was basified with sodium bicarbonate solution (10% w/v) and extracted with ether (5 × 25 mL). The organic extracts were combined and dried (anhydrous magnesium sulfate), and removal of the solvent gave a residue which was dissolved in anhydrous ether (100 mL). Excess of dry hydrogen bromide was passed into the ethereal solution at 0 °C, and the precipitate was collected, washed with anhydrous ether and chilled ethanol, and dried. The reaction products were recrystallized from 2-propanol to give the hydrobromide salts of the free bases from the following compounds: **11a**, mp 183−184 °C; **11c**, mp 182−184 °C; **11e**, mp 176−178 °C. The free bases of **11b**,**d** were obtained using the method described for the preparation of series **10** and **12** vide infra. Addition of dry hydrogen bromide gas to an ice-cooled solution of the Mannich base (0.001 mol) in ether (50 mL) led to precipitates which were collected, dried, and recrystallized from 2-propanol to give **11b**, mp 196−198 °C, or from ether−methanol leading to **11d**, mp 192−194 °C.

Synthesis of 10a,c,d and Series 12. A stirring solution of the piperidinols **9a**,**c**,**d** and **11a**−**e** (0.001 mol) in aqueous methanol (20% v/v, 25 mL) was cooled and maintained at less than 10 °C while basified with aqueous sodium bicarbonate solution (10% w/v). The mixture was extracted with ether (5 × 25 mL) and dried (anhydrous magnesium sulfate). Removal of the solvent under vaccuum gave an oil which was dissolved in anhydrous ether (50 mL) to which was added a 2.0 M solution of methyl bromide (0.01 mol) in tertiary butyl methyl ether at 0 °C. The reaction mixture was stirred at 0 °C for 6 h. The precipitates were collected, washed with dry ether, dried, and recrystallized from ethanol (95% v/v, **10a**,**d**) or ether−methanol (**10c**, **12a**−**e**). The yields (%) and melting points (°C) were as follows. **10a**: 77, 178−179. **10c**: 70, 202−204. **10d**: 78, 222−224. **12a**: 86, 192−194. **12b**: 73, 164−166. **12c**: 79, 168−170. **12d**: 80, 152−154. **12e**: 76, 188−190. TLC of the reaction products obtained in a similar manner from **9b**,**e** revealed the presence of an impurity. Recrystallization and column chromatography did not lead to the isolation of a pure compound.

The methiodide of the free base of **12d** was prepared in an identical manner except methyl iodide was used in place of methyl bromide and the reaction mixture was stirred at 0 °C overnight. The precipitate was collected and recrystallized from alcohol (95%) to give the desired compound, mp 177−178 °C, in 85% yield.

X-ray Crystallography of 10d. Compound **10d** was recrystallized from diethyl ether−methanol by vapor diffusion. A Nonius CAD-4 diffractometer with a ω scan was used for data collection, and the structure was solved by direct methods using NRCVAX[34] and refined using SHELX93.[35] Atomic scattering factors were taken from the literature.[36] All non-hydrogen atoms were found on the E-map and refined anisotropically. Hydrogen atom positions were calculated and not refined.

The data for **10d** were as follows: $C_{27}H_{33}BrNO_2$, M_r = 483.45; a = 8.0240(9), b = 10.253(2), c = 15.594(5) Å; α = 74.398(23)°, β = 83.549(16)°, γ = 86.847(14)°; Z = 2; space group = $P1$, triclinic; D_x = 1.308 g cm^{-3}, λ(Mo $K\alpha$) = 0.7093 Å; T = 123 K. Merging R is based on intensities of 0.015 for 447 replicate reflections. Refinement on F^2: $R[F^2 > 2\sigma(F^2)]$ = 0.0598 (2951 reflections), $wR(F^2)$ = 0.1797 (all data), S = 1.52. A total of 4790 reflections were measured of which 4343 were independent and used in the refinement of the structure. Parameters refined = 280, [$w = 1[\sigma^2(F_o^2) + (0.1222P)^2 + 0.000P]$, where $P = (F_o^2 + 2F_c^2)/3$. $\Delta\rho$ in the final difference map within +2.094 and −0.469 eÅ^{-3}.

Calculations of Atomic Charges. The molecular conformations were optimized using the semiempirical AM1 method. The electrostatic potential-derived charges were calculated using the CHELPG scheme at the RHF/3-21G level (restricted Hartree−Fock method with the 3-21G standard basis set). All computations were carried out using the Gaussian 92 program.[37]

B. Bioevaluation. Cytotoxicity Assays. Evaluation of the compounds using P388 D1 cells was undertaken by a literature procedure,[38] and the examination with L1210 cells was achieved using a previously reported method.[39] The assay

of various compounds using human tumors has been described.[40] Cell lines from the following diseases were employed: leukemia, non-small-cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancers. Compounds **9a**,**b**, **10a**, **11d**, and **12a**,**e** were not evaluated against prostate and breast cancers, but they were tested against small-cell lung tumors.

In Vivo Evaluation of 9d and 10a. The compounds were examined by the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. The murine P388 lymphocytic leukemia assay was conducted by a reported method,[41] and the maximum ILS figures for **9d** and **10a** were 5% and 20%, respectively, using doses of 54 and 6.7 mg/kg, respectively. An increase of 20% or more in the life spans is considered to be statistically significant. The reference drug 5-fluorouracil has an ILS of >35 using a dose of 20 mg/kg when given intraperitoneally for 5 days.[42] Passage of human tumors in athymic mice was undertaken by a published method.[43] No definitions of activity are available, but as a general rule compounds causing a 60% reduction in tumor weights in one of these screens would be evaluated further. For example, cyclophosphamide, while inactive toward COLO 205, SW-620, and NCI-H522 xenografts, reduced the growth of the LOX IMVI and CAKI-1 tumors by 100−150% and 60−100%, respectively.

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Supporting Information Available: Details of the X-ray structures of **8b**, **12d** (as the iodide salt), and **13a**,**b**; also atomic anisotropic displacement parameters, hydrogen positional and isotropic displacement parameters, and atomic positional and equivalent parameters (28 pages). Ordering information is given on any current masthead page.

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