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Evaluation of some Mannich bases of cycloalkanones and related compounds for cytotoxic activity

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Summary — A number of Mannich bases of cycloalkanones and related quaternary ammonium compounds were prepared for cytotoxic evaluation in order to examine the theory that sequential release of alkylating agents produces increased bioactivity compared to related compounds containing only 1 potential alkylating site. Many of the compounds had significant activity against murine L1210 cells and various human tumours. Some correlations between structure and activity were noted but the biological data did not support the view that potential sequential liberation of cytotoxic species produced compounds with increased potency. The formation of various oximes and oxime benzoates as candidate prodrugs was achieved but in general these compounds were not cytotoxic at the concentrations utilized. This observation may be due to the fact that the oximes were much more stable in deuterated phosphate buffered saline over a period of 48 h at 37°C than the Mannich bases, as revealed by ^{1}H-NMR spectroscopy.

Mannich bases / cytotoxic evaluations / prodrugs / ^{1}H-NMR spectroscopy / structure–activity relationships

Introduction

The theory of sequential cytotoxicity may be defined as the successive release of 2 or more cytotoxic compounds with a view to causing greater toxicity to malignant tissue rather than normal cells *(vide infra)*. In order to examine this theory, a number of Mannich bases and the corresponding quaternary ammonium compounds were chosen since they have previously demonstrated cytotoxicity towards tumour cells [1]. This property may be due to their capacity for deamination to the corresponding α,β-unsaturated ketones [2] which is a class of compounds known to have a marked affinity for thiol groups [3, 4] rather than hydroxy and amino groups which are present in nucleic acids. Thus a second reason for the choice of Mannich bases and related compounds used in this study is that these derivatives may prove to be bereft of the mutagenic [5] and carcinogenic [6] side effects of certain alkylating agents used in cancer chemotherapy.

In addition, this study has 2 further goals, namely the preparation and bioevaluation of some candidate prodrugs designed to liberate the related Mannich bases and quaternary ammonium compounds preferentially in malignant rather than normal tissues and also to evaluate whether, in compounds containing similar functional groups, rigidity or flexibility of the molecules is associated with greater cytotoxicity.

The reasons for wishing to evaluate the technique of sequential cytotoxicity with this class of compounds are as follows. First, on a number of occasions, lowering the concentration of cellular thiols prior to the administration of various anticancer agents enhances the cytotoxicity of these drugs towards tumours rather than normal cells [7, 8]. In other words, chemosensitization of tumour cells may cause the anticancer drug to exert a preferential toxicity to malignant tissue. Second, on occasions there are differences in the amounts of the different isozymes of glutathione *S*-transferase (GST) between malignant and normal cells [9, 10]. Hence it is possible that the initial formation of an alkylating agent may inhibit one or more of the isozymes of GST which could augment the cytotoxic action caused by the release of a second thiol alkylator. For example, if a certain group of tumours had lower levels of the α-GST isozyme than the corresponding normal cells

and the initially released cytotoxic agent had a high affinity for only this isozyme, the tumours would be expected to be more sensitive to the second alkylating agent than nonmalignant tissue. The recent reports of the potentiation of the cytotoxicity of different alkylating agents by the α,β-unsaturated ketone ethacrynic acid (a GST inhibitor) [11, 12] support this concept. Third, prior administration of the cellular energy metabolism inhibitor lonidamine potentiated cancer cell killing by various antineoplastic agents [13–15]. Since a number of Mannich bases of α,β-unsaturated ketones are mitochondrial inhibitors [16, 17] it is conceivable that initial inhibition of cellular energy followed by alkylation could occur, rendering useful therapeutic agents.

In order to explore the general viability of the concept of sequential cytotoxicity, the preparation of series **1** was envisaged. These compounds possess 2 latent alkylator groups and scheme 1 indicates how a representative compound **1a** could react under biological conditions. Since the Taft σ * values of the dimethylammonium and thiomethyl groups are 4.36 and 1.56 respectively [18], the avidity of (A) for thiols should be greater than in (C). Thus a rapid reduction of cellular thiols is likely followed by a more gradual depletion of these molecules. Compounds **1b**, **d** were suggested since quaternary ammonium salts may deaminate more rapidly than the corresponding hydrohalide salts [19]. The rates of deamination among **1e–h** would be expected to be similar; hence any variation in cytotoxicity among these 4 bis-Mannich bases would likely be attributed to differences in the hydrophobicities of the molecules.

Second, the formation of candidate prodrugs of ketones was considered with a view to enhancing toxicity for malignant tissues. Oximes are known to be labile under acidic conditions [20] and since the pH of a number of tumours is lower than the corresponding normal tissues [21], it is conceivable that preferential regeneration of the ketones in malignant tissues occurs. This process assumes that greater deamination occurs with ketones than oximes. Hence the preparation of **2a–d** was suggested. Since the polar hydroxy groups in these oximes may impede penetration through cell membranes, the ester **2e** may more easily transverse this barrier into the cells where hydrolysis can liberate the oxime **2a**.

If sequential release of cytotoxic species from the potentially bifunctional compounds **1**, **2** occurs, then analogues containing only 1 potential alkylating site should demonstrate significantly lower bioactivity. Hence series **3–5** were proposed in order to examine this supposition.

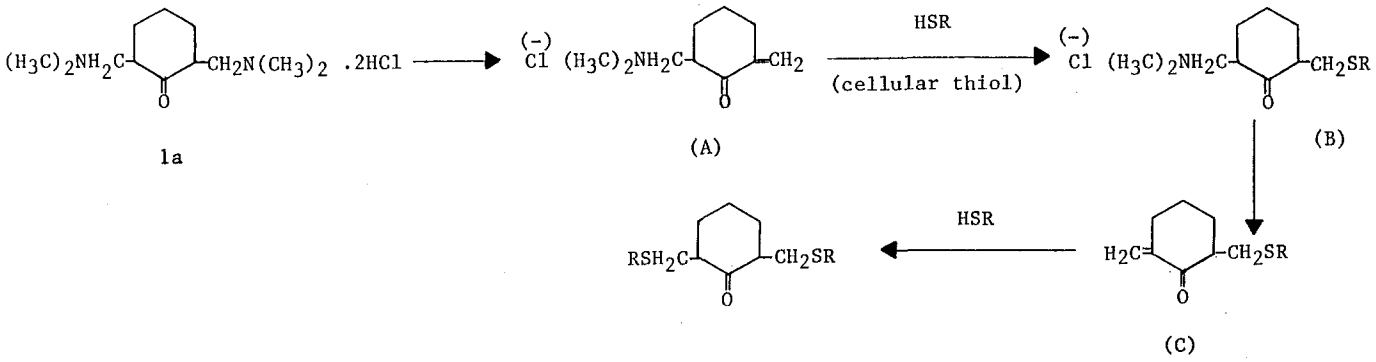
Third, on a number of occasions, flexible analogues of more rigid molecules have demonstrated higher bioactivity [22, 23]; conversely, the literature describes rigid compounds possessing greater activity than the corresponding derivatives which are more flexible [24, 25]. Series **6** and **7** were proposed in order to compare their cytotoxicities with their rigid counterparts in series **1–5**.

Finally the basic centre was changed for the following reason. The pK_a values of dimethylamine, dibenzylamine, morpholine and piperidine are 10.73 [26], 8.52 [27], 8.50 and 11.12 [28]. Since rates of deamination are inversely proportional to basicities, the predicted rates of formation of the corresponding α,β-unsaturated ketones are **8a**, **b** > **1a** > **8c**. Series **8** was therefore considered in an attempt to correlate rates of deamination with cytotoxicity.

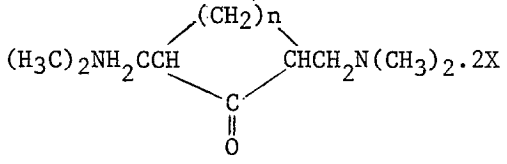
This report describes the synthesis and cytotoxic evaluation of these compounds along with stability studies on representative compounds.

Chemistry

The ketones **1a**, **c**, **e–h**, **3a**, **c**, **6a**, **7a**, **8a–c** were prepared by the Mannich reaction. Treatment of some of these ketones with hydroxylamine hydrochloride in

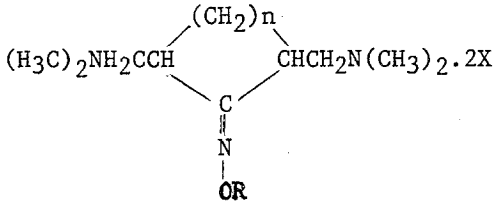


Scheme 1.



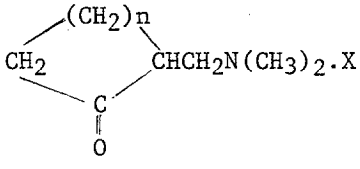
1

a: n=3; X=HCl
b: n=3; X=CH_3I
c: n=2; X=HCl
d: n=2; X=CH_3I
e: n=4; X=HCl
f: n=5; X=HCl
g: n=9; X=HCl
h: n=12; X=HCl



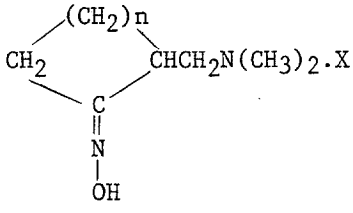
2

a: n=3; R=H; X=HCl
b: n=3; R=H; X=CH_3I
c: n=2; R=H; X=HCl
d: n=2; R=H; X=CH_3I
e: n=3; R=COC_6H_5; X=HCl



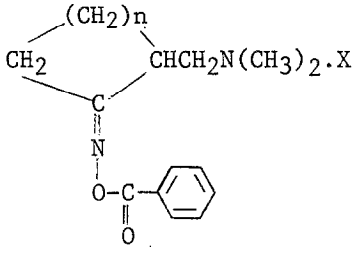
3

a: n=3; X=HCl
b: n=3; X=CH_3I
c: n=2; X=HCl
d: n=2; X=CH_3I



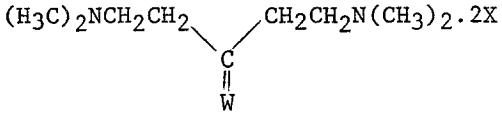
4

a: n=3; X= ---
b: n=3; X=CH_3I
c: n=2; X= ---
d: n=2; X=CH_3I



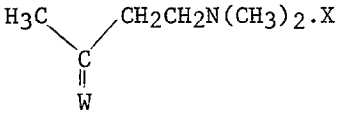
5

a: n=3; X=HCl
b: n=3; X=CH_3I
c: n=2; X=HCl
d: n=2; X=CH_3I



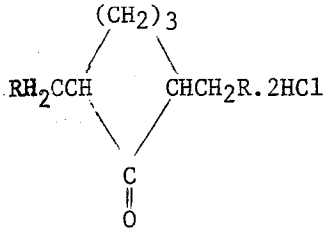
6

a: W=O; X=HCl
b: W=O; X=CH_3I
c: W=NOH; X=HCl
d: W=$NOCOC_6H_5$; X=HCl

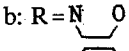
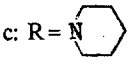


7

a: W=O; X=HCl
b: W=O; X=CH_3I
c: W=NOH; X=HCl
d: W=$NOCOC_6H_5$; X=HCl



8

a: R=$N(CH_2C_6H_5)_2$
b: R= 
c: R= 

the presence of base afforded the oximes **2a**, **c**, **4a**, **c**, **6c**, **7c**. Reaction of benzoyl chloride with the free bases of some of the oximes gave the corresponding benzoate hydrochlorides **2e**, **5a**, **c**, **6d**, **7d**. The free bases of selected ketones, oximes and oxime benzoates were treated with excess of methyl iodide to give the corresponding quaternary ammonium iodides **1b**, **d**, **2b**, **d**, **3b**, **d**, **4b**, **d**, **5b**, **d**, **6b**, **7b**. The melting points and percentage yields of compounds **1–8** are given in table I. The stabilities of solutions of representative compounds in deuterated phosphate buffered saline at 37°C for 48 h as determined by ^{1}H-NMR spectroscopy is presented in table II.

Cytotoxicity

Compounds **1–8** were evaluated *in vitro* against murine L1210 cells and most of the derivatives were examined in the NCI *in vitro* screen using human tumour cell lines; the data are presented in table I. In addition, several compounds were screened for selective toxicity towards 3 human colon cancers by comparison with a nontransformed fibroblast. The results of this study are given in table I.

Results

All of the compounds in series **1–8** were evaluated against L1210 cells and the cytotoxic activity of selected molecules against a large number of human tumours and human colorectal cancers was undertaken. The Mannich bases and the corresponding quaternary ammonium derivatives **1**, **3**, **6a**, **b**, **7a**, **b**, **8** demonstrated cytotoxicity towards L1210 cells with IC_{50} values ranging from 0.1–50 μM except for **1d**, **6a** which were inactive. In the NCI screen, 13 of these Mannich bases and related quaternary ammonium compounds were evaluated and shown to have similar or greater cytotoxicity than melphalan with the exception of **1g** which demonstrated little activity to the human tumours. Eleven of these 13 derivatives (85%) namely **1a–d**, **3b–d**, **6b**, **7b**, **8a**, **c** demonstrated selective toxicity towards leukemia lines of the $\approx$ 53 human tumour lines of the NCI screen. Fifteen of the Mannich bases and quaternary ammonium salts screened against 3 human colorectal tumours revealed that nearly 50% of the compounds had greater cytotoxicity than melphalan. In other words, the IC_{50} values for compounds **1a**, **b**, **e**, **f** were less than those determined for melphalan for all 3 human colorectal tumour cell lines and for 2 of those lines for compounds **1c**, **3b**, **8b**. In addition, 5 of these compounds were at least as selective for colorectal tumours as melphalan. Thus, **1a**, **b**, **e** proved to be at least 7- to 8-fold more effective against all 3 tumour cell lines than non-transformed fibroblasts (*ie*, selective), while 2 others (**3b**, **8b**) and melphalan were also selective for 2 of the 3 tumour cell lines.

The oximes of the Mannich bases and quaternary ammonium compounds, namely **2a–d**, **4**, **6c**, were inactive in the L1210 test system. In addition, various oximes demonstrated little or no cytotoxicity both in the NCI *in vitro* screen and against colorectal tu-mours. The only exception to this uniform lack of ac-tivity of the oximes was **7c** which had significant potency in both the L1210 and NCI screens. The oxime benzoates **2e**, **5**, **6d**, **7d** were virtually bereft of any cytotoxicity at the highest concentrations employed.

The stability of various compounds in deuterated phosphate buffered saline (PBS-d) at 37°C was measured by ^{1}H-NMR spectroscopy; the data are presented in table II. In deuterated solvents, pD = pH + 0.4. At pD 7.4 the bis Mannich bases **1a**, **6a**, **8b** decomposed entirely or nearly so during the 48-h period. Compound **1a** was 94% decomposed in a solution of PBS-d (pD = 7.4) containing 10% deuterated dimethylsulphoxide after incubation at 48 h at 37°C; hence it is unlikely that this organic solvent inhibits the breakdown of **4a** in solution. The monobasic derivatives **3a**, **c** decomposed more slowly. The quaternary ammonium iodides **1b**, **3b**, **d** had broken down completely in solution at the end of 48 h. Lowering the pD to 6.4 reduced the amount of decomposition of the Mannich bases **1a**, **3a**, **c** but not the corresponding quaternary ammonium derivatives **1b**, **3b**, **d**. Conversion of **1a**, **b**, **3a**, **b**, **6a** into the corresponding oximes **2a**, **b**, **4a**, **b**, **6c** markedly reduced or abolished any deamination from occurring using solutions of pD 7.4. In the case of **2a**, **b**, **4a**, **b** little or no change in the amount of decomposition occurred when the pD value was reduced to 6.4. The rates of deamination of the bis compounds **1a**, **b**, **6a**, **8b** and the mono derivatives **3a**, **b**, **d** were measured. Figure 1 illustrates the 2 stages of decomposition that occur with the bis compounds **1a**, **b**. The half-lives of **1a**, **b**, **6a**, **8b** for the first stage were 215, 8.7, 339 and 19 min respectively and for the second stage, the corresponding figures were 428, 11.8, 165 and 353 min respectively. The half-lives of **3a**, **b**, **d** were 1002, 20.0 and 18.8 min respectively.

Discussion

The cytotoxicity data revealed in table I indicate that in general the Mannich bases and related quaternary ammonium compounds demonstrated significant cytotoxicity while the corresponding oximes and benzoates are far less potent tumour inhibitors.

Three screens were employed to examine the cytotoxicity of all or some of the compounds in series **1–8**. The L1210 test system is widely used to detect

Table I. Physical data and *in vitro* evaluation of Mannich bases and congeners.

Compound	M.Pt. (°C)	Yield (%)	L1210 cells IC_{50} (μM)[a]	NCI screen $\log_{10}$ GI_{50} (M) MG MID values[b]	Colorectal tumours and a human fibroblast						
					COLO-320-DM		HT-29		OM-1		WI-38 IC_{50} (μM)
					IC_{50} (μM)	STI[c]	IC_{50} (μM)	STI[c]	IC_{50} (μM)	STI[c]	
1a	179	66	4.5	-5.50	1.6	9	2.1	7	0.53	26	14
1b	251-254	89	17.6	-5.78	1.4	11	1.8	9	0.43	37	16
1c	187	68	0.1	-5.05	4.2	4	5.4	3	2.3	7	16
1d	232	87	>50	-5.53	18	1	16	1	17	1	21
1e	170-171	42	0.2	---	1.8	7	1.4	9	1.6	8	12
1f	179-181	43	29	---	1.7	5	1.5	6	1.4	6	8.9
1g	144-144.5	39	40	-4.09	130	2	19	11	51	4	210
1h	138-139	35	4.4	-4.90	17	2	9.4	3	14	2	27
2a	222-224	64	>50	---	>30	---	>30	---	>30	---	>30
2b	272-274	85	>50	>-4.00	>30	---	>30	---	>30	---	>30
2c	160-162	57	>50	-4.07	---	---	---	---	---	---	---
2d	180-182	79	>50	-4.00	---	---	---	---	---	---	---
2e	244-247	39	>50	-4.02	>30	---	>30	---	>30	---	>30
3a	155	78	24.4	---	16	1	17	1	12	2	21
3b	210-212	91	6.9	-4.56	4.7	>5	2.1	>11	1.7	>14	>24
3c	149	74	2.1	-5.73	---	---	---	---	---	---	---
3d	209	93	50	-5.38	---	---	---	---	---	---	---
4a	106-107	71	>50	-4.01	>30	---	>30	---	>30	---	>30
4b	110-112	84	>50	>-4.00	---	---	---	---	---	---	---
4c	156-157	69	>50	>-4.00	---	---	---	---	---	---	---
4d	169-172	82	>50	-4.30	---	---	---	---	---	---	---
5a	157-158	57	>50	>-4.00	>30	---	>30	---	>30	---	>30
5b	210-211	79	>50	-4.01	---	---	---	---	---	---	---
5c	154-155	54	>50	>-4.00	---	---	---	---	---	---	---
5d	158-160	74	>50	>-4.00	---	---	---	---	---	---	---
6a	203-205	31	>50	---	>30	---	>30	---	>30	---	>30
6b	231-234	74	40.5	-4.67	23	---	>25	---	23	---	>30
6c	170-172	54	>50	>-4.00	---	---	---	---	---	---	---
6d	180-181	39	>50	>-4.00	---	---	---	---	---	---	---
7a	118-119	34	3.7	---	---	---	---	---	---	---	---
7b	149-150	69	0.8	-4.83	---	---	---	---	---	---	---
7c	146-148	72	0.7	-7.48	---	---	---	---	---	---	---
7d	170-172	68	>50	-4.04	---	---	---	---	---	---	---
8a	250	87	0.8	-5.20	>24	---	>23	---	10	>3	>30
8b	164.5	59	0.8	---	6.4	3	1.9	12	1.3	17	22
8c	177	37	2.3	-4.49	>30	---	25	---	28	---	>30
Melphalan	---	---	0.4	-4.58	3.0	22	20	3	6	11	65

[a]IC_{50} values refer to the concentration of compound required to inhibit 50% of the growth of murine L1210 cells; [b]log GI_{50} MG MID values refer to the concentration of compound required to inhibit the growth of approximately 53 human tumours by 50% (see *Discussion* section for further details); [c]the specific tumouricidal index (STI) figures were obtained by dividing the IC_{50} values for the WI-38 cells by each of the IC_{50} figures of the colorectal tumours.

Table II. Decomposition of selected compounds after 48 h in solution at 37°C.

Compound	Percentage decomposition[a]	
	pD 7.4	pD 6.4
1a	93	42
1b	100	100
2a	1.1	1.4
2b	1.7	1.3
3a	54	18
3b	100	100
3c	55	18
3d	100	100
4a	0	0
4b	0	0
6a	99	--
6c	3.1	--
8b	100	--

[a]Measurements were made in deuterated phosphate buffered saline. In order to achieve solubilisation, a solution of **4a** contained 10% dimethylsulphoxide-d_6.

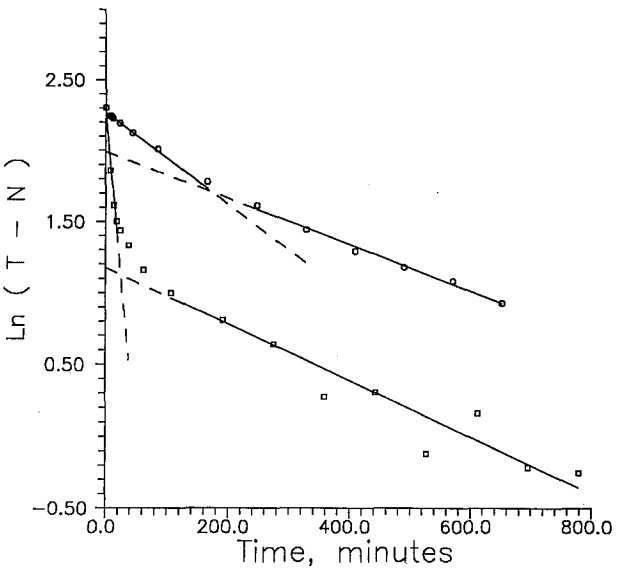


Fig 1. Decomposition of **1a** and **1b** in deuterated phosphate buffered saline (pD = 7.4) at 37°C. T = initial concentration of compound and N = new product formed, *ie* dimethylamine hydrochloride or trimethylammonium iodide. Legends –O–O– (**1a**) and –□–□– (**1b**).

promissing anticancer agents [29]. The results in table I indicate that the Mannich bases and quaternary ammonium compounds **1**, **3**, **6a**, **b**, **7a**, **b**, **8** are clearly a useful series of lead molecules in which **1c**, **e** are more cytotoxic than melphalan and **7b**, **8a**, **b** are half as active as this clinically useful drug. In fact, 90% of the Mannich bases and related quaternary ammonium salts have IC_{50} values of 50 μM or less. The NCI screen involved the assessment of various compounds against ≈ 53 human tumour cell lines from 8 specific disease states namely leukemia, melanoma, non-small cell lung, small cell lung, colon, CNS, ovarian and renal cancers [30]. The principal objective is to detect novel chemical entities with selective toxicity to one or more diseases and it is noteworthy that a number of the Mannich bases and related quaternary ammonium compounds demonstrated specificity to leukemia cell lines. Compounds are generally evaluated in this screen using 5 concentrations of derivatives, *ie* 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol. The molar quantities that cause 50, 0 and –50% growth of the tumours relative to the untreated cells are referred to as GI_{50}, TGI and LC_{50} figures which represent the growth-inhibiting, cytostatic and cytotoxic properties respectively of the compounds. Most of the derivatives listed in table I had GI_{50} figures > $\log_{10}$ –4.00 for 1 or more of the cell lines and this figure of $\log_{10}$ > –4.00 is used in calculating the average figure for all the cell lines. Hence the term MG MID (meangraph midpoint) rather than mean value is given in table I. With the exception of **1g**, **3b**, **8c**, the Mannich bases and related quaternary ammonium compounds had lower $\log_{10}$ GI_{50} figures than melphalan. The oximes and oxime benzoates had little activity towards these tumour cell lines although the remarkable potency of **7c** (approximately 1 000 times more active than melphalan) is clearly an exception to this general trend. A comparison of the cytotoxicities in the L1210 and NCI screens in table I revealed that except for **1d**, **g**, **4d**, compounds active in the L1210 screen (IC_{50} < 50 μM) had $\log_{10}$ GI_{50} figures < –4.10 and *vice versa, ie* those derivatives which were inactive in the L1210 screen had $\log_{10}$ GI_{50} figures > –4.10. The data in table I reveal that compounds **1a**, **b**, **e** were not only more potent than melphalan against the 3 human colorectal cancer cell lines but were also more selective. The significance of this observation lies in comparison to 4 established anticancer drugs which have been shown to be clinically effective against colorectal cancer: 5-fluorouracil [31–34], floxuridine [31, 35, 36], melphalan [33], and mitomycin C [32–34, 36]. When each of these drugs was evaluated against all 3 human colorectal cancer cell lines and the nontransformed human fibroblasts (WI-38) they were found to be selective (STI values in excess of 7–8) for at least 2 (melphalan and mitomycin C) or for all 3 (5-fluorouracil and floxuridine) colorectal tumour cell lines. Thirteen other established anticancer drugs which are known to

be ineffective against colorectal cancer (*eg*, adriamycin, hydroxyurea, vincristine, cytarabine) have also been evaluated. Three were found to be selective for only 1 of the 3 colorectal cell lines while the remaining drugs were not selective for any of the lines (GA Truitt, unpublished observations). While selectivity for 2–3 of the colorectal cell lines observed for some of the Mannich bases and derivatives is encouraging, the utility of any compound may yet be restricted by its pharmacokinetics and toxicity. One final observation in the context of colorectal cancer selectivity is that increasing the size of the cycloalkane ring of the Mannich bases in general diminished selectivity although high cytotoxicity was retained.

The following attempts at correlating the structures of many of these compounds described in this report with cytotoxicity were made. First, log–log plots were made between the IC_{50} figures in the L1210, COLO-320-DM, HT-29 and OM-1 screens against the *f* values of the ring methylene groups of the Mannich bases **1a**, **c**, **e–h** (n = 2–5, 9, 12). Using a test for zero correlation [37], correlations were noted using L1210 (p <0.1), COLO-320-DM (p < 0.1) and OM-1 (p < 0.05) tumours. When linear plots were made, correlations were obtained for HT-29 (p < 0.1) and OM-1 (p < 0.05) tumours. No correlation at the 90% confidence level was obtained for the HT-29 tumour (log–log plot) or for linear plots using L1210 and COLO-320-DM tumours. A plot of the $\log_{10}$ GI_{50} figures of **1a**, **c**, **g**, **h** against the ring methylene *f* values did not display any correlation at the 90% confidence level. Thus in over half of these evaluations, an increase in cytotoxicity with diminishing hydrophobicity was demonstrated and hence future molecular modifications should utilize small alicyclic rings.

Second, the theory of sequential cytotoxicity predicts that the bifunctional compounds should have greater bioactivity than the corresponding derivatives which have the potential for generating only one alkylating group. Thus a comparison of the cytotoxicity of the bifunctional derivatives **1a–d**, **2a–e**, **6a–d** with the analogous mono derivatives **3a–d**, **4a–d**, **5a**, **7a–d** was made. In the L1210 screen, compounds **1a**, **c** were more active than **3a**, **c**; however compounds **3b**, **d**, **7a–c** possessing only one basic centre had greater cytotoxicity than the corresponding bis amines **1b**, **d**, **6a–c**. In the remaining cases both bifunctional and monofunctional compounds were inactive at 50 μM. The NCI screen revealed that where comparisons could be made, greater cytotoxicity was displayed with 2 of the bis compounds (**1b**, **d**) and 4 of the monoamine analogues (**3c**, **4d**, **7b**, **c**). Two comparisons could be made using the results for the colorectal tumours whereby **1a**, **b** are clearly more cytotoxic than **3a**, **b**. Thus in general, the biological data generated does not support the sequential cytotoxicity theory which predicts greater activity with bis alkylators.

Third, a comparison of the cycloalkyl derivatives with the corresponding acyclic compounds was made. In general, when 2 basic centres were present in the molecule, the rigid compounds had greater cytotoxicity than the analogous flexible analogues while when one basic group was present the opposite trend was found. Thus the bis Mannich bases **1a**, **c**, **e–h** were more active than **6a** against L1210 leukemia cells and colorectal tumours. The data in table I revealed that the quaternary ammonium iodides **1b**, **d** had greater activity than **6b**; the only exception being the higher activity of **6b** than **1d** against L1210 cells. The lack of activity with the oximes **2a**, **c**, **6c** and oxime benzoates **2e**, **6d** precludes any conclusions being drawn pertaining to the advantages of rigidity or flexibility in these molecules. In the case of compounds containing one basic group, the orders of potencies against L1210 cells were **3c** > **7a** > **3a**, **7b** > **3b** > **3d** and **7c** > **4a**, **c** and in the NCI screen they were **3d** > **7b** > **3b** and **7c** > **4a**, **c**. The oxime benzoates **5a**, **c**, **7d** were all virtually inactive.

Fourth, the possibility of discerning structure–activity relationships from stability studies was considered. The data in table II indicate that using solutions of pD 7.4, the compounds may be classified into 3 groups depending on the percentage of decomposition at the end of 48 h, namely group A comprising **1a**, **b**, **3b**, **d**, **6a**, **8b** (93–100% decomposition), group B consisting of **3a**, **c** (≈ 50% decomposition) and **2a**, **b**, **4a**, **b**, **6c** (group C) which are much more stable (0–3% decomposition). Using figures of 50 μM, 10^{-4} M and 30 μM for inactive compounds in the L1210, NCI and colorectal screens respectively, calculations reveal that the order of cytotoxicity in the L1210 and NCI screens is B > A > C while using colorectal tumours, the order is A > B > C. Groups A and B consist of Mannich bases and related quaternary ammonium compounds and group C is comprised of oximes. Hence future drug design should expand groups A and B in order to evaluate whether a general trend is evident, *ie* that for promising activity in the L1210 and NCI screens, compounds decomposing slowly over the time of incubation with malignant cells are more preferable than when a more rapid rate of decomposition occurs. Conversely from the limited data available, the COLO-320-DM, HT-29 and OM-1 colorectal tumours may be more sensitive to compounds which deaminate readily. The fragility of some of these compounds at pD 6.4 reveals that the Mannich bases **1a**, **3a**, **c** (group D) deaminate more slowly under acidic conditions than at pD 7.4 while the corresponding quaternary ammonium compounds **1b**, **3b**, **d** (group E) are completely decomposed at the

end of 48 h. The order of cytotoxicity in the L1210 and NCI screens is D > E while against the colorectal tumours it is E > D. The second conclusion to be drawn from these stability studies is that the general inactivity of the oximes may be due to not only their being inactive *per se* but by failing to act as prodrugs for the corresponding bioactive ketones. It is of interest to note that incubation of solutions (pD 7.4) of **2a, b, 4a, b** at 37°C for 13 d caused only 1–10% decomposition of these compounds. In addition, even under acidic conditions, the oximes **2a, b, 4a, b** are unchanged or nearly so after 48 h incubation at 37°C using a solution of pD 6.4.

Two other features of the stability study in PBS-d (pD = 7.4) are as follows. First, the extent of decomposition of the compounds listed in table II was obtained by measurement of the *N*-methyl integrals of dimethylamine or trimethylammonium iodide. However the integrals for the corresponding olefinic protons were either less than predicted or absent. For example, in the case of **3a** the ratio of the *N*-methyl protons of dimethylamine with the olefinic protons of the putative decomposition product should be 6:2 but was in fact 23:1. Reduction in the size of the olefinic integrals was also found with **3c, d**. This observation may be due to the unsaturated olefine formed undergoing polymerization and/or dimerization which are reactions known to occur with conjugated α,β-unsaturated ketones [38, 39]. Support for this contention was obtained in the case of **1a, b**. Solutions of these compounds gave rise to precipitates which were collected, dried and solutions in deuterochloroform revealed absorptions in the range 0.8–2.5 ppm of which the principal broad peak from 1.0–2.2 ppm had a short T_2, suggesting that a polymer had been formed. Second, the measurement of the half lives of the bis compounds **1a, b, 6a, 8b** indicated that the first stage of decomposition proceeds quicker than the second phase for the alicyclic compounds **1a, b, 8b** while for the acyclic compound **6a** the opposite trend is noted. The mono derivatives **3a, b** decomposed more slowly than the corresponding bis analogues **1a, b**. The data for the bis derivatives **1a, b** as illustrated in figure 1 support the concept of biphasic deamination of these compounds which is a necessary feature for the sequential cytotoxicity theory to be viable. In addition, the kinetic data indicated that **8b** decomposed faster than **1a** and hence the postulated order of deamination **8a, b > 1a > 8c** is likely true. However, no correlation with cytotoxicity was apparent in considering these 4 compounds.

In conclusion, this study has shown that a series of Mannich bases and related quaternary ammonium salts which were designed to evaluate the theory of sequential cytotoxicity possess significant activity towards a wide range of malignant cells, particularly leukemia and colorectal tumours. In general the data indicate that while these molecules break down under simulated physiological conditions, the mono-Mannich bases are more active than the bis analogues which is at variance with the theory. A number of structural features associated with cytotoxicity have been found. Bioactivity was generally abolished when these Mannich bases and quaternary ammonium salts were converted into the related oximes and oxime esters which were designed as prodrugs of the corresponding ketones.

Experimental protocols

Chemistry

Melting points and boiling points are uncorrected. Elemental analyses (C, H, N) were undertaken on all of the compounds in series **1–8** and are within 0.4% of the calculated values. ^{1}H-NMR spectra were determined routinely on the compounds prepared using a Varian T-60 spectrometer and for the stability studies, a Bruker AM 300FT spectrometer was employed. This latter instrument was used in determining the spectra of representative compounds (s = singlet, dd = double doublet, t = triplet, m = multiplet, a = axial proton, e = equatorial proton).

*Syntheses of the Mannich base mono and bis hydrochloride **1a, c, e–h, 3a, c, 6a, 7a, 8a–c***

The general procedure for the preparation of **1a, c, 8a–c** was as follows. A mixture of cycloalkanone (0.10 mol), the appropriate amine hydrochloride (0.20 mol), paraformaldehyde (0.20 mol) and acetic acid (40 ml) was heated at 95°C (**1a, c, 8b, c**) or 55°C (**8a**) for 2.5 h. The solvents were removed *in vacuo* and the oily residue was dissolved in ≈ 100 ml of hot acetone. After refrigeration overnight at –20°C, the precipitate was collected and recrystallized from ether–methanol (**1a, 8c**), methanol (**1c**) or ethanol (**8a, b**). Compounds **1e–h** were prepared in a similar manner except that 50 ml acetic acid was used as the solvent and the reaction mixture was heated at 90°C for 3.5 h. The crude products were recrystallized from ethanol. Compounds **3a, c** were prepared in a similar fashion to **1a** except that the mol of ketone, formaldehyde and dimethylamine hydrochloride were 0.20, 0.10 and 0.10 respectively and the crude products were washed with cold acetone. The resultant solids were recrystallized from ether–methanol (**3a**) or methanol (**3c**). The Mannich base dihydrochloride **6a** was prepared in a similar way to **1a** except that the reaction mixture was heated at 95°C for 3 h and addition of hot acetone to the residue gave a solid which was separated and recrystallized from methanol to give **6a**. The Mannich base hydrochloride **7a** was prepared in a similar fashion to **3a, c** except that the crude product was dissolved in hot acetone and the resultant solid was recrystallized from methanol. The data for a representative compound **6a** determined in PBS-d were as follows: δ: 2.84(12, s, CH_3), 3.08(4, t, CH_2, J = 6.5 Hz) and 3.36(4, t, CH_2, J = 6.5 Hz).

*Syntheses of the oxime mono or bis hydrochlorides **2a, c, 6c, 7c** and oximes **4a, c***

Compound **2a** was prepared as follows. A mixture of **1a** (0.07 mol), hydroxylamine hydrochloride (0.25 mol), pyridine (30 ml) and ethanol (150 ml) was heated under reflux for 4 h. The solvents were evaporated and after dissolving the residue in water, the solution was basified with aqueous sodium

hydroxide solution (1 N), saturated with sodium chloride and extracted with ether. The dried ethereal extract was treated with hydrogen chloride yielding an oily precipitate which solidified on trituration with isopropyl alcohol. Recrystallization of the crude material from ether–methanol gave **2a**. Compounds **2c**, **6c** were prepared in a similar manner except in the case of **6c**, a relatively larger amount of ethanol was used (100 ml per 0.01 mol ketone) and it was recrystallized from ethanol.

The oxime **4a** was prepared by the following procedure. A mixture of **3a** (0.0052 mol), hydroxylamine hydrochloride (0.006 mol), potassium carbonate (0.0052 mol) and water (5 ml) was stirred at room temperature for 3 h. The solution was saturated with potassium carbonate, extracted with ether and the organic phase was dried. Evaporation of the solvent gave a solid which was recrystallized from methanol. Compounds **4c**, **7c** were prepared in the same manner except that **7c** recrystallized from ethanol. The 300 MHz ^{1}H-NMR spectrum of a representative compound **4a** in PBS-d: dimethylsulphoxide–d_6(9:1) was as follows: δ:1.30(1, m, 3a), 1.40(1, m, 5a), 1.55(1, m, 3e), 1.60(2, m, 4a, 5e), 1.85(3, m, 5e, 6a, 6e), 2.86(5.35, s, CH_3), 2.78–3.10(1, m, CH), 2.89(0.65, s, CH_3), 3.03(1, dd, CH_2, J = 13.0, 3.9 Hz) and 3.45(1, dd, CH_2, J = 13.0, 10.8 Hz).

Stability study of representative compounds in deuterated solvents

Solutions (10 mM) were prepared by dissolving the compounds in solvents preheated to 37°C namely PBS-d or, in the case of **4a**, in a mixture of PBS-d and dimethylsulphoxide–d_6 (see table II footnote). The 300 MHz ^{1}H-NMR spectra were recorded at 37°C as rapidly as possible and t_o was taken at the middle of the acquisition period which was ≈ 9 min after dissolution of the compounds. The solutions were incubated at 37°C for 48 h and the spectra recorded again. The percentage decomposition was obtained from the integrals of the methyl groups on the nitrogen atoms *ie* the integral of dimethylamine or trimethylammonium iodide/the integral of dimethylamine plus the integral of the methyl groups of the parent molecule x 100. The NMR spectra were acquired using 16 K TD and 32 K SI to which 16 K zero filling had been added to enhance resolution to 0.256 Hz/Pt. The pulse angle was ≈ 30 degrees yielding a 1.95-s acquisition time and a relaxation delay of 2 s. A total of 256 scans for each determination were made. The spectral window was set at 4201.681 to produce a 14-ppm spectrum range. The stability of **2a** was determined in quadruplicate in PBS-d (pD = 7.4) and the percent decomposition was 0.7, 1.6, 0.7 and 1.5 *ie* 1.1 ± 0.5%; in all other cases 1 determination only was made.

The kinetic determinations using 300 MHz ^{1}H-NMR spectroscopy were found by plotting the logarithm of the initial concentration of the compound minus the concentration of dimethylamine hydrochloride or trimethylammonium iodide in PBS-d (pD = 7.4) at 37°C against time. The information obtained was as follows (k in s^{-1}, r and $t_{1/2}$ figures in min); in the case of **1a**, **b**, **6a**, **8b** 2 sets of data are given. **1a**: (0.193 ± 0.008, 0.996, 215 ± 9; 0.097 ± 0.005, 0.996, 428 ± 20), **1b**: (4.8 ± 0.1, 0.999, 8.7 ± 0.2; 3.5 ± 0.1, 0.998, 11.8 ± 0.5), **3a**: (0.0415 ± 0.0008, 0.998, 1002 ± 19), **3b**: (2.077 ± 0.009, 0.999, 20.0 ± 0.1), **3d**: (2.21 ± 0.05, 0.999, 18.8 ± 0.4), **6a**: (0.123 ± 0.006, 0.995, 339 ± 16; 0.252 ± 0.005, 0.999, 165 ± 3), **8b**: (2.7 ± 0.3, 0.987, 19 ± 2; 0.12 ± 0.01, 0.964, 353 ± 35).

*Synthesis of the oxime benzoate mono and bis hydrochlorides **2e, 5a, c, 6d, 7d***

Compound **2e** was prepared as follows. Benzoyl chloride (0.01 mol) was added slowly to a solution of the free base from **2a** (0.01 mol) in aqueous sodium hydroxide solution (1 N, 10 ml) and ethanol (100 ml). The mixture was stirred for 2 h at 0°C and then a further 2 h at room temperature. After refrigeration overnight at –20°C, the mixture was extracted with ether and the combined ethereal extracts were washed with water, dried and treated with hydrogen chloride. A gummy precipitate was obtained which crystallized from ether–methanol on cooling (–20°C). The product was recrystallized from ethanol to give **2e**.

Compounds **5a**, **c** were synthesized by the following route. A solution of **4a**, **c** (0.01 mol) in benzene (≈ 50 ml) was prepared by heating the solution at ≈ 50°C. On cooling, benzoyl chloride (0.01 mol) was added slowly and the reaction mixture was stirred at room temperature for 4 h. The precipitates were collected and recrystallized from ethanol. The oxime benzoate dihydrochloride **6d** was prepared as follows. Benzoyl chloride (0.01 mol) was added dropwise to a solution of the free base of **6c** (0.01 mol) in pyridine (4 ml) at 0°C. The mixture was stirred at room temperature for 4 h and cooled overnight at –20°C. The deposited solid was collected, washed with ether, dried and recrystallization from ethanol afforded **6d**. Compound **7d** was synthesized by adding a solution of benzoyl chloride (0.01 mol) in benzene (4 ml) dropwise to an ice-cold solution of the free base of **7c** (0.01 mol) in benzene (5 ml). After stirring the solution at room temperature for 3 h, the precipitate was collected and recrystallized from ether–methanol to give **7d**.

*Synthesis of the quaternary ammonium iodides **1b, d, 2b, d, 3b, d, 4b, d, 5b, d, 6b, 7b***

Compounds **4a**, **c** were prepared as the free bases and in the other cases, aqueous solutions of the hydrochloride salts of the Mannich bases, oximes or oxime benzoates were basified with aqueous sodium carbonate solution (10% w/v) until basic to litmus. The free base was extracted with ether and the ethereal solution washed with water, dried and evaporation of the solvent afforded the free base.

To a solution of the free base (0.01 mol) in ice-cold ether (20 ml) was added dropwise a solution of methyl iodide (0.02 mol for monobasic amines; 0.03 mol for dibasic derivatives) in ether (10–15 ml). The reaction mixture was stirred at room temperature for 6–8 h and the precipitates were collected, dried and recrystallized from methanol. The ^{1}H-NMR spectrum (300 MHz) of a representative compound **4b** in PBS-d was as follows: δ: 1.66(5, d, 3a, 5a, 5e, 6a, 6e), 1.95(1, m, 3e), 2.50–2.65(2, m, 4a, 4e), 2.73(1, m, CH), 3.12(9, s, CH_3), 3.25(1, dd, CH_2, J = 13.7, 2.6 Hz) and 4.07(1, dd, CH_2, J = 13.7, 8.2 Hz).

Cytotoxicity screening

*Evaluation of **1–8** against L1210 cells*

The procedure for evaluating derivatives against L1210/C2 leukemia cells *in vitro* has been described in detail previously [40]. In brief, the compounds were dissolved in either dimethylsulphoxide or ethanol (95% v/v) and aliquots of these solutions were incubated for 48 h at 37°C. Each compound was examined in triplicate at 3 different concentrations and the IC_{50} values were determined graphically.

Evaluation in the NCI in vitro screen

Details of this assay have been described previously [30, 41]. The compounds described in this report were evaluated against an average of 53 human tumour cell lines. The $\log_{10}$ GI_{50} concentrations were < –4.00 M for all cell lines in the case of **1h**, **8a**; hence the figures in table I for these 2 derivatives are true mean values. For the remaining compounds, the $\log_{10}$ GI_{50} figures for some or all of the cell lines was > –4.00 M with the

following exceptions whereby the lowest concentrations used (compound, number of cell lines/total number of tumours) were –4.30 M (**1c**, 9/58) and –5.00 M (**1d**, 1/56; **2c**, 3/49; **3c**, 1/47; **3d**, 1/56; **6b**, 1/56; **7b**, 3/55). In addition, the $\log_{10}$ GI_{50} figures for 1 cell line for **1b** and **3d** were < –7.00 M and < –8.00 M respectively. Selective toxicity for leukemia was recorded for **1d**, **3d**, **6b**, **7b**, **8a**, **c** using the GI_{50} data and for **1a–c**, **3b**, **c** when the TGI figures were examined.

Evaluation against human colon tumours and a fibroblast

The data presented in table I were obtained by the following procedure. The culture media used for the 4 cell lines were RPMI 1640 for COLO-320-DM and OM-1 tumours, modified McCoy's 5A for HT-29 cells and Dulbecco's MEM for WI-38 fibroblasts. All tissue culture media was supplemented with 10% heat-inactivated fetal bovine serum. The compounds were dissolved in dimethylsulphoxide and aliquots were added to wells containing the cells in the appropriate media and incubated at 37°C in a humidified environment of carbon dioxide in air (5% v/v for 6 d). Quantification took place by a modification [42] of an assay [43] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the mean absorbance of triplicate drug-treated wells was compared to that of control wells. The IC_{50} values were calculated using a linear regression equation and were themselves used to ascertain tumour selectivity of any compound by calculating a specific tumouricidal index (STI) according to the formula: IC_{50} on non-transformed fibroblasts/IC_{50} on any colorectal tumor cell line. The experiment was repeated 3 times for compounds **1a**, **b**, **e**, **f**, **h**, **3b**, twice for **1c**, **g**, **8b**, **c** and once for **1d**, **6a**, **b**, **8a**.

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