Nuclear-Substituted Styryl Ketone Analogs: Effects on Neoplasms, Microorganisms, and Mitochondrial Respiration of Tumorous and Normal Cells

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Abstract □ Analogs of some antineoplastic and cytotoxic Mannich bases derived from conjugated styryl ketones were prepared and evaluated for activity in the P-388 lymphocytic leukemia screen. Most of the new compounds had lower antineoplastic and murine toxicity than the parent compounds. Antimicrobial evaluation of some oximes and alcohols related to the Mannich bases revealed activity against certain Gram-positive bacteria and fungi. Primary pharmacological evaluation showed that some compounds containing a dimethylaminomethyl group displayed analgesic and antihistaminic properties. Five of the Mannich bases were evaluated as respiratory inhibitors in mitochondria derived from hepatic tumors, liver tissue from tumor-bearing animals, and normal rat liver. No statistical difference between the sensitivity of the three tissues to the compounds was obtained.

Keyphrases □ Styryl ketones, nuclear substituted—effects on neoplasms, microorganisms, mitochondrial respiration in normal and tumor cells, structure–activity relationships □ Antineoplastic agents, potential—nuclear-substituted styryl ketones, effects on neoplasms, microorganisms, mitochondrial respiration in normal and tumor cells, structure–activity relationships □ Mitochondria—effect of nuclear-substituted styryl ketones on respiration, normal and tumor cells □ Mannich bases—derivatives, effects on neoplasms, microorganisms, mitochondrial respiration in normal and tumor cells, structure–activity relationships

Numerous Mannich bases derived from conjugated styryl ketones (I) have shown promising levels of antineoplastic activity accompanied by murine toxicity (1). The activity of these compounds is due, in part at least, to interference with mitochondrial function (2); examination of the effect of representative compounds on the electron transport chain of rat liver cells showed that competition with coenzyme Q_{10} is an important site of action (3). In addition, various studies revealed that Mannich bases have antimicrobial activity (4–7) as well as other bioactivities (8, 9), including analgesia in both Mannich bases (10) and their derivatives (11).

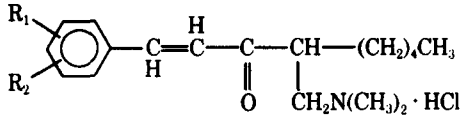
BACKGROUND

The aim of the present investigation was twofold. First, a study was contemplated involving a systematic modification of the lead compounds (I) to see if antineoplastic activity could be retained while toxicity was diminished. The assessment of the new derivatives for other bioactivities was also of interest. Second, this study compared the effects of representative Mannich bases on the mitochondrial electron transport chain derived from normal rat liver cells and from hepatomas. A structure–activity relationship to antineoplastic activity would permit the rational design of novel anticancer agents.

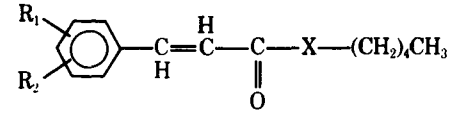
The modification of the Mannich bases (Ia–If) was through systematic alterations of the basic center, then of the lipophilic pentamethylene chain, and finally of the carbonyl group. Reaction of Ic and If with methyl iodide produced IIa and IIb, which are quaternary ammonium salts, representatives of which are known to cause lysis of cytoplasmic membranes (12). Furthermore, the addition of one bulky group onto the quadrivalent nitrogen atom (IIa and IIb) may reduce absorption onto serum proteins (13).

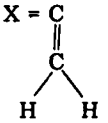
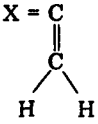
The activity of certain Mannich bases may be due to deamination to the corresponding conjugated carbonylene (14, 15), and thus IIc and IId may be breakdown products of the precursor derivatives Ic, If, IIa, and IIb *in vivo*. A comparison of the antitumor efficacies of these six compounds may allow certain conclusions about the active species. Compounds IIc and IId, while retaining the conjugated styryl ketone function permitting biological alkylation to occur (16–18), were expected to have considerably lower aqueous solubility than the precursor compounds. By contrast, Mannich bases IIIa and IIIb, in which the lipophilic pentamethylene chain of Ia had been removed, were predicted to have greater aqueous solubility than Ia. Recently, the biological activity of α,β-unsaturated ketones has been attributed partially to a judicious balance between their hydrophilic and hydrophobic portions (19).

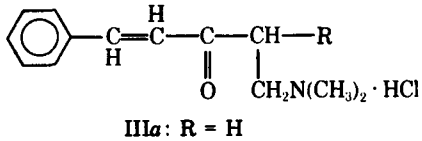
The final series of alkylating agents related to I involved ketone modification. Oximes may be ketone prodrugs (20), and the formation of IVa and IVb might allow selective release of the precursor ketones in tumorous tissue due to the acid lability of oximes (21); the claim has been made that the extracellular fluid of certain tumors is more acidic than



Ia: $R_1 = R_2 = H$
Ib: R_1 = 2-Cl, $R_2 = H$
Ic: R_1 = 4-Cl, $R_2 = H$
Id: R_1 = 2-Cl, R_2 = 4-Cl
Ie: R_1 = 2-Cl, R_2 = 6-Cl
If: R_1 = 3-Cl, R_2 = 4-Cl



IIa: R_1 = 4-Cl, $R_2 = H$, X = $CH(CH_2\overset{+}{N}(CH_3)_3\ \bar{I})$
IIb: R_1 = 3-Cl, R_2 = 4-Cl, X = $CH(CH_2\overset{+}{N}(CH_3)_3\ \bar{I})$
IIc: R_1 = 4-Cl, $R_2 = H$, X = 
IId: R_1 = 3-Cl, R_2 = 4-Cl, X = 



IIIa: R = H
IIIb: R = CH_3

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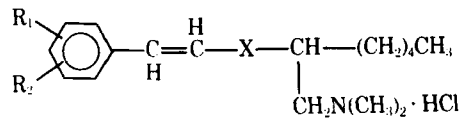
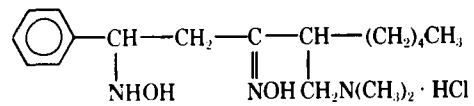


IV*a*: R_1 = 2-Cl, R_2 = H, X = C(=NOH)

IV*b*: R_1 = 3-Cl, R_2 = 4-Cl, X = C(=NOH)

IV*c*: R_1 = 2-Cl, R_2 = H, X = CH(OH)
IV*d*: R_1 = 4-Cl, R_2 = H, X = CH(OH)
IV*e*: R_1 = 2-Cl, R_2 = 4-Cl, X = CH(OH)
IV*f*: R_1 = 3-Cl, R_2 = 4-Cl, X = CH(OH)



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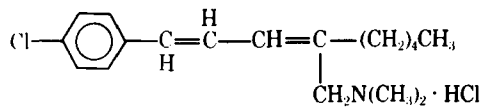
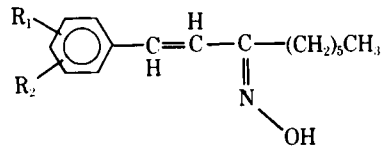
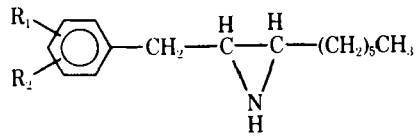


VI



VII*a*: R_1 = R_2 = H
VII*b*: R_1 = 2-Cl, R_2 = H
VII*c*: R_1 = 4-Cl, R_2 = H
VII*d*: R_1 = 2-Cl, R_2 = 6-Cl
VII*e*: R_1 = 3-Cl, R_2 = 4-Cl
VII*f*: R_1 = 4-N(CH$_3$)$_2$, R_2 = H



VIII*a*: R_1 = R_2 = H
VIII*b*: R_1 = 2-Cl, R_2 = H
VIII*c*: R_1 = 4-Cl, R_2 = H
VIII*d*: R_1 = 3-Cl, R_2 = 4-Cl

normal extracellular fluid (22, 23). In addition, an oxime derived from an α,β-unsaturated ketone had a ninefold increase in aqueous solubility over the precursor ketone (24). Reduction of the Mannich base ketone function would produce the substituted allyl alcohols IV*c*–IV*f*, expected to have alkylating properties due to the stability of the carbonium ion. In the case of these aryl-substituted alcohols, the carbonium ion would be expected to be further destabilized by delocalization of the positive charge on the ring. The murine toxicity of I may be associated with the dimethylaminomethyl function. Hence, nitrogenous analogs of conjugated styryl ketones that were not Mannich bases, were synthesized, namely the oximes VII as well as the aziridines VIII, representatives of which have displayed anticancer activities (25).

The second phase of the investigation involved comparing the effects of certain compounds on mitochondria derived from normal and from tumorous cells. Since the Mannich bases I*a* and I*d*–I*f* interfere with certain processes in the hepatocyte mitochondrial electron transport chain (3), an experimental rat hepatoma model was sought in which to examine the effects of these compounds on mitochondria from nontumorous tissue from normal and tumor-bearing rats.

Since 1960, various Morris hepatomas have become available (26). The 5123 TCH hepatoma chosen has a high mitochondria titer and is a highly differentiated stable growing tumor with an intermediate growth rate. This hepatoma grows in the pelvic region of the Buffalo rat strain, and the tumors develop within 4–5 weeks. Thus, by isolating the mitochondria from these three sources, the effects of I*a*, I*d*–I*f*, and III*a* in the presence of various substrates may permit conclusions about a mitochondrial sensitivity to the cytotoxic and antineoplastic Mannich bases.

RESULTS AND DISCUSSION

The first phase involved the synthesis and screening of derivatives I–VIII. In these compounds, the olefinic double bond of the styryl group was shown by ^{1}H-NMR and IR spectroscopy to have the (*E*)-configuration; ^{1}H and ^{13}C-NMR spectroscopy revealed that allyl alcohols IV*c* and IV*e* had the *threo*-configuration, while IV*f* was a 60:40 mixture of *erythro*- and *threo*-isomers. Purification of IV*d* led to the isolation of the *threo*-isomer (IV*d'*). After considerable difficulty, a small quantity of the *erythro*-isomer (IV*d"*) was isolated as the maleate salt. In the reaction between I*a* and hydroxylamine, the only product isolated was the hydroxylamino derivative, V. An attempt was made to form the IV*d* phosphate ester since the claim has been made that certain cancers contain high phosphatase (27). However, the product showed that IV*d* dehydration had occurred, producing the diolefin VI. NMR spectroscopy (100 MHz) showed that the aziridines VIII had the *cis*-configuration.

The activity of I–VIII against P-388 lymphocytic leukemia and their murine toxicity is summarized in Table I. The Mannich bases (I) increased the survival time in mice by an average 18%, and the related

Table I—Evaluation against P-388 Lymphocytic Leukemia and Murine Toxicity of Some Conjugated Styrenoid Ketones and Related Compounds

Compound	Maximum Increase in Mean Survival Time (dose in milligrams per kilogram)[a]	Murine Toxicity of Compound (dose in milligrams per kilogram)[b]	KB[c]
I*a*	105 (100)	0 (200), 6 (100)	1.5
I*b*	104 (6.25)	0 (200), 5 (100), 6 (50)	5.1
I*c*	113 (50)	0 (100), 6 (50)	1.7
I*d*	130 (18)	0 (200), 2 (100), 6 (50)	1.0
I*e*	115 (28)	2 (100), 6 (50)	2.8
I*f*	142 (6.25)	0 (55), 1 (35), 6 (12.5)	1.2
II*a*	113 (6.25)	0 (200), 4 (100), 6 (50)	2.1
II*b*	119 (12.5)	0 (200), 3 (50), 6(25)	—
II*c*	118 (400)	6 (200)	2.3
II*d*	109 (50)	0 (100), 6 (50)	5.0
III*a*	108 (50)	0 (200), 2 (100), 6 (50)	11
III*b*	109 (37.5)	0 (170), 3 (115), 6 (75)	1.2
IV*a*	112 (100)	2 (200), 6 (100)	—
IV*b*	116 (50)	1 (200), 5 (100), 6 (50)	—
IV*c*	117 (50)	0 (200), 3 (100), 6 (50)	3.2
IV*d'*	127 (25)	6 (50)[d]	2.6
IV*d"*	112 (25)	6 (50)[e]	2.5
IV*e*	108 (12.5)	0 (100), 5 (50), 6 (25)	—
IV*f*	109 (12.5)	0 (100), 3 (50), 6 (25)	—
V	106 (50)	2 (200), 6 (100)	—
VI	111 (50)	1 (200), 6 (100)	—
VII*a*	122 (50)	6 (200)	—
VII*b*	105 (200)	5 (200), 6 (100)	—
VII*c*	108 (100)	6 (200)	—
VII*d*	118 (400)	6 (400)	—
VII*e*	120 (200)	6 (200)	—
VII*f*	93 (100)	6 (400)	—
VIII*a*	115 (4.5)	0 (100), 5 (18), 6 (9)	—

[a] Figures are the ratios of the survival times of treated animals to those of control animals, expressed as a percentage. A compound is considered to be active if it increases the mean survival time by 25%. The data for I*d*–I*f* and III*b* are from Ref. 1 and are reproduced with permission of the copyright owner. [b] The murine toxicity of the compound is measured by the number of survivors out of six on the 5th day after the dosage schedule commenced. The data for Compounds I*a*, I*b* (200- and 100-mg/kg doses), I*d*–I*f*, II*a* (200- and 100-mg/kg doses), II*c*, III*b*, and IV*c* are from Ref. 1, and the data for II*d* (50-mg/kg dose) are from Ref. 2. These results are reproduced with permission of the copyright owner. [c] The figures, in micrograms per milliliter, in the KB cell culture screen indicate the dose inhibiting 50% of the growth of human epidermoid carcinoma of the nasopharynx in Eagle's medium. A compound is considered to be active if it inhibits 50% of the growth of this tumor at a concentration of 4 μg/ml. The data for I*c*–I*f*, II*a*, II*c*, III*b*, and IV*c* are from Ref. 1, and the result for III*a* is from Ref. 2. This information is reproduced with permission of the copyright owner. [d] The maximum dose administered with this compound was 50 mg/kg. [e] At 400 mg/kg, there were no deaths when the compound was administered twice on Days 2 and 6 to three animals.

Table II—Antimicrobial Evaluations of Some Oximes and Alcohols Derived from Conjugated Styryl Ketones [a]

Organism	IV*a*	IV*b*	IV*d'*	IV*e*	VII*a*	VII*b*	VII*c*	VII*d*	VII*e*	VII*f*
Escherichia coli (ATCC 8739)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Pseudomonas aeruginosa (ATCC 10145)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Klebsiella pneumoniae (ATCC 4352)	<100	<100	<100	<100	>500	>500	>500	>500	>500	>500
Haemophilus influenzae (ATCC 19418)	—	—	—	—	>500	>500	>500	—	>500	—
Staphylococcus aureus (ATCC 6538)	<100	<100	<100	<100	10	10	>500	<100	>500	>500
Streptococcus pyogenes (hospital isolate)	—	—	—	—	100	10	>500	—	>500	—
Streptococcus pneumoniae (ATCC 6303)	—	—	—	—	>500	10	>500	—	>500	—
Streptococcus faecalis (ATCC 8030)	<100	<100	<100	<100	100	10	250	<100	>500	>500
Bacillus subtilis (ATCC 6633)	<100	<100	<100	<100	10	10	10	<100	>500	>500
Salmonella typhimurium (G46)	<100	>500	>500	>500	>500	>500	>500	>500	>500	>500
Bordetella bronchiseptica (ATCC 4617)	<100	<100	<500	<100	—	—	—	>500	—	>500
Trichophyton mentagrophytes (ATCC 9533)	>500	<100	<100	<100	>500	>500	>500	<100	>500	<500
Microsporum gypseum (ATCC 14683)	>500	<100	<100	<100	>500	>500	>500	<100	>500	<500
Aspergillus niger (ATCC 10535)	>500	<500	<500	<500	>500	>500	>500	>500	>500	>500
Candida albicans (ATCC 10231)	>500	<500	<500	<500	>500	>500	>500	>500	>500	>500
Saccharomyces carlbergensis (ATCC 9080)	>500	<500	<500	<500	>500	>500	>500	>500	>500	>500
Average antimicrobial activity[b]	231	292	262	292	100	167	40	192	0	15

[a] Figures are the minimum inhibitory concentrations of the compounds in micrograms per milliliter. [b] Figures were calculated from (combined antimicrobial activity × 100)/number of microorganisms in the screen. The combined antimicrobial activity was determined by giving the following scores at the highest potency of the compound against the microorganisms: <500:1, 100:2.5, and <100:5.

synthons (II–VIII) caused an average 13% increase. Thus, antineoplastic activity was retained, albeit diminished. In general, murine toxicity also was retained but reduced except for the VII oximes, which did not contain the dimethylaminomethyl function. The oximes VII had an average 11% increase in mean survival time but were virtually bereft of murine toxicity.

All compounds examined in the *in vitro* KB screen displayed cytotoxicity. Quaternization of Ic gave IIa with similar effect in the P-388 screen and murine toxicity; the possible metabolite of both compounds, the $\alpha,\beta,\alpha',\beta'$-diolefinic ketone IIc, retained activity and decreased host toxicity. Quaternization of the highly active Mannich base If gave IIb with lower antineoplastic activity and lower murine toxicity, while the deaminated IId was virtually devoid of antileukemic potency, even though host toxicity was lower than in If. A comparison of the data for the unsubstituted Mannich bases Ia, IIIa, and IIIb revealed that while a similar low activity level in the P-388 screen was observed, murine toxicity increased as the chain length was shortened. While the average increase in mean survival time for Ib and If was 23%, the related oximes IVa and IVb increased survival time by 14% and host toxicity was reduced. The *threo*-isomeric allyl alcohol IV*d'* increased the mean survival time by twice that of either the *erythro*-isomer IV*d''* or the precursor Mannich base Ic.

The dichlorinated alcohols IVe and IVf had markedly inferior antineoplastic activity compared to the related Mannich bases Id and If. While three oximes, VIIa, VIId, and VIIe, showed an average 20% increase in the lifespan of the mice unaccompanied by host toxicity at the maximum doses administered, the aziridines VIIIa–VIIId proved lethal at 100 mg/kg. A representative aziridine, VIIIa, was examined further, but an increase of only 15% in mean survival time was found at the optimum dose level.

Some Mannich base derivatives were screened for antimicrobial activity (4, 5) and other pharmacological activities (11). Since α,β-unsaturated ketones have shown significant mammalian toxicity, it was felt that evaluating some related alcohols and oximes, in which the carbonylene group is masked, might lead to a greater chance of clinical acceptability if antimicrobial or pharmacological activity is found.

The antimicrobial results of 10 of the alcohols and oximes are listed in Table II. The results indicated that compounds containing a dimethylaminomethyl group, namely the oximes IVa and IVb and the alcohols IV*d'* and IVe, had the greatest activity and were virtually equipotent. In the VII series of oximes, the *o*-chloro and 2,6-dichloro oximes VIIb and VII*d* showed the greatest activity. The most sensitive bacteria to these compounds were the Gram-positive organisms *Staphylococcus aureus*, *Streptococcus faecalis*, and *Bacillus subtilis*. Compounds IVb, IV*d'*, and IVe all showed the same activity against the five fungi in contrast to IVa, which was inactive. The VII oximes were virtually devoid of antifungal activity.

Some Mannich bases have demonstrated analgesia (10), and the data generated from the primary pharmacological screening (Table III) indicate that the oximes and alcohols derived from these compounds have this property. Numerous narcotic analgesics have a common structural unit linked together, namely a tertiary nitrogen atom, a two-carbon chain, and a quaternary carbon atom to which an aryl ring is attached (28). The compounds bear some similarity to the general formula of narcotic analgesics, although a styryl group and not an aryl ring is attached to the quaternary carbon atom. The overlap of π-orbitals in this group of

Table III—Evaluation of Some Oximes and Alcohols Derived from Conjugated Styryl Ketones for Analgesic, Anti-Inflammatory, and Antihistaminic Activities

	Analgesic Activity[a]				Anti-Inflammatory Activity[b]			
	Percentage Protection[c]					Percentage Protection		
Compound	32	64	128	ED_{50}	Dose, mg/kg	3 hr	5 hr	Antihistaminic Activity[d]
IVa	75[e]	100	—	22	64	17	0	100[f]
IVb	7	42	96	64	—	—	—	79
IV*d'*	74	91	91	18	128	39	17	100[f]
					256	87	17	
IVe	52[e]	98	—	30	128	0	0	100[f]
VIIa	—	—	Algesic[g]	—	64	0	0	29
VIIb	—	—	Algesic[g]	—	64	25	50	13
VIIc	—	—	Algesic[g]	—	64	0	0	12
VII*d*	—	38	54	—	128	0	0	15
VIIe	—	—	6	—	64	0	0	7
VIIf	49	58	96	42	128	0	0	31

[a] Analgesic activity was measured by the percentage protection in the phenylquinone writhing test. Under these conditions, aspirin gave 50% protection at 52 mg/kg. A compound displaying an effect >50% is considered to be active. [b] Anti-inflammatory activity was measured by the percentage protection afforded by the compound to carrageenin-induced edema. The reference compound, indomethacin, gave 50% protection at 12 mg/kg under these conditions. Compounds showing >50% protection are considered active. [c] Dose levels in milligrams per kilogram. [d] Figures indicate the percentage protection of the compound at 0.1 mg/ml against a standard histamine dose. A compound is considered active if 20% protection is obtained. The reference compound, diphenylhydramine, gave 100% protection at 0.01 mg/ml. [e] Percentage protections at 16 mg/kg for IVa and IVe were 42 and 10, respectively. [f] At a dose level of 0.01 mg/ml, IVa, IV*d'*, and IVe inhibited the histamine action by 52, 70, and 100%, respectively. At 0.001 mg/ml, IVa, IV*d'*, and IVe inhibited the action of histamine by 29, 18, and 52%, respectively. [g] Percentage increases in the number of writhes for VIIa, VIIb, and VIIc were 18, 31, and 6, respectively.

Table IV—Inhibition of Respiration of Mitochondria Obtained from the Hepatic Tumor 5123 TCH, from the Liver of the Tumor-Bearing Animal, and from Normal Rat Liver by the Mannich Bases Ia, Id–If, and IIIa Using Succinate as the Substrate

Compound	Concentration, μmoles	Hepatic Tumor 5123 TCH				Hepatic Tissue from Tumor-Bearing Animal				Normal Rat Liver			
		Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE
Ia	5.0	96.69	1.07	−0.61	2.43	96.62	1.72	2.40	0.80	85.10	1.68	2.98	2.63
	2.5	66.35	6.15	2.44	6.38	84.27	2.88	−4.49	2.41	85.00	5.77	−4.74	2.34
	0.5	4.70	4.64	10.83	54.92	−4.57	12.30	−30.20	46.99	−25.42	22.71	−0.58	28.70
Id	5.0	97.38	1.00	−2.75	1.06	97.35	1.62	−1.87	1.21	94.27	2.26	−2.01	0.96
	2.5	95.62	1.52	−0.58	1.81	96.18	1.43	−1.00	1.13	91.47	2.99	−4.65	2.14
	0.5	85.09	3.05	11.96	2.36	83.26	2.84	10.29	2.98	71.08	6.26	2.59	2.53
	0.1	21.05	3.99	−4.62	41.17	51.06	10.70	−13.10	19.77	23.29	27.08	−12.37	7.97
Ie	5.0	97.14	1.06	−1.79	0.55	94.34	2.20	−2.37	1.93	97.49	1.64	0.21	0.38
	2.5	101.6	3.35	−1.05	0.94	89.69	2.08	−4.78	1.34	95.85	2.00	0.049	0.75
	0.5	83.29	4.07	14.53	3.71	74.40	3.92	2.10	0.97	74.44	6.59	8.81	4.20
	0.1	22.61	7.58	47.98	17.09	6.57	12.31	−29.02	23.33	21.92	13.24	7.37	10.42
If	5.0	95.60	2.12	−0.88	0.88	97.55	1.53	1.55	1.02	95.00	1.30	−3.73	1.45
	2.5	84.69	8.78	5.30	3.31	95.22	3.08	−1.20	1.03	94.27	1.85	−1.59	1.52
	0.5	90.78	4.24	5.81	3.59	78.68	8.47	3.45	1.49	76.76	5.37	−5.78	6.21
	0.1	44.53	17.23	−32.25	21.27	36.21	20.18	−37.64	15.16	49.92	8.81	−17.97	9.54
IIIa	5.0	10.83	4.50	−64.41	28.39	−10.08	7.02	−52.87	45.27	1.86	12.17	−44.33	31.12

[a] The percentage increase in respiration indicates the change in respiration after ubiquinone addition.

compounds may not preclude alignment at a nonpolar portion of the receptor, which is normally occupied by the aryl ring. The IV*a* and IV*d'* monochloro derivatives had, on the average, approximately 2.4 times the potency of IV*b* and IV*e*, which have two chlorine atoms in the aromatic ring and may exert some steric impedance to binding at a receptor. Codeine analgesia, expressed as an ED_{50} value, is 14.2 mg/kg in the hot plate test (29); and although the compounds listed in Table III were evaluated by the phenylquinone writhing test, it may be considered that IV*a* and IV*d'* are similar in potency to codeine, while IV*e* and IV*b* are approximately one-half and one-fifth as active as this widely used drug.

The VII oximes displayed a wide spectrum of response in the analgesia screen, ranging from algesia (VII*a*–VII*c*) to weak analgesic activity (VII*d* and VII*e*) to VII*f*, which had approximately one-third of the activity of codeine. The demonstration of analgesia by certain of these compounds would be enhanced if anti-inflammatory properties were also found in the active compounds. Table III indicates that none of the derivatives met the criterion for activity at 3 and 5 hr.

Table III indicates that all of the compounds examined in the antihistaminic screen displayed activity, with maximum potency shown by the dimethylaminomethyl compounds IV*a*, IV*b*, IV*d'*, and IV*e*. These derivatives are related structurally to the propylamine group of antihistaminics, in which halogenation of the aromatic ring enhances activity (30). Both of the alcohols IV*d'* and IV*e* as well as the oxime IV*a* showed histamine-inhibiting properties at a concentration as low as 0.001 mg/ml. None of the compounds listed in Table III demonstrated activity in the cardiovascular, hypoglycemic, and antianaphylactic screens, except IV*e* which inhibited passive cutaneous anaphylaxis in rats by 75% at a dose of 75 mg/kg.

The second phase of the work involved the examination of the effect of the Mannich bases Ia, Id–If, and IIIa on respiration of mitochondria obtained from the 5123 TCH hepatic tumor and from the tumor-bearing animal as well as from normal rat liver using different substrates (Tables IV–VII). Statistical evaluation showed no difference (at $p = 0.05$, analysis of variance) in sensitivity of the three types of tissue to the Mannich bases.

In summary, this work has shown that molecular modification of the Mannich bases (I) produced compounds of lower murine toxicity, generally accompanied by reduced anticancer activity. The results of mitochondrial respiration inhibition studies likewise showed that five of the compounds were not selective for mitochondria from tumor cells.

Table V—Inhibition of Respiration of Mitochondria Obtained from the Hepatic Tumor 5123 TCH, from the Liver of the Tumor-Bearing Animal, and from Normal Rat Liver by the Mannich Bases Ia, Id–If, and IIIa Using Glutamate as the Substrate

Compound	Concentration, μmoles	Hepatic Tumor 5123 TCH				Hepatic Tissue from Tumor-Bearing Animal				Normal Rat Liver			
		Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE
Ia	5.0	75.54	5.93	1.01	5.44	83.59	4.58	−6.83	6.51	89.16	1.03	−2.77	4.21
	2.5	67.39	3.85	−15.16	7.02	75.36	6.24	−8.14	3.66	61.30	3.54	−0.61	1.41
	0.5	42.88	9.81	1.44	11.85	7.17	9.36	19.77	26.91	31.44	11.22	−11.47	17.10
Id	5.0	81.78	4.33	−8.17	4.65	88.74	2.58	−1.22	2.79	84.19	4.11	−1.06	4.60
	2.5	77.24	2.90	−13.55	4.95	87.44	2.47	−5.86	2.77	81.64	2.02	−0.58	1.62
	0.5	50.05	3.11	−24.80	11.49	85.91	0.68	−1.89	1.83	73.77	4.72	6.45	9.52
	0.1	23.40	11.67	−13.39	6.74	78.58	3.16	−5.36	2.99	−22.38	30.43	−11.56	20.53
Ie	5.0	90.06	3.47	0.096	6.72	83.70	4.32	−4.90	2.90	81.69	4.41	−1.27	3.71
	2.5	75.73	3.22	−11.52	9.87	89.91	3.75	−0.46	1.91	81.22	2.61	−2.17	3.97
	0.5	68.45	2.50	−16.04	7.90	82.37	2.29	−2.78	3.19	75.23	2.48	−6.37	6.51
	0.1	44.42	9.06	−1.73	13.31	68.68	7.77	−9.82	1.52	29.12	23.30	−25.05	17.93
If	5.0	86.61	3.08	0.36	7.91	92.82	3.23	2.95	4.62	91.76	2.81	3.44	5.00
	2.5	80.06	6.89	−13.42	15.30	88.83	3.34	1.49	2.18	85.47	2.39	−3.71	2.70
	0.5	57.18	4.49	−27.68	5.91	81.08	3.65	−0.39	2.71	81.92	6.06	−5.89	2.13
	0.1	47.77	13.65	−24.71	8.95	78.09	4.93	−3.05	2.76	72.41	5.36	−7.14	3.36
IIIa	5.0	6.87	3.62	−78.15	17.10	18.75	12.85	7.54	13.21	4.45	11.14	−46.67	46.67

[a] The percentage increase in respiration indicates the change in respiration after ubiquinone addition.

Table VI—Inhibition of Respiration of Mitochondria Obtained from the Hepatic Tumor 5123 TCH, from the Liver of the Tumor-Bearing Animal, and from Normal Rat Liver by the Mannich Bases Ia, Id–If, and IIIa Using 3-Hydroxybutyrate as the Substrate

Compound	Concentration, μmoles	Hepatic Tumor 5123 TCH				Hepatic Tissue from Tumor-Bearing Animal				Normal Rat Liver			
		Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE
Ia	5.0	95.16	2.75	−1.38	1.13	94.89	2.07	−2.99	1.38	92.49	0.67	−2.20	1.16
	2.5	92.59	2.59	−4.47	2.39	96.06	2.00	−1.90	1.66	93.43	1.35	−0.19	1.70
	0.5	23.20	12.14	−125.9	31.37	−21.82	38.68	−8.55	5.13	−26.75	15.98	−19.23	17.44
Id	5.0	97.57	1.32	−1.72	1.36	92.79	2.22	−6.38	2.26	96.64	1.71	−1.27	1.14
	2.5	97.83	3.09	−0.73	2.53	95.28	2.24	0.64	2.14	94.73	2.00	−1.58	1.28
	0.5	93.72	3.01	−7.80	4.44	92.41	4.88	−8.92	6.95	98.02	0.80	−1.37	1.13
	0.1	54.19	10.61	−51.62	19.46	90.42	4.11	3.14	3.43	33.10	29.67	−1.54	1.54
Ie	5.0	99.70	0.27	0.27	0.27	96.15	1.51	−2.34	0.96	91.90	2.19	−2.67	1.68
	2.5	97.11	1.85	−0.51	3.51	94.53	1.47	−3.49	1.56	96.47	1.87	−1.81	1.05
	0.5	92.99	3.49	−3.33	1.65	95.74	2.40	−1.54	2.52	96.41	1.28	−0.94	0.95
	0.1	69.17	13.79	−20.79	12.11	93.79	1.65	−3.46	1.02	−55.73	28.36	−24.56	17.88
If	5.0	93.90	2.03	−4.07	2.28	97.76	0.84	−1.17	0.53	98.75	1.25	−0.33	0.33
	2.5	93.22	1.72	−6.47	1.85	97.56	0.99	−1.90	0.78	97.11	1.23	−1.87	1.90
	0.5	89.01	3.37	−4.90	3.82	97.67	0.83	−0.89	0.67	97.91	1.91	−1.70	1.70
	0.1	58.21	2.24	−52.97	21.57	95.56	1.09	−3.59	1.37	76.45	22.02	0.16	0.16
IIIa	5.0	18.27	8.18	−36.35	10.08	14.46	9.59	−81.10	27.19	−21.91	8.63	−16.77	12.37

[a] The percentage increase in respiration indicates the change in respiration after ubiquinone addition.

Table VII—Inhibition of Respiration of Mitochondria Obtained from the Hepatic Tumor 5123 TCH, from the Liver of the Tumor-Bearing Animal, and from Normal Rat Liver by the Mannich Bases Ia, Id–If, and IIIa Using Palmitylcarnitine as the Substrate

Compound	Concentration, μmoles	Hepatic Tumor 5123 TCH				Hepatic Tissue from Tumor-Bearing Animals				Normal Rat Liver			
		Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE	Inhibition, %	SE	Increase in Respiration[a], %	SE
Ia	5.0	87.97	6.02	2.05	9.84	88.84	2.23	4.22	2.75	89.04	4.39	19.86	5.11
	2.5	51.85	7.01	−29.37	12.39	77.88	10.15	−17.67	16.74	−30.02	33.32	−24.37	19.51
	0.5	−26.17	8.35	10.16	6.30	−61.49	17.48	51.99	24.57	−51.86	16.34	23.18	38.94
Id	5.0	85.24	4.29	−0.05	5.31	89.21	3.42	−7.13	4.15	82.12	5.54	−1.16	2.58
	2.5	81.74	5.07	−8.94	10.42	91.26	2.71	−1.44	4.49	77.65	5.87	−2.85	4.09
	0.5	2.38	16.93	−71.21	29.14	83.99	2.80	−9.38	2.96	−18.53	28.21	−31.67	20.80
	0.1	−25.77	4.74	79.23	28.93	−19.96	8.24	−55.53	37.91	−57.76	30.49	−128.4	61.18
Ie	5.0	89.35	3.71	−0.09	2.62	86.80	2.03	−1.77	2.15	71.89	6.02	−1.84	4.43
	2.5	86.70	4.67	−0.48	2.23	87.44	2.20	−1.28	2.45	61.85	7.40	−2.11	12.53
	0.5	22.93	11.17	−36.30	28.82	56.24	12.88	−14.94	12.11	−16.31	21.19	−11.41	28.94
	0.1	−20.13	6.02	74.52	18.14	−46.65	23.45	3.88	10.05	−69.09	9.42	64.25	10.90
If	5.0	96.45	1.60	3.07	1.60	96.13	2.13	−3.51	2.59	95.00	2.89	−2.62	2.48
	2.5	81.48	2.48	−4.46	6.38	90.93	0.57	−3.01	2.33	91.24	1.39	2.03	5.69
	0.5	74.67	8.74	−12.51	9.94	65.81	25.86	4.37	10.04	59.62	8.40	−31.72	14.01
	0.1	−68.39	13.27	71.82	60.09	−50.89	47.13	−9.36	674.6	−83.61	55.68	−19.41	45.80
IIIa	5.0	12.80	10.58	−56.56	47.96	−39.03	5.07	14.01	20.58	−57.71	30.26	−64.10	45.30

[a] The percentage increase in respiration indicates the change in respiration after ubiquinone addition.

EXPERIMENTAL

Compound Preparation—The preparation of Ia–If, IIa, and IIc was described previously (31). Compounds IIb, IId, and IIIa were prepared by the literature methodology (2) as was IIIb (1). The synthesis of IVa, IVb, IVe, IVf, and V–VIII was accomplished using a reported procedure (32), and the alcohols IVc and IVd were prepared by the method described previously (33). Compounds IVc and IVe possessed the *threo*-configuration; IVd was obtained as the pure *threo*-isomer, IVd′ (33), and a small quantity of the *erythro*-isomer (IVd″) was isolated as the maleate salt (33). The alcohol IVf was shown by spectroscopic means to consist of a 60:40 mixture of the *erythro*- and *threo*-isomers (32).

Compound Screening—The data summarized in Table I were generated by the Drug Research and Development Division of the National Cancer Institute, using their protocols (34). The compounds were administered in saline (Ia, Ib, Id, If, IIb, IIIa, IIIb, IVa–IVd′, IVe, and IVf), saline with polysorbate 80[1] (Ic, VIId, VIIf, and VIIIa–VIIId), hydroxypropylcellulose (Ie, IIa, IIc, IVd″, V, VI, VIIa–VIIc, and VIIe), and an unspecified solvent (IId) by the intraperitoneal route. The CD_2F_1 strain of mice was used for all compounds except Ie, If, IIa, IIc, IIIb, IVc, IVd′, and IVd″, in which cases the $B_6D_2F_1$ strain of mice was employed.

Injections were made daily for 9 consecutive days, except in the following cases. The maximum increase in mean survival times recorded in Table I for Ie was obtained by making three injections every 4 days; for IIa and IIc, only one injection was made. In addition, the murine toxicity data found in Table I for Id were obtained when three injections were made every 4 days, and the toxicity of IIa at the 50-mg/kg dose level was found when one injection was made. The toxicity data for IIb and IId at the 200- and 100-mg/kg dose levels, respectively, were derived from two injections separated by a 4-day period. The toxicity data for IVc at the 200-mg/kg dose level were obtained by making three injections every 4 days.

For the antimicrobial screen[2], the compounds were dissolved in water or dimethyl sulfoxide and diluted serially to various concentrations. The concentrations of stock solutions were prepared such that when 0.5 ml

[1] Tween 80, Atlas Chemical Industries, Wilmington, DE 19899.

[2] The antimicrobial and pharmacological screens were carried out by Bio-Research Laboratories Ltd., Montreal, Quebec, Canada.

was added to 15 ml of agar medium, the desired final concentrations were obtained. The growth medium for the bacteria was trypticase soy agar with 5% defibrinated rabbit blood added for the growth of *Haemophilus influenzae*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. Modified Sabouraud agar was employed for the growth of the fungi. The test organisms were grown previously for 2 days at 35° for bacteria and yeast and for 1 week at 24° for fungi on slants of the same media. The agar plates were streaked with a loopful of cell suspension, which had been washed off the slants and diluted to approximately 10^5 organisms/ml. The plates were incubated for 2–14 days at 24° for fungi and at 35° for bacteria. The results in Table II indicate the minimal inhibitory concentration of the compound that prevents visible growth in the media.

Unless otherwise stated, the same number of animals was used for controls as for pharmacological evaluation of the compound. The screen employed for examining the compounds for analgesic activity was the phenylquinone writhing test (35). Five male Swiss albino mice, 18–22 g, were used for each dose for each compound. The ED_{50} values for five of the compounds listed in Table III were obtained by using five mice at doses of 16, 32, and 64 mg/kg for IV*a* and IV*e* and at 32, 64, and 128 mg/kg for IV*b*, IV*d'*, and VII*f*.

The anti-inflammatory screen measured the antagonism of the test compound to carrageenin-induced rat paw edema (36). Six female Sprague–Dawley rats, 120–160 g, were used for the compounds listed in Table III, except for VII*a*–VII*c* and VII*e* where four animals were used. The edema volume was recorded at the end of 3 and 5 hr. The compounds were examined for antihistaminic activity using four guinea pig ileum preparations (37). The antianaphylactic screen measured the potential of a compound to inhibit passive cutaneous anaphylaxis in rats (38). Four female Sprague–Dawley rats were used for each compound. Compounds were administered intravenously at 125 mg/kg for IV*a*, 100 mg/kg for IV*b* and VII*d*, 25 mg/kg for IV*d'* and IV*e*, 128 mg/kg for VII*a*–VII*c* and VII*e*, and 50 mg/kg for VII*f*. A compound was considered active if it reduced wheal formation by 50%. The reference compound, chromolyn sodium, reduced the wheal area by 90% at a dose of 100 mg/kg.

The antidepressant screen measured the antagonism of a compound to tetrabenazine-induced ptosis in mice (39, 40). Six male Swiss albino mice were used for each compound; the compounds were administered subcutaneously, except VII*a*–VII*c* and VII*e*, which were given intraperitoneally. A dose of 128 mg/kg was used for all compounds except IV*a* and IV*e*, where the dose was 64 mg/kg. A compound should antagonize the tetrabenazine effect by 30% after 30 min and by 10% after 60 min to be considered active. The reference compound, amitriptyline, caused an antagonism of 90 and 50% after 30 and 60 min, respectively, at a dose of 8 mg/kg.

In the cardiovascular screen, a Sprague–Dawley rat of either sex, 250–400 g, was anesthetized by intraperitoneal urethan injection (1.9 g/kg). After cannulation of the femoral vein, the blood pressure was monitored *via* a pressure transducer[3] connected to a cannula in the left carotid artery. The arterial blood pressure and heart rate were recorded on a polygraph[4]. After the preparation had stabilized, the test compound was dissolved in saline and/or polysorbate 80[1] (1%) and administered in a volume of 0.01–0.05 ml. The changes in blood pressure and heart rate were expressed as percentage differences between the pre- and postdrug values. One or two determinations using one rat for each compound were made at the following dose levels: IV*a*, IV*b*, and VII*d* at 20 mg/kg; IV*d'*, IV*e*, and VII*f* at 10 mg/kg; and VII*a*–VII*c* and VII*e* at both 1 and 5 mg/kg. A compound should increase the blood pressure and heart rate by 30% for a time period considerably in excess of 5 min to be considered active.

The screen for hypoglycemic activity involved the measurement of blood sugar concentration in rats (41). Four male Sprague–Dawley rats were used for each compound. Compounds IV*b*, IV*d'*, IV*e*, VII*d*, and VII*f* were administered at a dose of 128 mg/kg po. A dose of 64 mg/kg po was employed for IV*a*. Compounds VII*a*–VII*c* and VII*e* were administered at 100 mg/kg iv. A 20% decrease in blood sugar concentration is required for a compound to be considered active in this screen.

Inhibition of Respiration in Mitochondria Obtained from Hepatic Tumor 5123 TCH and from Rat Liver—The inoculation of the hepatic tumor 5123 TCH into rats proceeded as follows. Adult, male Buffalo strain rats[5] containing a 4–5-week-old tumor[6] were sacrificed and the tumors were removed. The healthy tumor tissue was dissected from the remainder of the neoplasm. A 10% suspension of the tumor tissue in normal saline was prepared and placed in a glass hand homogenizer[7], after which two or three thrusts with a glass pestle were undertaken. The suspension (1 ml) was injected subcutaneously into each side of the inguinal regions of a male Buffalo rat, 100 g.

The mitochondrial pellet containing tumorous tissue was prepared as follows. Adult, male Buffalo rats containing a 4–5-week-old 5123 TCH tumor were sacrificed by decapitation and the tumors were removed. The healthy tumor tissue (5 g) was dissected from the remaining tumor mass and made into a 25% suspension using ice-cold 3.4 m*M* tromethamine hydrochloride[8] buffer (pH 7.40 at room temperature). The 3.4 m*M* tromethamine hydrochloride buffer contained 0.25 *M* sucrose and 1.0 m*M* ethylene glycol-bis(aminoethyl)tetraacetate. All of the following procedures were carried out at 0°.

The tumor tissue was homogenized with 15–20 thrusts with a pestle in a glass homogenizer[7] and then diluted to a 10% homogenate with buffer. The mitochondrial pellet, prepared from the homogenate using the reported methodology (3), was suspended in cold buffer (10 ml) to give a protein concentration of approximately 6 mg/ml; 0.5 ml of this suspension was used in each determination. Homogenates from 10 g of liver from tumor-bearing animals and normal Buffalo rats were prepared in a similar manner and suspended in 10 ml of buffer. One milliliter of this suspension was diluted with 4 ml of buffer, giving a suspension with a protein concentration of ~5 mg/ml; 0.5 ml of this suspension was used in each determination.

The media and methodology for measuring the effect of the Mannich bases on mitochondrial respiration were reported previously (3). The percentage increase in respiration was measured by adding 5 μl of a solution of ubiquinone (9 nmoles) in ethanol to the cell approximately 2 min after the addition of the Mannich base and noting the effect on respiration. The results in Tables IV–VII represent the mean values of five to eight separate determinations. In every case that mitochondria were used, the protein content was determined by a modification (42) of the previously described procedure (43). Results were evaluated statistically by the analysis of variance using the SPSS-Anova program (44) and a computer[9].

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Preparation of Diastereomeric Urethane Derivatives of Atropine and *l*-Hyoscyamine Using (−)-1-Phenylethylisocyanate

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Abstract □ Diastereomeric urethane derivatives of atropine (*d,l*-hyoscyamine) and *l*-hyoscyamine were prepared by reacting the alkaloids with (−)-1-phenylethylisocyanate. The derivatives, as the picrate ion-pairs, were characterized by their melting points, optical rotations, elemental analyses, and IR, NMR, and UV–visible spectra.

Keyphrases □ Diastereomers—atropine and *l*-hyoscyamine urethane derivatives, chemical synthesis □ Atropine—derivatives, urethane diastereomers, chemical synthesis □ Hyoscyamine—derivatives, urethane diastereomers, chemical synthesis

In an investigation designed to develop a procedure for the quantitation of *d*-hyoscyamine and *l*-hyoscyamine at therapeutic levels in finished drug dosage forms, a method for the preparation and isolation of stable diastereomeric urethane picrate derivatives was found. The derivatives were prepared by reacting the chiral isocyanate reagent (−)-1-phenylethylisocyanate with *l*-hyoscyamine and atropine (*d,l*-hyoscyamine).

The preparation of suitable alcohol derivatives generally is based on ester formation by reactions with acid chlorides or acid anhydrides (1–3) or on urethane formation by reaction with isocyanates (4–6). Little use of optically active isocyanates for the racemic mixture resolution has been reported in the literature. The reaction of (−)-menthylisocyanate with alcohols (7), the use of (−)-menthylisocyanate to prepare derivatives with *tert*-butyl alcohol, lactic acid, and amino acids (8), and the reaction of α-phenylethylisocyanates with amines, alcohols, and Grignard reagents (9) have been studied. Optical activity was retained during these reactions, and the respective derivatives possessed a larger optical rotation than the reagent itself.

Other investigators (10) reported that (−)-1-phenylethylisocyanate does not polymerize and can be stored for long periods, neat or in solution, without a change in the optical rotation value. GLC has been used (11) for the

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