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RESEARCH ARTICLES

Evaluation of Mannich Bases and Related Compounds as Inhibitors of Mitochondrial Function in Yeast and Inhibition of Blood Platelet Aggregation, Blood Clotting, and *In Vitro* Metabolism of 5-Dimethylamino-1-phenyl-1-penten-3-one Hydrochloride

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Abstract □ 5-Dimethylamino-1-phenyl-1-penten-3-one hydrochloride (Ia) and 32 analogs were tested for inhibition of respiratory-dependent growth in *Saccharomyces cerevisiae*. Thirteen of the 33 compounds tested appeared to affect mitochondrial function, since the inhibition of respiratory-dependent growth was statistically greater than the inhibition of growth on fermentable energy sources. Inhibition of mitochondrial function in yeast and growth inhibition of an *in vitro* culture of human epidermoid carcinoma (KB) were positively correlated since 83% of the compounds tested either had mitochondrial-inhibiting properties and significant activity in the KB test or were inactive in both tests. Similarly, 78% of compounds tested showed murine toxicity and mitochondrial inhibition or had no effect on murine toxicity and yeast mitochondrial function. Injection of Ia into rats resulted in the appearance of blood in the urine and feces. Compound Ia inhibited adenosine diphosphate and collagen-induced aggregation of rat platelets but had no effect on blood clotting. TLC, following incubation of Ia with a rat liver extract, showed that the structure of Ia was not enzymatically modified and indicated activity *per se* on platelet aggregation and mitochondrial function.

Keyphrases □ Mannich bases—effect on mitochondrial function in yeast, human epidermoid carcinoma cultures, blood platelet aggregation, blood clotting, rats □ Mitochondrial activity—effect of Mannich bases and related compounds, yeast □ Blood platelet aggregation—effect of Mannich bases and related compounds, rats □ Coagulation, blood—effect of Mannich bases and related compounds, rats □ Structure-activity relationships—effect of Mannich bases and related compounds on mitochondrial function in yeast, human epidermoid carcinoma cultures, blood platelet aggregation and blood clotting, rats

Mannich bases have a wide range of biological activity including antineoplastic properties (1, 2), antimicrobial effects (3–5), analgesic activity (6), local anesthetic properties (7, 8), and psychotropic effects (9). To pursue a continuing interest in Mannich reactions (10–12) and α,β-unsaturated ketones (13, 14), several properties of a simple Mannich base, 5-dimethylamino-1-phenyl-1-penten-3-one (Ia), derived

Table I—Effect on Yeast Mitochondria, Activity against the KB Tumor, and Mammalian Toxicity of Some Mannich Bases and Related Compounds

Compound	Concentration, mg/ml	Growth on Complex Ethanol Medium: Mean Area of Inhibition, mm²	Growth on Complex Ethanol Medium: SE	Growth on Complex Glucose Medium: Mean Area of Inhibition, mm²	Growth on Complex Glucose Medium: SE	KB[a]	Maximum[b] Tolerated Dose, Daily Injections, mg/kg	Maximum[b] Tolerated Dose, Injections Every 4 Days, mg/kg
Ia	5	32.4	4.5	0.0[c]	—	N.A.	100[d]	100[d]
Ib	5	70.4	9.4	0.0[c]	—	1.2	75	100
IIa	5	307.7	35.0	15.2	5.2	1.5	100	200
IIb	1	55.8	8.9	0.0[c]	—	2.4	<100	N.A.
IIc	1	102.2	16.5	0.0[c]	—	1.7	75	100
IId	1	123.2	10.5	41.5	15.4	1.0	12.5	50
IIe	1	41.5	5.6	0.0[c]	—	2.8	50	N.A.
IIf	1	128.7	8.9	27.9[c]	—	1.2	25	25
IIg	5	0	—	0	—	2.1	<100	100
IIh	5	11.16	6.83	27.9[c]	—	N.A.	N.A.	100[e]
IIIa	5	0	—	0	—	>100	N.A.	<100
IIIb	5	0	—	0	—	23	>400	N.A.
IIIc	5	0	—	0	—	>10	>400	>400[d]
IIId	5	0	—	0	—	30	>400	>400
IIIe	5	0	—	0	—	28	>400	>400
IIIf	5	0	—	0	—	N.A.	N.A.	>350[f]
IIIg	5	0	—	0	—	27	100	>400
IIIh	5	0	—	0	—	>100	N.A.	>400
IIIi	1	90.5	6.3	0.0[c]	—	24	50	100
IIIj	5	0	—	0	—	N.A.	N.A.	>400
IIIk	5	0	—	0	—	25	>400	>400
IIIl	5	0	—	0	—	N.A.	N.A.	>400
IIIm	5	100.9[c]	—	0.0[c]	—	N.A.	>175[g]	N.A.
IV	5	0	—	0	—	100	200	>400
Va	5	0	—	0	—	2.3	>200	>400
Vb	5	0	—	0	—	N.A.	N.A.	50[h]
VIa	5	369.5	15.7	46.4	8.8	6.4	>50	200
VIb	5	0	—	0	—	42	>10	10
VIc	1	194.9	11.8	0.0[c]	—	3.2	50	50
VId	5	0	—	0	—	>100	N.A.	>400
VIe	5	0	—	0	—	>10	200	200
VIIa	1	164.5	16.9	0.0[c]	—	N.A.	N.A.	>400[h]
VIIb	5	0	—	0	—	N.A.	>400	N.A.

[a] The figures, in micrograms per milliliter, in the KB cell culture screen indicate the dose inhibiting 50% growth of human epidermoid carcinoma of the nasopharynx in Eagle's medium. N.A. = result not available. With the exception of IV, the data are taken from Ref. 1 and are reproduced with permission. [b] In virtually all cases the compounds were injected intraperitoneally into the BDF_1 strain of mouse at doses of 400, 200, and 100 mg/kg. If toxicity appeared, the doses were reduced. The figures in the columns indicate 6/6 survivors. With the exception of Ia, IIh, IV, Vb, and VIIa, the data are taken from Ref. 1 and are reproduced with permission. N.A. = result not available. Daily injections were made for 9 days, and a total of three injections was given when injections were made every 4 days. [c] All five replicates gave the same value. [d] The CDF_1 strain of mouse was used. [e] Three BDF_1 strains of mice only were used. [f] One injection only at 350 mg/kg. [g] The DBA/2 strain of mouse was used. [h] Three BDF_1 strains of mice only were used, and two injections of compound were made.

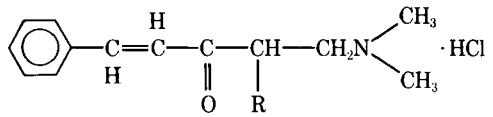
from an α,β-unsaturated ketone, were studied, and the bioactivity of closely related analogs was examined.

RESULTS AND DISCUSSION

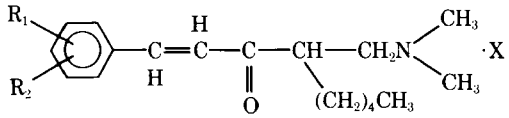
Compounds containing a benzylidene group attached to electron-withdrawing functions such as cyano and nitro moieties interfere with mitochondrial function (15), and certain anticancer agents interfere with mitochondria (16). To examine the possibility of Ia being a mitochondrial poison, a solution of this component was added to disks on complex ethanol medium and complex glucose medium which were inoculated with *Saccharomyces cerevisiae*. In the absence of mitochondrial function, the yeast will grow on complex glucose medium, which provides energy for growth by fermentation of glucose. However, the complex ethanol medium containing a nonfermentable energy source does not provide energy for the growth of the yeast if interference with mitochondrial function occurs. The antimitochondrial activity of Ia, an activity that is significantly more potent than established inhibitors such as chloramphenicol and tetracycline (17), is shown in Table I.

Further confirmation of Ia being a mitochondrial inhibitor was obtained by examining the effect on growth inhibition of *S. cerevisiae* when grown in liquid media containing nonfermentable energy sources (ethanol, glycerol, and acetate) and fermentable energy sources (glucose and galactose). Growth in complex galactose medium and minimal salts glucose medium containing fermentable energy sources required a concentration of Ia greatly exceeding that required for a 50% inhibition of growth in minimal salts ethanol medium, complex glycerol medium, and complex acetate medium, which contain nonfermentable energy sources (Table II).

Two structural analogs of Ia then were examined, in which one hydrogen atom of the methylene group vicinal to the ketone func-



Ia: R = H
Ib: R = CH_3



IIa: $R_1 = R_2 = H$, X = HCl
IIb: R_1 = 2-Cl, R_2 = H, X = HCl
IIc: R_1 = 4-Cl, R_2 = H, X = HCl
IId: R_1 = 2-Cl, R_2 = 4-Cl, X = HCl
IIe: R_1 = 2-Cl, R_2 = 6-Cl, X = HCl
IIf: R_1 = 3-Cl, R_2 = 4-Cl, X = HCl
IIg: R_1 = 4-Cl, R_2 = H, X = CH_3I
IIh: R_1 = 3-Cl, R_2 = 4-Cl, X = CH_3I

Table II—Growth Inhibition in Different Media[a] of *S. cerevisiae*

Compound	Medium	Concentration for 50% Inhibition of Growth, μg/ml
Ia	Minimal salts ethanol medium	51
	Complex glycerol medium	40
	Complex acetate medium	37
	Complex galactose medium	>1000
	Minimal salts glucose medium	780
Ib	Minimal salts ethanol medium	164[b]
	Minimal salts glucose medium	>1000
IIa	Minimal salts ethanol medium	90[c]
	Minimal salts glucose medium	385
IIIi	Minimal salts ethanol medium	73[d]
	Minimal salts glucose medium	287

[a] Cells were grown for 24 hr in a given liquid medium in the presence of the inhibitor. Growth is expressed as the percent of growth in the absence of inhibitor. [b] Mean of six determinations, SE = 5.36. [c] Mean of six determinations, SE = 4.28. [d] Mean of six determinations, SE = 2.72.

tion of Ia was replaced by a methyl group (Ib) or an *n*-pentyl function (IIa). Table I shows that both compounds inhibited mitochondrial function, a fact confirmed by growth inhibition studies using minimal salts ethanol medium and minimal salts glucose medium (Table II). Examination of Mannich bases related to IIa (namely, IIb–IIf) demonstrated that mitochondrial function was impaired. Quaternization of IIc gave IIg, which demonstrated no antimitochondrial properties. The related quaternary ammonium compound (IIh), which showed greater inhibition on complex glucose medium than on complex ethanol medium, presumably exerted its effect on yeast growth by interference with vital processes other than inhibition with mitochondrial function.

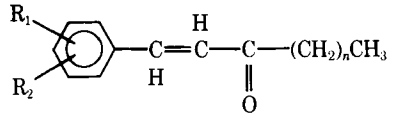
The ketones (III), which would be expected to be less water soluble than the related Mannich bases, did not inhibit mitochondrial function in yeast. The only exceptions were the quaternary ammonium compound (IIIi), which would be expected to be more water soluble than the related derivatives because it is fully ionized, and IIIm, which contains a shorter lipophilic aliphatic side chain than the corresponding unsubstituted compounds (IIIa and IIIj), indicating that solubility of these derivatives may play a part in the inhibition of mitochondrial function. Confirmation of the antimitochondrial activity of IIIi was found using liquid minimal salts glucose medium and minimal salts ethanol medium (Table II).

Mannich bases are capable of undergoing deamination to α,β-unsaturated ketones (10, 18), which may have possible significance *in vivo*. The potential breakdown products of IIc and IIf (namely, Va and Vb) were inactive as mitochondrial inhibitors under the test conditions. These results may indicate that the Mannich bases were active *per se* and did not break down to give the eliminated product. Alternatively, a hydrophilic dialkylamino group may be required to assist transportation to a site of action and liberate the corresponding unsaturated ketone (19).

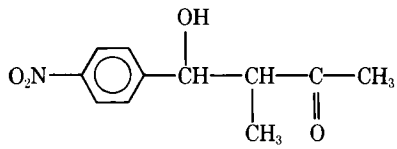
The higher chemical and biological activity of allyl alcohol compared to the saturated analog (20, 21) may be due to the ability of allyl derivatives to function as biological alkylating agents because of the formation of carbonium ions. In the case of the substituted allyl alcohols (VIa–VIc), the carbonium ion is further stabilized by resonance due to the adjacent aromatic ring. The reduction of IIa led to VIa with a retention of antimitochondrial activity, but quaternization of VIa gave VIb, which was inactive. Reduction of IIb led to VIc, with nearly a fourfold increase in activity.

When masking of the polar group of Va and Vb occurred, utilizing the concept of latentiation (22) leading to the formation of the esters VId and VIe, mitochondrial function was abolished. This finding indicated that breakdown to the allyl alcohols does not occur in yeast cells or, alternatively, that the ester does not enter the cell. Possible changes in activity against yeast mitochondria were sought by reducing the α,β-unsaturated keto group of some of these derivatives. Reduction of IIf gave VIIa with a slight increase in inhibitory activity against yeast mitochondria; reduction of IIIa gave VIIb, and both compounds had no activity.

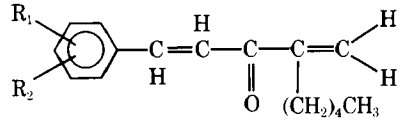
The KB test system gives an *in vitro* measure of the cytotoxicity of a compound and may indicate tumor-inhibiting properties (23).



IIIa: $R_1 = R_2 = H$, $n = 5$
IIIb: R_1 = 2-Cl, $R_2 = H$, $n = 5$
IIIc: R_1 = 3-Cl, $R_2 = H$, $n = 5$
IIId: R_1 = 4-Cl, $R_2 = H$, $n = 5$
IIIe: R_1 = 2-Cl, R_2 = 4-Cl, $n = 5$
IIIf: R_1 = 2-Cl, R_2 = 6-Cl, $n = 5$
IIIg: R_1 = 3-Cl, R_2 = 4-Cl, $n = 5$
IIIh: R_1 = 4-$N(CH_3)_2$, $R_2 = H$, $n = 5$
IIIi: R_1 = 4-$\overset{+}{N}(CH_3)_3\overset{-}{I}$, $R_2 = H$, $n = 5$
IIIj: $R_1 = R_2 = H$, $n = 1$
IIIk: R_1 = 4-Cl, $R_2 = H$, $n = 1$
IIIl: R_1 = 4-NO_2, $R_2 = H$, $n = 1$
IIIm: $R_1 = R_2 = H$, $n = 0$



IV



Va: R_1 = 4-Cl, $R_2 = H$
Vb: R_1 = 3-Cl, R_2 = 4-Cl

Significant activity in the KB test has been defined as the ability of a compound to inhibit 50% of the growth of human epidermoid carcinoma of the nasopharynx at a concentration of less than 4 μg/ml (24). The data in Table I were examined to see if a correlation existed between the effect on yeast mitochondria and KB toxicity. Of the 24 compounds for which data are available, 20 compounds (83%) either had mitochondrial-inhibiting properties and a minimal inhibitory concentration of less than 4 μg/ml in the KB test or failed to meet the criteria for activity in both tests.

The compounds in Table I were evaluated also for antineoplastic activity and murine toxicity (1) with the exception of Ia, IIh, IV, Vb, and VIIa, and the screening data for these compounds are given in Table III. Where data are available for mammalian toxicity, of the 19 compounds given daily at the maximum dose examined (*i.e.*, 400 mg/kg), 15 derivatives (79%) showed mitochondrial-inhibiting properties and mammalian toxicity or, alternatively, were inactive in inhibiting yeast mitochondria and caused no mortalities. Of the 26 compounds given every 4 days at the maximum dose administered (400 mg/kg), 21 (78%) showed toxicity and mitochondrial-inhibitory properties or showed no murine toxicity and did not inhibit yeast mitochondrial function. One may tentatively assume that interference with mitochondrial function is a factor in producing significant activity in the KB test system and also in producing mammalian toxicity.

Following injection of Ia into six rats at a dose of 100 mg/kg, the animals developed a paralysis of the hindlimbs within 2 hr. Although they recovered from this effect within 24 hr, death occurred in all animals within 36 hr. When Ia was administered at a dose of 50 mg/kg, blood was found in the feces and urine; this compound exerted a potent antidiuretic effect, a phenomenon that has been observed recently for IId and IIf (1). These results suggested that Ia might be interfering with normal hemostatic function of these animals, and studies were performed to determine if this compound was inhibiting blood clotting and/or platelet function.

The inhibitory effects of Ia on rat platelet aggregation induced by adenosine diphosphate and collagen are shown in Tables IV and V, respectively. Similar results were found when Ia was incubated for 5 min with human platelets. In this system, adenosine di-

Table III—Evaluation of Ia and Related Compounds against L-1210 Lymphoid Leukemia in Mice

Compound	Vehicle	Number of Animals Used	Dose Level[a], mg/kg	Frequency of Dose, days	Number of Injections	T/C, %[b]
Ia	Saline	6[c]	400 (0)	4	3	—
		6[c]	100	4	3	89
		6[c]	50	4	3	90
		6	100	1	9	88
		6	25	1	9	91
IIh	Hydroxypropylcellulose	3	400 (0)	4	2	—
		3	50	4	2	98
IV[d]	Hydroxypropylcellulose	6	400	4	3	98
		6	100	4	3	100
		6	400 (5)	1	9	92
		6	200	1	9	91
Vb	Dimethyl sulfoxide	3	400 (0)	4	2	—
		3	50	4	2	104
		3	25	4	2	114
VIIa	Hydroxypropylcellulose	3	400	4	2	117

[a] Drug was normally administered at 400, 200, and 100 mg/kg initially. The doses indicated are the maximum administered dose and the dose giving highest activity against L-1210 leukemia. If mortalities occurred, the number of survivors on Day 5 is indicated in brackets. [b] The value of T/C indicates the ratio of survival time of the treated mice over control mice, expressed as a percentage. A ratio of 125 or greater denotes significant activity. [c] The CDF_1 strain of mouse was used. [d] Compound inhibited 50% of the growth of human epidermoid carcinoma of the nasopharynx at 100 ppm (KB test).

Table IV—Reduction of Adenosine Diphosphate-Induced Aggregation Curve by Ia on Rat Blood Platelets

Concentration of Ia × 10^{-5} M	Curve Slope		Curve Height	
	Change, %	SE	Change, %	SE
13.2	8.6	±5.79	16.6	±2.35, $p < 0.005$
25.6	6.7	±9.55	31.4	±6.37, $p < 0.005$
48.8	37.9	±11.06, $p < 0.05$	68.6	±6.36, $p < 0.001$
88.9	62.1	±6.52, $p < 0.01$	91.9	±8.93, $p < 0.005$

phosphate-induced aggregation (curve height) was inhibited by 54.4% ± 4.37 *SEM* with 2.0 × 10^{-5} *M* Ia, by 76.3% ± 6.25 *SEM* with 6.0 × 10^{-5} *M* Ia, and by 100.0% ± 0.00 *SEM* with 15 × 10^{-5} *M* Ia. Collagen-induced aggregation of human platelets was completely inhibited by concentrations of Ia greater than 4 × 10^{-5} *M*.

The results of the prothrombin time, partial thromboplastin time, and activated partial thromboplastin time tests (Table VI) did not indicate the presence of a clotting defect in animals treated with Ia. There were no significant differences between the values found for these animals and for control rats. Compound Ia also did not have direct anticoagulant activity and did not hemolyze red cells *in vitro*. No metabolites of Ia were found following exposure of the compound to an active (determined by aniline hydroxylase activity) 9000×*g* supernatant fraction of rat liver for 20 min. The UV spectra obtained with the extract of the incubate and with a standard solution of Ia were identical, with one peak being present at 296 nm.

TLC did not reveal the presence of any metabolites from exposure of Ia to liver microsomes. The R_f value for Ia run on chloroform–methanol (95:5) was 0.19, and the R_f value for the extracted (alkaline) incubate was 0.18. In both cases, the spots were positive with Dragendorff's reagent and appeared as dark-brown spots following exposure to iodine vapor.

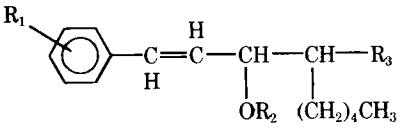
These observations suggest that Ia is active *per se* and that the presence of blood in the urine and feces of the treated rats is due in part to its inhibitory effect on platelet function.

Table V—Reduction of Collagen-Induced Aggregation Curve by Ia on Rat Blood Platelets

Concentration of Ia × 10^{-5} M	Curve Slope		Curve Height	
	Change, %	SE	Change, %	SE
10.9	21.1	±4.88, $p < 0.05$	26.3	±5.37, $p < 0.02$
21.3	52.4	±13.78, $p < 0.05$	64.8	±12.48, $p < 0.025$
40.8	94.6	±4.42, $p < 0.01$	97.5	±2.53, $p < 0.001$
75.5	100.0	±0.00, $p < 0.01$	100.0	±0.00, $p < 0.001$

Table VI—Effect of Ia on Prothrombin Time, Partial Thromboplastin Time, Activated Partial Thromboplastin Time, and Platelet Count in Rats

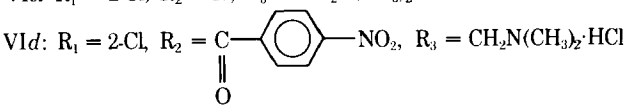
	Prothrombin Time, min	Partial Thromboplastin Time, min	Activated Partial Thromboplastin Time, min	Platelets				
				Count, mm^3	Lymphocytes, %	Polymorphs, %	Eosinophils, %	Monocytes, %
Untreated Rat 1	18.0	24.0	24.0	932,500	85	13	—	2
Untreated Rat 2	16.3	24.0	23.1	1,092,500	71	27	—	2
Rat 1	18.6	25.1	24.7	880,000	75	23	2	—
Rat 2	16.7	30.3	24.5	792,000	27	73	—	—
Rat 3	17.3	25.4	25.9	797,500	58	42	—	—
Rat 4	18.2	29.5	28.4	902,500	41	58	1	—



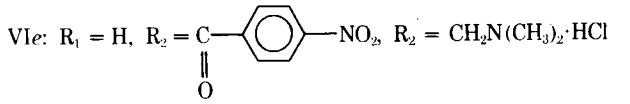
VI*a*: $R_1 = R_2 = H$, $R_3 = CH_2N(CH_3)_2 \cdot HCl$

VI*b*: $R_1 = R_2 = H$, $R_3 = CH_2\overset{+}{N}(CH_3)_3I$

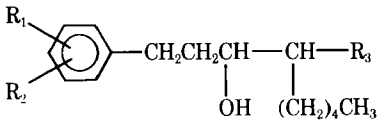
VI*c*: $R_1 = 2\text{-}Cl$, $R_2 = H$, $R_3 = CH_2N(CH_3)_2 \cdot HCl$



VI*d*: $R_1 = 2\text{-}Cl$, $R_2 = C(=O)$–C_6H_4–NO_2, $R_3 = CH_2N(CH_3)_2 \cdot HCl$



VI*e*: $R_1 = H$, $R_2 = C(=O)$–C_6H_4–NO_2, $R_3 = CH_2N(CH_3)_2 \cdot HCl$



VII*a*: $R_1 = 3\text{-}Cl$, $R_2 = 4\text{-}Cl$, $R_3 = CH_2N(CH_3)_2 \cdot HCl$

VII*b*: $R_1 = R_2 = R_3 = H$

EXPERIMENTAL

Syntheses[1]—The Mannich bases listed in Table I were prepared as follows. 5-Dimethylamino-1-phenyl-1-penten-3-one hydrochloride (I*a*) was prepared by heating, under reflux, a mixture of benzylidene acetone (35.04 g, 0.240 mole), paraformaldehyde (7.64 g, 0.255 mole), dimethylamine hydrochloride (19.70 g, 0.242 mole), and hydrochloric acid (0.2 ml) in anhydrous alcohol (24 ml) for 12 hr. The crystals deposited (11.68 g) were removed and triturated with alcohol. Evaporation of the mother liquor to dryness followed by recrystallization of the residue gave a further crop of crystals (19.25 g). Recrystallization of the crystals from alcohol gave I*a* as colorless crystals (22.58 g, 40%), mp 157–158° [lit. (25) mp 157°].

The preparation of the derivatives I*b* and II*g* was described previously (1), and the syntheses of the Mannich bases (II*a*–II*f*) were reported elsewhere (10). (*E*)-1-(3,4-Dichlorophenyl)-4-dimethylamino-1-nonen-3-one methiodide (II*h*), prepared in a similar manner to II*g*, crystallized from alcohol as pale-yellow crystals (85%), mp 162.5°.

Anal.—Calc. for $C_{19}H_{28}Cl_2INO$: C, 47.10; H, 5.78; N, 2.89. Found: C, 47.03; H, 5.90; N, 2.91.

The syntheses of the α,β-unsaturated ketones (III*a*–III*l*) were reported previously (10, 26). Compound III*m* was obtained commercially[2], and its purity was checked by GLC. Compound IV, having the *threo*-configuration, was synthesized by the literature method (27). It crystallized from benzene–petroleum ether (3:1 v/v) as pale-yellow crystals (17%), mp 93–94.5° [lit. (28) mp 93–94°]; IR (potassium bromide): 3400 s (OH), 1705 s (C=O), and 1695 s (C=O) cm^{-1}; NMR (deuterochloroform): δ 8.12 (m, 2, aromatic H), 7.50 (m, 2, aromatic H), 4.87 (d, 1, $J_{4,3} = 8$ Hz, C_4H), 3.33 (s, 1, C_4OH, exchanged with D_2O), 2.93 (m, 1, C_3H), 2.22 (s, 3, C_1H_3), and 1.00 (d, 3, $J = 7.5$ Hz, C_3CH_3) ppm.

The preparation of the dienone (V*a*) was described previously (10). Analog V*b*, prepared in a similar manner from II*h*, crystallized as orange-yellow crystals from alcohol (78%), mp 37°. The mass spectrum showed *m/e* 296 as the parent peak.

Anal.—Calc. for $C_{16}H_{18}Cl_2O$: C, 64.64; H, 6.06; Cl, 23.90. Found: C, 64.49; H, 5.96; Cl, 24.18.

The samples of allylic alcohols and related esters (VI*a*–VI*e*) examined on complex glucose medium and complex ethanol medium were identical to those used in antineoplastic evaluations described previously (1); VI*a* and VI*b* were predominantly the *threo*-isomers, VI*c* and VI*e* were pure *threo*-isomers, and VI*d* was isomerically pure but of unknown relative stereochemistry (1, 14).

The preparation of the saturated alcohol (VII*a*) also was described previously (14). A suspension of Raney nickel (0.10 g approximately) in absolute alcohol (29) was added to a solution of 1-(3,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride (1.00 g, 0.00264 mole) in absolute alcohol (25 ml), and the mixture was hydrogenated in a Paar apparatus for 24 hr at 10–12 lb of pressure. The solution was filtered through diatomaceous earth[3], and evaporation of the solvent gave a pale-yellow liquid. This liquid crystallized on storage under vacuum. Repeated recrystallization from acetone afforded 1-(3,4-dichlorophenyl)-4-dimethylaminomethylnonan-3-ol hydrochloride (VII*b*) (0.4 g, 40%) as a pale-yellow solid, mp 103°. The mass spectrum showed *m/e* 345 as the parent peak.

Anal.—Calc. for $C_{18}H_{29}Cl_2NO \cdot HCl$: C, 56.45; H, 7.90; N, 3.66. Found: C, 55.48; H, 7.79; N, 3.60.

Inhibition of Mitochondrial Function—Complex glucose medium contained yeast extract (1%), bactopeptone (2%), glucose (2%), and agar (2%) in water and complex ethanol medium contained ethanol (1%) in place of glucose. The compounds (5.0 mg) were dissolved in 95% ethanol (1 ml), and 0.02 ml of the solution was applied to antibiotic disks (12.7 mm) placed on the surface of the plates containing complex glucose medium and complex ethanol medium previously spread with 0.2 ml of a culture of *S. cerevisiae*[4] grown for 48 hr in liquid complex ethanol medium.

The plates were incubated at 28° for 3 days, and the area of inhibition (square millimeters) surrounding the disk was determined. The area of growth inhibition on complex ethanol medium in excess of growth inhibition on complex glucose medium is a quantitative estimation of the inhibition of mitochondrial function. Each compound was initially tested at 5 mg/ml. Compounds that inhibited growth were then tested five times at 5 or 1 mg/ml.

For 50% growth inhibition studies (Table II) in liquid media, a diploid strain of *S. cerevisiae*[4] was initially grown to an early stationary phase in a liquid complex glucose medium. The medium, placed in 8 × 150-mm tubes, was inoculated to an optical density (640 nm) of 0.1 unit/ml. The inhibitors were added at concentrations of 1, 2.5, 5, 7.5, 10, 50, 100, 250, 500, 750, and 1000 μg/ml. After 24 hr of growth at 30°, the percentage growth was ascertained by the change in optical density and expressed as a percentage of the control. Minimal salts ethanol medium and minimal salts glucose medium contained ethanol (1%) and glucose (5%), respectively; complex acetate medium, complex glycerol medium, and complex galactose medium employed potassium acetate (2%), glycerol (4%), and galactose (2%), respectively.

The antineoplastic evaluation[5] of the compounds in Table III was conducted by intraperitoneal injection into the BDF_1 strain of mouse (24).

Administration of I*a* to Rats—The Mannich base (I*a*) was dissolved in sterile saline (0.9% w/v) and injected intraperitoneally into 40 male albino Wistar rats, 150–200 g, at a dose of 50 mg/kg in volumes of less than 1 ml. Ten control animals were injected with sterile saline. The rats were placed in metabolic cages, and the urine and feces were collected separately. Blood was detected visually in the urine and confirmed in both urine and feces by microscopic examination and the benzidine test (30).

After 24, 48, 72, and 120 hr, the urine output from 40 treated rats was 55, 100, 160, and 300 ml, respectively; the urine output from 10 control rats was 55, 70, 60, and 60 ml, respectively. No deaths occurred during the 5 days. Administration of I*a* at a dose of 100 mg/kg was undertaken using six animals.

Effect of I*a* on Blood Platelets—Whole blood was collected from the inferior vena cava of white, male, Wistar rats, 250–300 g, anesthetized with chloroform. Nine milliliters of whole blood was

[1] Melting points and boiling points are uncorrected. Elemental analyses were performed by Dr. F. B. Strauss, Microanalytical Laboratories, Oxford, England. GLC analysis utilized a Hewlett Packard 5750B research instrument, using a OV-101 on Chromosorb G column, HP80/100, 1.2 m × 0.3 cm (4 ft × 0.125 in.), at 140 and 170°. IR spectra were recorded on a Unicam SP-200 G spectrophotometer calibrated with polystyrene. A strong band intensity is designated s. NMR spectra were determined with a Varian T-60 machine, and the mass spectra were recorded on an AE1 MS-12 single-focusing mass spectrometer operated by Mr. D. Bain of the Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[2] Aldrich Chemical Co.

[3] Celite, Anachemia, Montreal, Quebec, Canada.

[4] Strain GR13 constructed in the Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[5] Performed by the Drug Research and Development Division, National Cancer Institute, Bethesda, Md.

anticoagulated by mixing with 0.9 ml of aqueous trisodium citrate solution (3.8% w/v) in 13 × 100-mm Pyrex biological test tubes. The citrated whole blood was centrifuged at 750×*g* for 4 min to yield platelet-rich plasma.

Platelet aggregation was studied by a turbidimetric method (31) at 37°. Platelet-rich plasma, in 0.35-ml aliquots, was allowed to stand at room temperature for approximately 30 min before an experiment was begun. When adenosine diphosphate was the aggregating stimulus, a 1.35×10^{-5} *M* final concentration was employed. When collagen was the aggregation stimulus, a standard volume (0.1 ml) of collagen suspension was added. Experiments to determine the effects of I*a* on human blood platelets were performed as described previously (32).

Platelet aggregation was quantitated by measuring the slope of the aggregation curve and the maximum curve height attained within 5 min. The aggregation parameters of the curve employing I*a* were compared, by paired *t* test, to those of the control curve aggregated within 10 min (Tables IV and V).

Effects of I*a* on Blood Clotting—The hemolysis of red cells by I*a* was studied by a method outlined previously (33).

To determine if I*a* had any direct anticoagulant effect, saline solutions of I*a* and heparin were prepared (0.0, 0.1, 0.25, 0.5, 1.0, and 10.0 mg/ml). One-milliliter aliquots of these solutions were added to test tubes along with 0.9 ml of whole blood from male Wistar rats. The effects of I*a* were compared with those of heparin.

Prothrombin times were determined using a commercial rabbit brain thromboplastin[6], and partial thromboplastin[7] times and activated partial thromboplastin[7] times also were determined using commercial reagents.

In Vitro* Metabolism of I*a—*Tissue Preparation*—White, male Wistar rats, 200–350 g, which had been starved for 24 hr, were sacrificed by decapitation. Each liver was removed and placed in cold (4°) isotonic potassium chloride solution. To remove as much blood as possible, the liver was cut into small pieces into a second aliquot of cold isotonic potassium chloride solution.

Homogenization was carried out in 2 volumes of isotonic potassium chloride solution, using a homogenizer[8] operating at speed setting 40. Two grinding cycles of 5-sec duration, separated by 5 sec, were employed to produce a smooth liver homogenate. The homogenate was poured into two polycarbonate centrifuge tubes (28.7 × 103 mm) and centrifuged for 20 min (9000×*g*) in a refrigerated centrifuge[9]. The resulting supernatant layer was decanted and placed in a 50-ml beaker, which was kept in ice.

Substrates and Cofactors—The following solutions were prepared: 0.1 *M* phosphate buffer, 0.02 *M* magnesium chloride in distilled water, 0.02 *M* glucose 6-phosphate in 0.1 *M* phosphate buffer, 0.004 *M* nicotinamide adenosine dinucleotide phosphate in 0.1 *M* phosphate buffer, 0.005 *M* aniline hydrochloride in distilled water, and 0.01 *M* I*a* in 0.1 *M* phosphate buffer.

Enzyme Incubation—Three groups of incubation flasks were prepared.

1. To determine the enzyme activity of the 9000×*g* supernate, an aniline hydroxylase assay was performed by a modified method of LaDu *et al.* (34). The following items were added to five erlenmeyer flasks (25 ml): one glass bead (6 mm diameter), 1 ml of phosphate buffer, 1 ml of glucose 6-phosphate, 1 ml of nicotinamide adenosine dinucleotide phosphate, 1 ml of magnesium chloride, 1 ml of aniline hydrochloride solution, and 1 ml of the 9000×*g* supernatant homogenate.

2. To determine if enzymes present in the 9000×*g* supernate were altering the structure of I*a*, five incubation flasks were prepared as in Group 1, except that aniline hydrochloride was replaced with 1 ml of I*a* solution.

3. Five incubation flasks were also prepared to determine if I*a* had any effects on aniline hydroxylase activity. The contents of these flasks were similar to those in Group 1, except that 1 ml of I*a* solution replaced the 1 ml of phosphate buffer.

In all cases, control flasks were prepared in which the enzyme suspension was boiled prior to incubation. Incubation of aniline hydrochloride and aniline hydrochloride plus I*a* was of 20-min duration, while incubation of I*a* alone lasted 1 hr. All incubations were carried out in a shaking water bath at 37°. At the end of the incubation period, the reaction was stopped with 20% trichloroacetic acid, precipitated protein was removed by centrifugation, and the clear supernate was recovered. UV spectra were obtained on a control sample, a sample from the flask containing I*a*, and an aliquot of a standard solution of I*a*.

To isolate any metabolites of I*a* from the incubated samples, extractions were performed with chloroform at pH 2 and 9. The extracts were concentrated and spotted on TLC (silica gel G) and chromatographed using chloroform–methanol (95:5) as the developing solvent. Developed plates were treated with Dragendorff's reagent and exposed to iodine vapor. These experiments were repeated on five different occasions.

[6] Fibroplastin, BBL, Division of Bioquest, Cockeysville,.Md.
[7] Thrombofax and Activated Thrombofax, Ortho Diagnostics, Raritan, N.J.
[8] Virtis "45," Virtis Co., Gardiner, N.Y.
[9] International model B 20, International Equipment Co., Needham Heights, Mass.

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Controlled Drug Release from Polymeric Delivery Devices IV: *In Vitro–In Vivo* Correlation of Subcutaneous Release of Norgestomet from Hydrophilic Implants

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Abstract □ The *in vitro* and *in vivo* releases of norgestomet from hydrophilic implants were found to follow a matrix-controlled ($Q - t^{1/2}$) process. The sorption of drug onto the implants was observed to obey the same mechanism but with a much smaller magnitude of the $Q/t^{1/2}$ value. The effect of the extent of cross-linking on the magnitude of drug release ($Q/t^{1/2}$) profiles was analyzed both theoretically and experimentally. The release of norgestomet from hydrophilic implants was found to be an energy-linked process. Two energy terms were calculated; the activation energy for matrix diffusion was 7.71 kcal/mole, and the heat of drug crystal solvation was 25–28.6 kcal/mole.

Keyphrases □ Drug release—controlled, norgestomet from hydrophilic polymeric delivery devices □ Norgestomet—subcutaneous release from hydrophilic implants, matrix-controlled kinetic mechanism □ Implants—hydrophilic, subcutaneous release of norgestomet □ Delivery devices—polymeric, hydrophilic implants, subcutaneous release of norgestomet

The controlled release profiles of ethynodiol diacetate[1], a progestin, from silicone matrixes were reported previously (1). *In vitro* release of the drug from such silicone-type vaginal devices followed either of two kinetic mechanisms, matrix controlled or partition controlled, depending on whether the diffusion across the polymer phase or the partitioning across the polymer–solution interface was the rate-limiting step (2). An 8-week investigation on the intravaginal release of ethynodiol diacetate in rabbits (3) demonstrated that the matrix-controlled process was the predominant mode of drug release *in vivo*. Subsequently, the authors also investigated the controlled release of progestins from popularly used (Long–Folkman-type) polysiloxane[2] capsules (4). In contrast to the matrix-controlled ($Q/t^{1/2}$) mechanism seen in the drug release from silicone matrixes, they observed a constant (Q/t) drug release rate (5).

It was established that the carriers prepared from silicone polymer are permeable only to lipophilic drugs, *e.g.*, steroids. The rate of drug release is linearly proportional to the polymer solubility (partition-controlled process) (5) or to the square root of the polymer solubility (matrix-controlled process) (1, 2) of a given drug species when all other factors are constant. Apparently, the solubility of drug molecules in polymer plays a rate-limiting role in the controlled release of drug from the silicone-type drug delivery devices.

In addition to studying the lipophilic silicone polymer, researchers in this laboratory have had an ongoing interest in the development of a biocompatible, hydrophilic polymer (hydrogel) as the drug delivery carrier. First introduced by Wichterle and Lim (6) for prosthetic implants and contact lenses, this purified hydrogel is nontoxic, transparent, autoclavable, chemically stable, pliable, and moldable. The unique characteristics of this polymer (different from the silicone polymer discussed earlier) are its hydrophilicity, conductivity, and extreme wettability. Hydrogel will absorb and elute, in addition to neutral species, ionizable compounds with a molecular weight of 8000 or less (7). Through controlled alteration of the amount of cross-linking agent, the monomer-to-water ratio, and the polymerization conditions, a range of masses is obtainable from compact gel to cellular sponge, with varying physical properties.

The purposes of this study were to analyze the mechanisms and rates of the controlled release of

[1] SC-11800.
[2] Silastic.