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Original paper

Evaluation of acrylophenones and related *bis*-Mannich bases against murine P388 leukemia

Jonathan R. DIMMOCK[1], Shirish A. PATIL[1], Donald M. LEEK[2], Robert C. WARRINGTON[3] and Wei D. FANG[3]

[1] *College of Pharmacy,*
[2] *Department of Chemistry, College of Arts and Science, and*
[3] *Department of Biochemistry, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada*

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Summary — 2-Dimethylaminomethyl-1-(4-methoxyphenyl)-prop-2-en-1-one hydrochloride (**IIa**) was shown to have potent activity against P388 cells *in vitro*, whereas *in vivo* examination of this derivative and related acrylophenone hydrochlorides revealed little activity against P388 lymphocytic leukemia in mice. The preparation of a variety of *bis*-Mannich bases designed as prodrugs of certain acrylophenones gave rise to various compounds with *in vivo* activity. Measurement of the half-lives of seven *bis*-Mannich bases revealed some structural features affecting the rates of deamination. Both **IIa** and the related *bis*-Mannich base (**If**) produced 3-dimethylamino-1-(4-methoxyphenyl)-1-propanone under simulated physiological conditions and this process may represent degradation pathways of acrylophenones and *bis*-Mannich bases *in vivo*.

Résumé — **Evaluation d'acrylophénones et des bases *bis*-Mannich apparentées vis-à-vis de la leucémie P388.** *Le chlorhydrate de diméthylaminométhyl-2 (méthoxy-4 phényl)-1 propène-2 one-1,* ***IIa*** *manifeste* in vitro *une activité puissante vis-à-vis des cellules P388, tandis qu'*in vivo *ce dérivé ainsi que les chlorhydrates d'acrylophénones apparentés ont révélé une faible activité contre la leucémie lymphocytique P388 chez la souris. La synthèse d'une variété de bases* bis-*Mannich conçues comme «prodrogues» de certaines acrylophénones conduit à divers composés actifs* in vivo. *La détermination de la demi-vie de sept bases* bis-*Mannich a révélé que quelques caractéristiques structurales affectent les vitesses de désamination. Les deux composés,* ***IIa*** *et la base* bis-*Mannich apparentée (**If**) engendrent la diméthylamino-3 (méthoxy-4 phényl)-1 propanone-1 dans des conditions physiologiques simulées et cette transformation peut représenter les voies de dégradation* in vivo *des acrylophénones et de leurs bases* bis-*Mannich.*

acrylophenones / *bis*-Mannich bases / murine P388 lymphocytic leukemia screen / deamination rates of *bis*-Mannich bases

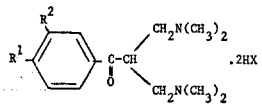
Introduction

Recently, the synthesis and anti-leukemic evaluation of a series of *bis*-Mannich bases **Ia**—**e** were reported from these laboratories [1]. Compounds with electron-releasing substituents in the aryl ring (**Ia**, **b**) increased the survival time of mice with P388 lymphocytic leukemia by 36 and 38%, respectively, **Ic**, **d** were marginally active and **Ie** was inactive. In addition, **If** was designated a Selected Agent Compound by the National Cancer Institute, U.S.A., for evaluation *versus* human tumor xenografts passaged in athymic mice as well as various murine tumors. This derivative was found to be active not only against P388 lymphocytic leukemia but to cause some reduction in tumor weights of the colon and lung xenografts [2].

A study of compounds **Ia**, **e** under simulated physiological conditions (phosphate buffer, pH 7.4, 37°C) revealed that deamination to the corresponding acrylophenones occurred very readily [1]. This process was of interest since α,β-unsaturated ketones and related compounds have a special avidity for thiol groups [3] and hence reaction of these acrylophenones with the amino and hydroxy functions of nucleic acids should be avoided. This, in turn may eliminate such problems as mutagenicity and carcinogenicity. However, this particular attribute might also eliminate the desired anti-tumor effects, if the mechanism of anti-neoplastic action resides predominantly, or solely, in a capacity to damage nucleic acids. Furthermore, the rate of deamination of **Ia** was slower than that of **Ie** [1] and hence bioactivities in series **I** may be influenced by the rate of formation of the corresponding acrylophenones, *i.e.*, the higher anti-neoplastic activities of **Ia**, **b** may be due to a more favorable, slower rate of formation of the unsaturated ketones *in vivo* than that of **Ic**—**e**.

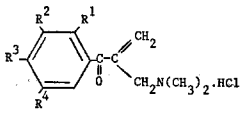
The objectives of the present study were 3-fold. Firstly, to evaluate whether the putative breakdown product of

If *in vivo*, namely **IIa**, was active *versus* P388 cells. Initially, an *in vitro* examination was planned in order to discern whether when experimental variables were minimized compared to *in vivo* conditions, a cytotoxic effect of **IIa** could be demonstrated. Secondly, if cytotoxicity was found, then **IIa** and related acrylophenones would be evaluated against P388 lymphocytic leukemia in mice. Thirdly, to expand the chemical synthesis program in light of the results obtained in screening the compounds in series **II**. Thus, if promising results were obtained, further acrylophenones would be prepared. On the other hand, should the results of **II** be unfavorable, reversion to the prodrug approach would occur by forming a variety of *bis*-Mannich bases with special reference to examining further the hypothesis that the antileukemic activity of these compounds is related to an optimal rate of release of the acrylophenones *in vivo*.



I

a: R^1=OCH_3;R^2=H;X=Br
b: R^1=CH_3;R^2=H;X=Br
c: R^1=R^2=H;X=Br
d: R^1=Cl;R^2=H;X=Br
e: R^1=R^2=Cl;X=Br
f: R^1=OCH_3;R^2=H;X=Cl
g: R^1=R^2=H;X=Cl



II

a: R^1=R^2=R^4=H;R^3=OCH_3
b: R^1=R^2=R^3=R^4=H
c: R^1=OCH_3;R^2=R^3=R^4=H
d: R^1=R^3=OCH_3;R^2=R^4=H
e: R^1=R^3=R^4=OCH_3;R^2=H
f: R^1=R^4=H;R^2=R^3=OCH_2O
g: R^1=CH_3;R^2=R^3=R^4=H
h: R^1=R^3=CH_3;R^2=R^4=H
i: R^1=R^4=CH_3;R^2=R^3=H
j: R^1=Cl;R^2=R^3=R^4=H

Discussion of the results

Initial experiments exposed P388 cells to **IIa** for 24 and 48 h (Fig. 1). Concentrations of 0.5 and 1.0 μg/ml of **IIa** were more potent than 5 μg/ml of *N,N-bis*(2-chloroethyl)-*N*-nitrosourea (BCNU), but with lower concentrations of the acrylophenone; cell viability was greater at 48 than 24 h. Since this latter observation could reflect increasing instability of **IIa** with time, the experiment was repeated whereby the leukemic cells were exposed to **IIa** for shorter duration. Higher concentrations of the compound were used in order to obtain rapid killing of the cells. The data illustrated in Fig. 2 revealed that this agent mediated both dose- and time-dependent reductions in survival and confirms the marked potency of this acrylophenone to P388 cells.

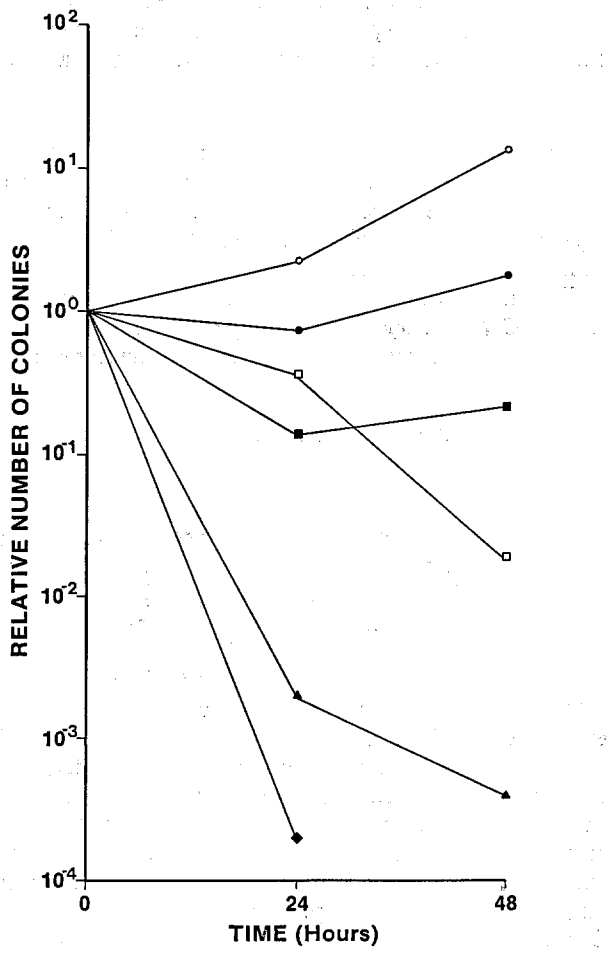


Fig. 1. Activity of **IIa** and BCNU *versus* P388 cells *in vitro*. Legends: ○ (control), □ (BCNU, 5 μg/ml), ● (**IIa**, 0.1 μg/ml), ■ (**IIa**, 0.25 μg/ml), ▲ (**IIa**, 0.5 μg/ml) and ✦ (**IIa**, 1 μg/ml).

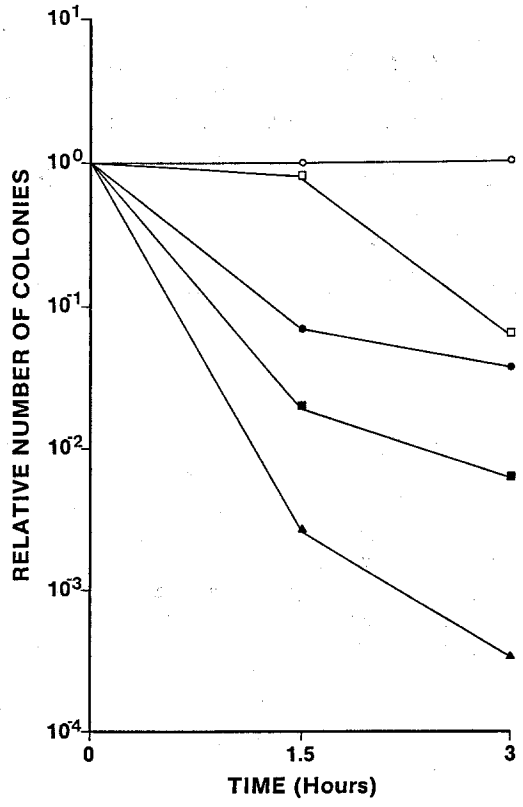


Fig. 2. Activity of **IIa** and BCNU, *versus* P388 cells *in vitro*. Legends: ○ (control), □ (BCNU, 50 μg/ml), ● (**IIa**, 1 μg/ml), ■ (**IIa**, 5 μg/ml) and ▲ (**IIa**, 10 μg/ml).

Flow cytometric analysis demonstrated that upon the addition of **IIa** at concentrations of 0.05 and 0.25 μg/ml (these quantities of compound show marked toxicity for P388 leukemia cells in culture), cell cycle transit was blocked totally, with no evidence of any phase-dependent effect.

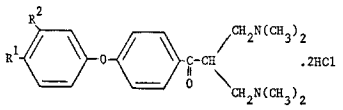
The second aspect of the study involved the synthesis and screening of the acrylophenones **II** in which the aryl ring had predominantly electron-releasing substituents, since this substitution pattern was found in the active *bis*-Mannich bases in series **I**. Table I indicates the results of

Table I. Physical data, evaluation against P388 lymphocytic leukemia in mice and murine toxicities of **II**—**VII**.

Compound	Yield (%)	M.P. (°C)	Maximum T/C%[a]	Murine Toxicity[b]
IIa	28	151-152[c]	113(1.87)	0(15),1(7.5),6(3.75)
b	45	156-158[d]	122(0.94)[e]	0(15),4(7.5),6(3.75)[e]
c	60	148-150	113(1.80)	0(15),6(7.5)
d	18	159-162	106(1.53)	0(25),3(12.5),6(7.5)
e	69	154-160	110(1.80)	0(15),6(7.5)
f	36	167-169	123(1.80)	0(15),3(7.5),6(3.75)
g	44	145-148	108(6.25)	0(25),1(12.5),6(6.25)
h	37	151-153	112(0.93)	0(7.5),6(3.75)
i	47	148-149.5	100(0.93)	0(15),1(7.5),6(3.75)
j	62	146-149	109(1.80)	0(15),6(7.5)
IIIa	60	170-172	123 (7.5)	0(60),4(30),6(15)
b	31	162-163	132(15)	0(60),6(30)
c	40	160-160.5	117(7.5)	0(60),6(30)
d	42	162-163	116(3.75)	0(30),5(15),6(7.5)
e	42	152-153	109(3.75)	0(30),2(7.5),6(3.75)
IVa	22	155	112(7.5)	0(30),6(7.5)
b	68	144	121(7.5)	0(30),4(15),6(7.5)
c	79	175	113(3.75)	0(30),3(15),6(7.5)
d	13	184	122(15)	0(120),3(60),6(30)
Va	41	157-158	130(30)	0(120),3(60),6(30)
b	60	162-163(dec.)	96(60)	0(240),5(120),6(60)
c	31	223(dec.)[f]	102(60)	6(240)
d	62	228-230(dec.)[g]	109(60)	6(240)
VIa	29	161-162.5	117(7.5)	0(30),6(15)
b	57	191-193(dec.)	107(120)	0(240),6(120)
VIIa	50	167-168	104(0.93)	0(15),1(7.5),6(3.75)
b	27	142-144.5	117(1.87)	0(30),1(15),3(7.5),5(3.75),6(1.87)

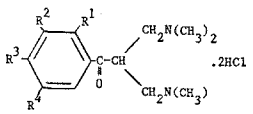
[a]The numbers are the ratios of the survival time of the treated (*T*) to control (*C*) animals expressed as a percentage. The dose (mg/kg) at which the maximum *T*/*C*% figure was found is given in brackets.
[b]The numbers are the number of survivors out of 6 on day 5 after dosing was started. The dose levels in mg/kg are in parentheses.
[c]Lit. [4] mp: 149—153°C.
[d]Lit. [5] mp: 158.16°C.
[e]Data taken from [6] and reproduced with the permission of the copyright owner.
[f]Lit. [7] mp: 226.5—227°C.
[g]Lit. [7] mp: 225—227°C.

the evaluation of compounds in series **II** *versus* P388 lymphocytic leukemia in mice. It is of interest to note that **IIa** was inactive at the dose levels tolerated by the mice and only two compounds in the series namely **IIb, f** increased the median survival time of mice by over 20%. High murine toxicity was noted with all members of series **II** whereby the compounds caused fatalities at 15 mg/kg and only approximately half of the acrylophenones could be administered at doses of 7.5 mg/kg without causing drug-induced mortalities. Thus, the general inactivity in series **II** may be due to the mice being unable to tolerate sufficient quantities of the compounds required to exert significant anti-leukemic effects. In addition, the presumed high chemical reactivity of these derivatives may cause them to be rapidly inactivated by a variety of body constituents prior to any interaction with malignant tissue. Thus, the decision was made to prepare *bis*-Mannich bases designed to liberate acrylophenones *in vivo*.



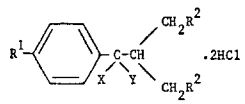
III

a: $R^1=R^2=H$
b: $R^1=OCH_3;R^2=H$
c: $R^1=CH_3;R^2=H$
d: $R^1=Cl;R^2=H$
e: $R^1=R^2=Cl$



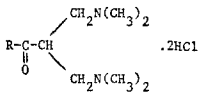
IV

a: $R^1=OCH_3;R^2=R^3=R^4=H$
b: $R^1=R^3=OCH_3;R^2=R^4=H$
c: $R^1=R^3=R^4=OCH_3;R^2=H$
d: $R^1=R^4=H;R^2=R^3=OCH_2O$



V

a: $R^1=OCH_3;R^2=$ morpholino; X=Y=O
b: $R^1=OCH_3;R^2=$ piperidino; X=Y=O
c: $R^1=OCH_3;R^2=N(CH_3)_2$;X=H;Y=OH
d: $R^1=H;R^2=N(CH_3)_2$;X=H;Y=OH



VI

a: $R=C_6H_{11}$
b: $R=C(CH_3)_3$

The third aspect of the project, namely the preparation of new *bis*-Mannich bases, involved the decision to use **If** as the prototype molecule and to prepare several series of compounds in which the rates of release of the acrylophenones *in vivo* would be predicted to vary with respect to **If**. In addition, an attempt would be made to measure the rates of deamination of selected compounds to see if these kinetic data correlated with activity against P388 lymphocytic leukemia. In series **III**, the methoxy group of **If** has been replaced by aryloxy functions containing ring substituents which would influence the rates of deamination and may permit a Topliss analysis [8] of the screening results. In order to retain structural features similar to **If**, compounds **IVa—c** were prepared which contain *ortho*-methoxy substituents in which the aryl ring would be expected to be forced out of coplanarity with the adjacent carbonyl group [9], thereby leading to altered rates of acrylophenone formation compared to **If**. The preparation and screening of the methylenedioxy derivative **IVd** was suggested in view of the activity of **IIf**. The rates of acrylophenone formation from Mannich bases vary inversely with the basicity of the amines liberated and since the pK_a values of dimethylamine, pyrrolidine and morpholine are 10.77, 11.27 and 8.70 [10], the predicted rates of decomposition are **Vb** > **If** > **Va**. In order to provide further support for the postulate that acrylophenones were responsible for the bioactivities of these compounds, the alcohols **Vc, d** were prepared in which deamination *in vivo* is unlikely. Consequently both **Vc, d** were predicted to be inactive and non-toxic. Finally in series **VI**, the aryl ring of **If** was replaced by electron-releasing aliphatic functions which should cause decreased rates of acrylophenone formation compared to **If**.

The stabilities of some of the compounds in series **III**—**VI** were examined in buffer solution at 37°C. Initially attempts

were made to measure the rates of deamination of the *bis*-Mannich bases at pH 7.4. However, under these conditions, with the exception of **VIb**, representative compounds in series **III**—**VI** decomposed at a rate too fast to be measured by the electronic absorption spectroscopic technique available. In the case of **VIb**, high resolution PMR spectroscopy recorded at various times over a 64 min period, showed that the *t*-butyl signal of **VIb** at 1.22 ppm disappeared completely between 43 and 53 min. In addition, the formation of the deaminated product from **VIb** was noted by an increase in the integral of the olefinic signals at 6.65 and 6.44 ppm accompanied by a corresponding increase in the intensity of the *t*-butyl group at 1.31 ppm of the vinyl ketone formed from **VIb** during this time frame. In order to discern the structural features affecting the rates of deamination of some of the other *bis*-Mannich bases, alteration of either the pH from 7.4 or the temperature from 37°C (as was carried out previously [1]) could be employed in order to slow down the reaction rate. After much experimentation, it was proposed to study the kinetics of deamination at 37°C and a pH of 3.50 under pseudo first order conditions. Preliminary experiments revealed that the absorbance of the *bis*-Mannich bases decreased steadily for about 5—7 half-lives and then behaved erratically *i.e.*, the absorbance either increased or decreased and invariably did not attain a constant value even at the end of 24 h. This problem may have been due to reactions of the acrylophenones formed *e.g.*, by the hydration of water to the olefinic bond. For this reason, Guggenheim's procedure [11] was followed. In addition, the kinetics of deamination at 37°C and pH 3.5 of **If** were measured by PMR spectroscopy which confirmed the formation of the corresponding acrylophenone.

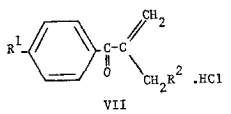
The results of the rates of deamination of seven *bis*-Mannich bases determined by electronic absorption spectroscopy are presented in Table II. These data indicate that the *para*-methoxy group of **If** retards deamination in comparison to the unsubstituted derivative **Ig**. However, the aryloxy derivatives **IIIb, d** have shorter half-lives than **If**, irrespective of whether electron-releasing (**IIIb**) or electron-attracting (**IIId**) substituents are present in the aryloxy ring. Since the *para*-phenoxy group has a slightly greater electron-releasing capability than the *para*-methoxy function (σ_p for the phenoxy and methoxy groups are —0.32 and —0.28 respectively [12]), the faster rates of deamination of **IIIb, d** than **If** in acidic media suggest that the ether oxygen atom of **IIIb, d** may be protonated to a greater extent than in **If**. The faster rates of deamination for the compounds containing an *ortho*-methoxy group (**IVa, c**) are probably due to inhibition of coplanarity of the aryl ring with the adjacent carbonyl function. In these compounds, the —I effect of the *ortho*-methoxy group may increase the acidity of the methine proton which is reflected by faster rates of deamination compared to **If**. Compound **Va** had a predictably longer half-life than **If**. Thus replacement of the *para*-methoxy group of **If** by a hydrogen atom (**Ig**) or aryloxy groups (**IIIb, d**) as well as inserting *ortho*-methoxy functions into the aryl ring (**IVa, c**) increased the rates of release of acrylophenones in contrast to **If**, whereas replacement of the basic centers of **If** by pyrrolidino groups (**Va**) retarded deamination 6-fold. The ionic strength of solutions can affect rate constants [13]. However, although a higher concentration of **If** was used in determining its half-life by PMR spectroscopy, it was found to be 23.90 min which is in good agreement with the data generated by electronic absorption spectroscopy.

Table II. Chemical half-lives of some *bis*-Mannich bases at 37°C in buffer (pH 3.5) determined by electronic absorption spectroscopy.

Compound	Pseudo first order rate constant $K\psi$ sec^{-1}	Standard deviation (S.D.)	Coefficient of variation (C.V.%)	Half life ($t_{\frac{1}{2}}$, min)	Standard deviation (S.D.)
If	4.707×10^{-4}	$\pm 0.043 \times 10^{-4}$	0.92	24.54	±0.226
Ig	2.462×10^{-3}	$\pm 0.016 \times 10^{-3}$	0.66	4.69	±0.030
IIIb	8.736×10^{-4}	$\pm 0.212 \times 10^{-4}$	2.43	13.23	±0.316
IIId	1.096×10^{-3}	$\pm 0.025 \times 10^{-3}$	2.28	10.54	±0.240
IVa	8.667×10^{-3}	$\pm 0.099 \times 10^{-3}$	1.15	1.33	±0.015
IVc	1.157×10^{-3}	$\pm 0.014 \times 10^{-3}$	1.17	9.98	±0.119
Va	7.613×10^{-5}	$\pm 0.360 \times 10^{-5}$	4.73	151.95	±7.353

The evaluation of compounds **III**—**VI** *versus* murine P388 lymphocytic leukemia is given in Table I. In contrast to the activities and murine toxicities of the acrylophenones **II**, whereby only one-fifth of the derivatives increased the median survival time by 20% or more and fatalities were invariably noted at 15 mg/kg, five of the thirteen Mannich bases produced significant increases in the lifespans of the mice and doses of 15 mg/kg were tolerated in the majority of cases. Replacement of the 4-methoxy group of the lead molecule **If** ($T/C\% = 128(2)$) by an aryloxy group gave **IIIa** with retention of activity and the insertion of a 4-methoxy group into the aryloxy ring (**IIIb**) gave a compound with increased activity in this screen. This derivative had $T/C\%$ values of 133 and 124, at 15 and 7.5 mg/kg, respectively. In order to verify this activity, doses of 15, 7.5 and 3.75 mg/kg were administered which gave $T/C\%$ values of 131, 133 and 121, respectively. The substitution pattern in the aryloxy ring of **III** does not conform to a Topliss parameter [8], although it is close to $-\sigma$ and $\pi - 3\sigma$ dependencies. A change in the position of the methoxy group of **If** gave **IVa**, whose activity was halved approximately, and the insertion of additional methoxy groups (**IVb, c**) did not increase activity compared to **If**. The methylenedioxy compound **IVd** had an activity similar to that of the corresponding acrylophenone. The pyrrolidino Mannich base **Va** had $T/C\%$ values of 128 and 121, at 30 and 15 mg/kg, respectively, and when the evaluation was repeated, the figures of 132 and 123 at these dose levels were recorded. Thus both **IIIb** and **Va** have confirmed activities *versus* P388 lymphocytic leukemia in mice and may serve as templates for further molecular modification with the goal of producing useful anti-neoplastic agents. Compounds **Vb, VIa, b** were inactive. The alcohols, **Vc, d**, as predicted, were inactive and non-toxic at the dose levels examined. In order to assess whether the acrylophenones in the new series of Mannich bases would be likely to have activity, compounds **VII** were synthesized which are the unsaturated ketones corresponding to **IIIa** and **Va**. As Table I indicates, marked anti-leukemic properties are

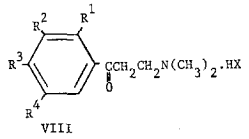
absent and **VIIa, b** show much higher murine toxicities than **IIIa** and **Va**. In comparing the half-lives of the seven *bis*-Mannich bases found in Table II with their activities in the P388 screen, it is of interest to note that the three active compounds (**If, IIIb, Va**) released acrylophenones at a slower rate than the four other derivatives.



VII

a: $R^1=OC_6H_5;R^2=N(CH_3)_2$

b: $R^1=OCH_3;R^2=N\square$



VIII

a: $R^1=R^2=R^4=H;R^3=OCH_3;X=Cl$

b: $R^1=OCH_3;R^2=R^3=R^4=H;X=Br$

c: $R^1=R^3=OCH_3;R^2=R^4=H;X=Br$

d: $R^1=R^3=R^4=OCH_3;R^2=H;X=Br$

e: $R^1=R^4=H;R^2=R^3=OCH_2O;X=Cl$

f: $R^1=R^3=CH_3;R^2=R^4=H;X=Cl$

g: $R^1=R^4=CH_3;R^2=R^3=H;X=Cl$

Finally, compound **If** was examined over a protracted period of time under simulated physiological conditions (buffer, pH 7.4, 37°C) in order to investigate its possible fate *in vivo*. While incubation of **If** for 5 min produced the acrylophenone **IIa**, after 96 h the *mono*-Mannich base **VIIIa** was isolated. As far as the authors are aware, apart from our earlier report [14], this is only the second example of a *retro*-Mannich reaction of a *bis*-Mannich base. Incubation of the acrylophenone **IIa** for 96 h under these conditions also produced **VIIIa** and hence both the *bis*-Mannich base **If** and the corresponding acrylophenone **IIa** eventually breakdown to **VIIIa** which has been shown previously (as the hydrobromide salt) to be bereft of activity towards murine P388 lymphocytic leukemia [1]. Compounds **VIIIb—g** have the same aryl substitution pattern as the acrylophenones **IIc—f, h, i** respectively, and **VIIIb—e** are related to the *bis*-Mannich bases **IVa—d**. The monobasic Mannich bases **VIIIb—g** were inactive towards P388 leukemia in mice, although some toxicity was retained. It is conceivable that, at least in part, the inactivity in general of the acrylophenones and some of the Mannich bases may be due to conversion into the corresponding *mono*-Mannich bases. It is of interest to note that incubation of certain concentrations of **IIa** with P388 cells *in vitro* showed a loss of activity after 48 h, which could be partially due to the conversion of **IIa** into **VIIIa** (Fig. 1).

In conclusion, this study has revealed the high potency of **IIa** against P388 cells *in vitro*. *In vivo*, this derivative and related acrylophenones demonstrated little activity against P388 lymphocytic leukemia and had high murine toxicity. However, among a variety of *bis*-Mannich bases designed as prodrugs of acrylophenones, several had anti-leukemic activity and in particular, **IIIb** and **Va** had confirmed *in vivo* potencies. Kinetic data on representative Mannich bases revealed some structural features likely to affect the rates of release of acrylophenones *in vivo*. In addition, both **If** and **IIa** were converted into a monobasic Mannich base which had no activity *versus* P388 leukemia *in vivo*. This process may possibly be an inactivation mechanism for both the *bis*-Mannich bases and acrylophenones *in vivo*.

Experimental protocols

Chemistry

Elemental analyses (C, H, N) were undertaken on **If, g, II—VIII** except **Vc, d, VIIIa, e** which have been reported previously. In addition, analyses were undertaken on 1-(4-methoxyphenyl)-2-(1-morpholinomethyl)-2-propen-1-one hydrochloride (C, H, N) required in the synthesis of **Vb** and the intermediate 1-(4-aryloxyphenyl)ethanones (C, H) produced for the preparation of **IIIb, c, e**. These analyses were performed by Mr. R. E. Teed, Department of Chemistry, University of Saskatchewan, Mr. S. S. Jonnalagadda at the Plant Biotechnology Institute, Saskatoon, Saskatchewan and Guelph Chemical Laboratories, Guelph, Ontario. They are within 0.4% unless otherwise indicated. Melting points and boiling points are uncorrected. Organic extracts were washed with water, dried and the solvent removed *in vacuo*.

Lyophilization was accomplished using a Labconco freeze—dryer 18. PMR spectra were determined using Varian T-60, Bruker AM-360-WB and AM 300 FT NMR spectrophotometers. The kinetic determinations were obtained using a Bruker AM 300 FT NMR instrument equipped with a variable temperature unit (BVT-1000) and an Aspect 3000 computer. PMR spectroscopy (generally 60 MHz) was used routinely in order to confirm the structures of the derivatives. Electronic absorption spectra were generated using a Gilford Response UV—VIS spectrophotometer equipped with a temperature controlling device. A computerized kinetic program featured with the instrument was used for the kinetic study. IR spectra were recorded on a Beckman Aculab 4 IR spectrophotometer. Thin—layer chromatography (TLC) used Eastman chromatogram sheets composed of silica gel and a solvent system of benzene:methanol (9:1). The P388 leukemia cells used for the *in vitro* studies were provided by the NCI-Frederick Cancer Research Facility, Frederick, MD., U.S.A. Flow cytometric analysis was undertaken using an EPICS IV flow cytometer/cell sorter purchased from Colter Electronics, Miami, FL., U.S.A.

Synthesis of compounds

1-Aryl-3-dimethylamino-2-dimethylaminomethyl-1-propanone dihydrochlorides (If, g, IV)

Compound **If** was prepared by the literature procedure [2] in 31% yield, mp: 175—177°C (lit. [2] mp: 175—177°C). The related derivative **Ig** was prepared in an identical manner in 20% yield, mp: 163—164°C (lit. [7] mp: 164—165°C).

Series **IV** was synthesized as follows. A mixture of the appropriate acetophenone (0.019 mol), *N,N*-dimethyl(methylene)ammonium chloride [15] (0.08 mol) in dry acetonitrile (50 ml) was stirred at 45—50°C for 24 (**IVa, d**), 90 (**IVb**) or 48 (**IVc**) h and in the case of **IVd**, the mixture was subsequently heated under reflux for 8 h. The precipitate was washed with hot acetonitrile, dried and crystallization from ether—methanol afforded **IVa**. The remaining compounds were purified by dissolving the crude solid in water and lyophilizing the solution and the resultant residue was crystallized from ether—methanol (**IVb, c**) or methanol (**IVd**). The structures were confirmed by PMR and IR spectroscopy as well as elemental analysis. Anal. for **IVd**. Calcd. C: 51.29%. Found C: 50.55%.

1-Aryl-2-aminomethyl-2-propen-1-one hydrochlorides (II, VII)

The compounds were prepared by a literature procedure [16], using times of heating under reflux of 1 (**IId**), 2 (**IIe**), 3 (**IIa, b, VIIa**), 6 (**IIc, f—j**) or 12 (**VIIb**) h. After removal of the solvent, the residue was triturated with ether or acetone (**IId, VIIb**) and crystallized from ether—ethanol (**IIa, b, d—f, VIIa**) or acetone (**IIc, g—j, VIIb**). The structures were confirmed by PMR and IR spectroscopy (except **VIIb**) in addition to combustion analyses. Anal. for **IIc**. Calcd C: 66.26%, Found C: 66.70%.

1-(4-Aryloxyphenyl)-3-dimethylamino-2-dimethylaminomethyl-1-propanone dihydrochlorides (III)

The intermediate 1-(4-aryloxyphenyl)ethanones required for the

synthesis of **IIIa**—**e** were obtained commercially (1-(4-phenoxyphenyl) ethanone was obtained from Trans World Chemicals, Inc., Chevy Chase, MD., U.S.A.) or prepared by a literature methodology [17] in yields of 80—82%. The structures were confirmed by PMR spectroscopy and/or elemental analysis. The hitherto unrecorded ketone, 1-[4-(3,4-dichlorophenoxy)phenyl]ethanone had a boiling point of 147—150°C/0.01 torr.

A mixture of the appropriate 1-(4-aryloxyphenyl)ethanone (0.014 mol), *N,N*-dimethyl(methylene)ammonium chloride (0.057 mol) and dry acetonitrile (60 ml) was stirred at 45—50°C for 24 h. The precipitate was removed, washed with hot acetonitrile, dried and crystallized from methanol (**IIIa, b**) or ethanol (**IIIc**—**e**). The structures were confirmed by PMR and IR spectroscopy and combustion analysis.

3-Amino-2-aminomethyl-1-(4-methoxyphenyl)-1-propanone dihydrochlorides (Va, b)

Compound **VIIb** was converted into the desired *bis*-Mannich base **Va** using a literature methodology [16] and purified by crystallization from ether—methanol.

1-(4-Methoxyphenyl)-2-(1-morpholinomethyl)-2-propen-1-one hydrochloride was prepared in 20% yield, mp: 171—173°C (dec.) (lit. [4] mp: 180—181°C) in a similar manner to the acrylophenones in series **II** and **VII** using a time of heating under reflux of 24 h. It was converted into **Vb** by a procedure used for preparing similar compounds [16] and crystallized from ethanol. The yields in Table I of **Va, b** are based on the quantity of acrylophenone used.

1-Aryl-3-dimethylamino-2-dimethylaminomethyl-1-propanol dihydrochlorides (Vc, d)

A solution of 3-dimethylamino-2-dimethylaminomethyl-1-(4-methoxyphenyl)-1-propanone (0.011 mol) in methanol (6 ml) was added over a 10 min period to a stirred solution of sodium borohydride (0.011 mol) in aqueous methanol (67% v/v, 6 ml) kept below 10°C. Stirring continued at this temperature for 10 min, then 75 min at room temperature and finally warmed at 45—50°C for 2.5 h. After removal of the solvents, water (12 ml) was added and the base extracted with ether. Removal of the organic solvent gave a residue which was dissolved in ethanol. This solution was acidified with ethanolic hydrochloric acid (3.3 N approximately), anhydrous ether was added and upon refrigeration, **Vc** crystallized.

The alcohol **Vd** was prepared as follows. A solution of 3-dimethylamino-2-dimethylaminomethyl-1-phenyl-1-propanone (0.014 mol) in methanol (25 ml) was added over a 30 min period to a stirred solution of sodium borohydride (0.014 mol) in aqueous methanol (50%, v/v, 65 ml) kept below 10°C. The solution attained room temperature after 1 h and was warmed to 45—50°C for 4 h. After removal of most of the methanol, the reaction mixture was processed as in the case of **Vc** to give **Vd** as the free base which was dissolved in anhydrous ether and upon acidification with anhydrous hydrogen chloride gas gave a precipitate which was crystallized from ethanol.

bis-*Mannich bases derived from cyclohexyl and* t-*butyl methyl ketones (VI)*

Compounds **VIa, b** were prepared in a manner similar to that of series **III**, except that the reagents were stirred for 48 h in the case of **VIb**. The reaction products were crystallized from methanol (**VIa**) and ethanol (**VIb**).

The procedure for measuring the stability of **VIb** by 300 MHz PMR spectroscopy was similar to that used for the kinetic studies of **If** *vide infra* except that phosphate buffer, pH 7.4, was used in place of formate buffer and the sample of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was not weighed accurately in this case. Spectra were recorded at the end of 5, 10, 21, 32, 43, 53 and 64 min. The *t*-butyl signal of **VIb** and the olefinic signals and *t*-butyl absorptions of the corresponding eliminated compound were integrated. The ratios of both of the latter two absorptions to DSS, which were made at the various time intervals in order to confirm that the *t*-butyl signal at 1.31 ppm was due to the olefinic compound, were found to be similar.

1-Aryl-3-dimethylamino-1-propanone hydrohalides (VIII)

Compound **VIIIa** was prepared as follows. A mixture of 4-methoxyacetophenone (0.0033 mol), *N,N*-dimethyl(methylene)ammonium chloride (0.0071 mol) in dry acetonitrile (7 ml) was stirred at 45—50°C for 2 h. The excess of the Mannich reagent was removed by filtration and the precipitate washed with hot acetonitrile. After refrigeration overnight, the deposited crystals were collected and crystallization from ethanol afforded **VIIIa**, mp: 165—167°C (lit. [7] mp: 182—184°C) in 14% yield. PMR (D_2O) δ: 3.03 [s, 6H, $N(CH_3)_2$]; 3.63 (s, 4H, CH_2CH_2); 3.97 (s, 3H, OCH_3); 7.13 (d, 2H, J = 9 Hz, aromatic H at C-3 and C-5) and 8.07 (d, 2H, J = 9 Hz, aromatic H at C-2 and C-6).

Compound **VIIIe** was prepared in a similar manner to **VIIIa** except that molar ratios of the appropriate ketone and Mannich reagent were 0.12 and 0.24, respectively, in acetonitrile (25 ml) and the reactants were stirred for 20 h. The desired compound was crystallized from methanol, mp: 189—192°C (lit. [18] mp: 192°C) and obtained in 41% yield.

Compounds **VIIIb**—**d, f, g** were prepared as follows. A mixture of the appropriate acetophenone (0.055 mol), paraformaldehyde (0.07 mol), dimethylamine hydrochloride (0.074 mol), hydrochloric acid (37%, w/v, 0.12 ml) and ethanol (45 ml) was heated under reflux for 7 (**VIIIb, c**), 8 (**VIIId**) or 16 (**VIIIf, g**) h. After removal of the ethanol, the residue was dissolved in water and extracted with ether to remove the unreacted acetophenones. The aqueous phase was basified with ammonia solution (10%, w/v), extracted with ether and removal of the solvent gave an oil which was dissolved in anhydrous ether and treated with the appropriate hydrohalide gas. The solid was collected, washed with ether and dried. In the case of **VIIId, f**, the compounds isolated were analytically pure, whereas the remaining derivatives were recrystallized from ether—ethanol. The melting points and yields of **VIIIb**—**d, f, g** were as follows: **VIIIb**: 116—118°C (lit. [19] mp: not stated), 33%; **VIIIc**: 141—143°C, 58%; **VIIId**: 185—186°C, 36%; **VIIIf**: 125—126°C, 36%; and **VIIIg**: 141—144°C; 34%.

Determination of the half-lives of certain bis-*Mannich bases at 37°C in aqueous formate buffer, pH 3.5*

The wavelengths of the absorption maxima (λ_{max}) of **If, Ig, IIIb, d, IVa, c** and **Va** were determined in formate buffer (0.25 M, pH 3.50) at 0°C and found to be 295.0, 255.5, 295.0, 293.5, 321.0, 348.0 and 296.5 nm, respectively. The rates of deamination were obtained using a concentration of compound of 10^{-5} to 10^{-6} mol/100 ml of buffer and by measuring the decrease in the λ_{max} of the *bis*-Mannich bases. The procedure followed was the same as that used previously in these laboratories [20], except that Δt was 1—2 half-lives, a minimum of 12 different O.D. (optical density) and O.D.′ (optical density after time t) values were employed, the temperature was 37 (± 0.2)°C and the rate constants of each compound were determined in triplicate.

The rate of deamination of **If** in formate buffer (pH 3.50) and 37 (± 0.1)°C was also determined by PMR spectroscopy. The sodium formate buffer (0.25 M, pH 3.50, pD 3.10) was prepared by dissolving sodium formate (0.34 g), DSS (0.10 g) and hydrochloric acid-d_1 (1.34 N, 3.10 ml) in deuterium oxide and adjusting the volume to 20 ml. Initial experiments showed that the pH of the buffer did not change over at least a 3 h period when 10 mg of **If** was added to 0.5 ml of the buffer. Compound **If** (50 μM) was added to 1 ml of buffer at 37°C and 0.5 ml of this solution was added to a PMR tube. As soon as the compound dissolved, a stopwatch was started and the tube inserted into the probehead. When the kinetic program commenced, the stopwatch was stopped and the delay time (between 1 and 2 min) was included in the calculation of the rate constant. Spectra were accumulated every 5—10 min and the reaction followed by integration of the olefinic signals of **IIa** at 6.37 and 6.60 ppm as they appeared with time. The reaction was terminated after 2—3 half-lives. The rate of deamination of **If** to **IIa** was obtained by plotting the logarithm of the mean integral value of the olefinic signals against time. The mean integral value of the olefinic signals was corrected each time by relating it to the integral of the DSS signal. During the kinetic experiment, PMR data acquisition was controlled by an automation program consisting of a data acquisition step, a data storage command, and a programmable time delay. This sequence was repeated until the reaction was complete. The experiment was undertaken in duplicate to yield $K\psi$ values of 4.84×10^{-4} ($\pm$ 0.155×10^{-4}) s^{-1} and a half-life of **If** of 23.90 ($\pm$ 0.76) min.

Stability studies of If and IIa in phosphate buffer (pH 7.40) at 37°C

A solution of **If** (1.00 g) in buffer (40 ml) was placed in an incubator at 37°C. After 5 min, the solution was cooled to room temperature, extracted with chloroform and the organic solvent was dried ($MgSO_4$). Removal of the solvent gave an oil (0.65 g) which was shown by PMR spectroscopy to be **IIa** as the free base. PMR($CDCl_3$): δ: 2.33 (s,

6H, $N(CH_3)_2$); 3.33 (s, 3H, CH_2N); 3.83 (s, 3H, OCH_3); 5.59 (s, 1H, $C=CH_2$); 5.83 (s, 1H, $C=CH_2$); 6.86 (d, 2H, J = 8.5 Hz, aromatic H at C-3 and C-5) and 7.77 (d, 2H, J = 8.5 Hz, aromatic H at C-2 and C-6). The oil was dissolved in anhydrous ether and addition of anhydrous hydrogen chloride gave a precipitate which upon recrystallization from ether—ethanol gave **IIa** (0.55 g, 73%), mp: 149—150°C which was confirmed by comparison of its melting point and PMR characteristics with those of an authentic sample prepared by an unambiguous route *vide supra.*

A solution of **If** (0.50 g) in buffer (400 ml) was incubated for 72 h at 37°C and at the end of this period, another 100 ml of buffer were added and the incubation continued for an additional 24 h. The solution was then cooled to room temperature, extracted with chloroform and the combined extracts washed with water and dried ($MgSO_4$). Removal of the solvent gave an oil (0.25 g) which was dissolved in anhydrous ether and treated with anhydrous hydrogen chloride to give a precipitate which upon recrystallization from acetone gave **VIIIa** (0.062 g, 17%) mp: 167—170°C. This derivative had a similar melting point and PMR spectrum to an authentic sample of **VIIIa** prepared by the method described earlier.

Compound **IIa** (0.50 g) was dissolved in phosphate buffer and treated in the same way as described in the previous paragraph to give an oil (0.32 g) which was converted into the hydrochloride salt. Crystallization from acetone gave **VIIIa** (0.032 g, 7%) mp: 164—167°C. The product isolated had identical TLC, mp and PMR characteristics to an authentic sample of **VIIIa** prepared by an unambiguous route *vide supra.*

Pharmacology

Effect of IIa on P388 cells in vitro

The effect of **IIa** on a clonal isolate of the P388 leukemia/MRI line was assessed by the clonogenic cell survival assay described previously [21]. A brief summary is as follows. The cells are exposed to the compound for various periods of time, washed and subsequently plated in soft agar. Cells which are capable of growing into macroscopic colonies are scored and reported as the number of surviving colonies in the experimental group *versus* the number obtained in control cultures (relative cell survival). Two determinations at each compound concentration were made and the results at each concentration were statistically indistinguishable. The effect of **IIa** on cell cycle transit in cultured P388 cells was assessed, using protocols described previously [22], which involve treating randomly proliferating P388 cells with vinblastine sulfate (0.05 μg/ml) with or without compound **IIa**. At various time intervals, the cells were fixed, stained with a propidium iodide—ribonuclease solution [23] and analyzed.

Evaluation of compounds versus *P388 leukemia in mice*

The evaluation of compounds against P388 lymphocytic leukemia in mice is the result of screenings performed under the auspices of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD., U.S.A., using their protocols [24]. The compounds were administered i.p. in an appropriate vehicle to CD_2F_1 mice. Five daily injections were given except for **IId** in which case, two injections on days 1 and 5 were given. Compounds are initially screened at 240 or 200 mg/kg as well as at doses one half and one quarter that of the maximum dose. If mortalities were induced by the compound at these doses, the amount of compound administered was reduced. The $T/C\%$ value, denoting activity, has varied, being previously 125 and 120 and is now 127. In this screen, 5-fluorouracil has a $T/C\%$ of > 135 at 20 mg/kg when given i.p. daily for 5 days [25].

The screening results for **II—VII** are given in Table I. The maximum $T/C\%$ values (dose in mg/kg) for **VIIIb—g** are 112(30), 108(60), 116(120), 100(60), 106(15) and 106(15), respectively. The doses at which there were 6 out of 6 survivors on day 5 (which denotes an absence of drug-induced fatalities), were 240 (**VIIId**), 120 (**VIIIe**) and 60 (**VIIIb, c, f, g**) mg/kg.

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