Eur. J. Med. Chem. **24** (1989) 379–383

Original paper

Intramolecular cyclization and cytotoxicities of some Mannich bases of styryl ketones

Jonathan R. DIMMOCK[1], Ercin ERCIYAS[1], Glen E. BIGAM[2], D. Lynn KIRKPATRICK[3] and Michelle M. DUKE[3]

[1]*College of Pharmacy, University of Saskatchewan, Saskatoon, S7N 0W0,*
[2]*Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, and*
[3]*Department of Chemistry, University of Regina, Saskatchewan S4S 0A2, Canada*

(Received August 22, 1988, accepted January 16, 1989)

Summary — The extent of intramolecular cyclization in a series of Mannich bases derived from 2-arylidene-1,3-diketones (series **5**) was probably influenced by the steric and electronic properties of the different basic groups. Replacement of one of the keto groups in series **5** by hydrogen, methyl, carboethoxy and phenyl functions gave series **6** in which the intramolecular cyclization process was abolished. Evaluation of the compounds *versus* the EMT6 cells *in vitro* indicated that cytotoxicity did not appear to be influenced by the ratio of cyclic and acyclic species but in series **6** bioactivity was correlated with the electronic nature of the substituents. Two compounds had approximately one-sixth of the activity of the reference compound, melphalan.

Résumé — **Cyclisation intramoléculaire et cytotoxicité de quelques bases de Mannich dérivées de styrylcétones.** *L'étendue de la cyclisation intramoléculaire dans une série de bases de Mannich d'arylidène-2 dicétones-1,3 (série* ***5****) est influencée probablement par les propriétés stériques et électroniques des différents groupes basiques. Le remplacement d'un des groupes cétoniques dans la série* ***5*** *par l'hydrogène, un groupement méthyle, carboéthoxy ou phényle donne la série* ***6*** *dans laquelle la cyclisation intramoléculaire est inexsitante. L'évaluation de ces composés contre les cellules EMT6* in vitro *a indiqué que la cytotoxicité n'était pas influencée par la proportion des formes cyclique et acyclique, mais en série* ***6****, l'activité biologique est en corrélation avec les effets électroniques des substituants. Deux composés sont six fois moins cytotoxiques que le composé de référence, le melphalan.*

Mannich bases / styryl ketones / intramolecular cyclization / EMT6 cells

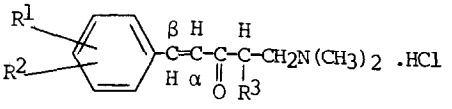
Introduction

A number of Mannich bases of α,β-unsaturated ketones **1**, designed to alkylate thiols rather than amino or hydroxyl groups found in nucleic acids, demonstrated anticancer activity both *in vitro* [1] and *in vivo* [2]. The hypothesis was advanced that replacement of the proton at the α position in series **1** by electron-attracting groups would increase the fractional positive charge on the β carbon atom, leading to greater alkylating ability which may result in the formation of compounds displaying improved antineoplastic activity. This consideration led to the preparation of 2 series of compounds **2** and **3**, some representatives of which demonstrated anticancer properties *in vivo* [3, 4]. However, high resolution ^{1}H NMR spectroscopy revealed that in the absence of *ortho* substituents, series **2** and **3** existed principally in the cyclic forms **4** in deuterium oxide.

The objectives of the present investigation were as follows: firstly, to prepare a number of compounds in which the dimethylamino group in **2** and **3** was replaced by other basic functions (**5b–d**) and also to replace one of the keto groups of **2** and **3** by other substituents (series **6**); secondly, to observe whether these molecular changes affected the cyclization process; thirdly, to examine the possibility that changes in the ratio of cyclic / acyclic species could be correlated with different toxicities to cancer cells.

Chemistry

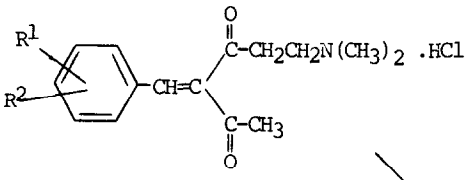
Initially **5a** was considered as the lead compound and the decision was made to prepare the analogous morpholino, piperidino and *N*-methylanilino derivatives. Preliminary experimentation showed that attempts to prepare the *mono*-morpholino compound analogous to series **2** gave only the dibasic compound **5b**; hence the other derivatives in series **5** were modelled on series **3**. Compound **5c** was prepared successfully, but reaction of *N*-methylaniline, formaldehyde and 3-(phenylmethylene)-2,4-pentanedione gave rise to a small quantity of product identified as



1

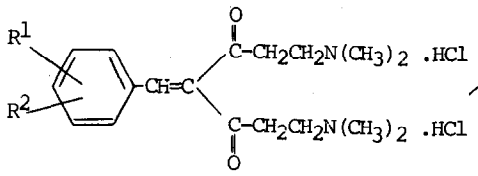
$R^1=R^2=H,Cl,OCH_3,CH_3$

$R^3=H,alkyl$



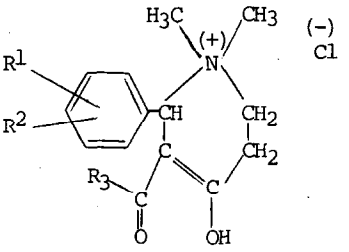
2

$R^1=R^2=H,Cl,F,NO_2,etc.$

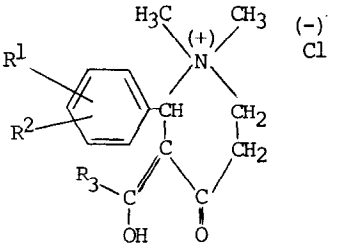


3

$R^1=R^2=H,Cl,CH_3,OCH_3$



A

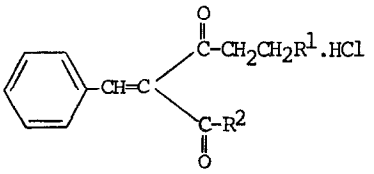


B

4

$R^1=R^2=H,Cl,CH_3,OCH_3$, etc.

$R^3=CH_3,CH_2CH_2N(CH_3)_2$ .HCl



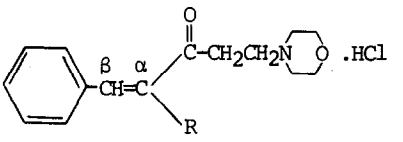
5

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

b: R^1=morpholino; $R^2=CH_2CH_2$-morpholino .HCl

c: R^1=piperidino; $R^2=CH_2CH_2$-piperidino .HCl

d: $R^1=N(CH_3)C_6H_5$; $R^2=CH_2CH_2N(CH_3)C_6H_5$ .HCl



6

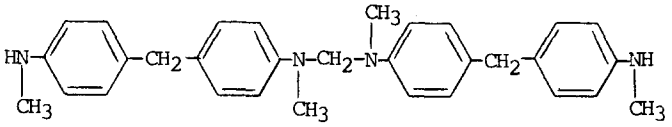
a: R =H

b: R =CH_3

c: R =$COOC_2H_5$

d: R =C_6H_5

This compound is likely to have been produced *via* formation of the 4-methylaminobenzyl carbonium ion as suggested by Grillot and Lau [5] which on interaction with *N*-methylaniline would give *bis*(4-methylaminophenyl)-methane. Condensation of 2 molecules of this compound with formaldehyde would furnish **7**. Since the morpholino derivatives **5b** displayed greater cytotoxicity to EMT6 cells than **5a**, **c**, *vide infra*, series **6** was prepared using this base.



7

Results and Discussion

High resolution ^{1}H NMR spectra of **5a**–**c** and **6** in deuterium oxide at 37°C were recorded 10 min and 2 h after dissolution of the compound. These times correspond to that of the initial spectra as well as the contact times of the compounds with the tumour cells, *vide infra*. At the end of 10 min, compounds **5a**–**c** existed in the cyclic forms to the extent of 79, 28 and 43% respectively, and the spectra were essentially unchanged at the end of 2 h. In contrast, there was no evidence of cyclization in series **6** over the time frame of 2 h. After 10 min, the spectra recorded for **6a**, **b**, **d** were in accord with the proposed structures and revealed the absence of geometrical isomers. Compound **6c** existed in an isomeric ratio of 68:27, as noted by integration of the olefinic protons at 8.06 and 8.10 ppm respectively, and assuming that the ester group is more shielding than the keto function, the olefinic proton at 8.06 ppm is likely that of the *Z* isomer. After 2 h, the percentage of **6a** in solution had fallen to 86% and the corresponding deaminated product, with the characteristic vinyl ABC system, was formed (14%). After 2 h, while the spectrum of **6b** was unchanged, the percentage of both **6c** and **d** had dropped to 70% and one or more compounds were present in solution as revealed by ^{1}H NMR spectroscopy. In both cases, there were a number of peaks in the olefinic region of the spectra (5.3–7.3 ppm with **6c** and 6.6–6.9 ppm in the case of **6d**), and thus the corresponding deaminated products may have been formed from **6c**, **d** as well as from **6a**.

One may deduce from these spectroscopic studies that firstly, replacement of one of the keto groups in **5a**–**c** by either a hydrogen atom or methyl, carboethoxy and phenyl functions abolished the cyclization process in series **6**; and secondly, these observations permit some general conclusions regarding the influence of steric and electronic factors in the cyclization process in **5a**–**c**. In regard to this latter consideration, Charton's steric parameter ν for the morpholino and piperidino groups are apparently unavailable and thus the constants for the analogous alkyl groups were employed. The ν values of the isopropyl (corresponding to dimethylamino) and cylcohexyl (considered analogous to both morpholino and piperidino) groups are 0.76 and 0.87, respectively [6]; and hence the reduced cyclization in **5b**, **c** in comparison to **5a** may be explained, at least in part, on steric grounds. Electronic considerations may influence the difference in extent of cyclization between **5b**, **c**. Thus the pK_a of *N*-methylmorpholine and *N*-methylpiperidine are 7.38 and 10.38, respectively [7]; and hence the greater basicity of the piperidino group with an increased nucleophilicity for the electron deficient carbon atom adjacent to the aryl ring may be responsible for the higher concentration of cyclized species in **5c** than in **5b**.

In view of the current emphasis in evaluating candidate antineoplastic agents initially *in vitro* rather than commencing studies with animal models [8], the proposal was made to screen the compounds against the EMT6 tumour

Table I. Percentage of surviving EMT6 cells[a] after exposure to various concentrations of **5a**–**c**, **6**.

Compound	Concentrations of compounds (μM)[b]						
	500	250	200	100	50	10	IC_{50} (μM)[c]
5a	37.4 ± 11.6	–	–	46.3 ± 11.2	61.5 ± 11.1	86.5 ± 15.4	85
5b	0	0	0	1.12 ± 1.15	6.56 ± 4.61	74.6 ± 12.2	16
5c	2.97 ± 1.76	–	–	67.1 ± 12.4	83.3 ± 26.1	93.2 ± 14.6	150
6a	0	0.08 ± 0.03	0.62 ± 0.30	17.3 ± 3.0	57.7 ± 7.3	93.1 ± 8.9	52
6b	17.5 ± 13.4	–	–	76.5 ± 18.3	91.2 ± 9.6	95.5 ± 5.1	200
6c	0	0	0	1.48 ± 0.73	6.66 ± 3.15	81.6 ± 23.9	18
6d	0	0.13 ± 0.01	0.43 ± 0.05	18.4 ± 5.5	52.9 ± 9.1	75.3 ± 11.9	51

[a]EMT6 cell plating efficiency = 0.72 ± 0.10.
[b]The symbol – means that the compound was not tested at this concentration.
[c]The IC_{50} is the estimated concentration of compound which will produce 50% survival of the cells.

in vitro. In particular, an examination of whether cyclic or acyclic species favored activity was sought. The effect of **5a–c**, **6** on EMT6 cells is summarized in Table I. The results indicate that no correlation between the extent of intramolecular cyclization and cytotoxicity is apparent. Thus the percentage of acyclic species in **5a–c** is 21, 72 and 57 respectively, but cytotoxicity is in the sequence **5b > 5a > 5c**. In addition, the average IC_{50} value for **5a–c**, which exist as a mixture of cyclic and acyclic species in solution is 84 μM which is comparable to the average IC_{50} value of series **6** (80 μM) in which only acyclic species were detected.

If the bioactivity of the compounds in series **6** is due to reaction with thiols at the methine function adjacent to the aryl ring, then the electronic properties of the R group should influence cytotoxicity. The *R* and *F* values for these groups are as follows, namely hydrogen (0.00, 0.00), methyl (−0.13, −0.04), carboethoxy (0.15, 0.33) and phenyl (−0.08, 0.08) [9]. Thus the predicted order of cytotoxicity is **6c> 6a, d > 6b**, which is found to be the case. Of particular interest in this series is compound **6c** which as one-sixth of the activity of the clinically useful alkylating agent melphalan. In addition, this Mannich base may be considered a soft drug [10] insofar as it contains an ester function which would be predicted to be hydrolyzed readily to the corresponding acid *in vivo*, thus permitting rapid excretion by conjugation. Hence **6c** serves as a useful lead compound for further molecular modification with the goal of producing cytotoxic agents with the potential for lower mammalian toxicity than the corresponding hard analogs [10] such as other compounds in series **5** and **6**.

In summary, procedures for preventing the process of intramolecular cyclization which occurs in the case of a number of Mannich bases have been found and these studies have revealed several compounds with useful cytotoxic properties.

Experimental protocols

Chemistry

All melting points are uncorrected. Elemental analyses were performed by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan and are within ±0.4% of the theoretical values. ^{1}H NMR spectroscopy was undertaken using either a Bruker WH 400 spectrometer in the Department of Chemistry, University of Alberta or Bruker AM 300 FT and Varian T-60 spectrometers in the Department of Chemistry, University of Saskatchewan. The mass spectrum was obtained from a VG Micromass MM16F mass spectrometer with a 2025 data system. Column chromatography was performed using Baker silica gel (60–200 mesh).

Synthesis of compounds

*Preparation of the unsaturated ketones required in the synthesis of **5** and **6***
3-(Phenylmethylene)-2,4-pentanedione (required in the preparation of **5**) and ethyl 3-*oxo*-2-(phenylmethylene)butanoate, which was a precursor for **6c**, were prepared by a reported procedure [11] except that the oils were not purified by distillation. The intermediate 4-phenyl-3-buten-2-one from which **6a** was prepared was obtained from the Aldrich Chemical Company, Inc. 3-Methyl-4-phenyl-3-buten-2-one was synthesized by a reported procedure [12] for use in the preparation of **6b** and used without purification by distillation while 3,4-diphenyl-3-buten-2-one required in the synthesis of **6d** was prepared by a literature method [13]. The ^{1}H NMR (60 MHz) of the synthesized compounds were in accord with the proposed structures.

*Preparation of the Mannich bases **5a–c**, **6***
Compound **5a** was synthesized by a literature method [3]. The Mannich reagents *N*-methylenemorpholinium chloride and *N*-methylenepiperidinium chloride (0.01 or 0.02 mol) required in the synthesis of **5b**, **c**, **6** were prepared by adding slowly a solution of acetyl chloride (0.25 mol) in dry methylene chloride (200 ml) to a stirring solution of the appropriate *bis*-aminomethane (0.25 mol) in dry methylene chloride (200 ml) cooled by an ice bath. The solid that precipitated was filtered, washed with dry methylene chloride (150 ml) and dried under vacuum to obtain the Mannich reagent. The structures of these compounds were confirmed by ^{1}H NMR spectroscopy (60 MHz) in dimethylsulphoxide–d_6.

The general procedure for preparing the Mannich bases **5b**, **c**, **6** was as follows. The Mannich reagent (0.01 or 0.02 mol) was added to a solution of the appropriate styryl ketone (0.01 mol) in dry acetonitrile (10–30 ml). The mixture was heated under reflux for a few minutes to ensure complete dissolution and the resultant clear solution was then shaken at 37°C (65°C for **5b**) for 18 (**5b**, **6a**), 24 (**6b–d**) and 48 (**5c**) h. After cooling at −11°C overnight, the precipitate was collected and recrystallized from ether–methanol to give the following compounds (mp °C, % yield): **5b** (198°, 55), **5c** as the dihydrate (166°, 58), **6a** (156°, 65), **6b** (167°, 65), **6c** (164°, 56) and **6d** (159°, 54). Compounds **5a–c**, **6** gave satisfactory combustion analyses (C,H,N) and ^{1}H NMR spectra (300 MHz) was in accord with the proposed structures.

*Attempted synthesis of **5d***
A mixture of 3-phenylmethylene-2,4-pentanedione (1.88 g, 0.01 mol), *N*-methylaniline (2.14 g, 0.02 mol) and an aqueous solution of formaldehyde (37% w/v, 0.6 g, 1.6 ml, 0.02 mol) was stirred at room temperature for 24 h. The solvent was removed *in vacuo* to give a residue which was triturated with aqueous sodium carbonate solution (10% w/v, 10 ml). Chloroform was added to the aqueous extract and evaporation of the solvent gave a residue (0.3 g). Chromatography using a column of silica gel and an eluting solvent of chloroform:methanol (8:2) gave a product which after washing with ether furnished *N*,*N*^{1}-dimethyl-*N*,*N*^{1}-*bis*[4-(4-methylaminophenylmethyl)phenyl]methylenediamine **7** as a pale yellow solid (0.18 g, 8%) mp 177°C. ^{1}H NMR ($CDCl_3$, 60 MHz): δ:7.07(m, 8, aryl H *ortho* to amino groups), 6.73 (m, 8, aryl H *ortho* to methylene functions), 4.40 (s, 6, 3 × CH_2), 3.49 (s, 2, 2 × NH) and 2.93 (s, 12, 4 × NCH_3). Mass spectrum: *m/z*:464 (M, 2.1%), 345 (M−CH_2-C_6H_4-$NHCH_3$, 5.1%) and 106 (C_6H_5-NCH_3, 40%). Anal. $C_{31}H_{36}N_4$ (C,H,N).

^{1}H NMR spectroscopy
The 400 MHz ^{1}H NMR spectra of solutions of **5a–c**, **6** in deuterium oxide (approximately 4 mg per ml) were determined at 37°C. The identification and quantitation of cyclic and acyclic species was made using our reported procedure [4]. Briefly this method involves integration at 2 different parts of the spectrum at least, principally the methine protons (benzylic in the cyclic species and olefinic in the acyclic form), the protons of the dimethylamino group and 2 methylene functions between the carbonyl group and the nitrogen atom. In the case of **5a**, after 10 min the ratio of cyclic:*E*:*Z* isomers was 79:13:8. The ratio of cyclic:acyclic species for **5c** was 43:57 after 10 min and 44:56 after 2 h. The spectra of **5a**, **b** did not change between 10 min and 2 h.

Quantitation took place in series **6** by integration of the olefinic protons. Compound **6a** gave rise to the deaminated product (14%) after 2 h. When the experiment was repeated using dimethylsulphoxide-d_6 as solvent (in which the compounds were dissolved prior to cytotoxicity screening), the compound remained stable at 37°C for 2 h. The spectrum of **6c** determined after 10 min showed the presence of *Z* and *E* isomers plus a third compound (present to the extent of 5%). After 2 h **6c** still with a *Z*:*E* isomeric ratio of 68:27 was present to the extent of 70% and at least one other decomposition product had been formed.

Cytotoxicity screening

*Evaluation of **5a–c**, **6** versus the EMT6 tumor* in vitro
The EMT6/RW mouse mammary carcinoma cells were maintained as described previously [14] in Waymouth's MB 752/1 media supplemented with Clex 15%*. The compounds were dissolved in dimethylsulphoxide

*Clex media supplement, obtained from bovine calf blood; Dextran Products Ltd., Scarborough, Ontario, Canada.

and control experiments used this vehicle alone, which was shown to be noncytotoxic at the amounts used.

Fresh media (10 ml) containing 1000 U of penicillin and 1 mg of streptomycin was added to EMT6 cells which were growing exponentially as monolayers in milk dilution bottles. The vehicle or solutions of the compounds (100 μl) were added to the media giving the final concentrations listed in Table I. After an exposure time of 2 h, the media was removed and the cells, after washing with phosphate buffered saline, were removed from the container by trypsinization. A sufficient number of cells were placed in plastic dishes (60 mm) containing fresh media (5 ml) so that approximately 100 colonies formed when cells were allowed to grow to a countable size for 10 days. Comparison of the plating efficiency of drug-treated *versus* vehicle-treated cells enabled the surviving fraction to be calculated. Each compound including the reference substance melphalan was tested in duplicate on at least 4 separate occasions and the numbers presented in Table I are the mean values ± SD.

The IC_{50} values were estimated from graphs of pooled data. In the case of melphalan, the percentage of surviving EMT6 cells (concentration in μM) was as follows namely 20.4 ± 4.5 (20), 35.8 ± 3.1 (10), 45.1 ± 6.9 (4) and 64.7 ± 4.2 (1). The IC_{50} value was 3 μM.

Use of the Student's *t*-test revealed that there were significant differences in the activities between the following pairs of compounds, namely **5a** and **6b** at 500 μM ($P=0.05$), **5b** and **6a** at 100 μM ($P\leq0.001$), **5c** and **6b** at 500 μM ($P\leq0.001$) and **6a** and **6d** at 250 μM ($P=0.001$). On the other hand, there were no significant differences between the cytotoxicities of **5b** and **6c** at 100 μM ($P=0.24$), **5c** and **6b** at 100 μM ($P=0.24$) and **6a** and **6d** at 200 μM ($P=0.24$).

Acknowledgments

The authors thank the Medical Research Council of Canada for a grant (MT-5538) to J.R. Dimmock and the Aegen University, Turkey who provided leave for E. Erciyas to undertake work at the University of Saskatchewan.

References

1 Nyathi C.B., Gupta V.S. & Dimmock J.R. (1979) *J. Pharm. Sci.* 68, 1383–1386
2 Dimmock J.R. & Taylor W.G. (1975) *J. Pharm. Sci.* 64, 241–249
3 Dimmock J.R., Raghavan S.K., Logan B.M. & Bigam G.E. (1983) *Eur. J. Med. Chem.* 18, 248–254
4 Dimmock J.R., Raghavan S.K. & Bigam G.E. (1988) *Eur. J. Med. Chem.* 23, 111–117
5 Grillot G.F. & Lau P.T.S. (1965) *J. Org. Chem.* 30, 28–33
6 Charton M. (1977) *in: Design of Biopharmaceutical Properties Through Prodrugs and Analogs* (Roche E.B., ed.), American Pharmaceutical Association, Washington, pp. 228–280
7 Albert A. & Serjeant E.P. (1984) *in: The Determination of Ionization Constants.* Chapman and Hall, London, 3rd edn., pp. 151, 152
8 Boyd M., Shoemaker R., Alley M., Scudiero N., Monks A., McLemore T., Gorelik E., Fine D., Mayo J. & Chabner B. (1987) *Invest. New Drugs* 5, 78
9 Hansch C., Leo A., Unger S.H., Kim K.H., Nikaitani D. & Lien E.J. (1973) *J. Med. Chem.* 16, 1207–1216
10 Bodor N. (1984) *in: Advances in Drug Research* (Testa B., ed.), Academic Press, London, vol. 13, pp. 255–331
11 Horning E.C., Koo J., Fish M.S. & Walker G.N. (1951) *Org. Synth.* 31, 56–58
12 Harries C. & Muller G.H. (1902) *Ber.* 35, 966–971
13 Pratt E.F. & Werbe E. (1950) *J. Am. Chem. Soc.* 72, 4638–4641
14 Rockwell S. (1977) *Lab. Anim. Sci.* 27, 831–838