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Mannich bases of phenolic azobenzenes possessing cytotoxic activity

JR Dimmock[1]*, E Erciyas[1], P Kumar[1], M Hetherington[1], JW Quail[2], U Pugazhenthi[2], SA Arpin[2], SJ Hayes[3], TM Allen[4], S Halleran[4], E De Clercq[5], J Balzarini[5], JP Stables[6]

[1]*College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan;*
[2]*Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5C9;*
[3]*Department of Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E5;*
[4]*Department of Pharmacology, University of Alberta, Edmonton, Alberta, T6G 2H7 Canada;*
[5]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium;*
[6]*National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892–9020, USA*

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Summary — A number of arylazophenols **1** were converted into the corresponding mono Mannich bases **2** from which two quaternary salts **3a,b** and an ester **3c** were prepared. A series of bis Mannich bases **4** were also synthesized. The angles (θ) made between one of the aryl rings and the adjacent azo linkage were determined by electronic absorption spectroscopy. X-ray crystallographic data were obtained for some of the Mannich bases. The compounds were evaluated against murine P388 D1 and L1210 cells and two human T-lymphocyte (Molt 4, CEM) lines, and most of the derivatives were also screened against a panel of human tumour cell lines. A number of correlations were noted between cytotoxicity and various physicochemical constants as well as some structural features determined by X-ray crystallography. Several of the Mannich bases were shown to have mutagenic properties using the λ RK mutatest; the compounds in series **2** and **4** have the ability to penetrate the central nervous system, as revealed by their anticonvulsant properties. While series **2–4** have the potential to deaminate forming *ortho* quinone methides which would be capable of alkylating cellular thiols, the results of stability studies suggest that the bioactivities noted were due to the molecules per se.

arylazophenol / Mannich base / cytotoxicity / mutagenesis / X-ray crystallography / anticonvulsant / CNS activity

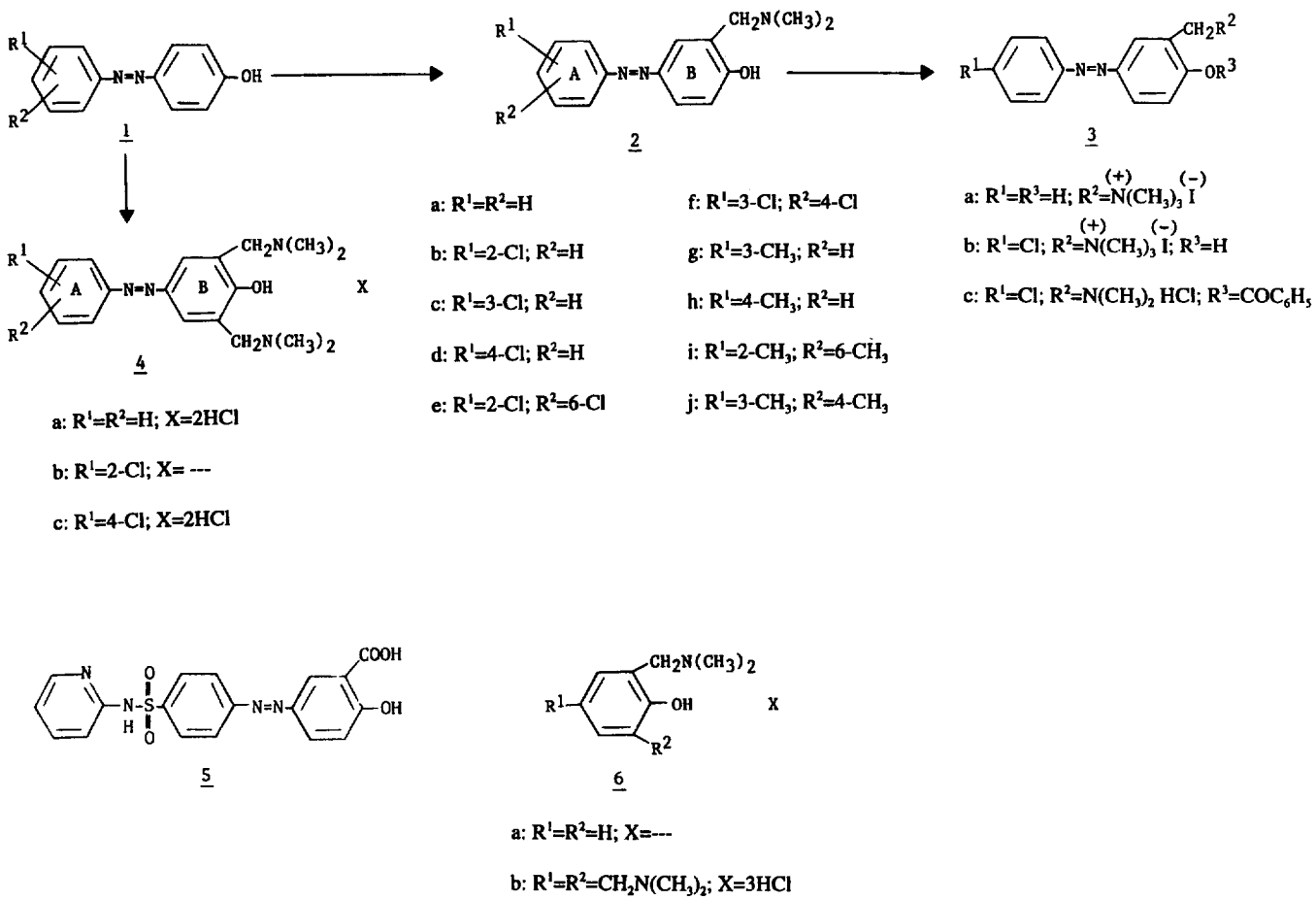
Introduction

A number of Mannich bases derived from aryl and arylidene ketones have demonstrated significant cytotoxicity and anticancer properties [1, 2]. The bioactivities have been attributed to the α,β-unsaturated ketones liberated from the Mannich bases by deamination [3]. These enones have high affinities for thiols which are absent in nucleic acids. Hence in contrast to currently available alkylating agents used in cancer chemotherapy, interactions with nucleic acids may be absent and side effects such as mutagenicity and carcinogenicity may be prevented.

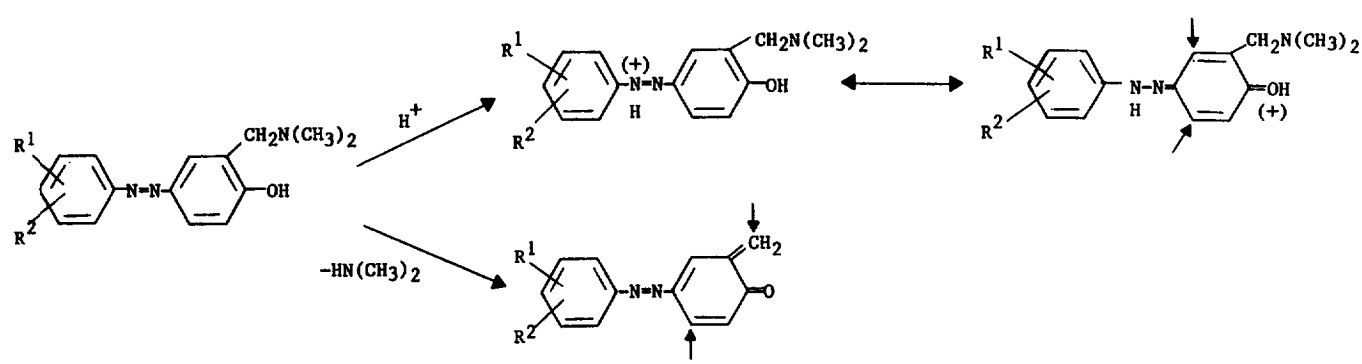
The initial aim of the present investigation was to prepare a number of Mannich bases of azobenzenes for cytotoxic evaluation (scheme 1). Thus the preparation of series **1** was planned, which would be converted to the corresponding mono Mannich bases **2**, representatives of which would give rise to the quaternary ammonium salts **3a,b** and ester **3c**. The preparation of the bis Mannich bases **4** was also considered.

The reasons for undertaking this study were as follows. First, certain cancer cells have a lower pH than the corresponding normal cells [4], and hence molecules which form alkylating species under acidic conditions may have selective toxicity towards neoplastic tissues. Azobenzenes containing an electron donor group in the *para* position of one of the aryl rings can produce protonated quinoneimines in an acidic milieu [5], which suggested that a series of compounds such as **2** would be of potential interest. In addition, deamination to the corresponding *ortho* quinone methides is conceivable. These possible reactions are illustrated in scheme 2. Second, sulfasalazine **5**, which contains the azobenzene nucleus, is claimed to have a special affinity for connective tissue and is used in treating chronic ulcerative colitis [6]. It is possible therefore that the compounds in series **2–4** may have a predilection for colonic cells and possibly colon cancers. Third, a number of studies have

*Correspondence and reprints



Scheme 1. Structure of compounds **1**–**6**.



Scheme 2. The putative pathways for the formation of alkylating species from series **2**. The arrows indicate the sites at which reactions with cellular thiols could occur.

revealed correlations between the relative planarities of different functional groups and the magnitude of biological responses [7, 8]. Hence the placement of different R^1 and R^2 substituents in ring A would cause variations in the interplanar angle θ formed between this ring and the adjacent unsaturated linkage. The θ value is defined as the angle between the two planes made firstly by the carbon atoms at positions 1 and 2 of ring A, and secondly the azo linkage. The observation of a relationship between cytotoxicity and θ values would be of value in subsequent drug design. Fourth, a noteworthy feature of a number of acyclic Mannich bases prepared previously was central nervous system (CNS) penetration, as revealed by their anticonvulsant [9, 10] and narcotic analgesic [11] properties. These results may have been due to the structural similarities of these Mannich bases with both established anticonvulsants [9] and narcotic analgesics [12]. This property may be advantageous insofar as the compounds would have the potential to treat CNS tumours, or it could constitute a significant unwanted side effect. The decision was made to attach the dimethylaminomethyl function at the *ortho* position to a phenolic hydroxy group producing series **2**. The question posed was whether these and the related molecules **3** and **4**, which are all structurally diverse from the previous series of acyclic Mannich bases, would penetrate the CNS. In order to observe whether bioactivity was due either entirely or in part to the aryl ring B, the preparation and screening of 2-dimethylaminomethylphenol and 2,6-*bis*(dimethylaminomethyl)-phenol were proposed.

Finally, a number of alkylating agents, including those used clinically in cancer chemotherapy, have been claimed to have carcinogenic and mutagenic properties [13]. Various in vitro tests have been introduced to detect carcinogens based on the fact that many cancer-causing chemicals are mutagenic. Although olsalazine, which is an azobenzene containing hydroxy groups in the *para* positions of the aryl rings, is found to be nonmutagenic in the Ames test (Pharmacia Montreal, 1995; private commun), other azobenzenes have marked carcinogenic properties [14, 15]. Hence in examining this novel series of candidate alkylating agents, the evaluation of representative compounds for mutagenic potential was considered to be an important aspect of this study.

Chemistry

The azophenols **1** were prepared by reacting the appropriate arylamine with nitrous acid to form the corresponding diazonium salt, which was then coupled with sodium phenate. Treatment of the azophenol with equimolar quantities of formaldehyde and dimethylamine led to the formation of the mono Mannich bases **2** in yields of approximately 50–70%. Quaternization of **2a,d** with methyl iodide produced **3a,b**. The ester **3c** was prepared by acylation of **2d** with benzoyl chloride. Reaction of the appropriate azophenol **1** with two molar equivalents of formaldehyde and dimethylamine gave rise to the related Mannich bases **4a**, isolated as the dihydrochloride salt and **4b**. A residue obtained by column chromatography from the reaction mixture from which **2d** was isolated was treated with hydrogen chloride to give **4c** in low yield. While the preparation of **6a** was successful, reaction of phenol with two molar equivalents of formaldehyde and dimethylamine in an attempt to prepare 2,6-*bis*(dimethylaminomethyl)phenol, produced an oil which on treatment with hydrogen chloride was shown to be **6b**.

The electronic absorption spectra of many of the compounds prepared in this study were examined, and the data have been summarized in table I. The ORTEP diagrams of **2a,h,j**, **4a,c** generated by X-ray crystallography are displayed in figs 1–5. The stabilities of two representative compounds under simulated physiological conditions were examined. Solutions of **2a** (in the form of the hydrochloride salt) and **4a** in phosphate buffer, pH 7.4 and 6.9, were incubated at 37 °C for 48 h, after which the free bases were isolated. TLC of the mother liquors revealed unreacted compounds. In addition, **2a** hydrochloride and **4a** were incubated at 37 °C for 48 h in deuterated phosphate buffered saline (PBS–d) using pD values of 7.4 and 6.4, and no changes in the ^{1}H-NMR spectra were observed. Incubation of the hydrochloride salt of **2a** with 2-mercaptoethanol in buffer, pH 7.4, for 48 h at 37 °C indicated that no thiol adducts were formed.

Bioevaluation

Evaluation of **1** ($R^1 = R^2 = H$) and series **2–4**, **6** against murine P388 D1 lymphocytic leukemia cells was undertaken. The same compounds were also screened against murine L1210 cells as well as Molt C/8 and CEM human T-lymphocytes. The azophenols in series **2** and **4** as well as **3c** were examined against 55–60 human tumour cell lines from the following neoplastic diseases, namely leukemia, melanoma, non-small cell lung, colon, CNS, ovarian, renal, prostate and breast cancers. The results of these cytotoxicity evaluations are presented in table II.

Compounds **1** ($R^1 = R^2 = H$), **2–4**, **6** were injected by the intraperitoneal route into mice and examined for anticonvulsant activity in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (scPTZ) screens [16]. In addition, evaluation of neurotoxicity in the rotorod test [16] was undertaken. These results are presented in table III.

Four representative Mannich bases **2a**, **2d**, **4a** and **4c** as well as an azophenol **1** ($R^1 = R^2 = H$) and a non-azophenol **6b** were evaluated for possible mutagenic activity at pH 7.6 in 20% (v/v) dimethylsulphoxide, except for **4c** in which case the solvent was 40% (v/v) dimethylsulphoxide using the RK–SM form of the λ RK mutatest [17]. The RK mutatest was chosen because it independently measures toxicity and mutagenicity. The test utilizes an *E coli* uvrB strain that has an integrated fragment of bacteriophage lambda (λ) in its chromosome [17–19] with the λ immunity region and the *O* and/or *P* genes of the defective prophage as the genetic target for mutation. The λ prophage has a temperature-sensitive repressor, CI, which is active at 30 °C, but at 42 °C this repressor is thermally denatured. Upon shifting parental RK^+ (replicative killing competent) cells to 42 °C, the trapped prophage fragment initiates replication which kills the host *E coli* cells. Rare spontaneous RK^- mutants defective for λ replication survive at 42 °C and form colonies at a frequency of ~ 3 x 10^{-8}. The frequency of the RK^- mutants is increased by exposing concentrated RK^+ cells (cultures grown at 30 °C) to mutagenic agents prior to shifting the cells to 42 °C. The results are summarized in table IV. Each of the Mannich bases were shown to be mutagenic, with **4c** being the most potent. Of interest is the observation that **4c** was inactive in the *Salmonella* mutagenicity test [20] in assays performed without the S9 microsomal fraction.

Results and discussion

Reaction of different aryl amines with nitrous acid led to the formation of the corresponding diazonium salts, which on treatment with sodium phenate gave rise to the compounds in series **1** [5]. Previous studies revealed that the predominant and most stable isomers in various azobenzenes had the *E* configuration [21]. In the present investigation a minor component, presumed to be the *Z* isomers, was noted in the initial reaction products which was removed by subsequent recrystallizations. The compounds were found to be homogeneous by TLC. The stereochemistry of the azo groups in **1**, $R^1 = R^2 = H$ and series **2–4** was therefore assigned the *E* configuration, a viewpoint which was confirmed in the case of **2a**,**h**,**j**, **4a**,**c** regarding which X-ray crystallographic data were available.

Equimolar amounts of the azophenol **1**, formaldehyde and dimethylamine underwent the Mannich reaction to form series **2**, while excess of the reagents led to the formation of **4a** and **4b**. The introduction of dialkylaminoalkyl groups into the aryl ring of the phenols during the course of the Mannich reaction preferentially occurs on the *ortho* position to the aryl hydroxyl groups; on occasions reaction at the *para* position also takes place [22]. On this basis, reaction, was assumed to occur at the *ortho* position to the hydroxy group, and the ORTEP diagrams of the compounds portrayed in figs 1–5 support this assignment.

The electronic absorption spectra of **1** ($R^1 = R^2 = H$), **2a–j** and **4a–c** were obtained with a view to determining the angle (θ) between the aryl ring A and the adjacent unsaturated linkage. Calculations of θ values were made using eq (1) developed by Braude et al [23, 24], whereby ε and ε_o refer to the molar absorbtivities of the substituted and unsubstituted compounds respectively.

$$\cos^2\theta = \frac{\varepsilon}{\varepsilon_o} \qquad (1)$$

The data in table I reveal that substituents in the *ortho* and *meta* positions of ring A inhibit its coplanarity with the adjacent unsaturated linkage. Compounds which have one *ortho* substituent, namely **2b** and **4b** had θ values of 22–28°, while the presence of two *ortho* groups as in **2e** and **2i** led to an approximately two-fold increase in the θ figures. The *meta* substituents in compounds **2c**, **2f**, **2g** and **2j** exert a buttressing effect, ie, the *meta* groups force the *ortho* hydrogens towards the azo linkage, impeding coplana-

Table I. Electronic absorption spectroscopy of **1**, $R^1 = R^2 = H$, **2** and **4** in methanol.

Compound	λ_{max}, *nm* (± *1 nm*)	ε (± *100*)	θ
1, $R^1 = R^2 = H$	347.1	25,971	–
2a	348.2	24,157	0.0
2b	362.0	22,278	22.3
2c	359.1	22,351	22.1
2d	358.3	25,765	0.0
2e	342.2	17,048	40.8
2f	366.7	24,699	12.1
2g	352.8	23,733	23.4
2h	353.9	27,541	0.0
2i	339.0	18,292	46.3
2j	355.4	26,794	10.1
4a	343.2	19,108	0.0
4b	372.7	19,153	28.4
4c	353.4	21,452	0.0

rity of the aryl ring with the adjacent azo group. The lowering of the θ values from 22–23° displayed by **2c** and **2g** to 10–12° when a *para* substituent was inserted into the aryl ring (**2f** and **2j**) is probably due to the groups in the *para* position increasing the delocalization of the electrons, thereby enhancing coplanarity in the molecules. Furthermore, the buttressing effect of the dimethylaminomethyl group in ring B was revealed by comparing the ε values of **2a** with **1**, $R^1 = R^2 = H$ as well as **4b** with **2b**.

The cytotoxicity data are presented in table II. The P388 screen was chosen since it is considered to be a good indicator for clinically effective compounds [25]. All of the azophenols were active except for the quaternary ammonium iodide **3a** and the compounds representing the partial structures in series **2** and **4** (namely **6a** and **6b**). A comparison of the cytotoxicity of **2a**, **3a** and **4a** with **2b–j**, **3b** and **4b**, **4c** respectively revealed that substitution in ring A increased bioactivity. Molecular modification of **2a** and **2d** producing the quaternary ammonium salts **3a** and **3b** led to a reduction in cytotoxicity, while acylation of **2d** led to a small increase in the IC_{50} value of the corresponding ester **3c**. The introduction of a second dimethylaminomethyl group into ring B produced variable results. Thus **2a** and **4a** had comparable potencies, **2b** was more active than **4b**, while **2d** was less potent than **4c**.

The second cytotoxic screen used murine L1210 cells, also claimed to predict useful cancer chemotherapeutic agents [26]. In addition, evaluation was undertaken using Molt 4 C/8 and CEM human T-lymphocytes with a view to detecting compounds with preferential toxicity to malignant cells. The data in table II reveal that, in contrast to P388 D1 cells, the faster-growing L1210 cells were on average approximately 11 times more refractory to the compounds **2–4**, **6**. With the exception of **2e** and **3b**, compounds containing substituents in ring A were more cytotoxic than the related unsubstituted analogues **2a**, **3a** and **4a**. Quaternization of **2a**, leading to **3a**, led to a threefold increase in cytotoxicity while quaternization and esterification of **2d** producing **3b** and **3c** caused a decrease and increase in activity, respectively. The introduction of a second dimethylaminomethyl group

Table II. Cytotoxic evaluation of various Mannich bases of phenolic azobenzenes and related compounds.

Compound	*P388 D1 cells* IC_{50} *(μM)*	*L1210 cells and human T-lymphocytes* IC_{50} *(μM)*					*Human tumours* IC_{50} *(μM)*
		L1210	*Molt 4 C/8*	*TI*	*CEM*	*TI*	
1, $R^1 = R^2 = H$	15.4	70.6 ± 12.1	73.2 ± 12.1	1.04	66.6 ± 6.6	0.94	–
2a	24.5	203.2 ± 22	314.5 ± 61	1.55	181 ± 5.9	0.89	41.69
2b	6.55	12.52 ± 0.6	12.6 ± 0.8	1.01	14.0 ± 1.1	1.12	17.78
2c	12.3	33.4 ± 8.7	66.3 ± 15.2	1.99	63.5 ± 18.6	1.90	10.96
2d	8.70	47.3 ± 3.1	54.2 ± 2.4	1.15	25.6 ± 10.8	0.54	14.79
2e	15.5	247.7 ± 17	216.2 ± 9.6	0.87	176.4 ± 20	0.71	69.18
2f	8.14	34.24 ± 2.5	45.3 ± 5.6	1.32	36.1 ± 8.6	1.05	7.76
2g	12.8	46.41 ± 3.7	65.7 ± 4.1	1.42	75.7 ± 12.3	1.63	17.78
2h	10.4	50.9 ± 2.2	65.7 ± 2.2	1.29	52.7 ± 4.5	1.04	19.05
2i	17.8	72.0 ± 3.9	108.3 ± 20	1.50	77.3 ± 11.6	1.07	40.74
2j	10.0	41.3 ± 1.8	72.0 ± 12	1.74	55.2 ± 5.3	1.34	22.39
3a	> 50	69.0 ± 10.1	46.3 ± 4.5	0.67	44.3 ± 2.5	0.64	–
3b	17.1	204.3 ± 17	176.7 ± 10	0.87	99.6 ± 11.1	0.49	–
3c	11.6	29.5 ± 4.4	41.4 ± 2.6	1.40	30.7 ± 3.5	1.04	14.45
4a	26.9	201.1 ± 11	211.5 ± 7.3	1.05	190.2 ± 27	0.95	74.13
4b	14.5	55.9 ± 17.3	191.7 ± 19	3.43	90.2 ± 17.6	1.61	46.77
4c	3.07	29.1 ± 7.4	10.1 ± 1.0	0.35	9.7 ± 0.9	0.33	6.91
6a	> 50	675 ± 95	> 734	–	701 ± 33	1.04	–
6b	> 50	> 530	> 530	–	> 530	–	–
Melphalan	0.22	2.13 ± 0.02	3.24 ± 0.56	1.52	2.47 ± 0.21	1.16	22.91

into **2a**, **2b** and **2d**, leading to **4a–c** respectively, revealed that **2a** and **4a** had the same activity, **2b** was more potent than **4b**, while **2d** was less active than **4c**. The data indicated that 77 and 56% of the compounds have therapeutic values > 1 when comparisons were made between the cytotoxicity data for the L1210 cells and the Molt 4 C/8 and CEM lymphocytes, respectively. Compounds **2c** and **4b** had the most favourable therapeutic indices which were greater than that of melphalan, and could therefore be considered as useful lead molecules.

Approximately 70% of the compounds evaluated against a panel of human tumours were more cytotoxic than melphalan. Of particular note were **2f** and **4c**, possessing approximately three times the potency of this clinically useful drug. The presence of different substituents in ring A of series **2** and **4** increased the cytotoxicity in general. Esterification of **2d** leading to **3c** had little effect on bioactivity. The introduction of a second dimethylaminomethyl group into **2a**, **2b** and **2d** gave varying results. While **2a** and **2b** had approximately twice the potency of **4a** and **4b** respectively, the bis Mannich base **4c** was more than twice as active as **2d**. Compound **4b** displayed selective toxicity towards melanoma cell lines when the total growth inhibition (TGI) panel was examined [27]. While the concentration of **4b** required to prevent an increase in cell growth was greater than 100 μM for 45/50 of the cell lines from the other eight neoplastic diseases, the average $\log_{10}$ TGI figures for six melanoma cell lines was 41.69 μM. Hence, this derivative with specificity for human melanoma cells is a useful prototypic molecule.

Linear and semilogarithmic plots of the IC_{50} figures of the compounds of series **2** generated in the P388 D1, L1210 and human tumour screens were made against the Hammett σ values, Hansch π figures and molar refractivity constants of the aryl substituents. Using the test for zero correlation [28], a significant relationship was observed whereby cytotoxicity towards human tumours rose as the Hammett σ values increased using linear ($P < 0.1$) and semilogarithmic ($P < 0.05$) plots. No other correlations were noted. Linear plots of the θ values against the IC_{50} figures of the compounds in series **2** in the three cytotoxicity screens were made. In addition, semi-logarithmic plots were undertaken, although in these cases, compounds having θ values of 0 were omitted. A lowering of the θ values was correlated with increased cytotoxicity in the P388 D1 ($P < 0.05$), L1210 ($P < 0.1$) and human tumour ($P < 0.05$) screens when semilogarithmic plots were made, whereas reduction in the θ values was correlated with increased cytotoxicity only in the human tumour assay ($P < 0.1$) when linear plots were made. These statistical analyses reveal that for the design of future analogues with increased potencies, a strongly electron-attracting substituent should be inserted into the *para* position of ring A.

Cytotoxicity is therefore influenced by the nature of the substituents in ring A. In order to examine whether the orientation of the dimethylaminomethyl groups in relation to aryl ring B also influenced bioactivity, X-ray crystallography of representative compounds was undertaken. In addition, confirmation of the assignments made for both the stereochemistry of the azo linkage and the locations of the dimethylaminomethyl group in the aryl ring may be provided by X-ray crystallography.

Figures 1–5 are the ORTEP diagrams of **2b**, **h**, **j**, **4a**, **c**. The Mannich base **2b** had two crystallographically independent molecules in the asymmetric unit,

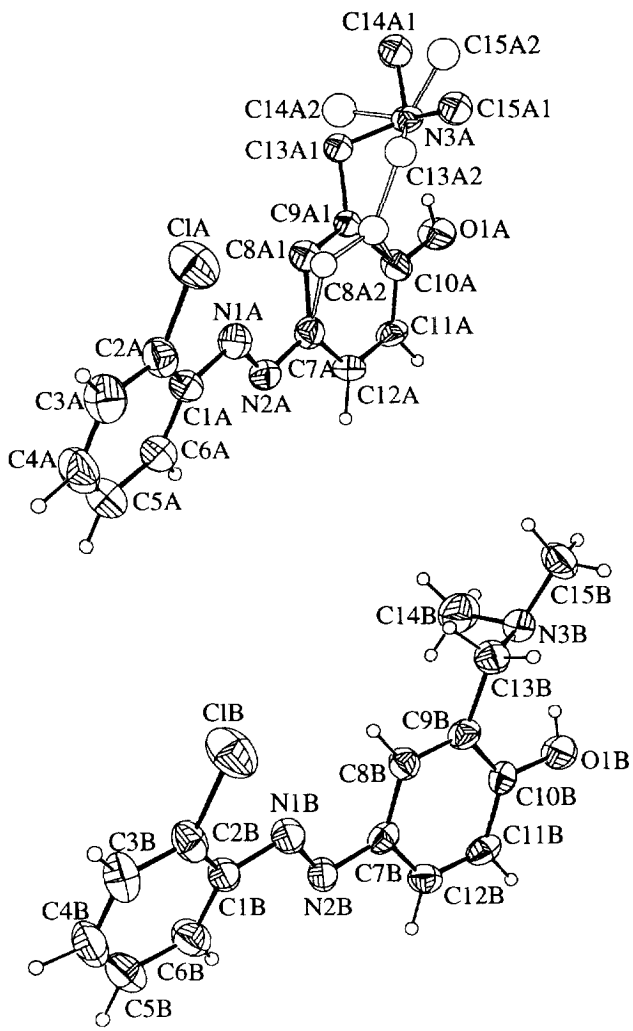


Fig 1. ORTEP diagrams of **2bA** and **2bB**.

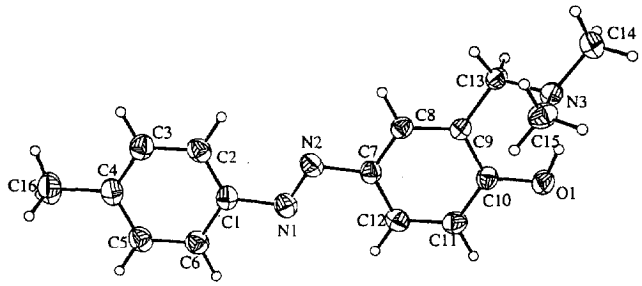


Fig 2. ORTEP diagram of **2h**.

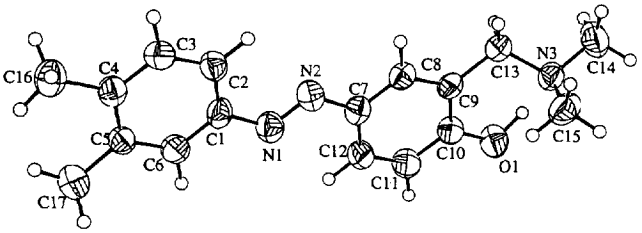


Fig 3. ORTEP diagram of **2j**.

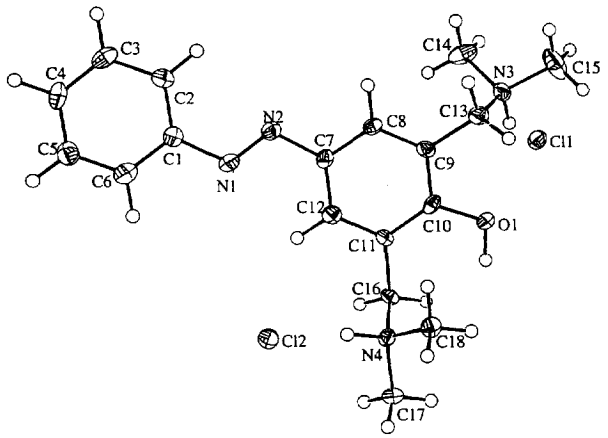


Fig 4. ORTEP diagram of **4a**.

which are referred to as **2bA** and **2bB**. In **2bA** the atoms C8A, C9A, C13A, C14A and C15A were disordered and occupied two different positions with occupancies of 0.63 (C8A1, C9A1, C13A1, C14A1, C15A1) and 0.37 (C8A2, C9A2, C13A2, C14A2, C15A2). The X-ray crystallographic data were obtained on **2b**, **h**, **j** as the free bases while **4a**, **c** were

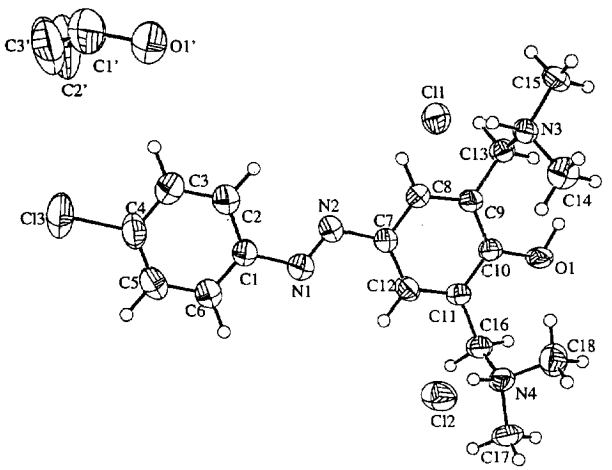


Fig 5. ORTEP diagram of **4c**.

examined as the dihydrochloride salts. In the cases of **2b**, **h**, **j**, strong hydrogen bonding between the hydroxyl protons and the amine nitrogen atoms was indicated by the short (~ 2.6 Å) N3–O1 distances in all three compounds (**2bA**, 2.611(7) Å; **2bB**, 2.605(8) Å; **2h**, 2.604(2) Å; **2j**, 2.628(4) Å. The assumption was made that for the dihydrochloride salts, **4a**, **c**, the amine nitrogens were both protonated with N–H distances of 0.90 Å. Interatomic distances indicated that hydrogen bonds existed between the chloride ions and the ammonium N–H (**4a**, d(C11–N3) = 3.065(4) Å, d(C12–N4) = 3.016(4) Å; **4c**, d(C11–N3) = 3.258(3) Å, d(C12–N4) = 3.027(3) Å). In **4a**, there was an indication of weak hydrogen bonds between N3 and O1 (3.205(4) Å) and between C11 and O1 (3.035(3) Å). In the structure of **4c** there was a short C11–O1 distance (3.116(3) Å), indicating an H-bonding interaction, but no N–O distance < 3.5 Å. The shapes of ring B and the substituents in the three compounds **2b**, **h**, **j** were virtually superimposable and ring B and the attached groups in **4a**, **c** were similar in shape. Hence the variation in cytotoxicity among **2b**, **h**, **j** and also **4a**, **c** may be attributed to the effects of different substituents in ring A and not to different orientations in the pendant groups of ring B.

Finally, as mentioned previously, the ORTEP diagrams confirm that the azo linkage adopts the *E* configuration and the dimethylaminomethyl groups are located *ortho* to the phenolic hydroxyl function.

The stability studies on two representative compounds **2a** and **4a** were carried out at 37 °C for 48 h, which were the temperature and time used in the P388 D1 and human tumour assays. At pH and/or pD values of 7.4, 6.9 and 6.4, the compounds were stable

and hence the deamination process envisaged in scheme 2 appears unlikely. No reaction occurred with a model thiol 2-mercaptoethanol and **2a** at pH 7.4. It is conceivable therefore that the bioactivities of the compounds are due to the molecules per se or that activity does not involve thiols.

The data in table III indicate that all the compounds except for the quaternary ammonium salts **3a** and **3b** and the monocyclic phenols **6a** and **6b** displayed anticonvulsant properties, and hence penetrate the CNS. While 88% of the azobenzenes were active in the MES screen, only 18% were effective in the scPTZ test. Hence development of these prototypic molecules may lead to clinically useful compounds which prevent the spread of seizures [16]. Neurotoxicity was demonstrated in the 30–300 mg/kg range for all of the azophenols except **2f** and **2j**. In general, the compounds afforded better protection at the end of 0.5 than 4 h. Compounds **2a–e**, **2g**, **2h**, **3c**, and **4a–c** were administered per os to rats using a dose of 30 mg/kg. Protection was afforded in the MES test for 25% of the animals by **2a–d**, **2h**, **3c**, **4a** and **4c**, while none of the compounds caused neurological deficit. Thus various Mannich bases of phenolic azobenzenes penetrate the CNS, and development of further analogues may lead to compounds of value in treating tumours of the CNS.

The mutagenic potential of Mannich bases **2a**, **2d**, **4a** and **4c**, the azophenol **1** ($R^1 = R^2 = H$) and a non-azophenol **6b** were assayed by the λ RK mutatest and the results are summarized in table IV. A substance is considered mutagenic in the assay if its average mutagenic index (MI) for several determinations is minimally two-fold greater than the MI of the solvent control, a point used as the threshold sensitivity for the assay. The results in table IV indicate that all of the azobenzenes displayed mutagenic activity, while the phenol **6b** was free from this property as assayed. The order of mutagenic activity was **4c** > **1** ($R^1 = R^2 = H$) > **2d** > **4a** > **2a**, from which one may deduce the

Table III. Evaluation of some Mannich bases of azophenols and related compounds for anticonvulsant activity.

Compound	*Anticonvulsant evaluation*[a]					
	MES screen		*scPTZ screen*		*NT screen*	
	0.5 h	*4 h*	*0.5 h*	*4 h*	*0.5 h*	*4 h*
1, $R^1 = R^2 = H$	300	–	300	–	300	–
2a	30	100	–	–	100	300
2b	30	300	–	–	300	300
2c	30	300	–	–	100	300
2d	300	100	–	–	300	–
2e	30	300	–	–	300	–
2f	–	300	–	–	–	–
2g	30	100	–	–	100	300
2h	100	300	–	–	300	300
2i	30	100	30	–	100	–
2j	300	300	–	–	–	–
3a	–	–	–	–	100	–
3b	–	–	–	–	100	100
3c	300	100	300	300	300	300
4a	30	30	–	–	100	–
4b	30	100	–	–	30	100
4c	30	30	–	–	100	300
6a	–	–	–	–	300	–
6b	–	–	–	–	–	–
Phenytoin	30	100	–	–	100	100

[a]Doses of 30, 100 and 300 mg/kg of the compounds were administered ip into mice and examined in maximal electroshock (MES), subcutaneous pentylenetetrazole (scPTZ) and neurotoxicity (NT) screens after 0.5 and 4 h. The figures indicate the minimum dose required to elicit a biological response in 50% or more of the animals. The symbol – indicates that anticonvulsant activity or neurotoxicity was absent at the maximum dose of 300 mg/kg.

Table IV. Evaluation of **1** ($R^1 = R^2 = H$), **2a**, **2d**, **4a**, **4c** and **6b** for mutagenic activity using *E coli* strain 832.

Compound	*Mutation indices (mM solutions)*[a]		
1 ($R^1 = R^2 = H$)	1.9 (0.25)	6.2 (0.5)	1400 (0.8)
2a	≤ 1 (4.9)	288 (9.8)	361 (19.6)
2d	1.6 (0.35)	47 (1.7)	705 (3.5)
4a	13 (1.3)	83 (2.6)	3666 (19)
4c	24 (0.24)	200 (1.2)	–
6b	≤ 1 (1.35)	≤ 1 (2.7)	≤ 1 (5.3)
Mitomycin C[b]	≤ 1 (0.15)	1.5 (0.3)	15 (0.6)
N-Methylnitrosourea	2.1 (0.1)	≤ 1 (0.2)	32.7 (0.4)

[a]Each mutation index (MI) is the ratio of the mutation frequency (MF) induced by the compound divided by the spontaneous mutation frequency (SMF) for untreated parental RK^+ assay cells. The MF figures are the titre of the spontaneous and induced RK^- mutants forming colonies at 42 °C divided by the titre of RK^+ parental assay cells at 30 °C; [b]concentrations are given in μM.

following correlations between structure and activity. First, the insertion of the 4-chloro atom into ring A led to an increase in mutagenic properties, since **2d** > **2a** and **4c** > **4a**. Second, the introduction of a second dimethylaminomethyl group into ring B increased mutagenicity, ie, **4a** > **2a** and **4c** > **2d**. Third, the mutagenic properties of **2a**, **2d**, **4a** and **4c** are likely due to the presence of the hydroxyazobenzene group common to all four molecules. This supposition is strengthened by the fact that **1** ($R^1 = R^2 = H$) is a potent mutagen. These data suggest that any development of this group of compounds should include toxicological assessments as potential mutagens.

Conclusions

The synthesis of a number of Mannich bases of phenolic azobenzenes has been described. The compounds demonstrated cytotoxicity towards murine and human cancers in vitro and a number of correlations between bioactivity and physicochemical properties were found. These Mannich bases penetrated the central nervous system as revealed by their anticonvulsant properties. While these compounds are a novel series of cytotoxic agents, their development should be accompanied by mutagenic screening since representatives described in this study were revealed to have this property in the RK mutatest.

Experimental protocols

Melting points are uncorrected. Elemental analyses (C, H, N) were undertaken on **1** ($R^1 = R^2 = H$), **1** (R^1 = 3-CH_3, R^2 = 4-CH_3), **2a** (and hydrochloride salt), **2b–j**, **3a–c**, **4a–c**, **6a** and **6b** and were within 0.4% of the calculated values except for **4c** (calc for $C_{18}H_{25}Cl_3N_4O$: N, 13.35%. Found: N, 12.62%). ^{1}H-NMR spectra were determined routinely using a Varian T-60 spectrometer, while for the stability study a Bruker AMX 500 machine was employed. TLC was performed routinely with plastic-backed silica gel plates using solvent systems of chloroform/methanol (4:1) for series **1** and **2**, while chloroform/methanol/ammonium hydroxide (4:1:0:1) was used for **4a–c**.

Synthesis of series 1

A solution of sodium nitrite (0.058 mol) in water (10 mL) was cooled to ~ 5 °C and added dropwise to a vigourously stirred solution of the appropriate arylamine (0.054 mol) in hydrochloric acid (37% w/v, 16 mL) and water (16 mL) at ~ 5 °C. A solution of phenol (0.054 mol) in aqueous sodium hydroxide solution (10% w/v, 45 mL) cooled to ~ 5 °C was added slowly to the diazonium salt solution. The reaction mixture stood at ~ 5 °C for 0.5 h and the precipitate was collected, washed with water, dried and recrystallized from ethanol except for **1**, $R^1 = R^2 = H$ and **1**, R^1 = 4-Cl, R^2 = H in which cases toluene and water/ethanol, respectively, were employed. The yields were in the 81–91% range. All of the compounds except for **1**, R^1 = 3-CH_3, R^2 = 4-CH_3 had been prepared previously and had melting points in accord with the literature values. The new azophenol, mp = 158 °C, was prepared in 88% yield.

Synthesis of series 2

A solution of the arylazophenol (0.025 mol), aqueous formaldehyde solution (37% w/v, 0.025 mol), aqueous dimethylamine solution (25% w/v, 0.025 mol) and ethanol (40 mL) was heated under reflux for 18 (**2a**), 24 (**2b**, **2e**, **2i**), 28 (**2c**, **2h**), 20 (**2d**, **2j**), 30 (**2f**) and 22 (**2g**) h. The solvents were removed in vacuo to give residues which were purified as follows. Compounds **2d** and **2h** were recrystallized from water/ethanol and ethanol respectively. The remaining compounds were obtained by chromatography using a column of silica gel 60 (70–230 mesh). A solvent system of ethyl acetate followed by either a mixture of ethyl acetate/methanol (7:3) (**2a–c**, **2e–g**, **2j**) or a chloroform/methanol solution (9:1) (**2i**) was utilized. The melting points (°C) and yields (%) were as follows: **2a**: 65, 68; **2b**: 84, 72; **2c**: 83, 55; **2d**: 118, 66; **2e**: 73, 56; **2f**: 99, 54; **2g**: 60, 63; **2h**: 103, 48; **2i**: oil, 51; **2j**: 123, 57. The ^{1}H-NMR spectra of **2h** were as follows: δ (D_2O): 2.30 [s, 6H, N $(CH_3)_2$], 2.40 (s, 3H, CH_3), 3.66 (s, 2H, CH_2), 6.75–7.72 (m, 7H, aryl H), 10.03 (s, 1H, OH).

Compound **2a** was converted into the corresponding hydrochloride salt as follows. The calculated quantity of hydrogen chloride gas was passed into a vigorously stirred solution of **2a** in anhydrous ether at ice bath temperature The precipitate was collected, washed with anhydrous ether and recrystallized from ether/methanol to give the hydrochloride salt of **2a**, mp = 169 °C.

Synthesis of series 3

Methyl iodide (0.015 mol) was added to a solution of **2a**, **d** (0.003 mol) and the reaction mixture was stirred at room temperature for 24 h. The precipitates were collected, washed with dry ether and dried. Compound **3a** was very hygroscopic,

and after evaporation of the solvent it was heated under vacuum on a steam bath for 0.5 h. The quaternary ammonium salts were recrystallized from ether/methanol to give the following compounds [mp (°C), yield (%)]: **3a**: 129–131 (dec), 64; **3b**: 174–175 (dec), 88.

A solution of benzoyl chloride (0.006 mol) in anhydrous ether (40 mL) was added dropwise to a stirred solution of **2d** (0.004 mol) in anhydrous ether (40 mL) at 0–5 °C. The reaction mixture was then stirred at room temperature for 24 h and the resultant precipitate was collected, washed with acetone and recrystallized from ethanol to give **3c**, mp = 198 °C in 72% yield. ^{1}H-NMR (dimethylsulphoxide-d_6): δ: 2.69 [s, 6H, $N(CH_3)_2$], 4.33 (s, 2H, CH_2), 7.36 (m, 12H, aryl H).

Synthesis of series ***4***

A solution of formaldehyde (37% w/v, 0.025 mol) was added dropwise to a solution of **1** ($R^1 = R^2 = H$) and **1** (R^1 = 2-Cl, R^2 = H) (0.01 mol) and aqueous dimethylamine (25% w/v, 0.025 mol) in ethanol (50 mL). The reaction mixture was heated under reflux for 24 h. Removal of the solvent in vacuo produced the crude Mannich bases. Compound **4a** was prepared as follows. The calculated quantity of hydrogen chloride gas was passed into a vigourously stirred solution of the free base in acetone at ice bath temperature. The resultant precipitate was collected, washed with acetone and recrystallization from ether/methanol gave **4a**, mp = 209–211 °C (dec) in 72% yield. Compound **4b**, mp = 53 °C, was prepared in 88% yield by recrystallization from water/ethanol. ^{1}H-NMR ($CDCl_3$): δ: 2.30 [s, 12H, 2 x $N(CH_3)_2$], 3.63 (s, 4H, 2 x CH_2), 7.15–7.74 (m, 6H, aryl H), 11.17 (s, 1H, OH).

Compound **4c** was prepared as follows. After isolation of **2d** vide supra, the silica gel column was eluted with a chloroform/methanol/ammonium hydroxide mixture (4:1:0.5). The solvents were removed in vacuo and the residue was dissolved in hot toluene, filtered to remove silica gel and evaporation of the solvent gave 4-(4'-chlorophenylazo)-2,6-bis(dimethylaminomethyl)phenol, which was treated with hydrogen chloride gas as per the method for preparing **4a** to give **4c**, mp = 222–224 °C (dec) in 16% yield. ^{1}H-NMR (D_2O): δ: 2.85 [s, 12H, 2 x N $(CH_3)_2$], 4.67 (s, 4H, 2 x CH_2), 7.37–7.84 (m, 6H, aryl H).

Synthesis of ***6***

An aqueous solution of formaldehyde (37% w/v, 0.05 mol) was added dropwise to a stirred mixture of phenol (0.05 mol) and aqueous dimethylamine solution (25% w/v, 0.05 mol) at 20–25 °C. After stirring at room temperature for 18 h, sodium chloride (0.014 mol) was added and the mixture was extracted with ether. The organic extracts were dried over anhydrous magnesium sulphate and the solvent removed, leaving an oil which on distillation produced **6a**, bp = 40–41 °C/0.1 torr (lit [29] 100–101 °C/12 torr) in 65% yield. ^{1}H-NMR: δ ($CDCl_3$): 2.36 [s, 6H, $N(CH_3)_2$], 3.72 (s, 2H, CH_2), 6.78–7.53 (m, 4H, aryl H), 10.25 (s, 1H, OH). The hydrochloride salt of **6a** was also prepared but it was extremely hygroscopic.

A mixture of phenol (0.05 mol) and aqueous solutions of formaldehyde (37% w/v, 0.1 mol) and dimethylamine (25% w/v, 0.1 mol) in ethanol (50 mL) was heated under reflux for 2 h. The solvents were then removed in vacuo to produce an oil which was dissolved in dry acetone (100 mL). Hydrogen chloride was passed into this solution to give **6b**, mp = 277–278 °C (dec) (lit [30] 277–278 °C (dec)) in 89% yield. ^{1}H-NMR (D_2O): δ: 2.90 [s, 6H, 4-$N(CH_3)_2$], 2.95 [s, 12H, 2 x $N(CH_3)_2$], 4.42 (s, 2H, 4-CH_2), 4.53 (s, 4H, 2 x CH_2), 7.79 (s, 2H, aryl H).

Stability studies on ***2a*** *(hydrochloride salt),* ***4a***

Solutions of the compounds (0.00065 mol) were shaken at 37 °C for 48 h. After incubating a solution of the hydrochloride salt of **2a** in phosphate buffer (pH 7.4, 8 mL), yellow needles of **2a** (87% recovery) were obtained. TLC of the filtrate revealed only the presence of the hydrochloride salt of **2a**. The experiment was repeated using phosphate buffer (pH 6.9, 8 mL) and TLC revealed only unreacted compound.

After incubating **4a** in phosphate buffer (pH 7.4, 8 mL), the mixture was extracted with chloroform to give the free base of **4a** (16%) and the aqueous phase revealed only unreacted compound. Similarly, in phosphate buffer pH 6.9, only unreacted compound was detected in the aqueous phase.

The isolated compounds were identified by comparison of their TLC and IR characteristics with authentic samples.

Solutions of **2a** (hydrochloride salt) and **4a** were dissolved in PBS–d [2] having pD values of 7.4 and 6.4. The ^{1}H-NMR spectra (500 MHz) recorded as rapidly as possible after dissolution, and at the end of 48 h incubation at 37 °C were identical.

Incubation of ***2a*** *hydrochloride with 2-mercaptoethanol*

2-Mercaptoethanol (0.0004 mol) was added to a solution of the hydrochloride salt of **2a** (0.0004 mol) in PBS (pH 7.4, 7 mL). After incubation at 37 °C for 48 h, the precipitate was collected and shown by mp, ^{1}H-NMR (60 MHz) and TLC characteristics to be **2a** (32% recovery). TLC of the aqueous phase revealed only the presence of the hydrochloride salt of **2a**.

Statistical analysis

The Hammett σ values were taken from the literature [31] and the π and molar refractivity (MR) values from previously published material [32]. Since the MR value of hydrogen is not unity but 1.03, this figure was subtracted from the MR constants of the chloro and methyl substituents. The test for zero correlation [28] was used to evaluate the significance of the different plots.

Determination of the θ values using electronic absorption spectroscopy

Stock solutions of all compounds were prepared in methanol at concentrations previously determined such that their absorbances were between 0.5 and 1. The λ_{max} values obtained for replicate samples were well within the accepted instrumental error of ± 1 nm. Error limits for the ε values are quoted on the basis of a 0.2% error in the concentration of solutions.

X-Ray crystallography of ***2b, h, j, 4a, c***

The compounds were recrystallized from butanol/chloroform (**2b**), water/acetone : methanol (1:1) (**2h**), isopropanol/methanol (**2j**), methanol/carbon tetrachloride (**4a**) and isopropanol/ethanol (**4c**) by vapour diffusion. An Enraf-Nonius CAD-4 diffractometer with an ω scan was used for data collection and the structures were solved by direct methods using NRCVAX [33]. Crystals of compound **2b** were poor diffractors, with intensities falling off rapidly with the diffraction angle. Attempts to obtain low temperature data resulted in shattered crystals. The data were collected at room temperature (287 K) on the best crystal found. Atomic scattering factors were taken from the literature [34]. All non-hydrogen atoms were found on the E-map and refined anisotropically. Hydrogen atoms were calculated and not refined. No absorption or extinction corrections were applied. Cell dimensions were obtained from 25 reflections in the 2θ range, 16–36°.

The data for **2b** were as follows: $C_{15}H_{16}N_3OCl$, M_r = 289.76, yellow plates, 0.45 x 0.30 x 0.075 mm, a = 9.1675(17), b = 28.159(5), c = 11.677(3) Å, β = 91.737(5)°, V = 3013.1(10) Å^3, Z = 8, space group = $P2_1/a$, monoclinic, D_x = 1.278 g cm^{-3}, $\lambda(MoK_\alpha)$ = 0.7093 Å, μ = 2.5 cm^{-1}, $F(000)$ = 217, T = 287 K, $(\sin\theta)/\lambda_{max}$ = 0.5190 Å^{-1}, $-9 \le h \le 9$, $0 \le k \le 29$, $0 \le l \le 12$. Merging R is based on intensities 0.015 for 203 replicate reflections, $R(F)$ = 0.067, R_w = 0.058, S = 4.14. A total of 3720 reflections were measured, of which 3517 were independent. The refinement of the structure used 2135 observed reflections [$I > 1\sigma(I)$]. Parameters refined = 356, [$w = 1/\sigma^2(F)$]; final $(\Delta/\sigma)_{max}$ = 0.000. $\Delta\rho$ in the final difference map within + 0.37 and – 0.24 e Å^{-3}.

The data for **2h** were as follows: $C_{16}H_{19}N_3O$, M_r = 269.34, yellow rods, 0.70 x 0.50 x 0.40 mm, a = 13.4420(8), b = 6.2256(4), c = 18.3301(12) Å, β = 104.884(5)°, V = 1482.48(16) Å^3, Z = 4, space group = $P2_1/a$, monoclinic, D_x = 1.207 g cm^{-3}, $\lambda(MoK_\alpha)$ = 0.7093 Å, μ = 0.7 cm^{-1}, $F(000)$ = 576, T = 287 K, $(\sin\theta)/\lambda_{max}$ = 0.5395 Å^{-1}, $-14 \le h \le 13$, $0 \le k \le 6$, $0 \le l \le 19$. Merging R is based on intensities 0.013 for 105 replicate reflections. $R(F)$ = 0.042, R_w = 0.067, S = 2.91. A total of 2040 reflections were measured, of which 1935 were independent. The refinement of the structure used 1623 observed reflections [$I > 2.5\sigma(I)$]. Parameters refined = 181, [$w = 1/\sigma^2(F) + 0.0003$], final $(\Delta/\sigma)_{max}$ = 0.000. $\Delta\rho$ in the final difference map within + 0.180 and – 0.180 e Å^{-3}.

The data for **2j** were as follows: $C_{17}H_{21}N_3O$, M_r = 283.37, yellow plates, 0.60 x 0.25 x 0.12 mm, a = 13.8237(13), b = 6.3043(8), c = 18.4624(19) Å, β = 105.353(9)°, V = 1551.6(3) Å^3, Z = 4, space group = $P2_1/a$, monoclinic, D_x = 1.213 g cm^{-3}, $\lambda(MoK_\alpha)$ = 0.7093 Å, μ = 0.7 cm^{-1}, $F(000)$ = 608, T = 287 K, $(\sin\theta)/\lambda_{max}$ = 0.5958 Å^{-1}, $-16 \le h \le 15$, $0 \le k \le 7$, $0 \le l \le 21$. Merging R is based on intensities 0.006 for 129 replicate reflections. $R(F)$ = 0.061, R_w = 0.064, S = 4.87. A total of 2853 reflections were measured, of which 2724 were independent. The refinement of the structure used 1668 observed reflections [$I > 2.5\sigma(I)$]. Parameters refined = 190, [$w = 1/\sigma^2(F)$], final $(\Delta/\sigma)_{max}$ = 0.000. $\Delta\rho$ in the final difference map within + 0.30 and – 0.30 e Å^{-3}.

The data for **4a** were as follows: $C_{18}H_{26}Cl_2N_4O$, M_r = 385.33, amber prisms, 0.50 x 0.38 x 0.06 mm, a = 8.7708(12), b = 24.3215(21), c = 9.9487(11) Å, β = 108.417(10)°, V = 2013.6(4) Å^3, Z = 4, space group = $P2_1/c$, monoclinic, D_x = 1.268 g cm^{-3}, $\lambda(MoK_\alpha)$ = 0.7093 Å, μ = 0.33 mm^{-1}, $F(000)$ = 813, T = 113 K, $(\sin\theta)/\lambda_{max}$ = 0.5588 Å^{-1}, $-9 \le h \le 9$, $0 \le k \le 27$, $0 \le l \le 11$. Merging R is based on intensities 0.013 for 212 replicate reflections, $R(F)$ = 0.047, R_w = 0.054, S = 3.64. A total of 3147 reflections were measured, of which 2935 were independent. The refinement of the structure used 2057 observed reflections [$I > 2.5\sigma(I)$]. Parameters refined = 226 [$w = 1/\sigma^2(F)$], final $(\Delta/\sigma)_{max}$ = 0.000. $\Delta\rho$ in the final difference map within + 0.35 and – 0.34 e Å^{-3}.

The data for **4c** were as follows: $C_{18}H_{25}Cl_3N_4O{\cdot}C_2H_5OH$, M_r = 459.80, yellow plates, 0.55 x 0.40 x 0.05 mm, a = 11.4534(9), b = 10.9304(9), c = 19.2150(17) Å, β = 93.810(7), V = 2400.2(3) Å^3, Z = 4, space group = $P2_1/a$, monoclinic, D_x = 1.272 g cm^{-3}, $\lambda(MoK_\alpha)$ = 0.7093 Å, μ = 3.90 cm^{-1}, $F(000)$ = 962, T = 287 K, $(\sin\theta)/\lambda_{max}$ = 0.6180 Å^{-1}, $-12 \le h \le 12$, $0 \le k \le 12$, $0 \le l \le 21$. Merging R is based on intensities 0.012 for 201 replicate reflections. $R(F)$ = 0.046, R_w = 0.065, S = 2.48. A total of 3550 reflections were measured, of which 3349 were independent. The refinement of the structure used 2367 observed reflections [$I > 2.5\sigma(I)$]. Parameters refined = 271 [$w = 1/\sigma^2(F) + 0.0003$], final $(\Delta/\sigma)_{max}$ = 0.024. $\Delta\rho$ in the final difference map was within + 0.390 and – 0.230 e Å^{-3}.

Cytotoxicity evaluations

The evaluation against murine P388 D1 cells was undertaken by a literature procedure [35] and the screening of various compounds using murine L1210 cells and human T lymphocytes (Molt 4 C/8 and CEM) has been reported previously [36]. The human tumour cell assay was conducted by a previously published procedure [27].

Anticonvulsant evaluation

The compounds were evaluated by the National Institute of Neurological Disorders and Stroke, USA, according to their protocols [16]. For the rat oral screen, four animals receiving a dose of 30 mg/kg of **2a–e**, **2g**, **2h**, **3c** and **4a–c** were examined after 0.25, 0.5, 1, 2 and 4 h in the MES and NT screens. Protection was noted in one of the four animals for the following compounds (time of protection in h): **2a** (1), **2b** (0.25, 1), **2c** (0.25), **2d** (0.5), **2h** (1), **3c** (4), **4a** (0.25) and **4c** (2).

Evaluation of compounds for mutagenic activity

Mutagenic determinations and toxicity evaluations (not shown) were undertaken as described for the RK–SM assay [17] using the *E coli* selector strain CHY832 [37]. Cells were exposed to the compounds at pH 7.6 in 20 or 40% dimethylsulphoxide which was required to retain the compounds in solution for 10 min at 30 °C (0.10 mL total vol/assay), then cell-treated dilutions were spotted to nutrient agar assay plates. The mutation indices of solutions containing 20 and 40% of dimethylsulphoxide (in the absence of compounds) were < 2 and 2.0 respectively.

The *Salmonella* mutagenicity test [20] was conducted in triplicate using concentrations of 0.1, 0.2 and 0.4 mg of **4c**. The average cfu/plate were 32, 41 and 37, respectively, which were similar to the untreated and dimethylsulphoxide controls, namely 43 and 31, respectively.

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