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Antimycobacterial arylidenecyclohexanones and related Mannich bases

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Several series of 2-arylidenecyclohexanones and related Mannich bases as well as various 2,6-bis(arylidene)cyclohexanones were evaluated against *Mycobacterium tuberculosis* $H_{37}Rv$. Using a concentration of 12.5 μg/ml, nearly half of the unsaturated ketones inhibited the growth of the microorganism by 21–66% while all of the Mannich bases achieved 99% or greater inhibition. The relative hydrophobicities and widths of the molecules may have been contributing factors as to whether bioactivity was present or absent. Two of the Mannich bases demonstrated noteworthy potencies towards *Mycobacterium avium*. The conclusion was drawn that Mannich bases of 2-arylidenecyclohexanones represent a novel class of antimycobacterials.

1. Introduction

In the not too distant past, tuberculosis was considered a disease which was diminishing in occurrence and its complete eradication was considered possible. However recently infections caused by *Mycobacterium tuberculosis* have increased dramatically. While a number of organic compounds are available to treat this micororganism, the problem of drug resistance has occurred. Consequently new classes of antitubercular agents are urgently required which possess novel modes of action in order to be effective against those bacteria which are resistant to available medication.

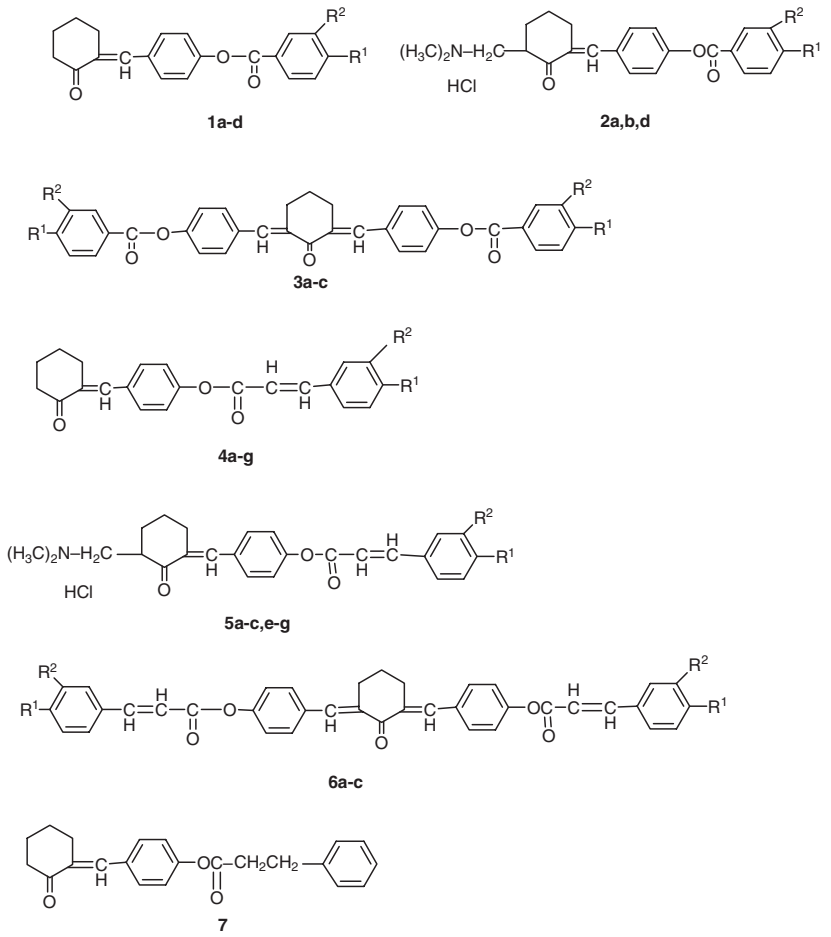
A variety of α,β-unsaturated ketones display antibacterial activity (Dimmock and Wong 1976; Opletalova 2000) and the mode of action has been attributed, *inter alia*, to alkylation of cellular thiols (Dimmock and Wong 1976). In addition, certain Mannich bases of conjugated enones possess antibacterial properties (Dimmock et al. 1975; Lorand et al. 2001) and evidence has been garnered that they too have thiol-alkylating properties (Mutus et al. 1989). Investigations as to the sites of action of various bioactive Mannich bases revealed that inhibition of respiration in mitochondria took place. This phenomenon was demonstrated in *Escherichia coli* (Khachatourians et al. 1984) as well as in mitochondria isolated from mouse and rat liver cells (Dimmock et al. 1983, 1986). Several Mannich bases exerted their effect on the electron transport chain in mitochondria at least in part by competition with coenzyme Q_{10} (Hamon et al. 1978) while an analog blocked electron flow between cytochromes b and c_1 (Hamon et al. 1982). Thus the manner in which α,β-unsaturated ketones and related Mannich bases exert their toxicities is divergent from the principal mechanisms of action of various antitubercular drugs such as inhibition of DNA-dependent RNA-polymerase (rifampin), interference with the synthesis of mycolic acids (isoniazid) and competition with paraaminobenzoic acid (aminosalicylic acid) (Martin, 1998). Furthermore, the decision to examine conjugated α,β-unsaturated ketones and Mannich bases as candidate antimycobacterials was strengthened by the fact that a few literature reports revealed the antitubercular properties of both groups of compounds (Meindle and Boehm 1987; Opletalova 2000; Taniyama et al. 1956).

The objective of the present investigation was to evaluate whether antitubercular properties were present in a series of related conjugated unsaturated ketones and the corresponding Mannich bases. If so, suitable guidelines for amplification of the project in the future derived from one or more lead compounds would be sought. The compounds considered in the initial exploration were **1**–**7** which were chosen for the following reasons. First, a previous study revealed that the compounds in series **1**, **2**, **4**, **5** and **7** inhibited the growth of a number of tumour cell lines (Dimmock et al. 2000) which may have been due to their acting as mitochondrial poisons and it was of interest to discern whether antitubercular properties would also be displayed. Second, the unsaturated ketones had one (**1**, **2**, **7**), two (**3**–**5**) and four (**6**) sites for thiol alkylation and hence a possible correlation between the putative extent of thiolation and antitubercular potencies may emerge. Third, of interest was whether differences in potencies to *Mycobacterium tuberculosis* would be demonstrated between the unsaturated ketones **1** and **4** and the corresponding Mannich bases **2** and **5**, respectively.

In summary, the object of the present study was to evaluate the compounds in series **1**–**7** for antitubercular properties.

2. Investigations, results and discussion

Initially the compounds in series **1**–**7** were examined against *Mycobacterium tuberculosis* $H_{37}Rv$ using a concentration of 12.5 μg/ml of each compound. The results are presented in Table 1. Antitubercular properties were displayed by both α, β-unsaturated ketones and Mannich bases; in fact approximately half of the compounds inhibited the growth of the microorganism by more than 50% at the concentration



Compounds evaluated for antitubercular properties. The aryl substitutions patterns in series 1–6 were as follows a : $R^1 = R^2 = H$; b : $R^1 = Cl$, $R^2 = H$; c : $R^1 = CH_3$, $R^2 = H$; d : $R^1 = OCH_3$, $R^2 = H$; e : $R^1 = R^2 = Cl$; f : $R^1 = F$; $R^2 = H$; g : $R^1 = Br$, $R^2 = H$.

Table 1: Evaluation of series 1–7 against *Mycobacterium tuberculosis* $H_{37}Rv$

Compd.	Percentage inhibition at 12.5 μg/ml
1a	66
1b	56
1c	58
1d	51
2a	100
2b	99
2d	100
3a	0
3b	0
3c	21
4a	45
4b	0
4c	3
4d	1
4e	47
4f	54
4g	3
5a	99
5b	100
5c	99
5e	99
5f	99
5g	99
6a	0
6b	0
6c	0
7	0

employed. No correlations were apparent between the number of potential sites of alkylation and antitubercular potencies. For example, the compounds in series **1**, **2** and **7** each have one olefinic double bond yet the average percentage inhibitions of bacterial growth in series **1** and **2** were 58 and 100, respectively, while **7** had no effect on the bacterium.

The following structure-activity relationships (SAR) were noted; the terms active and inactive refer to the observations made using 12.5 μg/ml of each compound. Initially the unsaturated ketones will be considered. First, molecular modification of **1a–c** leading to the 2,6-disubstituted cyclohexanones **3a–c** caused a reduction in potencies. In addition, **1a–d** were more effective against *Mycobacterium tuberculosis* than the vinylogues **4a–d**. Second, conversion of **4a–c** into the 2,6-disubstituted cyclohexanones **6a–c** led to compounds devoid of antitubercular properties while reduction of the cinnamoyl double bond of **4a** giving rise to **7** produced an inactive compound. Thus, in general, of the series of unsaturated ketones **1**, **3**, **4**, **6** and **7**, the greatest potencies were found in series **1**.

The differences in antitubercular potencies among the unsaturated ketones in series **1**, **3**, **4**, **6** and **7** may have been due to variations in certain physicochemical properties which influence both the rate of transportation to a locus of action and the sizes of the molecules which may govern the ability (or lack thereof) to align at a binding site. Accordingly the partition coefficients and maximum interatomic distances of the unsubstituted compounds in each of the series **1–7** were determined.

The calculated log P values of **1a** and **4a**, which demonstrated antitubercular properties, were 4.85 and 5.36, respectively. These figures were considerably lower than the data obtained for the inactive analogues **3a** and **6a** which possessed log P values of 8.27 and 9.30, respectively. The log P value of the inactive ketone **7** was 5.21. While a clearcut correlation between hydrophobicity and antitubercular properties did not emerge, it is conceivable that the huge differential in partition coefficients between the bioactive enones **1a** and **4a** with the inactive analogues of **3a** and **6a** may have contributed to the variations in activity towards *Mycobacterium tuberculosis*.

The maximum interatomic distances between the atoms in **1a**, **3a**, **4a**, **6a** and **7** were determined by molecular modeling. In the case of **1a**, **4a** and **7**, these distances were found to be between the equatorial hydrogen atom at position 6 of the cyclohexyl ring and the proton of the terminal aryl ring in either the para position (**1a**, **4a**) or the ortho location (**7**). The maximum interatomic distances (percentage inhibition of *M. tuberculosis* in parentheses) of **1a**, **3a**, **4a**, **6a** and **7** were 15.72 (66), 25.54 (0), 17.95 (45), 28.88 (0) and 13.71 (0) Å, respectively. Thus the widths of the two compounds displaying antitubercular potencies lay in between the analogues whose widths were either shorter (**7**) or larger (**3a**, **6a**) than the figures obtained for **1a** and **4a**. Conceivably an optimal span in these clusters of compounds exists and amplification of these groups of compounds should bear this possibility in mind.

The data in Table 1 revealed that conversion of **1a, b, d** and **4a–c, e–g** into the corresponding Mannich bases **2a, b, d** and **5a–c, e–g**, respectively, led to substantial increases in potencies. The pK_a value of a Mannich base of an unsubstituted conjugated arylidene ketone was 7.19 (Dimmock et al. 1989). Thus in solutions of pH 7.4, **2a** and **5a** will exist as a mixture of free bases and protonated species in a ratio of 60 : 40 approximately (Albert 1985). The log P values of **2a** were 4.87 (free base) and 4.52 (protonated species) and for **5a**, the relative figures were 5.38 and 5.03. These data are comparable to the log P values of **1a** and **4a**. Thus the log P values of **1a**, **2a**, **4a** and **5a**, which show antitubercular properties, have log P values in the 4.5–5.4 range which may have contributed to the bioactivity observed. The maximum interatomic distances in **2a** and **5a** were between one of the hydrogen atoms of a methyl group attached to the nitrogen atom and the proton at the para position of the terminal aryl ring and found to be 18.68 and 20.94 Å, respectively. Thus the widths of **1a**, **2a**, **4a** and **5a** which possess antitubercular properties have maximum interatomic distances between 15.7 and 20.9 Å while the inactive compounds (**3a**, **6a**, **7**) have figures which lie outside this range. Thus both partition coefficients and the widths of the compounds may play a part in determining whether antitubercular properties are displayed in these groups of compounds. Furthermore, certain Mannich bases of conjugated unsaturated ketones have greater thiol-alkylating properties than the precursor enones (Dimmock et al. 1980) which may have contributed to the increased antitubercular properties of the compounds in series **2** and **5**. The biodata in Table 1 indicated that further experimentation with the Mannich bases was warranted.

Table 2: MIC to inhibit the growth of *Mycobacterium tuberculosis* H_{37}Rv and percentage inhibition of the growth of *Mycobacterium avium* by various Mannich bases

Compd.	MIC vs *M. tuberculosis*		% Inhibition of the growth of *M. avium*[a]
	μg/ml	μM	
2a	0.39	0.98	87
2b	6.25	13.8	–
2d	0.78	1.81	15
5a	3.13	7.35	0
5b	6.25	13.6	0
5c	1.56	3.55	13
5e	0.39	0.77	4
5f	>12.5	>28.2	30
5g	1.56	3.04	87
Rifampin	0.16	0.19	–

[a] In this assay the concentration of the compounds was 12.5 μg/ml. The MIC of rifampin is 9.25 μg/ml (Collins and Franzblau 1997)

The minimum inhibitory concentration (MIC) values of **2a, b, d** and **5a–c, e, g** towards *Mycobacterium tuberculosis* H_{37}Rv are presented in Table 2. The most potent compounds were **2a** and **5e** with approximately 20–25% of the efficacy of rifampin towards this microorganism. Correlation analyses between the electronic, hydrophobic and steric properties of the substituents of the acyl aryl rings, and the MIC values (in μM) of **5a–c, e, g** were carried out. Linear and semilogarithmic plots were constructed between these figures and the sigma (σ), pi (π) and molar refractivity (MR) constants of the aryl substituents. However no significant correlations ($p > 0.05$) were noted.

In order to determine whether selective toxicity towards *Mycobacterium tuberculosis* was displayed by the compounds prepared in this study, two representative compounds **2a** and **2d** were evaluated towards Vero cells. The IC_{50} values for **2a** and **2d** were 0.21 and 16.4 μg/mL, respectively. Selectivity index (SI) figures were calculated, i.e., the ratios of the IC_{50} values towards Vero cells and *Mycobacterium tuberculosis* and found to be 0.54 and 21 for **2a** and **2d**, respectively. A SI figure of greater than 10 is considered to be significant and the datum for **2d** reinforces the viability of these compounds for development as candidate antitubercular agents.

In view of the potencies of the Mannich bases towards *Mycobacterium tuberculosis* H_{37}Rv, the decision was made to evaluate **2a, d, 5a–c, e–g** against *Mycobacterium avium* which is an opportunistic pathogen resistant to many antibacterial agents. The figures in Table 2 reveal that **2a** and **5g** exerted noteworthy potencies towards this microorganism and serves as prototypic molecules in the development of this series of compounds as candidate antimycobacterials.

In conclusion, the SAR observed were twofold. First, among the α,β-unsaturated ketones, series **1** displayed the highest potencies towards *Mycobacterium tuberculosis* H_{37}Rv. Second, conversion of various enones into the corresponding Mannich bases led to marked increases in potencies. These observations are of value in contemplating the expansion of these compounds as candidate antimycobacterials. It is important to state once again the spectre of the rise in the incidence of tuberculosis and the problem of increased drug resistance to this bacterium. Hence the fact that the compounds prepared in this study are chemically unrelated to current medication suggests that further work with analogues is clearly warranted.

3. Experimental

3.1. Chemistry

3.1.1. General procedures for syntheses and spectroscopy

Melting points are in Celsius degrees and are uncorrected. Elemental analyses (C, H) were undertaken on **3a–c** and **6a–c** by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan and were within 0.4% of the calculated values. ^{1}H NMR spectra were obtained using a Bruker

AMX 500 FT (500 MHz) instrument. TLC using silica gel precoated plastic sheets and a solvent system of hexane : ethyl acetate (7 : 3) was employed to monitor the progress of the reactions leading to series **3** and **6** and also to confirm the homogeneity of the purified products.

*3.1.2. Syntheses of series **1**, **2**, **4**, **5** and **7***

The preparation of **1a–d**, **2a, b, d**, **4a–g**, **5a–c, e–g** and **7** has been reported previously (Dimmock et al. 2000). Compound **2b** was isolated as the monohydrate and **5e** as the hemihydrate.

*3.1.3. Syntheses of series **3***

A mixture of 2,6-bis(4-hydroxyphenylmethylene)cyclohexanone (Borden, 1978; 0.005 mol), the appropriate aroyl chloride (0.01 mol), pyridine (0.5 ml) and dry acetonitrile (50 ml) was heated under reflux for 6 h with stirring. On cooling the precipitate was collected and washed successively with hydrochloric acid (1 N), aqueous sodium carbonate solution (10% w/v) and water. After drying, the product was recrystallized from dimethylsulphoxide to give **3a**, m.p. 215–217 °C, **3b**, m.p. 223–224 °C and **3c**, m.p. 191–193 °C in yields of 65, 65 and 68%, respectively. The ^{1}H NMR spectrum of a representative compound **3a** was as follows: δ ($CDCl_3$): 1.79–1.84 (m, 2 H, 4-CH_2), 2.93–2.96 (m, 4 H, 3-CH_2 and 5-CH_2), 7.27–8.22 (m, 18 H, aryl H), 7.80 (br s, 2 H, olefinic H).

*3.1.4. Syntheses of series **6***

The 3-aryl-2-propenoyl chlorides required in the synthesis of **6b, c** were prepared by literature methods (Dimmock et al. 2000; Furniss et al. 1989). The esters **6a–c** were synthesized from 2,6-bis(4-hydroxyphenylmethylene)cyclohexanone and the appropriate 3-aryl-2-propenoyl chloride by the same method used in the preparation of **3a–c** except the time of heating under reflux was 4 h. The products were recrystallized from dimethylsulphoxide to give **6a**, m.p. 220–221 °C, **6b**, m.p. 229–231 °C and **6c** mp 236–238 °C in yields of 70, 75 and 70%, respectively. The ^{1}H NMR spectrum of a representative compound **6a** was as follows: δ ($CDCl_3$) : 1.78–1.83 (m, 2 H, 4-CH_2), 2.91–2.95 (m, 4 H, 3-CH_2 and 5-CH_2), 6.62 (d, 2 H, OCOC<u>H</u>=CH, J = 16.0 Hz), 7.22–7.61 (m, 18 H, aryl H), 7.78 (s, 2 H, C<u>H</u>C_6H_4), 7.87 (d, 2 H, OCOCH=C<u>H</u>, J = 16.0 Hz).

3.1.5. Calculation of logP values

The structures of the compounds were minimized using the semi-empirical AM1 methodology which was followed by determinations of the logP (Ghose-Crippen) figures using Spartan'02 windows (Wavefunction 2002).

3.1.6. Measurement of interatomic distances

The MacroModel version 8.0 programme (MacroModel 2002) was used to minimize the structures of **1a, 2a, 3a, 4a, 5a, 6a** and **7** after which the maximum interatomic distances were obtained.

3.1.7. Statistical analyses

The Hammett sigma, Hansch pi and molar refractivity values were taken from the literature (Hansch and Leo 1979). Linear and semilogarithmic plots were constructed between each of these physicochemical constants and the MIC figures (in μM) of **5a–c, e, g** towards *Mycobacterium tuberculosis* H_{37}Rv using a commercial software package (Statistical Package for Social Sciences 1999).

3.2. Antitubercular and related assay

The evaluation of the compounds against *Mycobacterium tuberculosis*, Vero cells and *Mycobacterium avium* was undertaken through the auspices of the Tuberculosis Antimicrobial Acquisition Coordinating Facility which is coordinated by the Southern Research Institute, AL, USA under the direction of the National Institute of Allergy and Infectious Diseases, USA.

3.2.1. Evaluations against Mycobacterium tuberculosis and Vero cells

A concentration of 12.5 μg/ml of each compound was assessed against *Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue assay (Collins and Franzblau 1997). In order to obtain MIC values for **2a, b, d, 5a–c, e, g**, serial dilutions of each compound in CABTEC 460 medium were undertaken. The MIC figures are the lowest concentrations which inhibit 99% of the growth of the inoculum. Cytotoxicity towards Vero cells was undertaken using serial dilutions of **2a** or **2d** commencing with 10 times the concentration of the MIC figures towards *Mycobacterium tuberculosis*.

3.2.2. Evaluation against Mycobacterium avium

The screening of **5a–c, e–g** and rifampin against *Mycobacterium avium* (ATCC 25291) was carried out using the microplate Alamar blue assay (Collins and Franzblau 1997).

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