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Original article

Synthesis and antibacterial activity of fused Mannich ketones

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Abstract

New Mannich ketones of fused bicyclic ketones as 1-indanones and 1-tetralones were prepared using the classical acid-catalysed Mannich reaction. Known members of this family were used in comparative biological tests. Antibacterial activity of these new water-soluble compounds was reported against *Pseudomonas aeruginosa*, *Escherichia coli*, *E. coli* ReD31m4, *Salmonella minnesota* Re595, *Shigella sonnei* Re4350, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Micrococcus luteus* and *Bacillus subtilis* standard strains. Human cytotoxicity of our new compounds was evaluated against HeLa cell line. Some compounds showed low cytotoxicity (56.738 nM mL^{-1} for **24**, 47.497 nM mL^{-1} for **31** and 48.379 nM mL^{-1} for **26**) and proved to be efficient antibacterial agents against the Gram-positive and partly against *E. coli* strains. Minimum inhibitory concentrations (MIC) changed in the range of 1.56– > 200 μg mL^{-1}. The deep rough mutants showed (generally eight times) higher sensitivity toward the compounds than the smooth *E. coli*. Hence, the permeability of Gram-negative outer membrane can influence the MIC values of our compounds. A preliminary quantitative structure–activity relationship (QSAR) study indicated the maximum positive charge ($MaxQ^+$) as the parameter that most significantly affected antibacterial activity against *E. coli*. In *B. subtilis*, the influence of a topological descriptor (first-order valence-connectivity index, XV1) was also revealed; however, other strains did not yield meaningful QSAR with the set of descriptors employed.

Keywords: Mannich ketone; Antibacterial activity; Deep rough mutants; Cytotoxicity; QSAR

1. Introduction

Previously we have reported the synthesis and antibacterial study of several unsaturated Mannich ketones (**1**–**23**, Fig. 1) [1]. These water-soluble compounds were screened against some Gram-positive and -negative standard strains. The structure–activity relationship and the mechanism of action was also discussed at this class of compounds. In order to select possible candidates as antibacterial agents, we extended our studies to fused Mannich ketones. Some known compounds were prepared for the biological experiments and five new ones were synthesised. Quantitative structure–activity relationship (QSAR) calculations were performed using the antibacterial data of both the unsaturated and the fused Mannich ketones to obtain better predictions. The title compounds afford a more planar, rigid system when compared with the unsaturated Mannich ketones [1].

2. Chemistry

The title compounds were prepared from the corresponding bicyclic ketones, secondary amines and paraformaldehyde using the classical acid-catalysed Mannich reaction (Fig. 2). The products were isolated as hydrochlorides. The approach for the synthesis of 6-alkoxy derivatives (**37**–**39**) involved first the preparation of the

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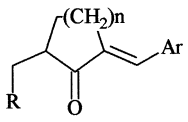

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No.	n	R	Ar
1	1	Pip	Ph
2	1	Mor	Ph
3	1	Pyr	Ph
4	1	Thik	Ph
5	1	Mor	4'-CH_3-C_6H_4
6	1	Pip	4'-OCH_3-C_6H_4
7	1	Mor	4'-OCH_3-C_6H_4
8	1	Pip	3'-OCH_3-C_6H_4
9	1	Mor	3'-OCH_3-C_6H_4
10	1	Pip	2'-OCH_3-C_6H_4
11	1	Mor	2'-OCH_3-C_6H_4
12	1	Pip	3',4',5'-$(OCH_3)_3$-C_6H_3
13	1	Mor	3',4'-OCH_2O-C_6H_3
14	2	Mor	Ph
15	2	Pyr	Ph
16	2	4-Pip	Ph
17	2	Mor	4'-CH_3-C_6H_4
18	2	Mor	4'-OCH_3-C_6H_4
19	2	Mor	3',4'-$(OCH_3)_2$-C_6H_3
20	3	Mor	Ph
21	3	Pip	Ph
22	4	Mor	Ph
23	4	Pip	Ph

Pip: 1-Piperidyl; Mor: 4-Morpholinyl; Pyr: 1-Pyrrolidinyl;

Thik: 2-(1,2,3,4-Tetrahydro)-isoquinolyl; 4-Pip: 4-methyl-1-piperidyl.

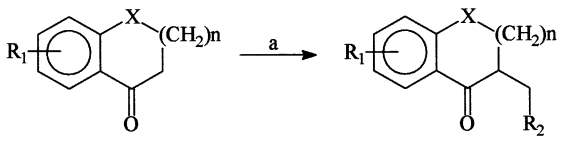
Fig. 1. (a) Ethanol, HCl, reflux.

corresponding 6-alkoxy-1-tetralones from the 6-hydroxy-1-tetralone by a base-catalysed alkylation according to procedures reported previously [2,3]. In order to purify our Mannich ketones a new method was introduced. This very mild procedure liberates the Mannich bases at 0 °C to remove the contaminating secondary amines (see Section 6 and [1]). Upon using this method, it is possible to partly avoid the deamination of the Mannich bases to methylene ketones. The physical data of the novel compounds are shown in Table 1. The isopropyl derivative **39** partly decomposed both in the acidic reaction mixture and on acidification during the purification procedure.

From the spectral data given in Tables 2 and 3, the postulated structures follow straightforwardly. (The NMR numbering is shown on Fig. 3.) Because of the very similar spectral behaviours of the recently studied (**27**, **30**, **37**–**39**) and the earlier reported Mannich ketones (**1**–**23**), only a few observations need explanation. Similarly to **1**–**23**, the bridging methylene hydrogens of the new compounds are chemically non-equivalent due to molecular asymmetry. Their signals (two double doublets) are the proof of the reaction leading to the expected products. The high difference in the chemical shifts of these methylene hydrogens (ca. 0.4 ppm) is due to the anisotropic neighboring effect [4] of the nearby carbonyl that influences one of the hydrogens. This observation suggests hindered rotation around the C-2–C-2α bond and a quasi-rigid structure for this part of the molecule. Compound **30** is an exception in this respect with the shift difference being only 0.16 ppm. Possibly, the rotation of the pyrrolidine substituent is less hindered than that of the bulkier piperidine and morpholine rings.

The IR carbonyl frequencies and the ^{13}C-NMR chemical shifts of the carbonyl carbons also depend on the ring size and lie in the expected ranges [5,6]. Ring strain is revealed at higher νC=O IR-frequency [7] for cyclopentanone **27** (1701 cm^{-1}). For all the other compounds containing *c*-hexanone rings, the corresponding band appears in the interval 1676–1678 cm^{-1}. The ^{13}C shift is 206.6 ppm for **27**, while 199.8 (**30**) and 198.5 ppm (**37**–**39**) for the *c*-hexanones. Again, the different values for **30** and **37**–**39**, respectively, suggest different conformation.

The downfield shift of the H-8 signals (7.66 for **27**, 7.99 for **30** and 7.89 ppm for **37**–**39**) are due to the anisotropy of the nearby carbonyl group and the difference in this shift, as compared to **37**–**39**, arises from the different ring size (**27**) and the difference in the conformational equilibrium (**30**). The ^{1}H-NMR spectra of **27** and **30** also contain two weak signals at about 5.5 and 6.3 ppm, which originate from a contamination of $<1\%$ concentration. These signals can be assigned to a terminal methylidene group in the decomposition product formed via deamination. A similar decomposition was also observed in the series **1**–**23**. Decomposition was not observed in the case of **37**–**39**; it is thus plausible that the morpholino substituent stabilises these Mannich bases.



No.	n	X	R_1	R_2
24	0	CH_2	H	Pip
25	0	CH_2	H	Mor
26	0	CH_2	H	Pyr
27	0	CH_2	5-OCH_3	Pip
28	1	CH_2	H	Pip
29	1	CH_2	H	Mor
30	1	CH_2	H	Pyr
31	1	CH_2	H	Thik
32	1	CH_2	5-OCH_3	Pip
33	1	CH_2	6-OCH_3	Pip
34	1	CH_2	6-OCH_3	Mor
35	1	CH_2	6-OCH_3	Pyr
36	1	CH_2	6-OCH_3	Thik
37	1	CH_2	6-OC_2H_5	Mor
38	1	CH_2	6-OC_3H_7	Mor
39	1	CH_2	6-O-isopropyl	Mor
40	1	CH_2	7-OCH_3	Pip
41	1	CH_2	7-OCH_3	Mor
42	2	CH_2	H	Pip
43	2	CH_2	H	Mor
44	1	S	H	Pip

Fig. 2. (a) Ethanol, HCl, reflux.

Table 1
Physical data of compounds **27**, **30** and **37**–**39**

Compound	General formula [a]	M.p (°C)	Yield (%)	Time of heating (h)	IR (KBr, cm^{-1})
27	$C_{16}H_{22}ClNO_2$	173–175	41	32	1701
30	$C_{15}H_{20}ClNO$	112 (dec., acetone)	30	12	1676
37	$C_{17}H_{24}ClNO_3$	125 (dec., acetone)	36	19	1677
38	$C_{18}H_{26}ClNO_3$	136 (dec., acetone)	36	18	1678
39	$C_{18}H_{26}ClNO_3$	142 (dec., acetone)	14	19	1676

[a] The combustion analyses were within ±0.4% of the theoretical values for C, H and N.

Table 2
^{1}H-NMR data [a] for compounds **27**, **30** and **37**–**39** [b]

Compound	H-2 m (1H)	CH_2 (Pos. 3) 2 × m (2 × 1H)	CH_2 (Pos. 4) 2 × m (2 × 1H)	NCH_2 [c] 2 × dd (4H)	NCH_2 [d] 2 × m (2 × 2H)	OCH_2 [d,e] m (4H)	Condensed benzene ring			CH_3 [i] (3H)
							H-5 [f]	H-7 [g]	H-8 [h]	
27	~2.88 [k]	–	3.08 [l], 3.24 [k]	2.43 [l], ~2.88 [m]	2.33, 2.51	1.57	6.88	6.88	7.66	3.87
30	2.68	1.95, 2.40	~2.99	2.76, 2.92	~2.5, ~2.6	1.78	7.21	7.26	7.99	–
37	2.58	1.85, 2.77	~2.85	2.44, 2.82	2.35, 2.47	3.63	6.58	6.71	7.88	1.34
38	2.58	1.85, 2.27	~2.86	2.44, 2.83	2.34, 2.48	3.64	6.60	6.72	7.89	0.96
39	2.58	1.87, 2.27	~2.86	2.45, 2.83	2.35, 2.50	3.64	6.59	6.71	7.89	1.28

Further signals: CCH_2C (R_1): 1.75, *sx* (2H, **38**); CH_2 (in γ-pos. to the N atom), R_2 group: 1.43 (**27**); OCH_2/OCH, R_1 group: 4.99, *qa* (2H, **37**), 3.89, *t* (*J*: 6.5, 2H, **38**), 4.56, *sp* (1H, **39**), H-6 (condensed benzene ring), *t* (1H): 7.42 (**30**).

[a] Chemical shifts (in ppm, $\delta_{TMS} = 0$ ppm) and coupling constants (in Hz) at 500 MHz in $CDCl_3$ solution.

[b] The assignments were supported (except for **37**) by HMQC and for **30** also 2D-COSY measurements.

[c] Side chain, ^2J: 12.2 (**27** and **30**), 12.7±0.1 (**37**–**39**), ^3J: 12.1 (**27**), 9.0±0.1 (**30**, **37**–**39**) and 4.7±0.1 (**30**, **37**–**39**) for the upfield and downfield *dd*, respectively. Further split to *td* by 2.2 Hz due to ^4J(H,H)-coupling.

[d] R_2 group.

[e] CH_2 in β-pos. to the N atom (**27** and **30**).

[f] Doublet, *J*: 7.5 (**30**), 2.2 (**37** and **38**), 1.8 (**39**).

[g] Coalesced (**27**), *t* (**30**), *dd* (**37**–**39**).

[h] Doublet, *J*: 9.1 (**30**), 8.7 (**37**–**38**), *dd*, *J*: 7.8 and 1.2 (**30**).

[i] Intensity: 6H for **39**, multiplicity: *s* (**27**), *t*, *J*: 7.0 (**37**), 7.4 (**38**), *d*, 6.0 (**39**).

[k] Overlapping signals.

[l] Double doublet, *J*: 17.5 and 3.0.

[m] Double doublet, 17.5 and 7.7.

Table 3
^{13}C-NMR chemical shifts [a] for compounds **27**, **30** and **37–39** [b]

Compound	C-1	C-2	C-3	C-4	C-4a	C-5	C-6	C-7	C-8	C-8a	NCH_2 [c]	NCH_2 [d]	OCH_2 [e]	CH_3
27	206.6	46.6	–	32.9	157.6	110.1	165.8	115.6	125.9	130.4	55.1	61.1	26.4	56.0
30	199.8	47.6	27.7	28.8	144.5	129.1	132.9	127.8	126.9	133.6	54.8	56.0	24.0	–
37	198.5	45.3	27.7	29.1	146.9	113.4	163.4	113.9	130.2	126.4	54.3	58.6	67.3	15.1
38	198.5	45.3	27.7	29.1	146.9	113.4	163.6	113.9	130.2	126.3	54.3	58.6	67.3	10.8
39	198.5	45.2	27.7	29.1	146.9	114.5	162.5	114.6	130.3	126.2	54.3	58.6	67.4	22.4

[a] In ppm ($\delta_{TMS} = 0$ ppm) at 125.7 MHz. Solvent: $CDCl_3$; further signals, CCH_2C: 24.7 (R_1, **27**), 22.9 (R_2, **38**); OCH_2/OCH (R_1): 64.1 (**37**), 70.1 (**38**), 70.4 (**39**).
[b] The assignments were supported by DEPT and HMQC (except for **37**) measurements.
[c] In R_2.
[d] Side chain.
[e] CCH_2C β-pos. to the N atom.

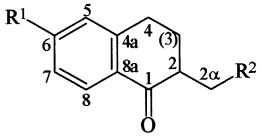


Fig. 3. NMR numbering of compounds.

3. Biology

Our purpose was to find the structure–antibacterial activity and cytotoxicity relationships in a series of fused Mannich ketones. Therefore, we varied here the size of the benzocycloalkanone ring, the secondary amine and the nature and position of the aromatic substituent. Regarding the 6-alkoxysubstituted Mannich ketones, we wished to study the effect of the hydrophobic side chain on the biological activity.

In vitro antibacterial activity of compounds **24–44** was examined on standard bacterial strains: *Pseudomonas aeruginosa* NIH Hungary 170000, *Escherichia coli* ATCC 25922, *E coli* ReD31m4, *Salmonella minnesota* Re595, *Shigella sonnei* Re4350, *Staphylococcus saprophyticus* NIH Hungary 120008, *S. aureus* NIH Hungary 118003, *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633. The minimum inhibitory concentration (MIC) and the minimum bactericidic concentration (MBC) of our compounds were determined by test tube dilution method. In vitro cytotoxicity tests were carried out on HeLa cell line growing on microplates. Finally we studied the connection between the capability of thiol depletion and the mechanism of action of the novel compounds (see Section 6).

4. Results and discussion

4.1. In vitro antibacterial activity

The antibacterial activity was determined (Tables 4 and 5) and compared to the activity of standard antibiotics (see Table 6 in Ref. [8]; MIC values from 0.20 to 100 μg mL^{-1}). From the 21 compounds studied, the least active ones are the 6-methoxy-1-tetralone derivatives **33** and **35** (for Gram-negative strains MIC: >200 μg mL^{-1}, for Gram-positive ones MIC 50–100 μg mL^{-1}). The compounds showing the highest activity were the five-membered indanone derivatives **24–26**. They were active both against the Gram-positive (MIC: 1.56–12.5 μg mL^{-1}) and the Gram-negative *E. coli* strains (MIC: 12.5–25 μg mL^{-1}). Compound **25** was the only one possessing a slight effect against the *P. aeruginosa* strain (MIC: 200 μg mL^{-1}). From the six-membered Mannich ketones, **29** and **44** showed highest antibacterial activity mostly on the Gram-positive strains. The nature of the amine substituent affects the antibacterial activity; more on the Gram-positive strains (**33–36**) and less on the Gram-negative ones (**24–26**). The alkoxy substituent on the aromatic ring apparently influences the activity against the Gram-positive strains. Thus, at the most active agent from this series was the 6-*O*-isopropyl-derivative and the more lipophilic Mannich ketones, as **38–39** showed the maximal activity in the *S. saprophyticus* strain.

The MIC values for the deep rough mutants (*E. coli* ReD31m4, *S. minnesota* Re595, *S. sonnei* Re4350) were lower (4–8 times) than for *E. coli* ATCC 25922 smooth strain (Table 6). These results show that the permeability of Gram-negative outer membrane plays an important role in antibacterial activity of our compounds. The outer membrane of the Gram-negative bacteria is less permeable than the cell wall of Gram-positives for some, mostly hydrophobic antibacterial molecules. Deep rough mutants of Gram-negative bacteria lost some components from their outer membrane; thus, they became more permeable and more susceptible for some antibacterial compounds [9–11]. In our experiments, we determined MIC values of a selected group of our compounds on deep rough mutants (Table 6) because we wanted to know whether the mutation of Gram-negative outer membrane could influence their antibacterial activity.

Table 4
In vitro antibacterial activity of five-membered (**24**–**27**) and six- and seven-membered Mannich ketones (**28**–**44**), expressed as MIC values (MIC, μg mL^{-1})

No.	*P. aeruginosa*	*E. coli*	*S. saprophyticus*	*S. aureus*	*M. luteus*	*B. subtilis*
24	> 200	25	12.5	12.5	12.5	12.5
25	200	12.5	6.25	6.25	6.25	6.25
26	> 200	25	6.25	6.25	3.125	1.56
27	> 200	50	25	6.25	12.5	12.5
28	> 200	200	6.25	50	6.25	25
29	> 200	100	12.5	12.5	3.125	12.5
30	> 200	> 200	25	25	25	50
31	> 200	50	12.5	12.5	12.5	25
32	> 200	> 200	50	25	25	50
33	> 200	> 200	200	200	12.5	100
34	> 200	> 200	25	25	25	25
35	> 200	> 200	100	200	100	100
36	> 200	> 200	25	50	25	25
37	> 200	> 200	12.5	25	25	50
38	> 200	> 200	12.5	12.5	12.5	25
39	> 200	> 200	6.25	12.5	12.5	25
40	> 200	> 200	25	25	12.5	12.5
41	> 200	200	25	12.5	12.5	12.5
42	> 200	> 200	12.5	12.5	12.5	12.5
43	> 200	> 200	12.5	12.5	12.5	12.5
44	> 200	100	12.5	3.125	12.5	25

Compared to the unsaturated Mannich ketones (**1**–**23**), the fused ones were more effective against the *E. coli* strains, but the activity toward the Gram-positive strains was similar. In comparison with standard commercial antibiotics [8], our compounds were slightly less active. Except the *B. subtilis* strain, about half of our compounds had MIC values of 6.25–12.5 μg mL^{-1} against the Gram-positive strains (Table 5). According to our experiments, the five-membered Mannich ketones proved to be the best antimicrobial agents for all of the investigated strains.

The MBC values were the same (compounds **24**, **28**, **29** and **44**) or near the same, two (**26**) or four times (**25**, **27** and **31**) higher than MIC values for compounds that were active against *E. coli* strain; so these agents are bactericidic on this strain. The MBC values for all of our compounds on Gram-positive *S. aureus*, *S. saprophyticus*, *M. luteus* and *B. subtilis* were > 200 μg mL^{-1}, these values are 8–16–32 times higher than their MIC values; therefore, these Mannich ketones are only bacteriostatic on these strains. The situation is similar for the *B. subtilis* strain: MBC changed from 50 to > 200 μg mL^{-1} and our agents were also bacteriostatic in this strain with MBC 16–32 times higher than the MIC values.

4.2. In vitro cytotoxicity tests

Compounds **24**, **26**, **27**, **31**, **36**–**41** and **44** fused Mannich ketones proved to be the least cytotoxic (Table

Table 5
Cummulative data of antibacterial sensitivity tests: number of compounds causing the given MIC value

MIC (μg mL^{-1})	*P. aeruginosa* NIH Hungary 170000	*E. coli* ATCC 25922	*S. saprophyticus* NIH Hungary 120008	*S. aureus* NIH Hungary 118003	*M. luteus* ATCC 9341	*B. subtilis* ATCC 6633
> 200	20	12				
200	1	2	1	2		
100		2	1		1	2
50		2	1	2		3
25		2	6	5	5	7
12.5		1	8	8	11	7
6.25			4	3	2	1
3.125				1	2	
1.56						1
	21 Gram-negative	21 Gram-positive	21	21	21	21

Distribution of the effective compounds.

Table 6
In vitro antibacterial activity of selected Mannich ketones, expressed as MIC values (MIC, μg mL^{-1}) against deep rough (Re) mutants

No.	*E. coli* ATCC25922	*E. coli* ReD31m4	*S. minnesota* Re595	*S. sonnei* Re4350
25	12.5	6.25	3.125	3.125
26	25	6.25	3.125	3.125
28	200	50	50	12.5
29	100	12.5	50	12.5
30	> 200	50	50	50
33	> 200	100	200	200
34	> 200	50	50	50
37	> 200	25	50	50
38	> 200	25	50	50
42	> 200	25	50	25

7). This low cytotoxicity was associated with a relatively high activity against the *E. coli* strain (**24** and **26**), while at the Gram-positive strains the same compounds reached the highest activity. The amine substituents of these Mannich ketones are very different, but none of them is morpholine. On the other hand, the most cytotoxic Mannich ketones to the HeLa cells are **25**, **28**–**30**, **32**–**35**, **37**–**39**; six of them have morpholine substituent. In this series, the 6-methoxy-substituted Mannich ketones are rather toxic except for the one with 1,2,3,4-tetrahydroisoquinoline sidechain.

4.3. Mechanism of action

All of the compounds examined contained potential thiol alkylating groups. The Mannich bases are considered latent thiol alkylators, because they give reactive vinyl ketones under physiological conditions by 1,2-elimination and can undergo addition reaction with thiols [12]. The Mannich ketones used in this study possess only one site for alkylation, in contrast to the unsaturated ketones investigated previously. The thiol alkylating action of these compounds may contribute to their biological activity, because the presence of free thiols is essential for the viability of the cells. To test this idea, we examined the degree of thiol depletion caused by a selected group of compounds administered at the MIC concentration using the DTNB (5,5′-dithiobis-2-nitrobenzoic acid) method [13]. These compounds can be divided into three groups based on their ability to deplete the free thiol content and their toxicity (Fig. 4). The first group (**24**, **26**, **27** and **29**) inhibits growth of *E. coli* already at low concentration and also causes free thiol depletion suggesting that the alkylating action contributes to the antibacterial activity in this case. The second group (**34**, **38**) comprises of compounds that cause significant thiol depletion but exert no antibacterial effect. The lack of antibacterial activity shows that thiol depletion alone up to that degree observed in this experiment does not decrease the viability of the cells. Compounds of the third group (**25**, **31**) are highly active against *E. coli*, but caused no thiol depletion. This result showed that the Mannich ketones have complex mechanism of action, and the alkylating ability does not explain their biological effect.

Table 7
In vitro cytotoxicity of compounds **24**–**44** on HeLa cell line, expressed as TD_{50}

No.	IC_{50}/TD_{50} (μg mL^{-1})	IC_{50}/TD_{50} (nM mL^{-1})
24	15.24	56.74
25	2.45	9.15
26	12.18	48.38
27	14.05	47.50
28	0.095	0.34
29	2.45	8.69
30	2.10	7.90
31	17.58	53.62
32	4.25	13.72
33	1.15	3.71
34	1.18	3.78
35	1.23	4.15
36	38.21	106.77
37	1.20	3.52
38	4.32	12.71
39	1.98	6.09
40	5.02	16.20
41	14.80	47.47
42	4.12	14.02
43	4.01	13.55
44	15.72	52.84

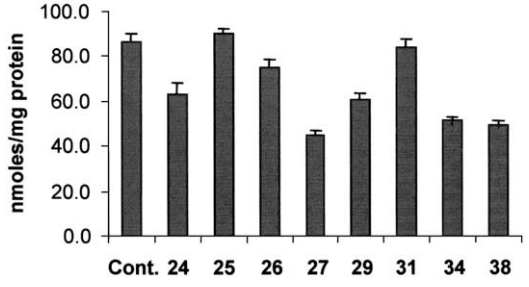


Fig. 4. Data of free thiol measurements (Contr., control).

4.4. QSAR study

Satisfactory single-descriptor QSAR was obtained against *E. coli* involving the Mannich ketones that showed MIC ≤ 200 mg L^{-1}:

$$pMIC = 5.71 - 0.0705\ Polar$$

$$n = 19, \quad RMSD = 0.25, \quad R = 0.70, \quad F = 16.07$$

$$pMIC = 5.85 + 50.52\ MaxQ^{+}$$

$$n = 19, \quad RMSD = 0.25, \quad R = 0.70, \quad F = 16.40$$

where pMIC is the negative logarithm of MIC expressed in molality [log(1/MIC)]. Polar, $MaxQ^{+}$, R, RMSD and F are the molecular polarisability [14], maximum positive charge on the molecule, correlation coefficient, root mean square of deviation and Fisher's statistic value, respectively. Polar and $MaxQ^{+}$ cross-correlated ($R = -0.88$). For *B. subtilis*—calculating for compounds producing MIC ≤ 200 mg L^{-1}—, an opposite effect of $MaxQ^{+}$ and the influence of a topological descriptor (first-order valence-connectivity index, XV1 [15]) were revealed, but the correlation was worse than that of *E. coli*:

$$pMIC = 20.96 - 0.46\ VX1 - 70.36\ MaxQ^{+}$$

$$n = 43, \quad RMSD = 0.34, \quad R = 0.46, \quad F = 5.38$$

However, Mannich ketones involved in this study did not afford meaningful QSAR for other strains of bacteria with the set of descriptors employed in this preliminary study.

5. Conclusions

New fused five-, six- and seven-membered Mannich ketones were prepared using the classical Mannich reaction. Their structure was verified by FT-IR and NMR methods. In addition several known bicyclic Mannich ketones were applied for antimicrobial tests. The antibacterial activity (MIC) of these compounds was examined both on Gram-positive and -negative strains and compared to the activity of standard antibiotics. MIC values for Gram-positive test strains differ between 1.56 and 200 μg mL^{-1} (mostly 6.25–12.5 μg mL^{-1}). Concerning the Gram-negative strains all compounds were ineffective against *P. aeruginosa* (MIC: > 200 μg mL^{-1}) except **25** (MIC: 200 μg mL^{-1}). The *E. coli* strain was relatively sensitive to compounds **24**–**27** (MIC: 12.5–50 μg mL^{-1}). The deep rough mutants were 4–8 times more sensitive to our compounds (MIC: 3.125–200 μg mL^{-1}) than the original smooth strain. These MIC values are similar to that ones gained for Gram-positive strains. It means that the permeability of Gram-negative outer membrane can markedly influence the antibacterial activity of our compounds. Our compounds are bactericidic on *E. coli* strain if they are active against this strain and bacteriostatic on Gram-positive strains.

According to the HeLa tests some of our compounds (**24**, **26**) showed low cytotoxicity coupled with good antibacterial activity. These agents can serve as future lead molecules. Compared to the unsaturated Mannich ketones (**1**–**23**) the fused derivatives (**24**–**44**) proved to be more toxic to the HeLa cells.

Our Mannich ketones are potential alkylating agents having one alkylation site, a latent one. The supposed mechanism of action can be the depletion of the free thiols. A group of toxic compounds really decreased the free thiol content of the cells but this is certainly not the only effect exerted by these ketones. A complex mechanism of action is suggested by the results obtained with compounds **25** and **31** that are highly active against *E. coli* without causing thiol depletion. QSAR study has shown that an increase of molecular polarisability adversely affected, while an increase of the maximum positive charge improved activity of our Mannich ketones against *E. coli*. The limited set of descriptors considered in this preliminary study has probably prevented us from obtaining reliable correlations for other bacteria. To clarify the mechanism of action and extend our (Q)SAR study, synthesis and biological screening of further derivatives are in progress.

6. Experimental

6.1. Chemistry

The reagents and solvents used were purchased from Aldrich Chemical Co. and Fluka and were not further purified. The majority of our starting fused ketones (**24**–**26**, **28**–**29**, **31**–**36** and **40**–**44**) are known compounds synthesised according to literature methods [16–28]. Thin-layer chromatography (TLC) was performed on Merck silica gel plates (60 F_{254}), and EtOAc–C_6H_6 (10:1 v/v) was applied as an eluent. Melting points were determined on a Boetius apparatus and are uncorrected. The analytical values were within $\pm 0.4\%$ of the theoretical values for C, H and N.

The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solution in 5 mm tubes at room temperature, on a Bruker DRX 500 spectrometer at 500.13 (^{1}H) and 125.76 (^{13}C) MHz, with the deuterium signal of the solvent as the lock and TMS as internal standard. DEPT spectra [29] were run in a standard manner [30], using only the $\Theta = 135^{\circ}$ pulse to separate CH/CH_3 and CH_2 lines phased 'up' and 'down', respectively. The 2D-COSY [31,32], and HMQC spectra [33,34] were obtained by using the standard Bruker pulse programs COSYGSSW, HXCO.AU (INV4GSSW) and HXXCO.AU (INV4GSLRNDSW), respectively.

FT-IR spectra were taken on a Nicolet Impact 400 spectrophotometer in KBr pellets. The starting 6-ethoxy-1-tetralone, 6-propoxy-1-tetralone and 6-isopoxy-1-tetralone were prepared according to literature methods [2,3].

*6.1.1. General procedure for the preparation of Mannich ketones (**24–44**)*

The detailed method was published previously [1]. Briefly, the corresponding bicyclic ketone (20 mmol), paraformadehyde (40 mmol) and the secondary amine (20 mmol) in the presence of concd. HCl (0.3 mL) were refluxed in EtOH (50 mL); the time of heating is displayed in Table 1. Our method reported earlier was applied to obtain pure Mannich bases [1]. The NMR measurements were done on the Mannich bases.

6.2. Biology

6.2.1. Determination of MIC and MBC

MIC values were determined by test tube dilution method as described previously [1]. Briefly, the test strains were: *P. aeruginosa* NIH Hungary 170000, *E. coli* ATCC 25922, *E. coli* ReD31m4 [35], *S. minnesota* Re595, *S. sonnei* Re4350 [36], *S. saprophyticus* NIH Hungary 120008, *Staphylococcus aureus* NIH Hungary 118003, *M. luteus* ATCC 9341 and *B. subtilis* ATCC 6633. Twofold dilution series of compounds were made in nutrient broth from 200 to 0.4 μg mL^{-1} concentration. The tested bacterial strains were added to each tube to achieve a final inoculum of ca. 5×10^5 colony-forming units per mL. The cultures were incubated for 24 h at 37 °C. The MIC values for each compound were determined as the lowest concentration of compounds that produce no visible bacterial growth after incubation time. Loopfuls (10 μL) of nutrient broth cultures from each tube were plated on nutrient agar to check the bacterial growth. MBC was determined as the lowest concentration of compound under investigation where we could not detect any living bacterium. All experiments were performed in triplicate [37].

6.2.2. In vitro effect of Mannich ketones on HeLa cell line

HCl, KCl, KH_2PO_4, NaCl, Na_2HPO_4, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), isopropanol, RPMI-1640, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated fetal calf serum low IgG was purchased from Gibco BRL-Life Technology Ltd. (UK). Streptomycin and penicillin were obtained from Richter Ltd. (Budapest, HU).

6.2.2.1. Cell microculturing. The procedure was published before [1]. Briefly, in vitro cultured HeLa cell line (ATCC number: CCL-2, USA) was used. The cells were cultured in RPMI-1640 culture medium containing 10% heat-inactivated fetal calf serum with antibiotics in 5% CO_2 humidified atmosphere at 37 °C (Forma Scientific, USA). The cells growing in the logarithmic phase had viability better than 98% by Tripan blue exclusion.

6.2.2.2. Modified tetrazolium assay. Cells were plated out in 100 μL of medium at concentration of $4–5 \times 10^3$ cells/flat-bottomed well in 96-well microtitre plates (Nunc, France). Hundred microlitres of media containing the drug dissolved in appropriate solvent were added to each well and incubated for 48 h. Hundred microlitres of medium was then removed from the wells. MTT was prepared as a 5 mg mL^{-1} stock solution 0.15 M phosphate-buffer saline (PBS, pH 7.2), filtered through a 0.22 μm filter and diluted in RPMI-1640 containing antibiotics to 1 mg mL^{-1}. Hundred microlitres of this solution added to each well and incubated for 4 h. All untransformed MTT (medium) removed by careful aspiration from the wells. The formazan crystals were dissolved in 100 μL of isopropanol–1 N HCl (24:1). Then the plate was shaken in order to ensure solubilisation of the blue formazan crystals. The absorbance was recorded in an enzyme-linked immunosorbent assay plate reader (Dynatech MR 7000) at a 560 nm test wavelength and a 690 nm reference wavelength.

6.2.3. Determination of free thiol content

The method was published previously [1]. Shortly, the bacterial cells were cultivated in shaker incubator (37 °C) to the mid log phase and treated with the different compounds at the MIC concentration or at 200 μg mL^{-1} in case of ineffective substance (MIC > 200 μg mL^{-1}) for 1 h. The treated cells were centrifuged (+4 °C), washed with physiological saline solution and stored at −80 °C as pellet. These cell pellets were resuspended in a buffer containing 50 mM Tris–HCl, 0.1% SDS of pH 7.4, and lysed by sonication. The cell lysates were clarified by centrifugation and used immediately for the determination of free thiol by the DTNB assay [13]. The protein concentration was determined by the BCA (bicinchoninic acid) assay [38]. The assay was repeated using eight selected compounds in three parallel cultures. Values represent the average of three independent determinations ± SD.

6.3. QSAR

QSAR analysis was done using the SCIQSAR 3.0 (SciVision, Lexington, MA) program and the equation:

$$\mathrm{pMIC} = \log(1/\mathrm{MIC}) = \sum_{i=1}^{17} K_i d_i$$

where K_i is the regression coefficient for the descriptor d_i. A semi-empirical quantum-chemical method (PM3),

available as a part of the Chem3D Ultra 5.0 (Cambridgesoft, Cambridge, MA) molecular modelling program linked with SCIQSAR, was used to obtain geometry-optimised structures as input for the calculation of 17 descriptors that included the calculated logarithm of the octanol–water partition coefficient (log P), as well as various topological and shape indices, electrostatic, geometrical and quantum-chemical descriptors. Satisfactory QSAR equations were cross-validated by comparing to regressions upon randomly removing 10% of the compounds.

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