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Original article

Synthesis and antibacterial study of unsaturated Mannich ketones

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Abstract – Several Mannich ketones of 2-arylmethylenecycloalkanones were synthesised using the classical acid-catalysed Mannich reaction. Antibacterial activity of these new water-soluble compounds was reported against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Micrococcus luteus* and *Bacillus subtilis* standard strains. Human cell line cytotoxicity of our new compounds was evaluated against HeLa cell lines. Some compounds showed low cytotoxicity (41.52 nM mL^{-1} for **14** and 46.60 nM mL^{-1} for **18**) and proved to be efficient antibacterial agents against the Gram-positive strains. Minimum inhibitory concentrations varied from 1.56 to 100 μg mL^{-1}. The mechanism of action was examined, too. © 2001 Éditions scientifiques et médicales Elsevier SAS

Mannich ketone / NMR / antibacterial activity / cytotoxicity

1. Introduction

The increasing number of the resistant bacterial strains, especially the highly resistant β-lactamase producing *Staphylococcus aureus* and Gram-negative strains require the development of new effective chemotherapeutic agents of low toxicity.

The family of the α,β-unsaturated ketones is known to possess antimicrobial effects [1]. The mode of action of this class of compounds has been proposed to be by their reaction with thiol groups of essential enzymes [2, 3]. Previously we have reported on our antifungal studies involving *E*-2-arylidene-1-tetralones, *E*-3-arylidenechroman-4-ones, and *E*-3-arylidene-1-thiochroman-4-ones—homoisoflavones [4]. These compounds were screened against human pathogenic yeasts showing marked antifungal effect. In order to prepare more efficient water-soluble unsaturated ketones as potential antimicrobial agents we wanted to prepare some Mannich ketones of arylidenecycloalkanones.

The Mannich ketones could show more selective toxicity toward microorganisms than the parent unsaturated ketones. Their breakdown can afford either in 1,2 elimination reactive vinyl ketones or they can undergo reverse Mannich reaction [1]. The vinyl ketones produced as intermediates have much higher affinity toward thiols than hydroxy and amino groups present in the nucleic acids, therefore they do not show the mutagenic side effect of some alkylating agents used in the therapy [5]. In addition the water solubility of the Mannich ketones can alleviate their

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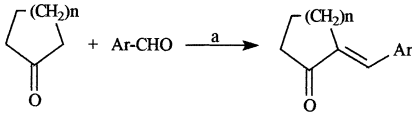
transport to the site of action, too. Several Mannich ketones are described having antibacterial, antifungal and cytotoxic activity [6–9].

Our objective was to study the structure–antibacterial activity relationship for this class of compounds. On the other hand we wished to examine our Mannich ketones from stereochemical—configuration and conformation—and electronical point of views as regards the conjugated enone structure. These factors can influence the possible reaction of these compounds with biological nucleophiles, e.g. thiol enzymes. In addition some attempts were made to find the mechanism of action, too.

2. Chemistry

The strategy for the synthesis of unsaturated Mannich ketones (**14**–**36**) involves the preparation of 2-arylidenecycloalkanones intermediates (**1**–**13**) by the base-catalysed aldol condensation (*figure 1*). The title compounds have been prepared from the corresponding 2-arylidenecycloalkanones, secondary amines and paraformaldehyde (*figure 2*). The products were isolated as hydrochlorides. Our method was the classical Mannich reaction applying ethanol as a solvent and HCl as a catalyst. Under these reaction conditions the **1**–**13** unsaturated ketones are able to undergo isomerisation to the thermodynamically more stable endocyclic enones [10, 11]. This means the loss of the starting ketones and the decrease of the possible yield. In addition it results in a reaction mixture enriched in the secondary amine component. 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). Using this method it is possible to avoid the deamination of the Mannich bases to methylene ketones. The physical data of the novel compounds are shown in *table I*.



No.	n	Ar
1	1	Ph
2	1	4'-CH_3-C_6H_4
3	1	4'-OCH_3-C_6H_4
4	1	3'-OCH_3-C_6H_4
5	1	2'-OCH_3-C_6H_4
6	1	3',4'-OCH_2O-C_6H_3
7	1	3',4',5'-$(OCH_3)_3$-C_6H_3
8	2	Ph
9	2	4'-CH_3-C_6H_4
10	2	4'-OCH_3-C_6H_4
11	2	3',4'-$(OCH_3)_2$-C_6H_3
12	3	Ph
13	4	Ph

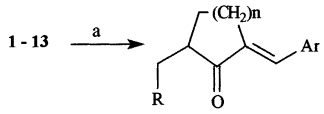
Figure 1. (a) $NaOH/H_2O$, 20 °C.

From the spectral data given in *tables II and III* the postulated structures follow straightforwardly.[1] Only the remarks below are necessary (*figure 3*):

The bridging methylene hydrogens (8α) are chemically non-equivalent due to molecular asymmetry. Their signals (two double doublets) are the proof of the expected reaction leading to the aimed products. The high difference in the chemical shifts of these methylene hydrogens (ca. 0.5 ppm) is due to the anisotropic neighbouring effect [12] of the near lying carbonyl, which influences one of the hydrogens. This fact suggests hindered rotation around the C-8–C-8α bond and a quasi-rigid structure for this part of the molecule.

The IR carbonyl frequencies and also the ^{13}C-NMR chemical shifts of the carbonyl carbons depend on the ring size and lie in the expected [16, 13] ranges. Ring strain reveals in higher IR-frequencies for cyclopentanones [17]; νC=O: 1700–1709 cm^{-1} for the c-pentanones, 1670–1686 cm^{-1} for all other compounds with six- to eight-membered rings; δC=O: 208.1±0.5 ppm (c-pentanones), 203.5±0.5 ppm (c-hexanones), 205.0 (**33**), 205.3 (**34**) and 208.8 ppm (**35**, **36**). The slightly different values suggest similar stereo structures (hardly different conformational situations).

[1] The NMR numbering is shown in *figure 3*. For easier comparison of analogous spectral data, the same numbering of *c*-octanones was used for all compounds in the text and in the tables. The correct (IUPAC) numbering is given in Section 6.



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No.	n	R	Ar
14	1	Pip	Ph
15	1	Mor	Ph
16	1	Pyr	Ph
17	1	Thik	Ph
18	1	Mor	4'-CH_3-C_6H_4
19	1	Pip	4'-OCH_3-C_6H_4
20	1	Mor	4'-OCH_3-C_6H_4
21	1	Pip	3'-OCH_3-C_6H_4
22	1	Mor	3'-OCH_3-C_6H_4
23	1	Pip	2'-OCH_3-C_6H_4
24	1	Mor	2'-OCH_3-C_6H_4
25	1	Pip	3',4',5'-$(OCH_3)_3$-C_6H_3
26	1	Mor	3',4'-OCH_2O-C_6H_3
27	2	Mor	Ph
28	2	Pyr	Ph
29	2	4-Pip	Ph
30	2	Mor	4'-CH_3-C_6H_4
31	2	Mor	4'-OCH_3-C_6H_4
32	2	Mor	3',4'-$(OCH_3)_2$-C_6H_3
33	3	Mor	Ph
34	3	Pip	Ph
35	4	Mor	Ph
36	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

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

The configuration *E* around the C=C double bond is plausible chemically (configuration *Z* is highly unfavourable because of strong steric interaction between the aryl and the carbonyl groups) and was proved earlier for very similar structures [14] by DNOE measurements [15, 19] which demonstrated the sterically close arrangement of the 3-methylene and aryl groups. The similar downfield shifted (due to anisotropy of the carbonyl [12]) H-8 signals in the spectra of compounds described in the recent paper confirm analogous configurations.

The chemical shifts of the H-2α signals (7.35±0.14 ppm) are, however, significantly smaller than the condensed benzocyclanones (7.84±0.29 ppm [18]). This may be arising, besides the absence of a condensed aromatic ring, from the higher probability of out-of-plane conformations of the aryl group in the conformational equilibrium. In contrast to benzocyclanones [18], there is no difference between compounds with c-pentanone ring on one hand and the ones containing six- to eight-membered cyclanone rings on the other hand (indanones have fully planar molecular skeletons [18]).

The narrow interval of the chemical shifts for H-2α (7.21–7.48 ppm) refers to similar dihedral angles O–C(1)–C(2)–C(2α) in the compounds investigated. The only exceptions are **23** and **24** (7.80 and 7.84 ppm) containing 2-methoxy substituted aryl groups. The downfield shift of the H-2α signal can be interpreted as the anisotropic neighbouring effect of the C–O bond [14]. This fact suggests the coplanar arrangement of the H-2α atom and the methoxy group, and beyond that, for **23** and **24**, the predominance of the rotamer containing the 3-methylene and methoxy groups in *S-trans* position.

Non-coplanar position of the aryl ring to the enone C=C double bond in the preferred conformation is reflected in the hardly different chemical shifts of the ring-hydrogens in the aryl group. Due to stronger conjugation, higher differences in shifts should be expectable. The commonly high polarisation of the enone groups resulting in significant shift differences ($\gg$10 ppm) for the α and β sp^2 carbon atoms to the carbonyl [13] is not observable in our compounds ($\Delta\delta$C-2, C-2α: 0.7–6.0 ppm). Because of the non-planar arrangement of the aryl group, the above fact cannot be interpreted by the buffering effect of the aromatic π-sextet. Thus, the suppressed polarisation refers to non-planar enone conformation in accordance with the X-ray measurement on **36**. Rotamers with non-planar aryl groups have somewhat more

Table I. Physical data of compounds **5**, **7** and **14**–**36**.

Compound	General formula [a]	M.p (°C)	Yield (%)	Time of heating (h)	IR (KBr, cm^{-1})
5	$C_{13}H_{14}O_2$	77 (dec., hexane)	53	–	1709
7	$C_{15}H_{18}O_4$	135–137 (methanol)	76	–	1706
14	$C_{18}H_{24}ClNO$	139 (dec., acetone)	22	5	1716, 1626
15	$C_{17}H_{22}ClNO_2$	135 (dec., acetone)	20	5	1707, 1623
16	$C_{17}H_{22}ClNO$	122 (dec., acetone)	26	5	1709, 1626
17	$C_{22}H_{24}ClNO$	130 (dec., acetone)	18	6	1709, 1624
18	$C_{18}H_{24}ClNO_2$	147 (dec., acetone)	20	5	1705, 1624
19	$C_{19}H_{26}ClNO_2$	147 (dec., acetone)	15	5	1710, 1625
20	$C_{18}H_{24}ClNO_3$	150 (dec., acetone)	14	3	1700, 1622
21	$C_{19}H_{26}ClNO_2$	128 (dec., acetone)	32	6	1701, 1621
22	$C_{18}H_{24}ClNO_3$	133 (dec., acetone)	26	6	1707, 1626
23	$C_{19}H_{26}ClNO_2$	117 (dec., acetone)	23	6	1708, 1625
24	$C_{18}H_{24}ClNO_3$	156 (dec., acetone)	27	4	1708, 1624
25	$C_{21}H_{30}ClNO_4$	144 (dec., acetone)	20	11	1700, 1621
26	$C_{17}H_{22}ClNO_2$	153 (dec., acetone)	12	7	1701, 1617
27	$C_{18}H_{24}ClNO_2$	127 (dec., acetone)	23	3	1673
28	$C_{18}H_{24}ClNO$	120 (dec., acetone)	43	5	1675
29	$C_{20}H_{28}ClNO$	119 (dec., acetone)	50	5	1686, 1615
30	$C_{19}H_{26}ClNO_2$	124 (dec., acetone)	30	3	1673
31	$C_{19}H_{26}ClNO_3$	136 (dec., acetone)	19	3	1675, 1604
32	$C_{20}H_{28}ClNO_4$	119 (dec., acetone)	20	4	1670
33	$C_{19}H_{26}ClNO_2$	185 (dec., acetone)	38	19	1684, 1608
34	$C_{20}H_{28}ClNO$	164 (dec., acetone)	46	24	1678, 1608
35	$C_{20}H_{28}ClNO_2$	166 (dec., acetone)	24	17	1676
36	$C_{21}H_{30}ClNO$	156 (dec., acetone)	32	24	1676, 1613

[a] The analytical values were within $\pm 0.4\%$ of the theoretical values for C, H and N.

importance in the conformational equilibrium in higher homologues **33**–**36** as shown by higher enone polarisation ($\Delta\delta$C-2, C-2α: 5.0–5.7 ppm) and narrower range of ArH signals.

In the c-octanone homologues **35** and **36** H-8 lies in the plane of the carbonyl group as proved by the significant downfield shift of H-8 (3.42 and 3.51 ppm, while these shifts are in the range 2.3–2.7 ppm for all the other compounds). This conformation becomes possible in the flexible eight-membered ring only.

Some ^{1}H-NMR spectra contain two weak signals at about 5.35 ± 0.1 and 6.15 ± 0.1 ppm, which originate from the contamination of $<1\%$ concentration. These signals can be assigned to a terminal methylidene group in decomposition product formed via deamination [20]. This fact supports the proposed mechanism of action of our compounds (see Section 4.3).

The X-ray structure of **36** is depicted in *figure 4*. The Cl^- ion is weakly bound by a hydrogen bridge to the nitrogen atom N1. The distance N1–H1N1···Cl1 is 3.048(1) Å and the bond angle at the H-atom is 177(1)°. Taking into account the experimental errors, all distances and angles are within the expected values [21]. The torsion angles about the double bond are: C1–C2–C2a–C11 177.01(9)°, C3–C2–C2a–C11 2.4(1)°, C1–C2–C2a–H2a 0.5(10)° and, C3–C2–C2a–H2a −174.1(10)°. Therefore, this is an almost planar surrounding. The conformation of the central eight-membered ring C1–C8 is crown-like. The torsion angle about the C11–C2a single bond C16–C11–C2a–C2 is 51.6(1)°. Therefore the double bond C2=C2a is inclined by this angle with respect to the plane of the phenyl ring.

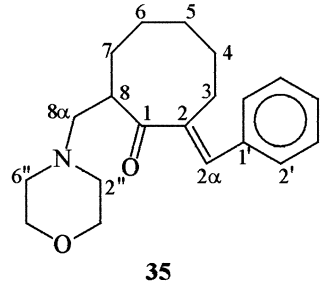

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Figure 3. NMR numbering of compound **35**.

Table II. [1]H-NMR data [a] for compounds **14–28** and **30–36** [b].

Compound	CH_2 (pos. 3–7) [c] m (4–10H)	H-8 m (1H)	CH_2 (8α) [d] 2 × dd (2H)	H-2α s (1H)	NCH_2 [e] m (4H)	CH_2 [f] m (4H)	CH_2 [g] m (2H)	Aryl group [h] 1–4 m (2–5H)	CH_3 [i] s (3H)
14	1.75, ~2.35, [j] ~2.8, [j] 2.97	2.55	~2.35, [j] 2.83 [j]	7.25	~2.35, [j] 2.45	1.55	1.41	7.33, 7.39, 7.51	–
15	1.70, ~2.28, [j] ~2.75, [j] 2.95	~2.5 [j]	2.35, 2.80 [j]	7.32	~2.32, [j] 2.45 [j]	3.63	–	7.30, 7.33, 7.45	–
16	~1.8 [j] 2.41, 2.80, 3.00 [j]	~2.55 [j]	~2.55, [j] 2.96 [j]	7.40	~2.55 [j]	~1.8 [j]	–	7.35, 7.39, 7.52	–
17	1.85, 2.40, ~2.7 [j] ~3.05 [j]	~2.7 [j]	2.63, ~2.9 [j]	~7.4 [j]	3.64, 3.74	~2.9 [j]	~2.9 [j]	7.37, 7.42, [j] 7.56	–
18	1.70, ~2.45, [j] 2.74, 2.93	~2.3 [j]	2.34, [j] 2.80	7.30	~2.35, [j] ~2.45 [j]	3.64	–	7.14, 7.36	2.30 [j]
19	1.62, ~2.2, [j] 2.62, 2.82	2.40	~2.2, [j] 2.72	7.21	~2.2, [j] 2.35	1.45	1.30	6.79, 7.35	3.68
20	1.74, 2.30, 2.76, 2.94	~2.5 [j]	~2.35, [j] 2.82	7.32	~2.35, [j] ~2.5 [j]	3.66	–	6.89, 7.46	3.79
21	1.72, ~2.32, [j] ~2.8, [j] 2.96	2.52	2.43, ~2.8 [j]	7.30	~2.32, [j] 2.45	1.55	1.38	6.86, 7.01, 7.08, 7.27	3.77
22	1.75, 2.32, ~2.78, [j] 2.99	~2.55 [j]	2.39, [j] 2.83 [j]	7.33	~2.4, [j] ~2.5 [j]	3.67	–	6.89, 7.03, 7.10, 7.29	3.79
23	1.71, ~2.35, [j] 2.76, 2.90 [j]	2.56	~2.35, [j] 2.87 [j]	7.80	~2.35, [j] 2.48	1.57	1.42	6.89, 6.96, 7.32, 7.46	3.86
24	1.75, 2.35, 2.81, ~2.95 [j]	~2.55 [j]	2.44, [j] 2.95 [j]	7.84	~2.45, [j] ~2.55 [j]	3.73	–	6.93, 6.99, 7.36, 7.49	3.88
25	1.80, ~2.35, [j] ~2.8, [j] 3.00	2.55	~2.35, [j] ~2.8 [j]	7.28	~2.35, [j] 2.45	1.55	1.40	6.76	3.83
26	1.80, 2.35, 2.78, 2.98	~2.55 [j]	~2.42, [j] 2.86	7.32	~2.42, [j] ~2.55 [j]	3.71	–	6.85, 7.06	6.01
27	1.57, 1.62, 1.78, 2.10, 2.61, 2.85	2.48	2.38, [j] 2.72	7.31	2.28, 2.40 [j]	3.59	–	~7.2, ~7.27	–
28	1.67, ~1.75, [j] 1.90, 2.25, ~2.7, [j] 2.95	~2.6 [j]	2.75, [j] 2.88	7.38	2.50, ~2.6 [j]	~1.8 [j]	–	7.3–7.45	–
30	~1.6, [j,k] 1.81, 2.10, 2.65, 2.88	2.50	2.40, [j] 2.75	7.32	2.32, [j] 2.45 [j]	3.62	–	7.10, 7.21	2.29 [j]
31	~1.6, [j,k] 1.83, 2.10, 2.66, 2.88	~2.48 [j]	2.41, 2.74	7.34	2.30, 2.45 [j]	3.62	–	6.83, 7.30	3.75
32	~1.7, [j,k] 1.90, 2.18, ~2.75, 2.98	~2.57 [j]	2. 50, [j] 2.82	7.40	2.38, ~2.55 [j]	3.70	–	6.88. 6.93, 7.02	3.88
33	1.29, 1.38, 1.60, ~2.0, [j,k] 2.16, 2.78, 3.00	~2.4 [j]	2.33, [j] 3.10	7.48	~2.4, [j] 2.50	3.67	–	7.25–7.37	–
34	1.29, 1.34, ~1.6, [j] 1.95, 2.00, 2.15, 2.80, ~3.1 [j]	~2.4 [j]	~2.4 [j] 3.08 [j]	7.47	~2.4, [j] 2.5 [j]	~1.55 [j]	1.40	7.25–7.35	–
35	~1.47, [j,k] ~1.6, [j,k] ~1.85, [j,k] 2.66, 2.98	3.42	2.35, [j] 2.89	7.43	~2.4 [j]	3.64	–	~7.37	–
36	~1.55, [j,k] ~1.85, [j,k] 2.68, ~2.92 [j]	3.51	~2.35, [j] 2.96 [j]	7.43	~2.35 [j]	~1.55 [j]	1.35	7.28, ~7.34	–

[a] Chemical shifts (in ppm, $\delta_{TMS} = 0$ ppm) and coupling constants (in Hz) at 500 MHz in $CDCl_3$ solution.
[b] Compound **29** is a ca. 2:1 mixture of diastereomers having overlapped signals. The only exactly determinable signal, CH_3, d (J: 6.5): 0.94 > 0.96. The assignments were supported (except for **14**, **17**, **18**, **31**, **33**, **34** and **36**) by 2D-HSC (HMQC) and for **19** and **27** also 2D-COSY measurements.
[c] $4 \times m$ (4H) for **14–26**, $5/6 \times m$ (6H) for **27**, **28**, **32**, $7/8 \times m$ (8H) for **33**/**34** and $5/4 \times m$ (10H) for **35**/**36**.
[d] J: 12.5 ± 0.3, 8.7 ± 0.2 and 4.5 ± 0.3, for downfield dd (**33**): 15.0 and 6.4.
[e] R group, two (2×2H) or one signal (4H). Pos. 1 for **17**.
[f] R group, CH_2 (piperidyl, Pos. 3,5) for **14**, **19**, **21**, **23**, **25**, **34** and **36**, OCH_2 (morpholyl) for **15**, **18**, **20**, **22**, **24**, **26**, **27**, **30–33** and **35**, CH_2 (pyrrolidinyl, Pos. 3,4) for **16** and **28**, and NCH_2 (Pos. 3) for **17** (1H) with a second m at 3.05 [j](1H).
[g] R group, CH_2 (piperidyl and compound **17**, Pos. 4.
[h] δH-2′,6′ (~d, 2H) > δH-3′,5′ (~t, 2H) > δH-4′ (~t, 1H) for **14–17**, δH-2′,6′ (~d, 2H) > δH-3′,5′ (~d, 2H) for **18–20**, **30** and **31**, J: 8.1 (**18**, **30**), 8.8 (**19**, **20**, **31**), δH-3′ (~t) > δH-6′ (~d) > δH-2′ (~s) > δH-4′ (~d) for **21** and **22**, δH-2′ (~d) > δH-4′ (~t) > δH-5′ (~t) > δH-3′ (~d) for **23** and **24**, δH-3′,5′ (s) for **25**, δH-6′ (~d) > δH-2′ (~s) > δH-5′: 7.03 (for **26** and **32**, δH-3′–6′ (m, 4H) > δH-2′ (m, 1H) for **27** and **36**, coalesced multiplets (5H) for **28**, **33** and **34**, singlet-like signal for **35**. Further aromatic signals (**17**), H-5′: 7.03 (~d), H-6–8: ~7.12 (coalesced ms, 3H).
[i] Pos. 4 for **25**, further signal (Pos. 3,5) at 3.86, s (6H), $2 \times$ s (2×3H) for **32**, other signal at 3.90.
[j] Overlapping signals.
[k] Intensity: 2/4/10H.

Table III. ^{13}C-NMR chemical shifts [a] for compounds **14**–**36** [b].

Compound	C-1	C-2	C-3	C-4	C-8	C-2α	C-8α	C-1′	C-2′	C-6′	C-3′	C-5′	C-4′	C-2″	C-3″	C-4″	CH_3
14	208.6	136.1 [c]	28.0	27.3	47.5	132.8	59.9	136.7 [c]	132.8		129.0		129.6	55.2	26.4	24.7	–
15	208.4	135.9 [c]	28.0	27.1	47.1	133.2	59.5	136.4 [c]	131.0		129.1		129.8	54.3	67.3	–	–
16	208.2	136.0 [c]	28.0	27.9	49.0	133.1	56.9	136.6 [c]	131.0		129.7		129.7	55.0	24.0	–	–
17	208.5	136.0 [c]	28.1	27.1	47.7	133.1	56.9	136.6 [c]	131.0		129.1 [d]		129.1 [d]	58.9 [e]	51.5	29.6	–
18	208.4	135.4	28.0	27.1	47.0	133.3	59.6	133.1	129.9		131.0		140.2	54.3	67.3	–	21.9
19	207.6	133.4	27.1	26.6	46.5	131.8	59.3	127.9	131.9		113.8		160.1	54.4	25.6	24.0	54.9
20	208.0	134.1	27.9	27.0	47.0	132.9	59.6	128.7	132.7		114.7		161.1	54.4	67.4	–	55.7
21	208.0	136.4 [c]	27.5	26.8	46.9	132.2	59.4	136.8 [c]	115.6	122.9	159.6	129.5	114.9	54.7	25.9	24.2	55.1
22	207.7	136.1 [c]	27.5	26.5	46.6	132.5	59.0	136.7 [c]	115.7	123.0	159.6	129.5	114.9	53.8	66.8	–	55.1
23	208.6	136.5	28.2	27.7	47.5	131.2	60.0	125.0	159.3	127.5	111.1	120.6	130.1	55.2	26.4	24.7	55.9
24	208.2	136.3	28.2	27.4	47.2	131.3	59.6	124.9	159.3	127.9	111.2	120.7	130.2	54.3	67.3	–	55.9
25	208.5	135.6	27.9	27.3	47.4	133.1	59.9	135.6	108.4		153.6		131.5	55.2	26.3	24.6	56.6 [f]
26	208.2	134.4	27.9	27.0	47.0	133.2	59.6	130.3	110.0	126.9	148.5	109.1	149.2	54.3	67.4	–	101.9 [g]
27	203.0	137.0	27.5	22.2	46.7	134.9	59.0	135.5	130.0		128.1		128.2	53.7	66.8	–	–
28	203.3	137.3[c]	27.9	22.5	48.9	135.1	56.8	135.8[c]	130.2		128.4		128.3	54.4	23.6	23.6	–
29	204.1	137.8	28.3	22.8	47.9	135.4	59.6	136.2	130.6		128.7		128.3	53.4 [h]	34.8 [h]	31.2	–
30	203.8	136.7	28.2	22.8	47.2	136.0	59.7	133.3	130.7		129.5		139.1	54.3	67.4	–	21.8
31	203.0	134.8	27.5	22.2	46.7	135.5	59.3	128.3	132.1		113.8		159.8	53.9	67.0	–	55.2
32	203.4	135.5	28.0	22.7	47.1	136.2	59.8	129.0	111.2	124.2	149.0	113.9	149.9	54.3	67.4	–	56.3 [d]
33	205.0	141.3	27.4	29.9	48.4	135.8	60.1	136.5	129.8		128.8		128.5	54.5	67.4	–	–
34	205.3	141.4	27.3	29.8	48.7	135.7	60.4	136.6	129.8		128.7		128.4	55.4	26.2	24.6	–
35	208.8	141.6	26.2	26.2	44.2	136.6 [d]	61.9	136.6 [d]	130.0		128.8		128.7	54.4	67.4	–	–
36	208.8	141.5	26.3	26.8	44.2	136.5	62.1	136.7	130.1		128.8		128.7	55.2	26.1	24.5	–

[a] In ppm ($\delta_{TMS} = 0$ ppm) at 125.7 MHz. Solvent: $CDCl_3$; Further signals, OCH_3 (**25**, Pos. 4): 61.3; CH_2 (Pos. 5): 28.7 (**27**), 29.0 (**28**), 29.4 (**29**), 29.4 (**30**), 28.9 (**31**), 29.5(**32**), (Pos. 5–6): 30.7, 31.1 (**33**), 30.6, 31.7 (**34**), (Pos. 5–7): 26.8, 30.8, 35.3 (**35**), 26.8 [d], 31.0, 35.8 (**36**); isoquinoline ring (**17**), C-5″: 126.0, C-6″,7″: 127.0, 129.8, C-8″: 126.6, C-4a″: 134.7, C-8a″: 135.2.

[b] The assignments were supported by DEPT, 2D-HSC (HMQC, except for **14**, **17**, **18**, **31**, **33**, **34** and **36**), and for **28** also 2D-COLOC (HMBC) measurements. Superscripts in C-1′–6′ and C-2″–4″ refer to substituents Ar and R, respectively.

[c] Interchangeable assignments.

[d] Two overlapping lines.

[e] C-1″ (isoquinoline).

[f] Pos. 3,5.

[g] OCH_2O group.

[h] Doubled signals with the second lines at 56.0 (C-2″) and 34.9 (C-3″) due to diastereomeric mixture.

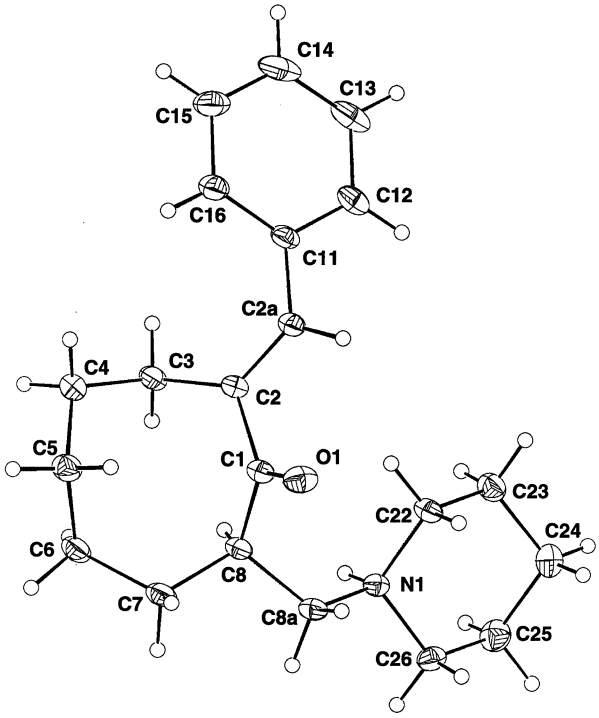

Figure 4. X-ray structure of **36** showing displacement ellipsoids with 50% probability.

3. Biology

In vitro antibacterial activity of the compounds synthesised was examined on standard bacterial strains: *Pseudomonas aeruginosa* NIH Hungary 170000, *Escherichia coli* ATCC 25922, *Staphylococcus saprophyticus* NIH Hungary 12008, *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

Our purpose was to find the structure–antibacterial activity and cytotoxicity relationships in the series of the new Mannich ketones. Therefore, we varied here the size of the cycloalkanone ring, the secondary amine and the quality of the aromatic substituent.

4.1. In vitro antibacterial activity

The antibacterial activity was determined (*tables IV* and *V*) and compared to the activity of standard antibiotics (see *table VI* in Ref. [22]; MIC values from 0.20 to 100 $\mu g\,mL^{-1}$). Out of the unsaturated Mannich ketones examined the seven- and eight-membered ketones were the least active (**30**–**36**). The quality of the amine substituent generally did not influence the antibacterial activity neither against the *E. coli* strains, nor against the Gram-positive strains. The changes in the position of the aromatic methoxy group did not affect the activity neither against *E. coli* strains (**19**, **21**, and **23**) nor against the Gram-positive ones (**20**, **22**, and **24**). As for the Gram-positive strains **14**, **18**, **27** and **28**—unsaturated Mannich ketones—proved to be the most effective compounds. More than half of the strains had MIC values of 6.25–12.5 $\mu g\,mL^{-1}$ (see *table V*). In comparison with standard commercial antibiotics [22] our compounds were slightly less active. For these strains we did not find any special structural characteristics required by an active compound. As regards the Gram-negative strains all our compounds were inactive against *P. aeruginosa*. Mostly the Mannich ketones of cyclopentanones showed activity against *E. coli* strain, compound **15** produced the highest activity (MIC: 25 $\mu g\,mL^{-1}$). As for the effect of the electrondonating aromatic substituents on the antibacterial activity, the introduction of the methyl or methoxy groups decreases the efficiency against *E. coli* strain (see **14**, **15** and **18**, **19**). The MBC values were also same or near the same—two or four times higher—as MIC values for sensitive *E. coli* strains, so the compounds were mostly bactericidic. The MBC values for Gram-positive strains were 8, 16 or 32 times higher than the MIC values. So the compounds proved to be bacteriostatic. We are sure that the permeability of bacterial cell wall for our compounds is also an important factor in their antibacterial activity. The Gram-negative bacteria (*P. aeruginosa*, *E. coli*, etc.) in comparison with Gram-positive ones (*S. aureus*, *M. luteus*, etc.) have a more complicated cell wall structure. Their thick outer membrane consists of lipoproteins, lipopolysaccharides, etc. It is difficult for the compounds to penetrate through this membrane into the bacterial cytoplasm, to the site of action. We suppose that some of our compounds cannot penetrate the Gram-negative cell wall and sometimes that may explain

their ineffectivity. Further structure–activity studies (e.g. QSAR calculations) are in progress.

4.2. In vitro cytotoxicity tests

Compounds **14**, **18**, **20**, **22**, **24**, **26**, **29**, and **33**–**36**—the Mannich ketones of seven- and eight-membered ring—showed the lowest cytotoxic activity and in fact this effect was not measurable in the case of **33** (*table VI*). The low cytotoxicity of **14**, **18** is coupled with an efficient antibacterial activity against the Gram-positive strains. On the other hand for the seven- and eight-membered derivatives (**33**–**36**) the antibacterial effect was generally diminished both against the Gram-positive and the Gram-negative strains, too. Out of the above compounds several representatives are morpholine derivatives; while compounds **16**, **17**, **25**, **27** and especially **32** proved to be rather toxic to the HeLa cells. These results demonstrate that the introduction of more OCH_3 groups into the aromatic side chain dramatically

Table IV. In vitro antibacterial activity of Mannich ketones, expressed as minimum inhibitory concentration values (MIC, $\mu g\ mL^{-1}$).

No.	*P. aeruginosa* 170000	*E. coli* 25922	*S. saprophyt.* 120008	*S. aureus* 118003	*M. luteus* 9341	*B. subtilis* 8833
14	>200	100	6.25	6.25	6.25	12.5
15	>200	25	6.25	50	25	6.25
16	>200	100	12.5	12.5	25	12.5
17	>200	200	25	12.5	50	50
18	>200	>200	3.125	12.5	6.25	3.125
19	>200	200	12.5	12.5	12.5	3.125
20	>200	>200	25	12.5	25	12.5
21	>200	200	25	6.25	6.25	25
22	>200	>200	50	12.5	25	12.5
23	>200	>200	12.5	12.5	12.5	6.25
24	>200	200	12.5	12.5	6.25	12.5
25	>200	>200	12.5	12.5	6.25	25
26	>200	200	12.5	6.25	6.25	12.5
27	>200	>200	6.25	1.56	6.25	12.5
28	>200	200	3.125	3.125	6.25	6.25
29	>200	>200	3.125	3.125	6.25	12.5
30	>200	>200	6.25	12.5	6.25	>200
31	>200	>200	12.5	25	6.25	12.5
32	>200	>200	3.125	12.5	12.5	12.5
33	>200	200	12.5	50	25	25
34	>200	>200	25	100	100	50
35	>200	>200	25	50	25	12.5
36	>200	>200	50	100	100	100

Table V. Cumulative data of antibacterial sensitivity tests: number of compounds causing the given MIC value.

MIC ($\mu g\ mL^{-1}$)	*P. aeruginosa* NIH H. 170000	*E. coli* ATCC 25922	*S. saprophyticus* NIH H. 120008	*S. aureus* NIH H. 118003	*M. luteus* ATCC 9341	*B. subtilis* ATCC 6633
>200	23	13	–	–	–	1
200	–	7	–	–	–	–
100	–	2	–	2	2	1
50	–	–	2	3	1	2
25	–	1	5	1	6	3
12.5	–	–	8	11	3	11
6.25	–	–	4	3	11	3
3.125	–	–	4	2	–	2
1.56	–	–	–	1	–	–
	Gram-negative		Gram-positive			

Table VI. In vitro cytotoxicity of compounds **14–36** on HeLa cell line, expressed as TD_{50}.

No.	IC_{50}/TD_{50} (μg mL^{-1})	IC_{50}/TD_{50} (nM mL^{-1})
14	12.7	41.52
15	9.8	31.86
16	4.6	15.76
17	4.05	11.44
18	15	46.6
19	7	20.84
20	17.2	50.91
21	9.2	27.39
22	35.3	104.48
23	11.7	34.83
24	14.1	41.78
25	7.1	19.75
26	32	90.95
27	0.7	2.17
28	11.6	37.92
29	15.4	46.12
30	7.55	22.47
31	13.6	38.65
32	2.1	5.49
33	[a]	[a]
34	16.5	48.67
35	16.5	49.41
36	25	71.85

[a] Non-toxic/non measurable data.

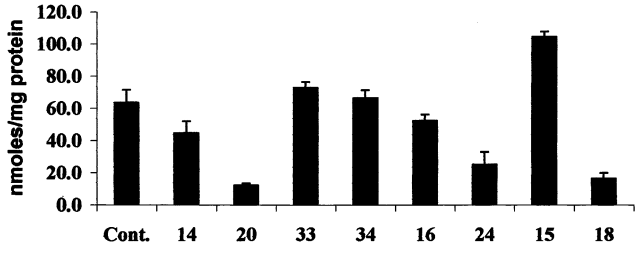

Figure 5. Data of free thiol measurements (Cont.: control).

increases the cytotoxicity. According to the possible mode of action—mentioned above—the Mannich ketones afford in deamination reaction vinyl ketones that react with the cellular thiols. The rate of deamination is inversely proportional to the basicity of the amine side chain [9]. Thus the pK_a values of morpholine, piperidine, pyrrolidine, 1,2,3,4-tetrahydroisoquinoline and 4-methyl-piperidine are 8.40 [23], 11.12 [23], 11.30 [23], 9.41 [24] and 10.78 [25]. The predicted rates of deamination of the five-membered Mannich ketones are **15**>**17**>**14**>**16**. The rates of deamination (see above) can be correlated with the cytotoxicity of these compounds. The order of potencies against the HeLa cells is **17**>**16**>**15**>**14** that do not show correlation with the expected order; while for the six-membered Mannich ketones the predicted rates of deamination are **27**>**29**>**28** that can be correlated with the cytotoxicity of these compounds: **27**>**28**>**29**.

4.3. Study of mechanism of action

All the compounds examined contained potential thiol alkylating groups. The Mannich bases are considered latent thiol alkylators because under biological conditions in 1,2 elimination they yield reactive vinyl ketones that can undergo addition reaction with thiols [9]. Our Mannich ketones possess two sites for alkylation (C=C and the latent position). Therefore, we examined the degree of thiol depletion caused by the selected group of compounds administered at the MIC concentration using the DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) method [26].

Our results (*figure 5*) revealed that these compounds can be divided into four classes. Those compounds (**33**, **34**) belong to first group, which had no toxicity and cause no thiol depletion. Most probably, they do not enter the cells. The second group comprises mildly toxic and mildly thiol depleting compounds (**14**, **16**, and **24**). In this group the depletion of thiols may certainly contribute to the toxicity. The third group (**20**, **18**) displayed strong alkylating effect. They decreased the intracellular thiol content down to 20% of the control value, hence they had no effect on the growth of the cells showing, that living cells have a huge capacity to protect the essential thiols even if the bulk thion, glutathion, is greatly depleted. Finally, among these compounds we also found a highly toxic one, which caused no thiol depletion (**15**). Interestingly the free thiol content was significantly elevated when cells were grown in the presence of **15**.

5. Conclusions

Some new unsaturated Mannich ketones of arylidenecycloalkanones were prepared by using the classical Mannich reaction. Their structure was proved by FT-IR and NMR spectroscopic methods. Generally in the preferred conformation there is a non-coplanar aryl ring to the enone C=C double bond. These results were corroborated by the X-ray diffraction study showing a crown-like conformation of the central eight-membered ring C1–C8 in **36**.

The antibacterial activity (MIC) of these compounds was examined both on Gram-positive and on Gram-negative strains and compared to the activity of standard antibiotics. All compounds were ineffective against *P. aeruginosa* (minimum inhibitory concentration: MIC: >200 μg mL^{-1}). Thirteen compounds were inactive and ten others were active (MIC: 25–200 μg mL^{-1}) against *E. coli* and were bactericidic. All the Gram-positive strains were sensitive to all the compounds (MIC: 1.56–200 μg mL^{-1}) and the compounds were bacteriostatic. All the compounds contained two alkylation sites a direct and a latent one. We observed that these two sites are not of equal importance regarding their biological action. This fact is proposed by the antibacterial activity of the **19**, **21** and **23**, the *para*-, *meta*-, and *ortho*-methoxy substituted compounds. In this group the MIC values are rather similar. In the latter one the *ortho*-methoxy group is very close to the β-carbon making it less accessible toward the attack of thiols. Since the antibacterial activities are very similar for these *p*-, *m*- and *o*-substituted compounds, therefore, the direct alkylating activity of these compounds does not correlate with the antibacterial activity. This suggests a secondary importance for the direct alkylating site in the toxicity.

The studies on HeLa cells demonstrated that some highly active antibacterial agents (**14**, **18**), displayed low cytotoxicity. The latter fact could be important as a base for a possible drug development.

All these compounds are potential alkylating reagents; therefore their supposed mechanism of action can be a depletion of free thiols. We examined this issue by determining the free thiol content of the cells grown in the presence of minimum inhibitory concentration of these compounds. Some toxic compounds depleted the cellular free thiol content, but other non-toxic compounds decreased the free thiol even stronger, showing that the thiol depletion cannot be the only reason for the toxicity. The fact, that a compound from the same family displayed high toxicity, without thiol depletion gave further support to the idea, that these compounds have other biological effects besides alkylation.

6. Experimental protocols

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 unsaturated ketones (**1**–**4**, **6** and **8**–**13**) are known compounds synthesised according to literature methods [27–34]. Thin-layer chromatography (TLC) was performed on Merck silica gel plates (60 F_{254}), and as an eluent ethylacetate–benzene (10:1 v/v) was applied. Melting points were determined in a Boetius apparatus and are uncorrected. The analytical values were within ±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, in 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 an internal standard. The standard Bruker microprogram NOEMULT.AU to generate NOE [35] was used with a selective pre-irradiation time. DEPT spectra [36] were run in a standard manner [37], using only the $\Theta = 135°$ pulse to separate CH/CH_3 and CH_2 lines phased 'up' and 'down', respectively. The 2D-COSY, [38, 40], 2D-HSC (HMQC) [39, 41] and 2D-COLOC (HMBC) spectra [42, 43] were obtained by using the standard Bruker pulse programs COSYGSSW, HXCO.AU (INV4GSSW) and HXXCO.AU (INV4GSLRNDSW), respectively.

FT-IR spectra were taken in a Nicolet Impact 400 spectrophotometer in KBr pellets.

6.1.1. General method for the preparation of 2-arylidenecyclopentanones (**5** and **7**)

The appropriate aldehyde (0.08 mmol) was added dropwise to the mixture of cyclopentanone (0.4 mmol) and 1000 mL aqueous sodium hydroxide (0.4%) under stirring. After the addition was completed the reaction mixture was stirred for an additional 4 h. Its pH was adjusted to 7 using 10% acetic acid. The yellow oily product was extracted with toluene and washed with distilled water until neutral. The toluene solution was dried over anhydrous magnesium sulphate for 12 h. The solvent and the excess of cyclopentanone were evaporated in vacuo and the residue was recrystallised from hexane.

6.1.2. General procedure for the preparation of Mannich ketones (**14**–**36**)

The mixture of the appropriate unsaturated ketone (20 mmol), paraformadehyde (40 mmol) and the secondary amine (20 mmol) in the presence of concentrated hydrochloric acid (0.3 mL) was refluxed in ethanol (50 mL). The time of heating is indicated in *table 1*. The reaction

was monitored by TLC. The hot reaction mixture was filtered and the solvent was evaporated. The residue was treated with dry acetone yielding crystalline material. The products were recrystallised from dry acetone–methanol mixture. The following purification method gave pure compounds. To the cold (0 °C) methanolic solution (100 mL) of Mannich ketones as hydrochlorides (10 mmol) an aqueous solution (50 mL) of anhydrous sodium carbonate (5 mmol) was added dropwise at 0 °C (ice bath) under magnetic stirring. Thereafter, ice and sodium chloride (20 g) were added to the solution. The oily Mannich ketones were extracted with cold chloroform (3×100 mL). The chloroform phase was washed with cold brine (4×50 mL) and was dried over anhydrous magnesium sulphate in refrigerator for 1 h. After filtration the solvent was removed in vacuo using a bath temperature of 15–20 °C. The oily residue was dissolved in dry acetone and cooled to 0 °C then treated dropwise with cold methanolic hydrochloric acid (4 mL, 2 N). The mixture was refrigerated and the crystals separated were filtered and dried. This procedure was repeated if it was necessary. The NMR measurements were done on the Mannich bases.

6.1.3. X-ray structure determination of **36**

$C_{21}H_{30}ClNO$, $M_r = 347.91$, colourless crystal of size $0.25 \times 0.30 \times 0.40$ mm, triclinic, space group $P\bar{1}$ (No. 2 of IT [44]). Lattice parameters are: $a = 7.099(1)$ Å, $b = 10.065(2)$ Å, $c = 13.901(3)$ Å, $\alpha = 99.14(3)°$, $\beta = 101.23(3)°$, $\gamma = 97.77(3)°$ and $V = 947.6(3)$ Å^3 (determined from ~ 1000 reflections with $3 < \theta < 33.5°$), $Z = 2$, $D_{calc} = 1.219$ g cm^{-3} and μ_x (synchrotron radiation, $\gamma = 0.71$ Å) $= 2.09$ cm^{-1}. Intensity data were collected with a 2D-CCD detector (SMART 1K, Bruker) using synchrotron radiation from the bending magnet of station F1 at HASYLAB/Hamburg and a Si (1 1 1) monochromator at $T = 120$ K. Data collection was carried out in the range $-10 \le h \le 10$; $-15 \le k \le 13$; $-20 \le l \le 20$ with $(\sin(\Theta)/\lambda)_{max} = 0.755$. The total number of reflections measured is 19 052, from which 5905 are unique ($R_{merged} = 3.0\%$, completeness: 86.6%). Intensity data were integrated, reduced and scaled using SAINT V5 [45]. A total of 5466 reflections have $I(h) > 2\sigma(I(h))$ and were retained for further analysis. The structure was solved by direct methods (SHELXS [46]). All non-hydrogen atoms were refined anisotropically using full-matrix least-squares [47] based on $|F|^2$ with weights $1/(\sigma^2(F_o^2) + (0.0555P)^2 + 0.2864P)$ using $P = (F_o^2 + 2F_c^2)/3$. Hydrogen atoms H1N1 and H2a were refined isotropically. The positions of all other H-atoms were calculated with an appropriate C–H-distance and a hydrogen displacement parameter of 1.2 times that of the corresponding C-atom. The shifts $(\Delta/\sigma)_{max}$ of the final least-square cycle were smaller than 0.001. R-values are $R_w(|F|^2) = 0.102$ and $R(|F|) = 0.039$ for 225 refined parameters. Goodness-of-fit is 1.041. The final difference Fourier map is featureless ($\Delta\rho_{min} = -0.28$; $\Delta\rho_{max} = 0.48$ e Å^{-3}).

6.2. Biology

6.2.1. Determination of minimum inhibitory concentration and minimum bactericidic concentration

MIC values were determined by test tube dilution method. The test strains were: *P. aeruginosa* NIH Hungary 170000, *E. coli* ATCC 25922, *S. saprophyticus* NIH Hungary 120008, *S. aureus* NIH Hungary 118003, *M. luteus* ATCC 9341 and *B. subtilis* ATCC 6633. The compounds investigated were dissolved in nutrient broth at a concentration of 200 μg mL^{-1}. Double dilution series of compounds were made in nutrient broth. A nutrient broth starter culture (2 μL) of test bacterial strains was added to each tube to achieve a final inoculum of ca. 5×10^5 colony forming units per millilitre. Control tubes without compounds were used to check the inoculums. The cultures were incubated for 24 h at 37 °C. The MIC values were determined from the lowest concentration of compounds where the tubes remained clear, showing that the bacterial growth was inhibited. Loopfuls (10 μL) of nutrient broth cultures from each tube were plated on nutrient agar to check the bacterial growth. MBC value was determined for the lowest concentration of compound under investigation where we could not detect any living bacterium. All experiments were performed in triplicate [48].

6.2.2. In vitro anticellular activity of Mannich ketones against 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 foetal calf serum low IgG was obtained from Gibco BRL™—Life Technology Ltd (UK). Streptomycin and penicillin were obtained from Richter Ltd (Budapest, Hungary).

6.2.2.1. Cellmicroculturing

In vitro cultured HeLa cell line (Flow-Labs. Ltd, UK) was used. The cells were cultured in RPMI-1640 culture

medium containing 10% heat-inactivated foetal calf serum with antibiotics streptomycin (25 μg mL^{-1}) and penicillin (50 μg mL^{-1}) in 5% CO_2 humidified atmosphere at 37 °C (tissue culture incubator, Forma Scientific, USA). The cells growing in the logarithmic phase, had viability better than 98% as proved by Trypan blue exclusion.

6.2.2.2. Modified tetrazolium assay

The cells growing in the logarithmic phase, showed viability better than 98% as proved by Trypan blue exclusion. Cells were plated out in 100 μL of medium at concentration of $4–5\times10^3$ cells per flat-bottomed well in 96-wells microtiter plates (Costar Co. Cambridge, MA, USA). Plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Hundred microlitres of media containing the drug dissolved in appropriate solvent were added to each well and incubated for further 48 h. Hundred microlitres of medium were then removed from the wells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was prepared as a 5 mg mL^{-1} stock in 0.15 M phosphate-buffer saline (PBS, pH adjusted to 7.2 with NaOH) containing in 5.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl and 2.7 mM KCl. This stock solution can be stored for no more than 2 weeks in dark at 4 °C. Before use it was filtered through a 0.22 μm filter to remove any blue formazan products 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) was 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 the free thiol content

Cells grown in shaker incubator (37 °C) to the mid log phase were 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 collected by centrifugation (+4 °C), washed with 150 mM NaCl solution and stored at −80 °C as a 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 [26]. The protein concentration was determined by the BCA (bicinchoninic acid) assay [49]. The assay was repeated using eight selected compounds in three parallel cultures. Error bar means e.s.d. of three measurements.

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