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Short communication

2-Substituted indazoles. Synthesis and antimicrobial activity

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Abstract – 2-Isothiocarbamoyl substituted fused pyrazolines and their S-alkyl derivatives were prepared as potentially antimicrobial agents. Conventional methods were used to synthesize the novel derivatives starting from cyclic unsaturated ketones and thiosemicarbazide under acidic catalyst. These cyclizations yielded only one diastereoisomer of 3-H, 3a-H *cis*. The alkylations were performed applying alkyl halides. The structures of the new compounds, including configurations and conformations, were elucidated by NMR spectroscopy, also making use of 2D-HSC, DEPT and DNOE measurements. The *S*-alkyl derivatives were evaluated for activity against Gram-negative and Gram-positive bacteria and their in vitro toxicity was determined on HeLa cells. The structure-activity relationship was also studied. © 1999 Éditions scientifiques et médicales Elsevier SAS

indazole / alkylation / stereostructure by NMR / antibacterial effect / in vitro toxicity

1. Introduction

Some thiosemicarbazides are known antibacterial compounds, like thiosemicarbazones of 5-nitrofurfurylideneacetone [1] and dodecanone [2]. The 1-methylindole-2,3-dione 3-thiosemicarbazone, called Metisazone, was used as an antiviral agent [3]. Because of their relatively high toxicity these agents are not widely used drugs. Similarly, some members of the family of the fused pyrazolines and pyrazoles show antimicrobial effects [4–6]. This prompted us to find an effective antimicrobial agent of relatively low toxicity having a thioamide moiety attached to a pyrazoline ring. Recently we have published the synthesis of several bi- and tricyclic pyrazolines starting from unsaturated ketones and hydrazine derivatives [7–9]. Some of these compounds can be considered as cyclic thiosemicarbazide derivatives too. Our aim was to prepare water-soluble thiosemicarbazide derivatives for microbiological investigations. Therefore, 3,5-diarylidene-1-methyl-4-piperidones were also used as starting ketones.

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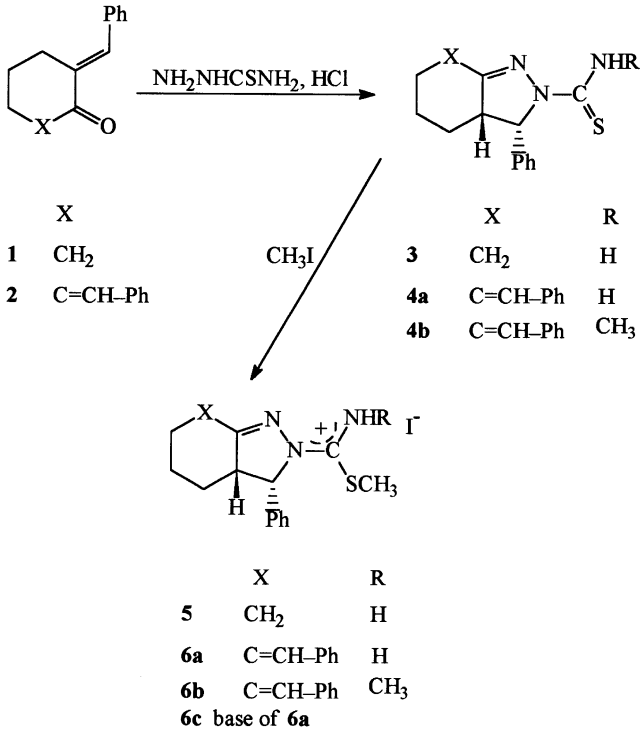
2. Chemistry

In order to obtain potentially antibacterial compounds starting from 2,6-dibenzylidenecyclohexanone, 3,5-diarylidene-1-methyl-4-piperidones or 2-arylidene-1-tetralones and semicarbazide or thiosemicarbazides, ten new bi- and tricyclic pyrazolines (**4b, 8a–d** and **11b–f**) have been prepared (*figures 1–3*). The cyclizations performed with thiosemicarbazides under acidic conditions yielded only one diastereoisomer of 3-H, 3a-H *cis*. While the reactions with semicarbazide afforded the mixture of the 3-H, 3a-H *cis* and *trans* diastereoisomers, which have been separated. The structure and the relative configuration of the compounds have been determined by ^{1}H-NMR and ^{13}C-NMR spectroscopic methods.

To increase the water solubility of the 2-isothiocarbamoyl pyrazolines they have been alkylated with alkyl halides. These reactions gave the corresponding *S*-alkyl derivatives (**5, 6a–b, 9a–d** and **12a–g**) depicted in *figures 1–3*. From **6a** hydroiodide, the free base **6c** was liberated to provide a compound of better solubility for NMR study. At the alkylation of **8c–d** pyrazolo[4,3-*c*]pyridines the corresponding hydrochloride was used to avoid the quaternarization of the pyridine ring. On the



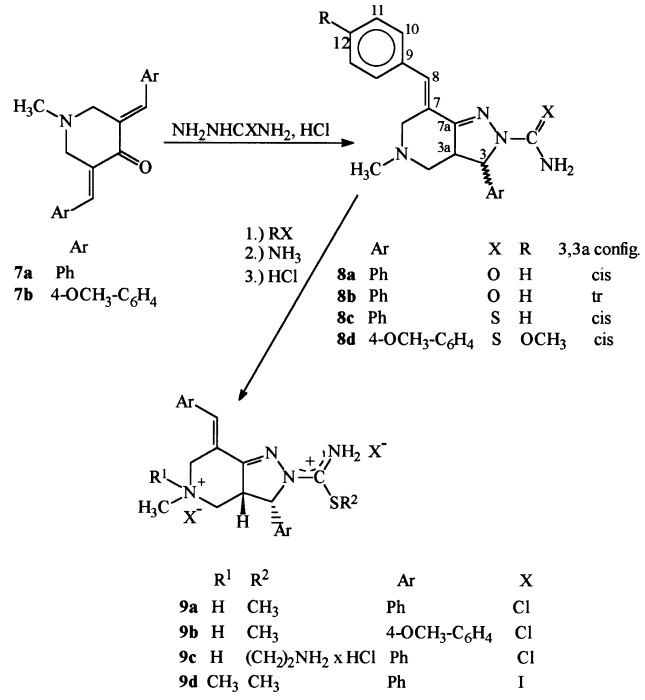
	X	
1	CH_2	
2	C=CH–Ph	

	X	R
3	CH_2	H
4a	C=CH–Ph	H
4b	C=CH–Ph	CH_3

	X	R
5	CH_2	H
6a	C=CH–Ph	H
6b	C=CH–Ph	CH_3

6c base of **6a**

Figure 1. Synthesis of substituted hexahydroindazoles.



	Ar
7a	Ph
7b	4-OCH_3-C_6H_4

	Ar	X	R	3,3a config.
8a	Ph	O	H	cis
8b	Ph	O	H	tr
8c	Ph	S	H	cis
8d	4-OCH_3-C_6H_4	S	OCH_3	cis

	R^1	R^2	Ar	X
9a	H	CH_3	Ph	Cl
9b	H	CH_3	4-OCH_3-C_6H_4	Cl
9c	H	$(CH_2)_2NH_2$ x HCl	Ph	Cl
9d	CH_3	CH_3	Ph	I

Figure 2. Synthesis of hexahydropyrazolo[4,3-c]pyridines.

contrary, allylic halides, like allyl bromide and benzyl chloride, yielded the **13** and **14** *N*-alkyl derivatives (*figure 4*). *N*-alkylation was observed at thioamides with alkylating agents favouring the carbonium ion formation [10]. The structures of the *N*-alkylated and *S*-alkylated derivatives were proven by their ^{1}H- and ^{13}C-NMR spectra. Two of the *S*-alkyl derivatives (**6a** and **9a**) were prepared via an alternative route using *S*-methylthiosemicarbazide hydroiodide and the corresponding α,β-unsaturated ketone. The products of this one-step synthesis were identical in every respect with those prepared in a two-step method. The physical data of the novel compounds are displayed in *table I*.

The ^{1}H- and ^{13}C-NMR data on the new compounds are listed in *tables II* and *III*. As the spectra inequivocally prove the expected constitutions, the following discussion is focused on the steric aspects of the structures, on the configurations and conformations.

Two problems have to be considered: the *cis* or *trans* configuration of the H-3, H-3a hydrogens and the hindered rotation of the carbamide or thiocarbamide moiety.

Concerning the first problem, the spectral data of the *cis-trans* pair **8a** and **b** can serve as a starting point. The vicinal H-3, H-3a coupling is 9.3 and 11.5 Hz for the isomers. While the values are in accordance with the expected ratio $^3J_{cis} > {}^3J_{trans}$ [11], the difference is far too small for a firm differentiation of the *cis* or *trans* configurations in the case of single compounds without their counterparts. Moreover, because of broadened signals, it was not possible to determine the value of this coupling constant for all compounds and in most cases the splitting was between the two values (about 10–11 Hz) measured for the pair **8a** and **b**. On the contrary, the ^{13}C–NMR field effect [12, 13] arising in the *cis* isomers is a firm base to identify the C-3,3a configurations.

The C-3a chemical shifts for the *cis* and *trans* isomers **8a** and **b** are 48.4 and 55.4 ppm. The significant upfield shift (8.2 ppm) for C-3a in **8a** and the similar δC-3a values (48.4–51.0 ppm) measured for the other compounds proved their *cis*-configuration unambiguously.

Signal splitting or broadening in the ^{1}H- and ^{13}C-NMR of most of the salts investigated refers to the equilibrium of rotamers or other differences in the structures. Because of the absence of these phenomena in the spectra of bases, the origin can also be found in different protonation sites: besides exocyclic, the basic cyclic sp^2-N can also be protonated. The double splitting of H-8 and C-7a signals for **12**-type salts supports this latter assumption, while splitting or broadening of H-3 and C-3 signals can be

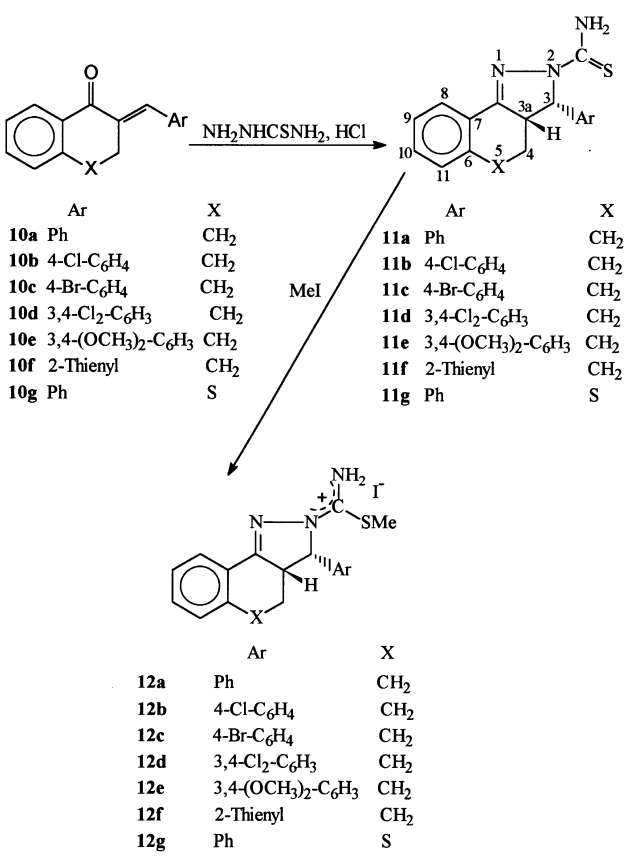


Figure 3. Synthesis of tricyclic pyrazolines.

interpreted by hindered rotation only. Probably, the above facts have to arise from hindered rotation and/or site isomers of protonated molecules, depending on the structure as well as medium effects (solvent, concentration, temperature, pH, etc.).

Two further aspects of stereostructures should be considered: the geometrical isomerism of the benzylidene moiety and the conformation of the partly saturated condensed ring. As regards the geometrical isomers, the *Z*-isomer (*S-cis* Ar and N-1) is to be precluded due to unfavourable steric structure. The *S-cis* (to N-1) position of the hydrogen in the exocyclic =CH- group reveals a high downfield shift of its ^{1}H-NMR signal (this appears to overlap with the multiplets of the phenyl hydrogens) due to anisotropic deshielding of the non-bonded electron pair on N-1 [14].

The condensed, partly saturated six-membered ring can exist in two preferred conformations. These chair- and boat-like forms with out-of-plane C-5 and C-7a atoms are both distorted approaching the sofa-

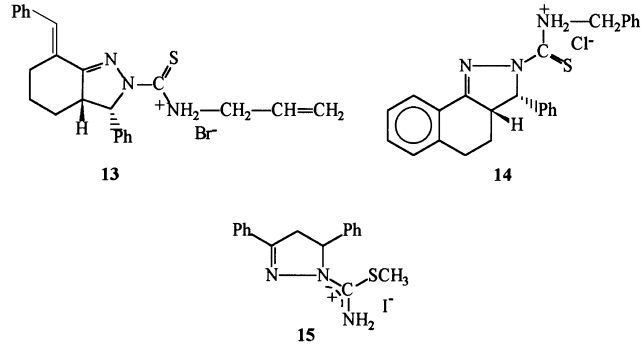


Figure 4. *N*-alkyl derivatives **13**, **14** and monocyclic pyrazole derivative **15**.

arrangement with coplanar C-7a. It is to be noted that the inversion of the six-membered ring hardly influences the envelope form of the pyrazoline ring (with an out-of-plane C-3 atom) bearing the Ar group in quasi-equatorial position, cf. Ref [7]. Nevertheless, the quartet-like signal of H-4*ax* (for **4b**, **5**, **6a–c**, **11c**, **13** and **14**) refers to three large H,H-splittings, one of which is due to geminal coupling [15] 4*ax*,4*eq* and the two others to 3a,4*ax* and 4*ax*,5*ax* diaxial vicinal interactions [16, 17] which can be present only in the chair-form. This conformation is also preferred in the case of **8a** and **c**, but the H-4*ax* signal shows a triplet splitting in the absence of H-5*ax* here. Due to signal-overlap, it was not possible to identify the multiplicity of the H-4*ax* signal for **9a** and **d**, but in the TFA-*d* solution of **9a** it is separated with triplet-like multiplicity and the two large splits are proof of the same conformation. In the ^{1}H-NMR spectra of **12a**,**c** and **g** the signals are broadened and consequently, it was not possible to identify the multiplicities. It is conceivable that the signal-broadening is the consequence of a slow ring inversion combined with the hindered rotation of the protonated thiocarbamide group. That is, the conformationally homogeneous quasi-rigid system of the other compounds is substituted by a flexible equilibrium of ring isomers for **9**-type derivatives. Again, this equilibrium can be influenced in **12g** by the incorporated sulphur in position 5.

3. Biology

Antibacterial activity tests of the synthesized compounds were carried out on twenty cultures of *Staphylococcus aureus* and *Escherichia coli* strains isolated from different clinical samples (see Experimental protocols). Eight of the most effective antibacterial compounds (**5**,

Table I. Physical data of compounds **4b**, **5**, **6a–c**, **8a–d**, **9a–d**, **11b–f**, **12a–g** and **13–15**.

Compound	General formula[a]	M.p. (°C)	Yield (%)	Method
4b	$C_{22}H_{23}N_3S$	155 (dec., methanol)	62	–
5	$C_{15}H_{19}N_3S\times HI$	172 (dec., acetone)	58	–
6a	$C_{22}H_{23}N_3S\times HI$	180–183 (acetone)	79	A, C
6b	$C_{23}H_{25}N_3S\times HI$	242 (dec., acetone)	47[b]	A
6c	$C_{22}H_{23}N_3S$	160 (dec., methanol)	92	–
8a[c]	$C_{21}H_{22}N_4O$	163–165 (methanol)	33	–
8b[c]	$C_{21}H_{22}N_4O$	130 (dec., methanol)	6	–
8c	$C_{21}H_{22}N_4S$	174–177 (methanol)	70	–
8c × HCl	$C_{21}H_{22}N_4S\times HCl$	213–216 (methanol)	60	–
8d	$C_{23}H_{26}N_4O_2S$	181 (dec., methanol)	39	–
8d × HCl	$C_{23}H_{26}N_4O_2S\times HCl$	189 (dec., methanol)	42	–
9a	$C_{22}H_{24}N_4S$ 2×HCl	200–204 (ethanol)	47	B, C
9b	$C_{24}H_{28}N_4O_2S$ 2×HCl	195 (dec., ethanol)	39	B
9c	$C_{23}H_{27}N_5S$ 3×HCl	114 (dec., ethanol)	38	B
9d	$C_{23}H_{27}IN_4S\times HI$	198–202 (ethanol)	42	B
11b	$C_{18}H_{16}ClN_3S$	225–227 (methanol)	90	–
11c	$C_{18}H_{16}BrN_3S$	232–233 (methanol)	93	–
11d	$C_{18}H_{15}Cl_2N_3S$	118 (dec., methanol)	74	–
11e	$C_{20}H_{21}N_3O_2S$	130–132 (methanol)	40	–
11f	$C_{16}H_{15}N_3S_2$	190 (dec., methanol)	80	–
12a	$C_{19}H_{19}N_3S\times HI$	158–162 (acetone)	65	A
12b	$C_{19}H_{18}ClN_3S\times HI$	168–171 (acetone)	41	A
12c	$C_{19}H_{18}BrN_3S\times HI$	160 (dec., acetone)	43	A
12d	$C_{19}H_{17}Cl_2N_3S\times HI$	170–173 (acetone)	65	A
12e	$C_{21}H_{23}N_3O_2S\times HI$	158–160 (acetone)	69	A
12f	$C_{17}H_{17}N_3S_2\times HI$	140–143 (acetone)	71	A
12g	$C_{18}H_{17}N_3S_2\times HI$	183 (dec., acetone)	36	A
13	$C_{24}H_{25}N_3S\times HBr$	176–180 (acetone)	75	A
14	$C_{25}H_{23}N_3S\times HCl$	192–195 (acetone)	49	A
15	$C_{17}H_{17}N_3S\times HI$	116 (dec., acetone)	25	A

[a]The analytical values were within ± 0.4% of the theoretical values for C, H and N. [b]36% of **4b** recovered. [c]IR (KBr) 1 685 cm^{-1}.

6a, **9a**, **9d** and **12a–d**) were examined for determination of their minimum inhibitory concentration (MIC) values by the test tube dilution method (see Experimental protocols). In these experiments we used standard reference strains. The MIC values of these five standard strains were tested on eight well-known antibiotics to compare our compounds with antibiotics used in therapy as well. In addition, the MIC values of compound **9a** were investigated on five *E. coli* strains cultivated from urine. In vitro cytotoxicity tests of the compounds on HeLa cell-line OHIO, was carried out on microplates (see Experimental protocols).

4. Results and discussion

Our aim was to study the structure-biological activity relationship for these fused pyrazolines by varying the type and size of the ring system, the aromatic substituent at position 3 and the substituents on the isothiocarbamoyl group.

4.1. Antibacterial results

Our work provided us with information about the structure-antimicrobial activity relationship connected to the class of fused pyrazolines (*table IV*). Opposed to the Metisazone they are ineffective without an *S*-alkyl substituent, like **8c.** An arylidene substituent or a third (aromatic) ring is needed for effectiveness (see **6a** and **12a** versus **5**), in addition, without fused ring the pyrazolines are ineffective, like **15**. Replacement of the 5-CH_2 group with sulfur in the benz[*g*]indazole series (**12g**) removed the antimicrobial effect. This can be explained in part by the different conformation of compounds **12a** and **12g** and the different electron structure. As for the

Table II. [1]H-NMR data (chemical shifts, in ppm, δ_{TMS} = 0 ppm, and coupling constants in Hz) of compounds **4b**, **5**, **6a–c**, **8a–d**, **9a, d, 11b–f**, **12a–g** and **13**–**15** in $CDCl_3$ or DMSO-d_6 solution[a] at 250 MHz[b].

Compound[c]	H-3 *d* (1H)[d]	H-3a *m* (1H)[e]	NMe *s* (3H)[f]	SMe *s* (3H)	NH 1 or 2 *s* (2H)[g,h]
4b	6.08	3.45	3.18	–	–[i]
5	5.75	3.75	–	2.57	8.9 9.5
6a	5.87	≈ 3.9[g]	–	2.65	9.2 9.7
6b	6.15	3.85	3.25	2.45	9.2
6c	5.70	3.45	–	2.28	6.5
8a	5.60	3.70	2.25	–	5.65
8b	4.82	3.05[k]	2.25	–	6.5
8c	6.09	3.77	2.25	–	6.2 7.1
8d	6.02	3.74	2.27	–	–[i]
9a	6.07	4.70	2.70	2.76	10.05 12.4
9d	6.12	≈ 4.7[k]	3.15	2.66[l]	9.5 10.0
11b	6.10	3.67	–	–	6.3 7.1
11c	6.09	3.70	–	–	6.2 7.1
11d	6.08	≈ 3.7[g]	–	–	6.3 7.1
11e	5.93	3.73	–	–	7.75 7.95
11f	6.26	3.77	–	–	7.74 8.02
12a	5.95	4.18	–	2.67	9.2 9.7
12b	5.96[g]	4.13	–	2.63	9.15 9.75
12c	6.02	4.18	–	2.67	9.2 9.7
12d	≈ 6.0[g]	4.14	–	2.64	9.2 9.8
12e	5.86	4.07	–	2.63	9.10 9.65
12f	6.27	4.10	–	2.64	9.25 9.70
12g	≈ 6.0[g]	4.50	–	2.64	9.5 10.0
13	6.05	3.95	4.00	–	8.35 8.9
14	5.75	3.98	4.42	–	–[i]
15	5.95	–	–	2.67	9.15 9.75

[a]Solvent was $CDCl_3$ for **4b**, **6c**, **8a–d** and **11b–d**, TFA-*d* for **14**. Compounds **6b** and **13** were measured in $CDCl_3$ and DMSO-d_6 (**13** in DMSO-d_6 at 100 °C), **9a** in DMSO-d_6 and TFA-*d*. [b]Measuring frequency was 500 MHz for **8d**, **11b** and **d–f**, **12b** and **d–g** and **15.** Further signals: CH_2 (position 4–7), 2–8 signal (6H for **4b**, **6a–c**, **13**, 8H for **5**, 2H for **12g** and **15** and 4H for all other compounds): 0.4–4.7, OCH_3, *s* (3H): 3.78 and 3.82 (**8d**), 3.67 (6H, **11e**), 3.69 and 3.71 (**12e**), ArH + C(sp^2)H, 1–6 m's [11H or 5H (**5**), 8H (**11c** and **12c**), 9H (**12a** and **g**), 10H (**15**) and 14H (**14**)]: 6.8–8.1, allylic group (**13**), C(sp^3)H_2: 4.10, ≈ *d* and 4.25, ≈ *dqa*, C(sp^2)H_2: 5.20, *d* and 5.30, *d*, C(sp^2)H: 5.65 br and 5.90 br (doublet signals due to rotamers). [c]Assignments were proved by DR- (**8a**), 2D-COSY (**8d**, **11f**) and DNOE-measurements (**11c**). A sample containing ca. 5% of *cis*-isomer was measured for compound **14,** δH-3: 5.90 for the minor isomer. [d]*J*: 11.0 (**4b**, **6a** and **8c**), 9.4 (**6b** in DMSO-d_6, **8a**), ≈ 10 (**6c**, **9a** and **d**, **13** and **14**), 11.5 (**8b**), 10.7 (**8d**, **11b** and **d**), 8.3 (**11c**), 10.5 (**11e**), 10.2 (**11f**), broadened signal (**5**, **12a**,**c** and **g** and **15**). Doubled signals due to rotamers with the second *d* at 6.05 (**6a** and **12b**), 6.62 (**9a**), 6.25 (**9d**), 6.1 (**12a**), 6.00 (**12e**) and 6.35 (**12f**).[e]*dt* (**5**, **6c**, **8a** and **c**, **12a**, **c** and **g**), *ddd* (**11c**, **13** and **14**), broadened (**6a**). [f]*d* for **4b** (*J*: 4.8), **9a** (*J*: 8.5) and **13** (*J*: ≈ 7), NCH_2 (intensity 2H) for **13** and **14** (*N*-allyl and *N*-benzyl group), two singlets (2×3H) for **9d** with the second signal at 3.19. [g]Broad signals. [h]Intensity: 1H (**5** and **6a–c** and for the signal at 12.4 in **9a**). The split to two signals due to rotamers for **5**, **6a**, **8c**, **9a** and **d**, **11b–f**, **12a–g**, **13** and **15**. The intensity ratio is ca. 1:1, for **5**, **6a**, **9d**, **12a** and **d** and **15** ca. 2:1 for **12e** and **f** ca. 5:2. [i]Not identifiable. [k]In overlap with the CH_2-signals. [l]Doubled signal with the second singlet at 2.67.

3-aryl group, the best effect has been shown with the 4′-halogen substituted derivatives (**12b–d**). With respect to the *S*-alkyl group, the replacement of the nonpolar methyl group removed the antibacterial effect as in the case of **9c**. This effect requires an *N*-unsubstituted thioamide moiety (see **6b**). **9a** proved to be the only effective compound against both Gram-negative and Gram-positive strains. Its quaternarization decreased its effect both against the Gram-negative and Gram-positive strains.

4.2. Conclusion

We have synthesized a series of 2-isothiocarbamoyl substituted bi- and tricyclic pyrazolines and their *S*-alkyl derivatives as potent antibacterial compounds. For this class of compounds a certain substitution pattern is necessary for the optimal antibacterial effect. Thus the 7-benzylidene-3,3a,4,5,6,7-hexahydro-5-methyl-2-*S*-methylthiuronyl-3-phenyl-2*H*-pyrazolo[4,3-*c*]pyridine dihydrochloride (**9a**) is an acceptably potent compound

Table III. ^{13}C-NMR chemical shifts (δ_{TMS}= 0 ppm) of compounds **4b**, **5**, **6a–c**, **8a–d**, **11b–f, 12a–g** and **15** in $CDCl_3$ or DMSO-d_6 solution[a] at 63 or 126 MHz[b].

Compound	C–3	C–3a	C–4	C–5	C–6	C–7	C–7a	C–8	C(sp^2)X[c]	XMe[d]
4b	67.7	49.7	28.2	23.6	25.5	137.3	159.0	127.3[e]	176.4	31.1
5	65.7[f]	50.7	27.1[e]	22.9	24.7	27.6[e]	≈ 164[f]	–	≈ 171[f]	14.5[f]
6a	66.7[g]	51.0	27.8	22.7	25.0	135.0	163.7	126.2	165.6[g]	14.2[g]
6b	67.5	50.6	27.8	22.9	24.7[e]	135.1	159.0	127.2[e]	176.1	25.4[e]
6c	66.2	49.9	28.0	23.7	25.5	136.5	155.4[e]	127.2[e]	159.9[e]	12.8
8a	62.6	48.4	55.7[e]	–	54.2[e]	135.2	153.2	127.7	154.3	45.3
8b	66.5	55.4	56.6[e]	–	56.0[e]	142.9	157.2	129.6[i]	152.8	45.1
8c	66.5	48.8	55.8[e]	–	54.3[e]	135.0	158.0	127.7	176.0	45.5
8d	66.3	48.9	54.3	–	56.0	?[f]	159.0	129.7	175.9	45.5
11b	66.6	49.2	29.3	24.3	140.3	126.5	157.6	126.9	176.1	–
11c	66.4	48.9	29.1	24.1	140.1	126.3	157.5	126.7	175.8	–
11d	66.1	49.1	29.2	24.3	140.2	126.3	157.6	126.9	176.1	–
11e	67.2	49.1	29.4	24.7	141.1	127.7	157.1	125.7	176.6	–
11f	63.7	49.3	29.3	24.0	141.2	?[f]	157.0	125.8	176.6	–
12a	66.5[g]	49.8	28.3	23.8	141.4	126.0	163.0[g]	127.0[i]	165.0	14.5[g]
12b	66.5[g]	50.6	29.2	24.7	142.4	126.8	163.3	127.8	164.8	15.5[g]
12c	66.1[f]	49.7	28.3	23.9	141.4	125.9	163.2[g]	127.0	≈ 165[f]	14.7[f]
12d	66.3	50.6	29.2	24.7	142.3	126.5	≈ 164[f]	127.8	≈ 165[f]	15.4
12e	67.6[g]	50.6	29.2	24.6	142.4	126.8	?[f]	126.1	164.4[g]	14.8
12f	64.2	50.7	29.1	23.9	142.4	126.0	≈ 164[f]	127.7	≈ 165[f,g]	15.5
12g[k]	67.3	49.5	26.5	–	137.8	123.7	159.4	126.4[e]	164.8	14.6
15	63.6	44.5	–	–	–	–	162.5	–	165.1	14.6

[a]Solvent was $CDCl_3$ for **4b**, **6b** and **c**, **8a**,**c** and **d** and **11b–d** and DMSO-d_6 for **5**, **6a**, **8b**, **11e** and **f**, **12a–g** and **15.** Compounds **8d**, **11b** and **d–f**, **12b** and **d–f** and **15** were measured at 126 MHz. Assignments were supported by DEPT (for **8d**, **11b** and **d–f**, and **12b** and **d–g**) and 2D-HSC (for **8d** and **11e** and **f**) measurements. [b]Futher lines, OMe: 55.2 and 55.3 (**8d**), 56.3 and 56.4 (**11e**), 56.4 and 56.6 (**12e**). Aromatic carbons (because of poor quality of the ^{13}C-NMR spectra due to hindered rotation and bad solubility it was not possible to measure exact chemical shifts for some broad and weak lines of these carbons in the cases of **6a** and **b**, **12b** and **d–f** and **15**), 3-phenyl/aryl or in **11f** and **12f** 3-(2-thienyl) and the conjugated phenyl/aryl groups in the side chain and in position 3 (for **15**): C-1´: 135.0–137.3 (non-conjugated rings), 126–130 (cr: conjugated rings), 141.8 (**11f**), C-2´,6´: 125.0–130.8, C-3´,4´,5´: 126.0–130.0, except for the following cases: **8d**: C-3´,5´: 114.1, C-2´,6´: 131.7 (cr), C-4´: 159.0, 159.7 (cr), **11b** and **12b**: C-4´: 133.5, **11c** and **12c**: C-3´,5´: 131.7, 132.6, C-4´: 121.4, 121.9, **11d** and **12d**: C-3´: 132.9, 132.2, C-4´: 131.8, C-5´: 130.7, **11e** and **12e**: C-1´: 131.2, C-2´: 111.0, C-3´: 149.6, C-4´: 148.7, 149.7, C-5´: 112.6, 113.0, C-6´: 118.4, **11f**: C-3´,5´: 125.2, 125.5. Condensed benzene ring in **11b–f** and **12a–g** (the numbering is given in the figure), C-9: 125.1–126.6; C-10: 131.2–134.1; C-11: 129.0–130.4. [c]Thiocarbamide (X = S) for **4b**, **5**, **6a–c**, **8c** and **d, 11b–f**, **12a–g** and **15**, carbamide (X = O) for **8a** and **b**. [d]X = N for **4b, 6b** and **8a–d,** X = S for **5, 6a** and **c, 12a–g** and **15**. [e]Interchangeable assignments (with an aromatic line for C-8 in **4b, 6b** and **c** and **12g**). [f]Broadened signal due to hindered rotation of the *N*-acyl group. Broadening was also observed for some aromatic carbon lines in **12b, d** and **f** and **15**. [g]Split signals due to hindered rotation with the second line at 67.4 (C-3), 166.7 (Csp^{2}S) and 14.8 (SMe) for **6a**, 66.9 (C-3), 163.4 (C-7a) and 14.8 (SMe) for **12a,** 67.0 (C-3) and 14.9 (SMe) for **12b**, 163.9 (C-7a) for **12c,** 67.0 (C-3) and 164.9 (= CS) for **12e** and ∼ 167[f] for **12f**. [i]Two overlapping lines, one originates from an aromatic carbon. [k]Measurement temperature was 70 °C.

inhibiting both Gram-positive and Gram-negative bacteria. **12c** was the best agent against the Gram-positive strains (cf. *table V*; MIC values 12.5–25 μg/mL.) Either a fused aromatic ring or a planar arylidene substituent is also crucial. It is very important that the thioamide moiety can not be substituted. In comparison with antibiotics commonly used in therapy our most effective compounds show similar or slightly less antibacterial activity (*tables V* and *VI*). This class of compounds having relatively low toxicity (cf. *table VII*; LD_{t50} > 250 μg/mL) could be new potent antibacterial agents. Resistance to antimicrobial drugs is increasing all over the world. Both Gram-negative and Gram-positive strains are involved in this process. New types of compounds with antimicrobial activity could diminish this negative tendency.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Boetius apparatus and are uncorrected. Microanalyses were carried out at the Central Research Laboratory, University Medical School, Pécs. IR spectra were run in KBr discs on a Bruker IFS-55 FT-IR spectrometer controlled by Opus 2.0 software. ^{1}H- and ^{13}C-NMR spectra were recorded in

Table IV. Microbial screening of the antimicrobial effects of the novel compounds.

Compound[a]	Number of *S. aureus* inhibited / tested	Number of *E. coli* inhibited / tested
5	1/20	0/20
6a	19/20	0/20
6b	0/20	0/20
8a	0/20	0/20
8c[b]	0/20	0/20
9a	15/20	13/20
9b	0/20	0/20
9c	0/20	0/20
9d	20/20	0/20
12a	20/20	0/20
12b	18/20	0/20
12c	19[c]/20	0/20
12d	19/20	0/20
12e	0/20	0/20
12f	0/20	0/20
12g	0/20	0/20
15	0/20	0/20

[a]Concentration, 50 μg/mL. [b]Used as a hydrochloride. [c]The effect was bactericidal against some strains.

different solutions (*tables II* and *III*) in 5 mm tubes at room temperature, on a Bruker WM-250 FT-spectrometer equipped with an Aspect 2000 computer at 250.13 (^{1}H) and 62.89 (^{13}C) MHz, respectively, using the deuterium signal of the solvent as the lock and TMS as internal standard. Conventional CW irradiation of ≈ 0.15 W was used in the DR experiments. DEPT spectra [18] were run in a standard way [19], using only the θ = 135° pulse to separate the CH/CH_3 and CH_2 lines phased up and down, respectively. For DNOE measurements [20, 21] the standard Bruker microprogram 'DNOEMULT.AU' to generate NOE was used. The 2D-HSC spectra [22] were obtained by using the standard Bruker pulse program 'XHCORRD.AU'.

Analytical thin layer chromatography (TLC) was applied to monitor the reactions using precoated plates (Silica gel 60 F-254, Merck), and spots were visualized with UV light. The synthesis of some starting tricyclic pyrazolines (**3**, **4a**, **11a** and **11g**,) [7, 8], 2-isothiocarbamoyl-3,5-diphenyl-2-pyrazoline [23], 2-arylidene-1-tetralones [24, 25, 26 and unpublished data], 2-benzylidene-1-thiochroman-4-one [27] and 3,5-diarylidene-1-methyl-4-piperidones [28] has been reported earlier. The analytical values were within ± 0.4% of the theoretical values for C, H and N.

5.1.1. General procedure for the preparation of 2-isothiocarbamoyl- or 2-carbamoyl- substituted compounds (**4b**, **8a–d** *and* **11b–f**)

The mixture of thiosemiocarbazide or semicarbazide (30 mmol) and the corresponding unsaturated ketone (10 mmol) was refluxed in ethanol (110 mL) containing 9% concentrated hydrochloric acid until the disappearance of the starting unsaturated ketone. The reaction mixture was cooled down, the precipitate was filtered and washed with cold ethanol and water until neutral. As

Table V. Minimum inhibitory concentration (MIC) values.

	Concentration μg/mL					
STRAINS	> 200	200	100	50	25	12.5
S. aureus NIH HUNGARY 118 003	**5** **9d**	**6a** **12a**		**9a** **12b**	**12c** **12d**	
S. saprophyticus NIH HUNGARY 120 008		**5**	**9d**	**9a** **12a**	**12c** **12d**	**6a** **12c**
M. luteus ATCC 9341				**9a** **9d** **12a**	**5** **6a** **12b** **12d**	**12b** **12c**
B. subtilis ATCC 6633	**9d**	**9a**	**5**	**6a** **12a**	**12d**	**12b** **12c**
E. coli ATCC 25922	**5**, **6a**, **9a**, **9d**, **12a**, **12b**, **12c**, **12d**					
E. coli from 1–2 urine 3–5	**9a**			**9a**		

Table VI. Minimum inhibitory concentration (MIC) values of standard commercial antibiotics measured on standard bacterial strains.

	Concentration (μg/mL)										
STRAINS	> 100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.20	< 0.20
S. aureus NIH HUNGARY 118 003	T				C		CRO	CXM	G A		OXA P
S. saprophyticus NIH HUNGARY 120 008					CRO	C CXM		T	A		G OXA P
E. coli ATCC 25922	OXA P	G		CXM A		C T					CRO
M. luteus ATCC 9341							G	C	T		CXM CRO A OXA P
B. subtilis ATCC 6633						C CXM		A	CRO		G T OXA P

P = penicillin-G; OXA = oxacillin; T = oxytetracycline; A = ampicillin; CRO = ceftriaxone; CXM = cefuroxime; C = chloramphenicol; G = gentamicin.

for the preparation of *cis*-7-Benzylidene-2-carbamoyl-3,3a,4,5,6,7-hexahydro-5-methyl-3-phenyl-2H-pyrazolo-[4,3-c]pyridine (**8a**), at the end of the reaction the solution was made alkaline. The precipitate separated was filtered and washed with cold ethanol. Diastereoisomers **8a** and **8b** were separated by fractional recrystallization from methanol.

Table VII. In vitro cytotoxicity test of compounds on a HeLa cell-line.

Compound	Cell death % at 250 μg/mL concentration	LD_{t50} μM/L
5	87	155.7
6a	35	> 510.8
9a	90	22.2
9d	73	96.7
12a	72	278.2
12b	29	> 516.7
12c	8	> 473.3
12d	17	> 482.4

5.1.2. General procedure for the preparation of 2-iso-thiocarbamoylpyrazolo[4,3-c]pyridine hydrochlorides (**8c × HCl** *and* **8d × HCl**)

5.51 mmol of the free base (**8c** or **d**) was dissolved in ethanol and 5 mL of 6 N HCl in ethanol was added. The salt separated was filtered off and recrystallized from methanol.

5.1.3. General procedure for the alkylation of 2-iso-thiocarbamoyl-substituted compounds (**3, 4a–b** *and* **11a-g**)

Method A: an alkyl halide (9.3 mmol) was added to the solution of the 2-thiocarbamoyl compound (8.4 mmol) in anhydrous ethanol (100 mL). The reaction mixture was refluxed for 2 h with the exclusion of moisture till the disappearance of the starting 2-isothio-carbamoyl compound. The solvent was removed in vacuo and the residue was recrystallized from acetone.

5.1.4. cis*-7-Benzylidene-3,3a,4,5,6,7-hexahydro-2-S-methyl-thiuronyl-3-phenyl-2H-indazole* (**6c**)

6a (0.50 g, 1 mmol) was dissolved in ethanol (100 mL) and it was treated with concentrated ammonia solution (5 mL). The solution was poured into water and the separated precipitate was filtered off. It was recrystallized from methanol.

5.1.5. General procedure for the alkylation of 2-isothiocarbamoyl-substituted compounds (**8a–d**)

Method B: alkyl halide (1.93 mmol) was added to the solution of the hydrochloride of the 2-isothiocarbamoyl compound (1.75 mmol) in anhydrous ethanol (65 mL). The reaction mixture was refluxed for 2.5 h with the exclusion of moisture until the disappearance of the starting 2-isothiocarbamoyl compound. The reaction mixture was made alkaline by using concentrated ammonia solution (0.7 mL) and poured into water. The precipitate was filtered off and washed with water until neutral. After drying the product was dissolved in ethanol and triturated with HCl gas.

5.1.6. General method for the preparation of S-methyl derivatives from dibenzylidene ketones and S-methylthiosemicarbazide hydroiodide (**6a** and **9a**)

Method C: the unsaturated ketone (20 mmol) and *S*-methylthiosemicarbazide hydroiodide (30 mmol) were dissolved in the mixture of ethanol (300 mL) and concentrated hydrochloric acid (10 mL). After 12 h boiling the reaction mixture was cooled down. The precipitate was filtered off, washed with cold ethanol and water until neutral. It was recrystallized from the mixture of acetone and methanol. The salt formed in the case of **9a** was converted to the corresponding base that was treated with HCl to yield a dihydrochloride. These samples were in every respect identical with the product of the alkylation.

5.2. Biology

5.2.1. Microbial screens

Twenty each of *S. aureus* and *E. coli* isolates (the most common representatives of Gram-positive and Gram-negative bacterial pathogens) of various clinical sources were selected for screening the antimicrobial effect of the compounds. The strains were maintained on nutrient agar medium. The test compounds were dissolved in nutrient broth (Difco) medium at a concentration of 50 μg/mL, and 2 μL of a Nutrient Broth starter culture of the bacterial strain to be tested was added to achieve a final inoculum of ca. 5×10^5 colony forming units per mL [25]. The cultures were incubated overnight at 37 °C. Inhibition was shown by no change in optical density. Nutrient broth medium without the compound served as control. Loopfuls of nutrient broth cultures were plated on nutrient agar to show if the effect of the compounds was bacteriostatic or bactericidal [29].

5.2.2. Determination of minimum inhibitory concentration (MIC) value by test tube dilution method

Eight of the most effective antibacterial compounds (**5**, **6a**, **9a**, **9d** and **12a–d**) were tested on reference strains: *S. aureus* NIH Hungary 118003, *Staphylococcus saprophyticus* NIH Hungary 120008, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 25922. The MIC values of these five standard strains were tested on eight antibiotics commonly used in therapy: penicillin-G (P), Biogal, Debrecen, Hungary; oxacillin (OXA) and ampicillin (A), Bristol Myers Squibb Co., USA; oxytetracycline (T) and gentamicin (G), Chinoin, Budapest, Hungary; ceftriaxone (CRO) F, Hoffmann-La Roche AG, Basel, Switzerland; cefuroxime (CXM), GlaxoWellcome, Greenford, UK; chloramphenicol (C), EGIS, Budapest, Hungary. In addition the **9a** compound was investigated on five *E. coli* strains isolated from urine. The test conditions were similar as mentioned earlier. The exceptions: the compounds in 200 μg/mL concentrations were diluted in medium containing 2.5% DMSO. Using backwards dilution of DMSO all tubes contained the same concentration of DMSO. Control tubes without compounds were used to check the effect of DMSO. Double dilution series of compounds were made in test tubes. After inoculation and 24 h incubation at 37 °C, the MIC values were obtained from the lowest concentration of compound where the tubes remained clear, where the bacterial growth was inhibited. All experiments were performed in triplicate [29].

5.2.3. In vitro cytotoxicity tests of compounds on HeLa cell-line OHIO

A microplate technique was used. The DMEM1 (Sigma, Missouri, USA) growth medium contained 10% foetal bovine serum (Sigma, Missouri, USA). The tested compounds were solved in growth medium containing 2.5% DMSO. Double dilution series were set up from 250 μg/mL concentrations. 1.5×10^4 cells/well were incubated for 24 h at 37 °C. Cells killed by compounds were washed out, living cells were fixed and stained by crystal violet in methanol. The remnants of stain were washed out. One hundred μL of 1% SDS/well solved the cells under slow shaking (20 min). The cell lysates were measured in a Dynatech MR7000 photometer at 595 nm. Controls without compounds (100% cell) were used in determination of cell death percent. A graph of cell death percent vs. concentration was drawn. From this graph the LD_{t50} values were determined [30].

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