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Synthesis and anti-microbial activity of isothiosemicarbazones and cyclic analogues

E. Maccioni [a,*], M.C. Cardia [a], L. Bonsignore [a], A. Plumitallo [a], M.L. Pellerano [b], A. De Logu [b]

[a] *Dipartimento farmaco chimico tecnologico, Via Ospedale, 72, 09124 Cagliari, Italy*
[b] *Dipartimento di scienze chirurgiche e trapianti d'organo, Via Palabanda, 14, 09123 Cagliari, Italy*

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Abstract

It is known that some derivatives of both thiourea and thiosemicarbazide exhibit potent anti-microbial activity. In order to investigate the effects on the biological properties of structural modifications of such structures, we have synthesised and studied some arylidenisothiosemicarbazones. In this paper we report on the synthesis and structure–activity relationships of some isothiosemicarbazones, where the arylidene group has been replaced with a cycloalkyl group and the sulfur atom has been either differently substituted or enclosed in a thiazole ring.

Keywords: Isothiosemicarbazones; Thiazole; Biological activity

1. Introduction

During the past years an increasing interest has been devoted to the study of new and more selective anti-microbial agents. Due to this, not only have new synthetic methods been developed, but a greater amount of interest has also been devoted to the comprehension of their mechanisms of action and structure–activity relationships.

In a previous paper we reported on the synthesis and biological properties of some arylidenisothiosemicarbazones [1].

In particular the influence of structural modifications of both the sulfur and the nitrogen atom on the biological properties of these molecules were investigated (see Fig. 1).

The substitutions on the nitrogen atom (R″ in Fig. 1), not only did not lead to a significant enhancement of the anti-microbial activity, but, in some cases, led to a complete loss of activity.

Our efforts were therefore concentrated on the introduction of different subtituents on the sulfur atom, thus leading to some interesting results. We observed that the introduction of a benzyl group bearing chlorine atoms on the aromatic ring always led to the most potent compounds. Surprisingly the same activity was not observed on replacing chlorine with other halogens. Moreover, a chlorine position-depending influence was observed, the para-substituted compounds being always more active than the meta and ortho ones.

In the light of the above we now wish to investigate the influence of structural modifications on these kinds of structures further. In particular the arylidene group will be replaced with a cycloalkyl group. This change will lead to the elimination of the aromatic planar lipophile centre, and to the introduction of an aliphatic flexible cyclic system.

Two different kinds of changes will be operated on the sulfur atom: the introduction of the substituents that led to the highest activity in the case of the previously studied arylidenisothiosemicarbazones, and the introduction of the sulfur itself in a thiazole ring.

Furthermore we wish to investigate the structure–activity relationships by measuring the lipophilic character of the synthesised molecules and by relating it to

* Corresponding author
E-mail address: maccione@unica.it (E. Maccioni).

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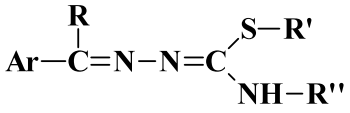


Fig. 1.

their biological properties. It has been reported that pharmaceutical properties are strongly related to the lipophilic character [2–5]. In particular Boyce and Milborrow introduced the chromatographic value R_M [6,7], which can be related to the partition coefficient and calculated from the equation:

$$R_M = \log(1/R_f - 1)$$

Many different methods to determine the values of R_M have been reported in the literature by either reversed or non-reversed (straight) phase systems and also by paper or thin layer chromatographic methods [8–10].

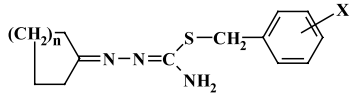
R_M calculation method to obtain an index of the lipophilic character of a drug presents some advantages with respect to direct partition methods, e.g. the simultaneous analysis of a large number of samples, which also enables the direct comparison of the obtained results, the fact that a very little quantity of products is required (very important when biological-originating molecules are under investigation), and the fact that there is no need for a quantitative analysis of the solute [11].

2. Results and discussion

2.1. Chemistry

Several new isothiosemicarbazone derivatives have been synthesised with the aim of obtaining, not only effective anti-microbial agents, but also structure–activity relationship data that may make up the basis for the design of new and more efficient derivatives.

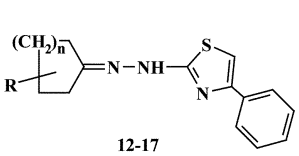
Two different lines of products have been prepared and tested against a number of microbial species, the first bearing a substituted benzyl group on the sulfur atom (**1**–**11**), while the second is characterised by the inclusion of both the sulfur and the nitrogen atom in a thiazole ring. Compounds **1**–**11** are reported in Fig. 2a, while compounds **12**–**17** are depicted in Fig. 2b.



1-11

1: n = 3; X = 2,4-Cl
2: n = 3; X = 2-Cl
3: n = 2; X = 2,4-Cl
4: n = 2; X = 2-Cl
5: n = 1; X = 2,4-Cl
6: n =3; X = 3-Cl
7: n = 2; X = 3-Cl
8: n = 3; X = H
9: n =1; X = 2-Cl
10: n = 2; X = H
11: n =1; X = H

(a)



12-17

12: n =1; R = H
13: n =2; R =H
14: n =3; R = H
15: n =4; R = H
16: n =2; R = 2-CH_3
17: n =2; R = 4-CH_3

(b)

Fig. 2.

The synthetic pathway to compounds **1**–**17** is illustrated in Scheme 1.

The synthesis of compounds **1**–**17** was accomplished by functionalisation of the thiosemicarbazide moiety.

In the case of compounds **1**–**11** the thiosemicarbazide was reacted, under reflux in isopropyl alcohol, with the appropriate benzyl chlorides.

The obtained isothiosemicarbazides were then refluxed in isopropyl alcohol in the presence of the required cycloketones and catalytic amounts of acetic acid.

These conditions proved to be the best, especially because the required product directly precipitates from the reaction mixture.

Only in the case of compounds **5**, **9** and **11**, where the cyclopentanone is reacted with benzylisothiosemicarbazides, are some precautions required. In particular the temperature should not be higher than 50–60 °C to avoid the formation of undesired side products.

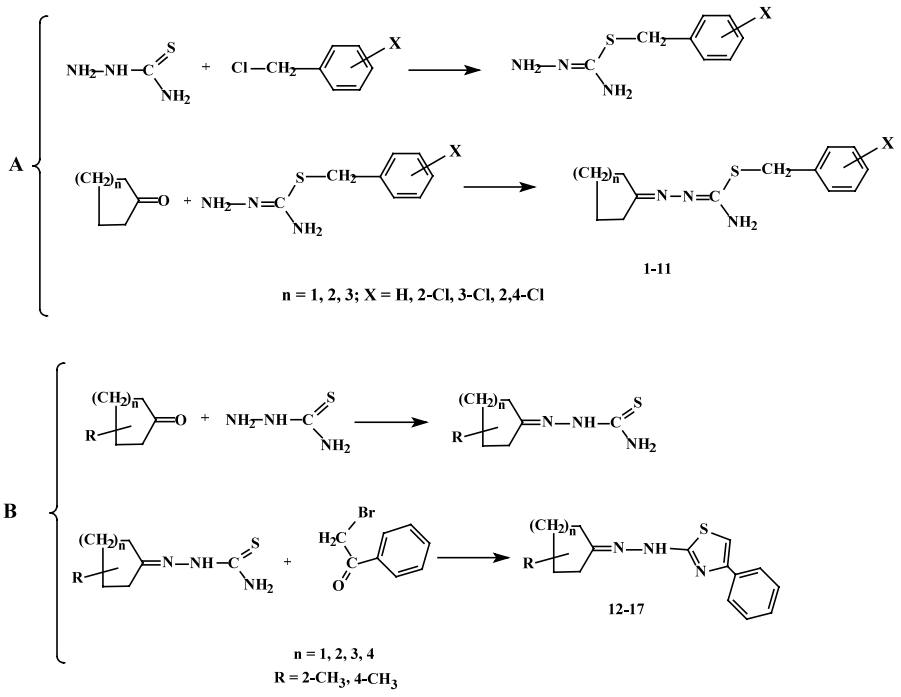
In the case of compounds **12**–**17** the cycloketone is directly reacted with thiosemicarbazide.

The obtained cycloalkylthiosemicarbazones are then reacted with ω-bromoacetophenone to achieve the formation of the 4-phenylthiazole ring. Also in this case, isopropyl alcohol proved to be the most appropriate solvent for our purpose. In fact, the reaction products can easily be separated as a white foam, which after cooling down can be filtered and purified by crystallisation from ethanol or water/ethanol. All the synthesised products have been fully characterised by means of analytical methods, ^{1}H NMR, and Mass Spectrometry in electron ionisation (e.i.) conditions. As an example the fragmentation pathway of compound **6** is depicted in Scheme 2.

2.2. Microbiology

All the synthesised products have been tested to evaluate their anti-microbial activity against several microbial species.

In the case of compounds (**1**–**11**), as well as for the previously reported compounds [1], almost no activity was found against fungi. The MFC (Minimum Fungicidal Concentration) values where too high for all compounds **1**–**11** (higher than 200 μg/ml). Surprisingly, while in the case of arylidene derivatives [1] the activity observed against *Staphylococcus aureus*, *S. epidermidis*, *B. subtilis*, and *B. thurigensis*, was higher than that against *Streptococcus agalactiae* and *S. faecalis*, in the case of the cycloalkyl derivatives **1**–**11** an almost selective activity against *S. agalactiae* was observed. In order to obtain further information on the influence of the structural modifications on biological activity, the



Scheme 1.

R_M values of compounds **1**–**11** have been measured and related to their biological activity against *S. agalactiae*. Particular effort has been placed in the measurements of the R_M values in order to avoid adsorption mechanisms. For this purpose the measurements were achieved on thin layer chromatography, where the stationary phase was previously saturated with liquid paraffin, and the mobile phase was a water/acetone mixture. With this procedure, reproducible results were obtained by using either Al_2O_3 or silica gel thin layers pre-impregnated with paraffin. This means that the stationary phase, either Al_2O_3 or silica gel, is acting only as a support for the non-aqueous solvent, and it cannot adsorb the compounds under investigation.

The relations between the R_M values and the anti-microbial activity of the most active compounds (**1**, **2**, **5**, **6**, **8**, **9**, **11**) are reported in Fig. 3.

A higher anti-microbial activity was generally observed for those compounds where the number of carbon atoms on the cycloalkyl ring is odd.

Moreover, as can be seen in Fig. 3, the higher the R_M value, the lower the MIC's, meaning that an increase in the lipophilic character leads to an increase in anti-microbial activity. However, the measured R_M values are not absolute data but can only be related to these series of compounds.

Unlike in the other odd cycloalkyl compounds, the activity observed in compound **11** was rather low. This is reasonably due to the absence of a chlorine atom on the benzyl group, which is necessary to increase the lipophilic character of the molecule. In fact the need for chlorine atoms becomes less important on increasing the number of carbon atoms.

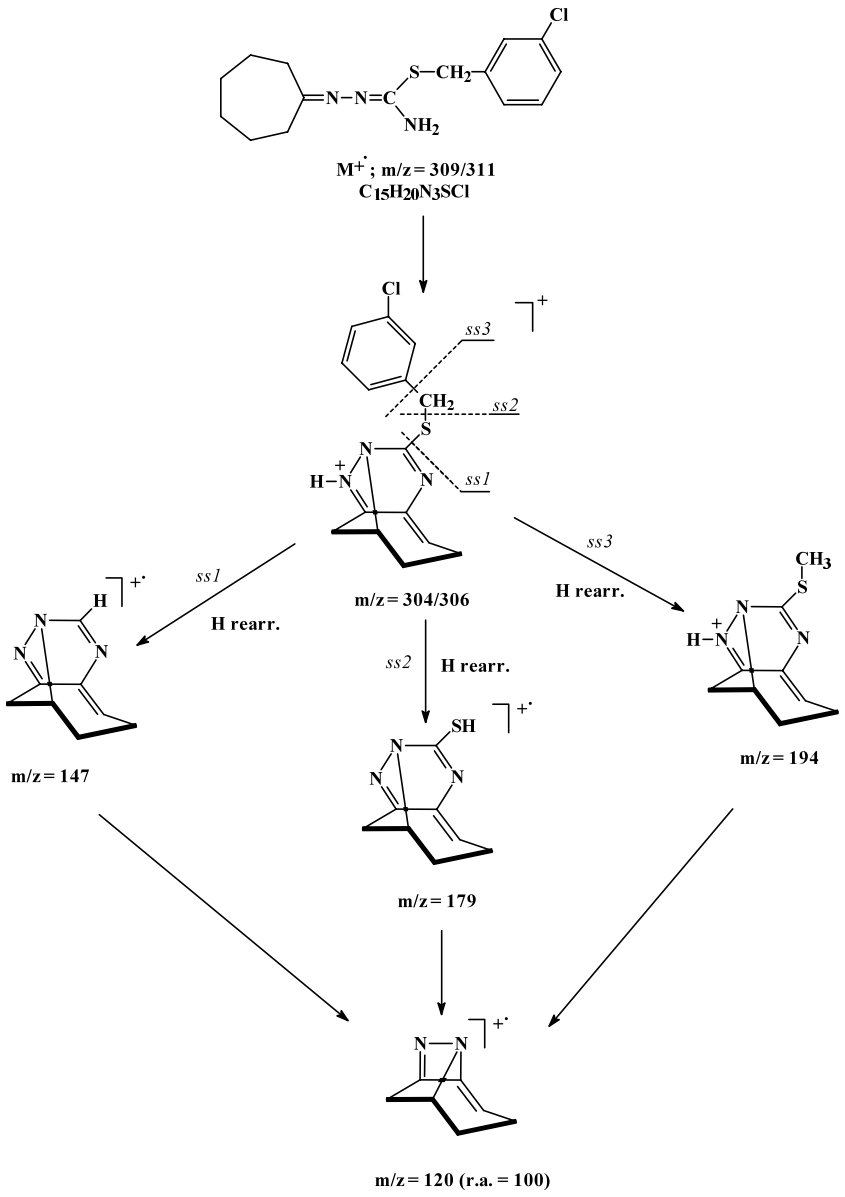
In the case of the cyclic compounds **12**–**17**, quite a different behaviour was observed. All the compounds showed almost no activity against all the tested bacteria. Cyclisation completely suppresses the anti-bacterial activity.

On the contrary their activity against fungi is more than encouraging.

In particular their activity against *C. albicans* and *C. krusei* is very interesting (Table 1). The MNTD (maximum non-toxic dose) varied between 62.5 μg/ml for compound **15** and 500 μg/ml for compound **12**. In particular the most active compounds showed the lowest toxicity.

In the case of *C. albicans* most of the compounds exhibit an activity comparable or higher than that of the reference compounds. This data is even more interesting if we consider that although many Candida species have been identified as etiologic agents of opportunistic infections, *C. albicans* and *C. krusei* are the most involved pathogens in these infections [12]. In the case of compounds **16** and **17** a chiral centre is present. In this study only the racemic mixture has been employed in the biologic tests.

In the light of these data some SAR should be taken into consideration. In the case of *C. albicans*, com-



Scheme 2.

pounds **12** and **16** exhibited the highest fungicidal activity (measured as MFC). These two compounds differ from the cycloalkyl ring. Compound **12** bears a cyclopentyl, while compound **16** bears a cyclohexyl, but both showed a comparable antifungal activity.

Surprisingly, also in the case of compound **13**, a cyclohexane ring is present, but the absence of the methyl group leads to a dramatic decrease in the fungicidal activity. On comparing the biological properties of the two isomers **16** and **17**, in which the methyl group is in position 2 and 4, respectively, it can be observed that the position of the methyl group on the cyclohexane ring is important for fungicidal activity. In fact, the MFC values shown by compound **17** are much higher than those shown by its isomer **16**.

Along the series **12**, **13**, **14**, and **15**, two different behaviours can be observed. On the one hand fungicidal activity decreases on increasing the size of the cycloalkyl ring, while on the other fungicidal activity is higher for the odd carbon number cycles, indicating that the higher the molecular symmetry the lower the fungicidal activity.

Also in the case of compounds **16** and **17**, a higher molecular symmetry seems to lead to a decrease in fungicidal activity.

3. Conclusions

An easy and economic synthetic pathway to both antibacterial and antifungal agents has been set up. In

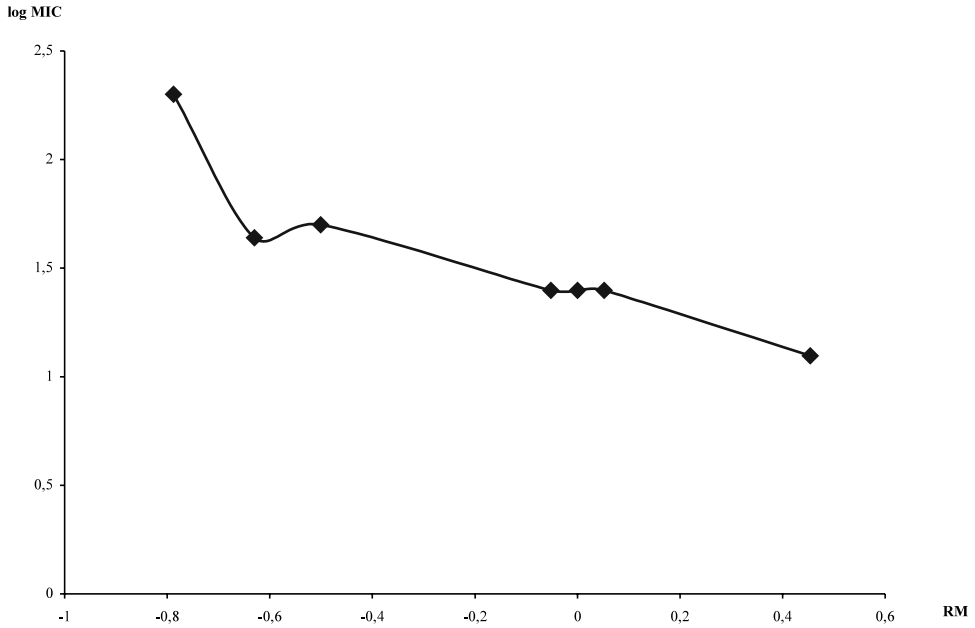


Fig. 3. Relationships between the MIC values and the measured R_M values of the seven most acive isothiosemicarbazones listed in order of increasing R_M values. Compounds: **11**, **9**, **8**, **2**, **6**, **5**, **1**.

the case of compounds **1**–**11**, the structure–activity data suggests that a higher lipophilic character will probably lead to more effective products.

However, steric and electronic factors could also play an important role in determine biological properties, and they will be considered in the design of future molecules.

In the case of thiazole derivatives, a potent antifungal activity, comparable with commercially available compounds, has been observed, in particular against *C. albicans* and *C. krusei*.

These results are very encouraging to place greater efforts in this research.

4. Experimental

4.1. Materials and methods

M.p. are uncorrected and were determined on a Reichert Kofler thermopan apparatus. IR spectra were recorded on a Perkin–Elmer 1640 FT spectrometer (KBr discs, in cm^{-1}). ^1H NMR spectra were recorded on a Bruker AMX (300 MHz) using tetramethylsilane (TMS) as internal standard (chemical shifts in δ values). Electron ionisation (EI) mass spectra were obtained by a Fisons QMD 1000 mass spectrometer (70 eV, 200 μA, ion source temperature 200 °C). The samples were introduced directly into the ion source. Elemental analyses were obtained on a Perkin–Elmer 240 B microanalyser.

4.2. Chemistry

Analytical data of compounds **1**–**17** are reported in Table 2.

4.3. Synthesis of starting isothiosemicarbazides

Starting isothiosemicarbazides have been synthesised according to previously reported methods [1]: 1 equiv. of thiosemicarbazide is refluxed in isopropyl alcohol with 1 equiv. of the appropriate benzyl chloride. The mixture is refluxed until complete dissolution (30–90 min) and then allowed to cool down to room temperature (r.t.). The required isothiosemicarbazide precipitates. After filtration the product is crystallised from isopropyl alcohol.

General method for the synthesis of compounds **1**–**11**.

4.4. Synthesis of cycloheptyliden-S-(2,4-dichlorobenzyl)-isothiosemicarbazone (*1*)

In a two-necked flask, equipped with a reflux condenser, a mixture of cycloheptanone 2 g (0.018 mol) and *S*-(2,4-dichlorobenzyl)-isothiosemicarbazide (0.018 mol) are reacted in 80 ml of isopropylalcohol in the presence of a catalytic amount of AcOH. The suspension is refluxed until complete dissolution (30–90 min) and then allowed to cool down to r.t. The product is obtained as a crystalline solid.

^1H NMR ($CDCl_3$): δ 1.60–1.74 (m, 8H, c-CH_2,); 2.47 (t, 2H, c-CH_2–C=N); 2.62 (t, 2H, c-CH_2–C=N); 4.59 (s, 2H, CH_2–S); 7.19–7.59 (m, 3H, phenyl–H); 8.92, 9.93

Table 1
Activity of compounds **12**–**17** against different fungi in comparison with Miconazole and Clortrimazole

Compound activity	12		13		14		15		16		17		Miconazole		Clotrimazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	3.12	3.12	0.78	12.5	3.12	6.25	> 100	> 100	3.12	3.12	0.39	25	3.12	12.5	3.12	12.5
C. glabrata	25	> 100	50	> 100	> 100	> 100	> 100	> 100	25	> 100	50	> 100	6.25	25	6.25	25
C. krusei	0.78	6.25	1.56	100	3.12	100	> 100	> 100	1.56	12.5	1.56	50	0.78	50	0.09	1.56
C. parapsilosis	0.78	3.12	0.78	> 100	3.12	100	> 100	> 100	0.78	3.12	1.56	50	0.09	25	0.04	3.12
S. cerevisiae	12.5	> 100	50	> 100	> 100	> 100	> 100	> 100	25	> 100	50	> 100	12.5	50	12.5	50
C. tropicalis	0.78	6.25	0.78	100	25	> 100	> 100	> 100	1.56	3.12	3.12	50	0.39	25	3.12	25

Table 2
Analytical and physicochemical properties of compounds **1**–**17**

Compound	M.p. (°C)	Crystal/solvent	Formula	C% [a]	H% [a]	N% [a]	*m*/*z*	Yield
1	184–187	Water/ethanol	$C_{15}H_{19}Cl_2N_3S$	52.33 (52.52)	5.56 (5.58)	12.20 (12.16)	344	> 95
2	191–192	Water/ethanol	$C_{15}H_{20}ClN_3S$	58.15 (57.91)	6.51 (6.49)	13.56 (13.59)	309	> 95
3	175–177	Water/ethanol	$C_{14}H_{17}Cl_2N_3S$	50.91 (50.79)	5.19 (5.21)	12.72 (12.69)	330	> 95
4	199–200	Ethanol	$C_{14}H_{18}ClN_3S$	56.84 (57.00)	6.13 (6.15)	14.20 (14.17)	295	> 95
5	193–195	Ethanol	$C_{13}H_{15}Cl_2N_3S$	49.37 (49.17)	4.78 (4.80)	13.29 (13.31)	316	> 95
6	151–153	Ethanol	$C_{15}H_{20}ClN_3S$	58.15 (58.25)	6.51 (6.53)	13.56 (13.59)	309	> 95
7	147–149	Ethanol	$C_{14}H_{18}ClN_3S$	56.84 (57.02)	6.13 (6.14)	14.20 (14.17)	295	> 95
8	192–193	Ethanol	$C_{15}H_{21}N_3S$	65.42 (65.60)	7.69 (7.71)	15.26 (15.30)	275	> 95
9	186–189	Ethanol	$C_{13}H_{16}ClN_3S$	55.41 (55.28)	5.72 (5.69)	14.91 (14.88)	247	> 95
10	200–202	Ethanol	$C_{14}H_{19}N_3S$	64.33 (64.18)	7.33 (7.30)	16.08 (16.04)	261	> 95
11	186–189	Ethanol	$C_{13}H_{17}N_3S$	63.12 (62.93)	6.93 (6.90)	16.99 (17.02)	247	> 95
12	215	Water/ethanol	$C_{14}H_{15}N_3S$	65.34 (65.13)	5.88 (5.90)	16.33 (16.31)	259	> 95
13	189–190	Water/ethanol	$C_{15}H_{17}N_3S$	66.39 (66.25)	6.32 (6.29)	15.48 (15.51)	273	> 95
14	208–209	Ethanol	$C_{16}H_{19}N_3S$	67.33 (67.17)	6.71 (6.69)	14.72 (14.75)	287	> 95
15	177–179	Water/ethanol	$C_{17}H_{21}N_3S$	68.19 (67.95)	7.07 (7.05)	14.03 (14.07)	301	> 95
16	160–162	Water/ethanol	$C_{16}H_{19}N_3S$	67.33 (67.21)	6.71 (6.73)	14.72 (14.68)	287	> 95
17	191–192	Water/ethanol	$C_{16}H_{19}N_3S$	67.33 (67.17)	6.71 (6.70)	14.72 (14.69)	287	> 95

[a] Found values in parentheses.

(ds, 1H, C–NH_2, D-exch.); 12.08 (s, 1H, =C–N^2H, D-exch.).

According to the same procedure, the following listed compounds have been synthesised.

4.5. Cycloheptyliden-S-(2-chlorobenzyl)-isothiosemicarbazone (**2**)

^1H NMR ($CDCl_3$): δ 1.59–1.76 (m, 8H, c-CH_2,); 2.45 (t, 2H, c-CH_2–C=N); 2.63 (t, 2H, c-CH_2–C=N); 4.51 (s, 2H, CH_2–S); 7.21–7.56 (m, 4H, phenyl–H); 8.15, 10.15 (ds, 1H, C–NH_2, D-exch.); 12.34 (s, 1H, =C–N^2H, D-exch.).

4.5.1. Cyclohexyliden-S-(2,4-dichlorobenzyl)-isothiosemicarbazone (**3**)

^1H NMR ($CDCl_3$): δ 1.56–1.70 (m, 6H, c-CH_2,); 2.32 (t, 2H, c-CH_2–C=N); 2.59 (t, 2H, c-CH_2–C=N); 4.55 (s, 2H, CH_2–S); 7.19–7.52 (m, 3H, phenyl–H); 8.47, 9.81 (ds, 1H, C–NH_2, D-exch.); 12.35 (s, 1H, =C–N^2H, D-exch.).

4.5.2. Cyclohexyliden-S-(2-chlorobenzyl)-isothiosemicarbazone (**4**)

^1H NMR ($CDCl_3$): δ 1.59–1.69 (m, 6H, c-CH_2,); 2.29 (t, 2H, c-CH_2–C=N); 2.57 (t, 2H, c-CH_2–C=N); 4.56 (s, 2H, CH_2–S); 7.21–7.56 (m, 4H, phenyl–H); 8.55, 9.82 (ds, 1H, C–NH_2, D-exch.); 12.45 (s, 1H, =C–N^2H, D-exch.).

4.5.3. Cyclopentyliden-S-(2,4-dichlorobenzyl)-isothiosemicarbazone (**5**)

^1H NMR ($CDCl_3$): δ 1.79–1.90 (m, 4H, c-CH_2,); 2.45 (t, 2H, c-CH_2–C=N); 2.55 (t, 2H, c-CH_2–C=N); 4.62 (s, 2H, CH_2–S); 7.22–7.57 (m, 3H, phenyl–H); 9.11, 9.64 (ds, 1H, C–NH_2, D-exch.); 12.00 (s, 1H, =C–N^2H, D-exch.).

4.5.4. Cycloheptyliden-S-(3-chlorobenzyl)-isothiosemicarbazone (**6**)

^1H NMR ($CDCl_3$): δ 1.57–1.74 (m, 8H, c-CH_2,); 2.47 (t, 2H, c-CH_2–C=N); 2.59 (t, 2H, c-CH_2–C=N); 4.44 (s, 2H, CH_2–S); 7.20–7.44 (m, 4H, phenyl–H); 8.69, 9.81 (ds, 1H, C–NH_2, D-exch.); 12.08 (s, 1H, =C–N^2H, D-exch.).

4.5.5. Cyclohexyliden-S-(3-chlorobenzyl)-isothiosemicarbazone (**7**)

^1H NMR ($CDCl_3$): δ 1.60–1.70 (m, 6H, c-CH_2,); 2.27 (t, 2H, c-CH_2–C=N); 2.55 (t, 2H, c-CH_2–C=N); 4.45 (s, 2H, CH_2–S); 7.22–7.48 (m, 4H, phenyl–H); 8.90, 9.83 (ds, 1H, C–NH_2, D-exch.); 12.34 (s, 1H, =C–N^2H, D-exch.).

4.5.6. Cycloheptyliden-S-benzyl-isothiosemicarbazone (**8**)

^1H NMR ($CDCl_3$): δ 1.60–1.81 (m, 8H, c-CH_2,); 2.46 (t, 2H, c-CH_2–C=N); 2.63 (t, 2H, c-CH_2–C=N); 4.47 (s, 2H, CH_2–S); 7.29–7.51 (m, 5H, phenyl–H); 8.49, 9.96 (ds, 1H, C–NH_2, D-exch.); 12.16 (s, 1H, =C–N^2H, D-exch.).

4.5.7. Cyclopentyliden-S-(2-chlorobenzyl)-isothiosemicarbazone (**9**)

^1H NMR ($CDCl_3$): δ 1.77–1.90 (m, 4H, c-CH_2,); 2.41 (t, 2H, c-CH_2–C=N); 2.58 (t, 2H, c-CH_2–C=N); 4.64 (s, 2H, CH_2–S); 7.21–7.59 (m, 4H, phenyl–H); 8.96, 9.69 (ds, 1H, C–NH_2, D-exch.); 12.07 (s, 1H, =C–N^2H, D-exch.).

4.5.8. Cyclohexyliden-S-benzyl-isothiosemicarbazone (**10**)

^{1}H NMR ($CDCl_3$): δ 1.62–1.81 (m, 6H, c-CH_2,); 2.31 (t, 2H, c-CH_2–C=N); 2.59 (t, 2H, c-CH_2–C=N); 4.47 (s, 2H, CH_2–S); 7.24–7.47 (m, 5H, phenyl–H); 8.40, 9.74 (ds, 1H, C–NH_2, D-exch.); 12.44 (s, 1H, =C–N^2H, D-exch.).

4.5.9. Cyclopentyliden-S-benzyl-isothiosemicarbazone (**11**)

^{1}H NMR ($CDCl_3$): δ 1.76–1.92 (m, 4H, c-CH_2,); 2.44 (t, 2H, c-CH_2–C=N); 2.56 (t, 2H, c-CH_2–C=N); 4.63 (s, 2H, CH_2–S); 7.23–7.52 (m, 5H, phenyl–H); 9.01, 9.54 (ds, 1H, C–NH_2, D-exch.); 12.03 (s, 1H, =C–N^2H, D-exch.).

4.6. Synthesis of starting thiosemicarbazones

The starting thiosemicarbazones have been prepared by slightly modifing the procedures reported in the literature [13]. In a flask equipped with a reflux condenser, equimolar amounts of thiosemicarbazide and of the appropriate ketone are reacted in isopropyl-alcohol in the presence of a catalytic amount of AcOH. The mixture is then refluxed for 1 h. and the obtained solid is filtered and used without further purification.

General method for the synthesis of compounds **12**–**17**.

Equimolar amounts of cycloalkylthiosemicarbazone and α-halogen ketone are reacted under reflux in isopropyl alcohol. The mixture is refluxed until complete dissolution, and then after a period ranging between 30 and 120 min, a foaming product is formed. The mixture is then allowed to cool down and the solid filtered. The product is crystallised from ethanol or water/ethanol.

According to this method, the following listed compounds have been synthesised.

4.6.1. 2-Cyclopentylidenhydrazo-4-phenyl-thiazole (**12**)

^{1}H NMR ($CDCl_3$): δ 1.83–1.97 (m, 4H, c-CH_2,); 2.53 (t, 2H, c-CH_2–C=N); 2.66 (t, 2H, c-CH_2–C=N); 6.73 (s, 1H, CH–thiaz.–H_5); 7.29–7.74 (m, 5H, phenyl–H); 12.22 (s, 1H, =C–N^2H, D-exch.).

4.6.2. 2-Cyclohexylidenhydrazo-4-phenyl-thiazole (**13**)

^{1}H NMR ($CDCl_3$): δ 1.69–1.80 (m, 6H, c-CH_2,); 2.41 (t, 2H, c-CH_2–C=N); 2.65 (t, 2H, c-CH_2–C=N); 6.73 (s, 1H, CH–thiaz.–H_5); 7.29–7.74 (m, 5H, phenyl–H); 12.51 (s, 1H, =C–N^2H, D-exch.).

4.6.3. 2-Cycloheptylidenhydrazo-4-phenyl-thiazole (**14**)

^{1}H NMR ($CDCl_3$): δ 1.64–1.84 (m, 8H, c-CH_2,); 2.55 (t, 2H, c-CH_2–C=N); 2.73 (t, 2H, c-CH_2–C=N); 6.75 (s, 1H, CH–thiaz.–H_5); 7.29–7.74 (m, 5H, phenyl–H); 12.26 (s, 1H, =C–N^2H, D-exch.).

4.6.4. 2-Cyclooctylidenhydrazo-4-phenyl-thiazole (**15**)

^{1}H NMR ($CDCl_3$): δ 1.45–1.99 (m, 10H, c-CH_2,); 2.49 (t, 2H, c-CH_2–C=N); 2.64 (t, 2H, c-CH_2–C=N); 6.71 (s, 1H, CH–thiaz.–H_5); 7.29–7.76 (m, 5H, phenyl–H); 12.40 (s, 1H, =C–N^2H, D-exch.).

4.6.5. 2-(2-Methylcyclohexyliden)hydrazo-4-phenylthiazole (**16**)

^{1}H NMR ($CDCl_3$): δ 1.16–1.18 (d, 3H, CH_3), 1.59–2.49 (m, 6H, c-CH_2,); 3.07 (t, 2H, c-CH_2–C=N); 3.09 (m, 1H, c-CH–C=N); 6.73 (s, 1H, CH–thiaz.–H_5); 7.29–7.74 (m, 5H, phenyl–H); 12.22 (s, 1H, =C–N^2H, D-exch.).

4.6.6. 2-(4-Methylcyclohexyliden)hydrazo-4-phenylthiazole (**17**)

^{1}H NMR ($CDCl_3$): δ 1.00–1.02 (d, 3H, CH_3); 1.25–2.55 (m, 5H, c-CH_2, CH–CH_3); 3.12 (t, 2H, c-CH_2–C=N); 3.17 (t, 2H, c-CH_2–C=N); 6.71 (s, 1H, CH–thiaz.–H_5); 7.22–7.75 (m, 5H, phenyl–H); 12.52 (s, 1H, =C–N^2H, D-exch.).

4.7. Microbiology

4.7.1. Compounds

Compounds for anti-microbial studies were dissolved in DMSO at 10 mg/ml and stored at −20 °C.

The working solutions were prepared in the same medium employed for the tests. To avoid interference from the solvent [14], the highest DMSO concentration was 1%.

4.7.2. Bacteria

The anti-microbial activity of compounds **1**–**17** was evaluated against five Gram positive species (*S. aureus*, *S. epidermidis*, *S. agalactiae*, *S. faecalis*, and *B. subtilis*), and five Gram negative species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Proteus mirabilis*, and *Klebsiella pneumoniae*) isolated from clinical specimens.

C. albicans, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *Saccharomyces cerevisiae*, and *C. tropicalis*, all isolated from clinical specimens, were employed in the evaluation of antifungal activity.

4.7.3. Determination of MICs

The MICs of the compounds against Gram positive bacteria, Gram negative bacteria, and fungi, were determined by a standard broth macro dilution method [15,16]. Tests with Gram positive and Gram negative bacteria were carried out in Mueller Hunton broth (Difco Laboratories, Detroit, MI, USA). Antifungal activity was evaluated in Sabouraud Dextrose broth (Difco Laboratories) [17]. In the case of fungi also MFC values, or more commonly MLC (minimum lethal concentration) values were measured.

The compounds were diluted in the test medium to obtain a final concentration ranging between 100 and 0.19 μg/ml. Tubes containing 1 ml of the diluted compounds were inoculated with 1×10^5 bacteria, and were incubated at 37 °C for 18 or 24 h.

4.8. Toxicity

Cellular toxicity of compounds **12**–**17** was tested in vitro incubating 2×10^5 Vero cells in six-well tissue culture plates in the presence of compounds at concentrations ranging between 1000 and 15.6 μg/ml in RPMI 1640 medium (Gibco) with 10% foetal bovine serum for 72 h at 37 °C. The medium was then removed, the cells trypsinized, and the viable cells counted by the trypan blue dye exclusion test [18]. The MNTD varied between 62.5 μg/ml for compound **15** and 500 μg/ml for compound **12**.

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