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European Journal of Medicinal Chemistry 43 (2008) 2029–2034
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Short communication

Synthesis and *in vitro* antibacterial activity of new steroidal thiosemicarbazone derivatives

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Received 19 May 2007; received in revised form 4 December 2007; accepted 6 December 2007
Available online 23 December 2007

Abstract

We investigated the antibacterial activity of some new steroidal thiosemicarbazone derivatives, prepared from the reaction of cholest-5-en-7-one with thiosemicarbazides, in ethanol in the presence of a few drops of HCl at 80 °C in high yield. All the compounds have been characterized by means of elemental analyses, IR, ^{1}H NMR and mass spectroscopic data, to find an effective antibacterial agent. The antibacterial activity was first tested *in vitro* by the disk diffusion assay against two Gram-positive and two Gram-negative bacteria, and then the minimum inhibitory concentration (MIC) of compounds was determined. The results showed that the steroidal thiosemicarbazones derivatives inhibit growth of both types of the bacteria (Gram-positive and Gram-negative). The acetoxy and chloro derivatives of cyclopentyl and cyclohexyl amine thiosemicarbazones were found to have more antibacterial activity than the other derivatives.

Keywords: Thiosemicarbazone; Steroids; Antibacterial activity

1. Introduction

The diverse parasitic bacteria such as *Staphylococcus aureus*, *Staphylococcus pyogenes*, *Salmonella typhimurium*, and *Escherichia coli* have significant impact on the mucosal health of humans. Infection with *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli* may have resulted in massive destruction of host tissue and life-threatening diseases. These bacterial parasites cause food poisoning, rheumatic fever and diarrhea that affect millions of individuals in developing countries. More than 50 million people worldwide are infected and up to 110,000 of these die every year. Amoxicillin, Norfloxacin, Ciprofloxacin are the most common drugs used for this bacterial infection but are associated with severe side effects. Toxicity and resistance to the drugs also play an important role in the failure of treatment [1,2]. Hence, the present strategy for new drug development is directed towards identifying the essential enzyme systems in the bacterial and developing molecules to inhibit them. The present work is aimed towards developing novel molecules with improved potential for treating bacterial infections and with decreased probability for developing drug resistance. Considerable attention has been focused on substituted thiosemicarbazone derivatives due to their interesting biological activity. Compounds with a thiosemicarbazone structure are known to possess tranquilizing, muscle relaxing, psychoanaleptic, hypnotic, ulcerogenic, antidepressant, antibacterial, antifungal, analgesic and anti-inflammatory properties [3–10]. Steroidal thiosemicarbazones dramatically increase the diversity of certain biological properties [11–13]. As evident from the literature, it was noted that a lot of research has been carried out on thiosemicarbazone derivatives but no work has been done on steroidal (cholesterol) thiosemicarbazone derivative screening on bacteria. In this paper the steroidal thiosemicarbazone derivatives (Fig. 1) have been synthesized by the condensation of the steroidal ketones with thiosemicarbazide. The activities of these compounds were screened *in vitro* against bacteria *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli*. To the best of our knowledge this is the first report that thiosemicarbazone analogues having

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0223-5234/$ - see front matter © 2008 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2007.12.004

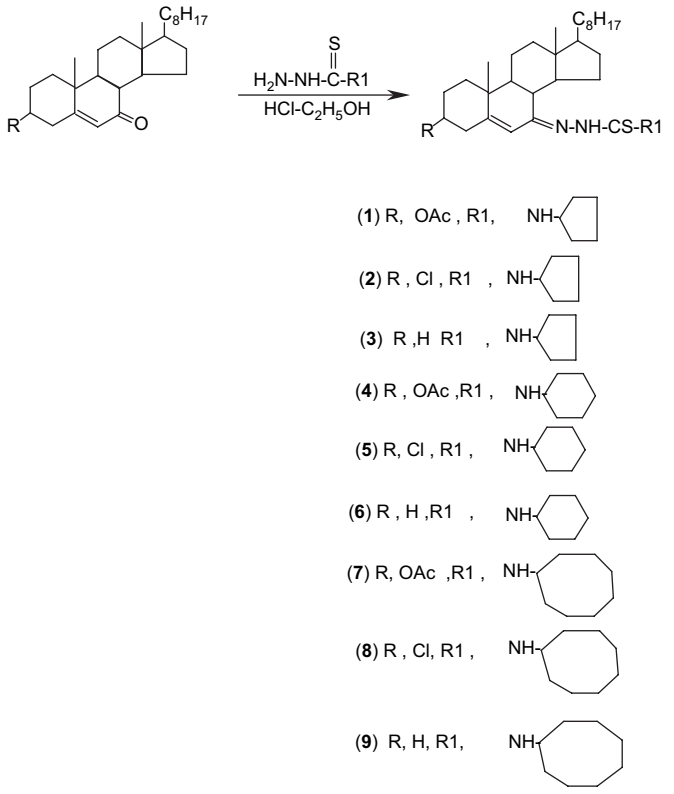


Fig. 1. Showing the number of thiosemicarbazone compounds.

steroidal moiety inhibit the growth of bacteria (Gram-positive and Gram-negative). The present work deals with the synthesis of new thiosemicarbazones (Scheme 2). The structures of all compounds were elucidated by IR, ^{1}H NMR, mass spectra and elemental analysis. These compounds were tested *in vitro* against bacteria such as *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli*.

2. Results and discussion

The yields of thiosemicarbazide products were in the range of 60–82%. The thiosemicarbazide derivatives were synthesized by using the literature procedure [14]. All thiosemicarbazone derivatives (**1**–**9**) were prepared by the corresponding ketones (**c**, **d** and **f**) and thiosemicarbazide in dry ethanol in the presence of a few drops of HCl and gave 62–70% yield (Scheme 2) [15]. The obtained compounds are stable in the solid state as well as in the solution state. Analytical data of these compounds are in good agreement with their composition (see Section 4). The compounds are insoluble in water but soluble in most of the organic solvents. The structures of all compounds were established by means of their spectral data (IR, ^{1}H NMR, FAB mass) and elemental analysis. These compounds were tested *in vitro* against bacteria such as *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli*.

2.1. IR spectral studies

Selected diagnostic bands of the IR spectra of the steroidal thiosemicarbazone derivatives (**1**–**9**) showed useful information about the structure of the compounds. All the compounds showed intense bands in the region 1138–1185 cm^{-1} due to the ν(C=S) stretching of the thiocarboxamide group. In addition, the absorption bands at 1528–1590 cm^{-1} were attributed to the ν(C=N) stretching vibration, which also confirms the formation of desired thiosemicarbazones in all the compounds. The compounds (**1**–**9**) showed additional sharp bands in the region 3345–3388 cm^{-1} due to the ν(N–H) stretching.

2.2. Nuclear magnetic resonance spectral studies

Further evidence for the formation of steroidal thiosemicarbazone compounds was obtained from the ^{1}H NMR spectra, which proved to be a diagnostic tool for the elucidation of position of the protons. Assignments of the signals are based on the chemical shift and intensity pattern. The N–H protons of all the thiosemicarbazones (**1**–**9**) are singlets in the range of 9.50–10.50 ppm. Other spectral studies of thiosemicarbazones (**1**–**9**) are given in Section 4.

2.3. FAB mass studies

Characteristic peaks were observed in the mass spectra of all thiosemicarbazone derivatives (**1**–**9**), which followed a similar fragmentation pattern. The spectrum of compounds **1**, **4** and **7** showed molecular ion peak ($M^{+\bullet}$) at *m/z* 584, 598 and 626. The other fragments within the mass spectra of thiosemicarbazones (**1**–**9**) are given in Section 4.

2.4. In vitro evaluation of antibacterial activity against Gram-positive and Gram-negative bacteria

The *in vitro* antibacterial activities of thiosemicarbazone derivatives (**1**–**9**) were carried out using the culture of *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli* by the disk diffusion method [16] and then the minimum inhibitory concentration (MIC) of all the compounds were determined. Amoxicillin (30 μg) was used as the standard drug, whereas DMSO poured disk was used as negative control and then minimum inhibitory concentration (MIC) was evaluated by the macro-dilution test using standard inoculums of 10^{-5} CFL mL^{-1}. Serial dilutions of the test compounds, previously dissolved in dimethyl sulfoxide (DMSO) were prepared to final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 μg mL^{-1}. To each tube was added 100 μL of 24 h old inoculums. The MIC, defined as the lowest concentration of the test compound, which inhibits the visible growth after 18 h, was determined visually after incubation for 18 h, at 37 °C, The thiosemicarbazone derivatives **1**, **4** and **7** have acetoxy group at 3β position and compounds **2**, **5** and **8** have chloro group at 3β position, respectively. The *in vitro* study results showed that the compounds chloro and acetoxy derivatives of cyclopentyl and cyclohexyl thiosemicarbazones were found to be more active among all the thiosemicarbazone derivatives. The susceptibility of the bacteria to the test compounds was determined by the formation of an inhibitory zone after 18 h of incubation at 36 °C. Table 1 reports the inhibition zones (mm) of each compound and the results are

Table 1
Antibacterial activity of steroidal thiosemicarbazone derivatives, positive control (Amoxicillin), and negative control (DMSO) measured by the Halo Zone Test (unit, mm)

Compounds	Corresponding effect on microorganisms			
	S. aureus	*S. pyogenes*	*S. typhimurium*	*E. coli*
1	12.5 ± 0.5	11.8 ± 0.4	14.6 ± 0.2	12.4 ± 0.4
2	16.2 ± 0.2	13.4 ± 0.5	12.5 ± 0.4	15.6 ± 0.3
3	10.4 ± 0.5	9.5 ± 0.5	9.2 ± 0.5	9.4 ± 0.4
4	14.6 ± 0.2	16.8 ± 0.4	14.5 ± 0.5	13.2 ± 0.2
5	13.4 ± 1.4	12.2 ± 1.6	14.4 ± 0.6	11.8 ± 1.6
6	10.2 ± 1.4	9.2 ± 0.3	10.4 ± 0.8	7.8 ± 0.4
7	12.6 ± 0.6	11.4 ± 0.4	13.6 ± 0.5	13.8 ± 0.4
8	15.6 ± 0.4	14.0 ± 0.4	14.6 ± 0.5	12.4 ± 0.4
9	9.9 ± 0.4	11.3 ± 0.2	10.2 ± 0.4	10.0 ± 0.4
Amoxicillin	21.0 ± 0.5	22.2 ± 0.4	25.2 ± 0.8	20.0 ± 0.2
DMSO	–	–	–	–

presented in Table 2. The tests use DMSO and Amoxicillin as negative and positive controls. The molecular structure of these active compounds showed enhanced activity. The distinct difference in the antibacterial property of the thiosemicarbazones further justifies the purpose of this study. The importance of such work lies in the possibility that the new compound might be more effective against bacteria for which a thorough investigation regarding the structure–activity relationship, toxicity and the biological effects which would be helpful in designing more potent antibacterial agents for therapeutic use is required.

3. Conclusion

This research examined the antibacterial activities of new steroidal thiosemicarbazones prepared by the reaction of steroidal ketones with thiosemicarbazides substituted by different amines. The *in vitro* antibacterial activity of these compounds revealed that the compounds chloro and acetoxy derivatives of cyclopentyl and cyclohexyl thiosemicarbazones were found to be more active among all the thiosemicarbazone compounds.

4. Experimental

Reactions were monitored by TLC analysis using Merck silica gel 60F-254 thin layer plates. All the chemicals were purchased from Aldrich chemical company (USA). Elemental analysis (C, H, N) was carried out by Central Drug Research Institute, Lucknow, India, and the results were within 0.6% of calculated values. Melting points were recorded on a KSW melting point apparatus and were uncorrected. Electronic spectra were recorded in DMSO. IR spectra were recorded in KBr on a Perkin–Elmer model 1620 FTIR spectrophotometer. ^{1}H NMR spectra were recorded at ambient temperature using a Brucker spectroscopin DPX-300 MHz spectrophotometer in DMSO. The following abbreviations were used to indicate the peak multiplicity: s-singlet, d-doublet, t-triplet and m-multiplet. FAB mass spectra were recorded on a JEOL SX 102 mass spectrometer using argon/xenon (6 kV, 10 mB gas). Steroidal compounds **a**–**f** were synthesized by the published method [17–19].

Table 2
Minimum inhibitory concentration (MIC) of steroidal thiosemicarbazone derivatives and positive control (Amoxicillin)

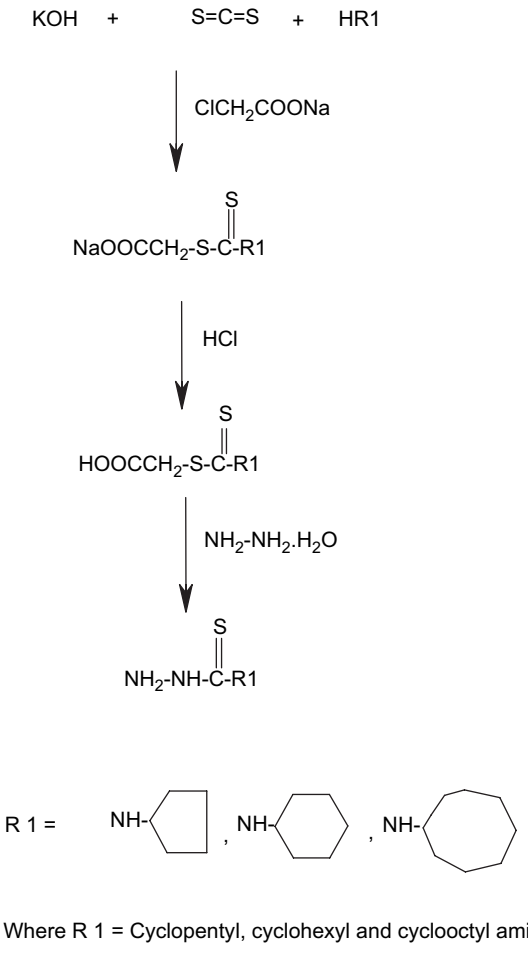
MIC (μg mL^{-1}) Strain	Compounds **1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	Positive control
S. aureus	32	64	256	64	32	128	128	64	256	32
S. Pyogenes	64	64	128	64	64	256	64	128	128	32
S. typhimurium	64	128	128	64	128	256	128	128	128	32
E. coli	32	64	256	32	64	128	128	64	256	32

4.1. Synthesis of thiosemicarbazides: a general method

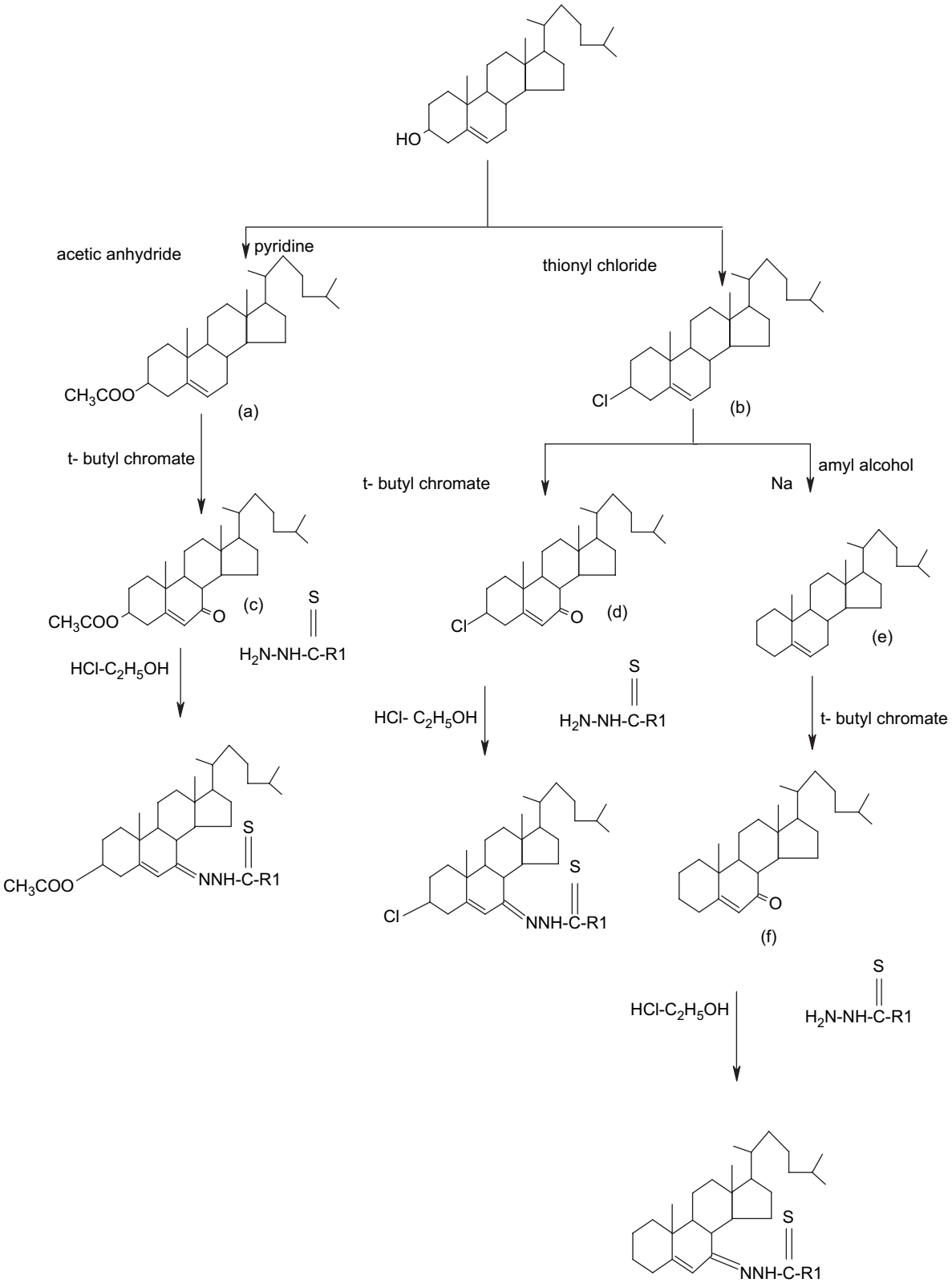
All the thioglycolic acids were prepared by the same method (Scheme 1). Cycloalkyl amino and thio carbonyl hydrazines were prepared by refluxing the alkaline solution of thioglycolic acid with hydrazine hydrate [20].

4.2. Synthesis of thiosemicarbazones: a general method

Steroidal thiosemicarbazones were synthesized (Scheme 2) by refluxing the solution of cyclopentyl, cyclohexyl and cyclooctyl thiosemicarbazide (0.03 mol) in ethanol in the presence



Scheme 1. Schematic diagram showing the synthesis of thiosemicarbazide.



Scheme 2. Showing the synthesis of compounds **1**–**9**.

of a few drops of HCl and the alcoholic solution of steroidal ketones (0.03 mol) at 60 °C for 5 h with continuous stirring. After cooling the compounds were filtered and recrystallized from the appropriate solvent.

*4.2.1. 3β-Acetoxy cholest-5-en-7-one cyclopentyl thiosemicarbazone (**1**)*

Yield: 53.6%; m.p. 172 °C; Anal. calc. for $C_{35}H_{57}N_3O_2S$: C, 72.04; H, 9.77; N, 7.20. Found: C, 72.02; H, 9.75; N,

7.18%. IR (KBr) ν_{max} cm^{-1}: 3380 (NH), 1725 ($OCOCH_3$), 1625 (weak, C=C), 1590 (C=N), 1162 (C=S). ^1H NMR (DMSO) (δ): 10.5 (s, 1H, NH), 7.54 (d, 1H, −NH), 6.08 (s, 1H, C6−H), 4.5 (br m, 1H, $J = 17$ Hz, C3α−H, axial), 2.50 (s, 3H, $OCOCH_3$), 1.2 (C10−CH_3), 0.71 (C13−CH_3) 0.97, 0.87 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 584, 525 (M − AcO), 515 (M − C_5H_9), 500 (M − $C_5H_{10}N$), 456 (M − $C_6H_{10}NS$), 441 (M − $C_6H_{11}N_2S$), 427 (M − $C_6H_{11}N_3S$).

4.2.2. 3β-Chloro cholest-5-en-7-one cyclopentyl thiosemicarbazone (**2**)

Yield: 53.6%; m.p. 164 °C; Anal. calc. for $C_{33}H_{54}N_3SCl$: C, 70.52; H, 9.94; N, 7.47. Found: C, 70.48; H, 9.97; N, 7.44%. IR (KBr) ν_{max} cm^{-1}: 3345 (NH), 1620 (C=C), 1585 (C=N), 1145 (C=S), 725 (C−Cl). ^1H NMR (DMSO) (δ): 10.46 (s, 1H, NH), 7.38 (d, 1H), 6.06 (s, 1H, C6−H), 4.05 (br m, 1H, $J = 15$ Hz, C3α−H, axial), 1.20 (C10−CH_3), 0.69 (C13−CH_3), 0.94 and 0.84 (remaining methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 560, 525 (M − Cl), 491 (M − C_5H_9), 476 (M − $C_5H_{10}N$), 432 (M − $C_6H_{10}NS$), 417 (M − $C_6H_{11}N_2S$), 403 (M − $C_6H_{11}N_3S$).

4.2.3. Cholest-5-en-7-one cyclopentyl thiosemicarbazone (**3**)

Yield: 53.6%; m.p. 142 °C; Anal. calc. for $C_{33}H_{55}N_3S$: C, 76.50; H, 10.00; N, 7.65. Found: C, 76.45; H, 9.92; N, 7.62%. IR (KBr) ν_{max} cm^{-1}: 3386 (NH), 1628 (C=C), 1568 (C=N), 1185 (C=S), 2928 (C−H). ^1H NMR (DMSO) (δ): 9.7 (s, 1H, NH), 5.9 (d, 1H, NH), 5.22 (s, 1H, C6−H), 0.07 (C10−CH_3), 0.66 (C13−CH_3), 0.90 and 0.82 (remaining protons). Mass spectra ($M^{+\bullet}$) at *m/z* 526, 457 (M − C_5H_9), 442 (M − $C_5H_{10}N$), 398 (M − $C_6H_{10}NS$), 383 (M − $C_6H_{11}N_2S$), 369 (M − $C_6H_{11}N_3S$).

4.2.4. 3β-Acetoxy cholest-5-en-7-one cyclohexyl thiosemicarbazone (**4**)

Yield: 53.6%; m.p. 146 °C; Anal. calc. for $C_{36}H_{59}N_3O_2S$: C, 72.36; H, 9.88; N, 7.03. Found: C, 72.32; H, 9.81; N, 7.02%. IR (KBr) ν_{max} cm^{-1}: 3375 (NH), 1728 ($OCOCH_3$), 1612 (C=C), 1582 (C=N), 1152 (C=S). ^1H NMR (DMSO) (δ): 10.05 (s, 1H, −NH), 7.26 (d, 1H, −NH), 6.04 (s, 1H, C6−H), 4.3 (br m, $J = 17$ Hz, C3α−H axial), 2.2 (s, 3H, $OCOCH_3$), 4.5 (m, 10H, $-CH_2$), 1.20 (C10−CH_3), 0.73 (C13−CH_3), 0.94, 0.87 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 598, 539 (M − AcO), 515 (M − C_6H_{11}), 500 (M − $C_6H_{12}N$), 456 (M − $C_7H_{12}NS$), 441 (M − $C_7H_{13}N_2S$), 427 (M − $C_7H_{13}N_3S$).

4.2.5. 3β-Chloro cholest-5-en-7-one cyclohexyl thiosemicarbazone (**5**)

Yield: 53.6%; m.p. 126 °C; Anal. calc. for $C_{34}H_{56}N_3SCl$: C, 70.89; H, 10.0; N, 7.29. Found: C, 70.84; H, 9.82; N, 7.25%. IR (KBr) ν_{max} cm^{-1}: 3380 (NH), 1622 (C=C), 1566 (C=N), 1155 (C=S), 715 (C−Cl). ^1H NMR (DMSO) (δ): 10.27 (s, 1H, NH), 7.3 (d, 1H, NH), 4.2 (br m, 1H, $J = 15$ Hz, C3α−H), 4.28 (m, 10H, $-CH_2$), 5.85 (s, 1H, C6−H), 1.21 (C10−CH_3), 0.68 (C13−CH_3), 1.09 and 0.88 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 574, 539 (M − Cl), 491 (M − C_6H_{11}), 476 (M − $C_6H_{12}N$), 432 (M − $C_7H_{12}NS$), 417 (M − $C_7H_{13}N_2S$), 403 (M − $C_7H_{13}N_3S$).

4.2.6. Cholest-5-en-7-one cyclohexyl thiosemicarbazone (**6**)

Yield: 53.6%; m.p. 122 °C; Anal. calc. for $C_{34}H_{57}N_3S$: C, 76.73; H, 10.12; N, 7.46. Found: C, 76.68; H, 10.08; N, 7.42%. IR (KBr) ν_{max} cm^{-1}: 3388 (NH), 1618 (C=C), 1546 (C=N), 1185 (C=S). ^1H NMR (DMSO) (δ): 9.9 (s, 1H, NH), 6.2 (d, 1H, NH), 5.4 (s, 1H, C6−H), 1.06 (C10−CH_3), 0.68 (C13−CH_3), 0.86, 0.80 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 540, 457 (M − C_6H_{11}), 442 (M − $C_6H_{12}N$), 398 (M − $C_7H_{12}NS$), 383 (M − $C_7H_{13}N_2S$), 369 (M − $C_7H_{13}N_3S$).

4.2.7. 3β-Acetoxy cholest-5-en-7-one cyclooctyl thiosemicarbazone (**7**)

Yield: 53.6%; m.p. 108 °C; Anal. calc. for $C_{38}H_{63}N_3O_2S$: C, 72.96; H, 10.08; N, 6.72. Found: C, 72.92; H, 10.04; N, 6.70%. IR (KBr) ν_{max} cm^{-1}: 3365 (NH), 1715 ($OCOCH_3$), 1605 (C=C), 1570 (C=N), 1148 (C=S). ^1H NMR (DMSO) (δ): 10.3 (s, 1H, NH), 7.4 (d, 1H, NH), 4.2 (br m, $J = 17$ Hz, C3α−H, axial), 2.18 (s, 3H, $OCOCH_3$) 4.12 (m, 14H, $-CH_2$), 1.22 (C10−CH_3), 0.74 (C13−CH_3) 0.98, 0.87 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 626, 567 (M − AcO), 515 (M − C_8H_{15}), 500 (M − $C_8H_{16}N$), 456 (M − $C_9H_{16}NS$), 441 (M − $C_9H_{17}N_2S$), 427 (M − $C_9H_{17}N_3S$).

4.2.8. 3β-Chloro cholest-5-en-7-one cyclooctyl thiosemicarbazonee (**8**)

Yield: 53.6%; m.p. 114 °C; Anal. calc. for $C_{36}H_{60}N_3SCl$: C, 71.5; H, 10.2; N, 6.95. Found: C, 71.2; H, 9.08; N, 6.88%. IR (KBr) ν_{max} cm^{-1}: 3355 (NH), 1618 (C=C), 1554 (C=N), 1142 (C=S), 705 (C−Cl). ^1H NMR (DMSO) (δ): 9.86 (s, 1H, NH), 7.1(d, 1H, NH), 5.53 (s, 1H, C6−H) 4.23 (br m, $J = 15$ Hz: C3α−H, axial), 3.6 (m, 14H, $-CH_2$), 1.24 (C10−CH_3), 0.67 (C13−CH_3), 0.91, 0.89 (remaining methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 602, 567 (M − Cl), 491 (M − C_8H_{15}), 476 (M − $C_8H_{16}N$), 432 (M − $C_9H_{16}NS$), 417 (M − $C_9H_{17}N_2S$), 403 (M − $C_9H_{17}N_3S$).

4.2.9. Cholest-5-en-7-one cyclooctyl thiosemicarbazone (**9**)

Yield: 53.6%; m.p. 102 °C; Anal. calc. for $C_{36}H_{61}N_3S$: C, 77.15; H, 10.32; N, 7.10. Found: C, 77.12; H, 10.26; N, 7.06%. IR (KBr) ν_{max} cm^{-1}: 3362 (NH), 1623 (C=C),1518 (C=N), 1138 (C=S). ^1H NMR (DMSO) (δ): 9.5 (s, 1H, NH), 5.4 (d, 1H, NH), 4.8 (s, 1H, C6−H), 1.16 (C10−CH_3), 0.70 (C13−CH_3), 0.92 and 0.86 (other methyl protons). Mass spectra ($M^{+\bullet}$) at *m/z* 568, 457 (M − C_8H_{15}), 442 (M − $C_8H_{16}N$), 398 (M − $C_9H_{16}NS$), 383 (M − $C_9H_{17}N_2S$), 369 (M − $C_9H_{17}N_3S$).

4.3. Organism culture and in vitro screening

Antibacterial activity was done by the disk diffusion method with minor modifications. *S. aureus*, *S. pyogenes*, *S. typhimurium*, and *E. coli* were subcultured in BHI medium and incubated for 18 h at 37 °C, and then the bacterial cells were

suspended, according to the McFarland protocol in saline solution to produce a suspension of about 10^{-5} CFU mL^{-1}: 10 μL of this suspension was mixed with 10 mL of sterile antibiotic agar at 40 °C and poured onto an agar plate in a laminar flow cabinet. Five paper disks (6.0 mm diameter) were fixed onto nutrient agar plate. One milligram of each test compound was dissolved in 100 μl DMSO to prepare stock solution and from the stock solution different concentrations 10, 20, 25, 50, and 100 μg/μl of each test compound were prepared. All these concentrations of the different compounds **1–9** were poured over disk plate. Amoxicillin (30 μg) was used as a standard drug (positive control). DMSO poured disk was used as negative control. The susceptibility of the bacteria to the test compounds was determined by the formation of an inhibitory zone after 18 h of incubation at 36 °C. Table 1 reports the inhibition zones (mm) of each compound and the controls. The minimum inhibitory concentration (MIC) was evaluated by the macro-dilution test using standard inoculums of 10^{-5} CFL mL^{-1}. Serial dilutions of the test compounds, previously dissolved in dimethyl sulfoxide (DMSO) were prepared to final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 μg mL^{-1}. To each tube was added 100 μL of a 24 h old inoculum. The MIC, defined as the lowest concentration of the test compound, which inhibits the visible growth after 18 h, was determined visually after incubation for 18 h, at 37 °C, and the results are presented in Table 2. The tests use DMSO and Amoxicillin as negative and positive controls.

Acknowledgment

Authors are thankful to Dr. Amir Azam, Department of Chemistry, Jamia Millia Islamia, New Delhi for providing laboratory facilities and chemicals for research.

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