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Novel Steroidal (6*R*)-Spiro-1,3,4-thiadiazoline Derivatives as Anti-bacterial Agents

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Novel steroidal (6*R*)-spiro-1,3,4-thiadiazoline derivatives have been synthesized by the cyclization of steroidal thiosemicarbazones. Thiosemicarbazones have been synthesized by the reaction of steroidal ketones with thiosemicarbazide. All the compounds have been characterized by IR, ^{1}H NMR, mass and elemental analyses. The antibacterial activities of these compounds have been 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) values have been determined with the reference of standard drug amoxicillin. The results showed that steroidal thiadiazoline derivatives exhibited better antibacterial activity than the steroidal thiosemicarbazone derivatives. Chloro and acetoxy substituents on the 3β-position of the steroidal thiadiazoline ring increased the anti-bacterial activity. Among all the compounds, compounds **7** and **8** were found better inhibitors as compared to the respective drug amoxicillin.

Keywords steroidal thiosemicarbazones, steroidal thiadiazolines, cholesterol, amoxicillin, antibacterial activity

Introduction

Food poisoning, rheumatic, salmonellosis and diarrhoea are the serious health problem mainly in developing country. It is caused by the bacterial infection such as *S. aureus*, *S. pyogenes*, *S. typhimurium* and *E. coli*.[1] More than 50 million people worldwide are infected and up to one hundred fifty thousand die every year due to these bacterial infections.[2] Amoxicillin norfloxacin, ciprofloxacin and chloramphenicol are most common drug for the treatment of these bacterial infection but it is associated with some side effects.[3] Hence, the present work is aimed towards developing novel molecules with improved potential for treating bacterial infections and with decreased probability for developing drug resistance. Heterocyclic compounds with reference of S and N containing are of great importance in treating biological systems. Compounds such as thiazole, thiazolidinone, thiadiazoline are abundant in nature and are of great significance to life because their structural subunits exist in many natural products such as vitamins, hormones, antibiotics *etc*. Compounds with a thiadiazoline structure are known to possess tranquilizing, muscle relaxing, psychoanaleptic, hypnotic, ulcerogenic, antidepressant, antibacterial, antifungal, analgesic and anti-inflammatory properties.[4-9] Recently a number of thiazolidinone derivatives were synthesized and their potential antibacterial activity has been studied in our laboratory.[10] It is evident from the literature that no work has been done on steroidal (cholesterol) thiadiazoline derivative screening on bacteria. Considering the facts that nearly all the classes of the cyclic thiosemicarbazones are biologically active and as a part of our continuous efforts towards the development of more potent antibacterial agents, we herein report the synthesis, characterization and *in vitro* antibacterial activity of novel spiro-1,3,4-thiadiazoline derivatives. In continuing our previous works to report steroidal thiosemicarbazones as antibacterial agents[11] and heterocyclic chemistry,[12] herein, we have developed the steroidal thiosemicarbazone as key starting materials to form novel heterocyclic compounds, to exhibit antibacterial activitives of new substituted steroidal compounds and to synthesize some new steroidal thiadiazolne derivatives for their antibacterial screening.

Experimental

All chemicals were purchased from Aldrich Co. and were used without further purification. Precoated aluminium sheets (silica gel 60 F254, Merck) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. All melting points were measured with a capillary apparatus and were uncorrected.

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All the compounds were routinely checked by IR, ^{1}H NMR and mass spectrometry. IR spectra were recorded on a Perkin-Elmer model 1620 FTIR spectrophotometer as KBr discs. ^{1}H NMR spectra were recorded on Brucker spectroscopin DPX-400 MHz spectrophotometer in $CDCl_3$ and DMSO. The following abbreviations were used to indicate the peak multiplicity s—singlet, d—doublet, t—triplet, m—multiple. FAB mass spectra were recorded on a JEOL SX102 mass spectrometer using Argon/Xenon (6 kV, 10 mB gas.) Column chromatography was performed on silica gel (Merck Co.). Anhydrous sodium sulfate was used as a drying agent for the organic phase.

Synthesis of steroidal thiosemicarbazones (4—6): a general method

Steroidal thiosemicarbazones were synthesized by refluxing the solution of thiosemicarbazide (0.03 mol) in methanol (10 mL) and the alcoholic solution (10 mL) of steroidal ketones (0.03 mol) at 60 ℃ for 5 h with continuous stirring. After cooling the compounds were filtered and recrystallized from methanol (**4**—**6**).

3*β*-Acetoxy-5*α*-cholestan-6-one-*m*-toluidinethiosemicarbazone (4) Yield 65%; m.p. 168 ℃; ^{1}H NMR (DMSO) δ: 10.42 (s, H, NH), 7.25—8.38 (m, 4H, aryl protons), 4.72 (m, *W*1/2 18 Hz, 1H, C3 α axial), 1.96 (s, 3H, CH_3), 1.10 (s, 3H, 10-CH_3), 0.98 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 0.82 (s, 3H, 13-CH_3); IR (KBr) ν_{max}: 3247 (NH), 1735 (AcO), 1564 (C=N), 1625 (C=C), 1118 (CN), 1032 (C=S) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 608, 593 (M−CH_3), 517 (M−C_7H_7), 502 (M−C_7H_8N), 458 (M−C_8H_8NS), 443 (M−$C_8H_9N_2S$), 549 (M − AcO). Anal. calcd for $C_{37}H_{57}N_3O_2S$: C 73.14, H 9.39, N 6.91; found C 73.09, H 9.25, N 6.55.

3*β*-Chloro-5*α*-cholestan-6-one-*m*-toluidinethiosemicarbazone (5) Yield 74%; m.p. 152 ℃; ^{1}H NMR (DMSO) δ: 10.38 (s, 1H, NH), 7.28—8.30 (m, 4H, aryl protons), 4.48 (m, br, 1H, *W*1/2 17 Hz, C3α-H, axial), 1.92 (s, 3H, CH_3), 1.05 (s, 3H, 10-CH_3), 0.98 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3), 0.76 (s, 3H, 13-CH_3); IR (KBr) ν_{max}: 3246 (NH), 1572 (C=N), 1624 (C=C), 1118 (CN), 1028 (C=S), 718 (CCl) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 584, 569 (M−CH_3), 493 (M−C_7H_7), 478 (M−C_7H_8N), 458 (M−C_8H_8NS), 419 (M − $C_8H_9N_2S$), 549 (M − Cl). Anal. calcd for $C_{35}H_{54}N_3SCl$: C 72.04, H 9.26, N 7.20; found C 71.96, H 9.16, N 7.18.

5*α*-Cholestan-6-one-*m*-toludinethiosemicarbazone (6) Yield 78%; m.p. 218 ℃; ^{1}H NMR (DMSO) δ: 10.35 (s, 1H, NH), 7.28—8.60 (m, 6H, aryl protons), 1.95 (s, 3H, CH_3), 1.08 (s, 3H, 10-CH_3), 0.99 (s, 3H, 18-CH_3), 0.88 (s, 3H, 19-CH_3), 0.78 (s, 3H, 13-CH_3); IR (KBr) ν_{max}: 3256 (NH), 1576 (C=N), 1636 (C=C), 1132 (CN), 1024 (C=S) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 550, 535 (M−CH_3), 459 (M−C_7H_7), 441 (M−C_7H_8N), 400 (M−C_8H_8NS), 385 (M−$C_8H_9N_2S$). Anal. calcd for $C_{35}H_{55}N_3S$: C 76.50, H 10.01, N 7.65; found C 75.52, H 9.96, N 7.62.

General procedure for oxidative cyclization of steriodal 6-ketone thiosemicarbazones (4—6) to steroidal 6*R*-spiro-1',3',4'-thiadiazolines (7—9)

Steroidal thiosemicarbazones **4**—**6** (1.0 mmol) were dissolved in chloroform (25 mL) and treated with freshly distilled acetic anhydride (11.0 mmol) and pyridine (2.5 mmol) and the mixture was stirred for 3—4 h over an oil bath at 80 ℃. Reaction progress was monitored by TLC. After completion of the reaction, solvent was removed under reduced pressure and the residue was purified by column chromatography over silica gel (petroleum ether∶diethyl ether, 8∶2) to give the respective steroidal (6*R*)-spiro-1',3',4'-thiadiazolines **7**—**9**.

3*β*-Acetoxy-5*α*-cholestan-(6*R*)-spiro-6,4'-acetyl-2'-(acetylaminomethylbenzene)-$\Delta^{2'}$-1',3,4'-thiadiazoline (7) Yield 78%; m.p. 146 ℃; ^{1}H NMR (400 MHz, CDCl3) δ: 4.88 (m, *W*1/2 18 Hz, C3 α-H), 2.28, 2.16 (s, 3H Ac), 7.38—8.42 (m, 4H, aryl protons), 1.96 (s, 3H, CH3), 0.78 (s, 3H, 13-CH_3), 0.92 (s, H, 18-CH_3), 1.10 (s, 3H, 10-CH_3), 0.80 (s, 3H, 19-CH_3); ^{13}C NMR ($CDCl_3$) δ: 171.6, 169.0, 156.5, 135.5, 133.5, 129.2, 51.8, 46.4, 30.3, 26.2, 20.8, 17.9; IR (KBr) $\nu_{max:}$ 2968 (CH), 1728, 1698 (amide), 1654 (C=N), 1738 ($OCOCH_3$), 1168 (CN), 646 (CS) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 691, 677 (M−CH_3), 601 (M−C_7H_7), 650 (M−CH_3CO), 545 (M−$C_9H_{10}O$), 428 (M−$C_{12}H_{13}N_3SO_2$), 632 (M−CH_3COO). Anal. calcd for $C_{41}H_{61}N_3O_4S$: C 71.20, H 8.82, N 6.07; found C 70.95, H 8.78, N 6.07.

3*β*-Chloro-5*α*-cholestan-(6*R*)-spiro-6,4'-acetyl-2'-(acetylaminomethylbenzene)-$\Delta^{2'}$-1',3,4'-thiadiazoline (8) Yield 65%; greenish solid; m.p. 136 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.56 (m, *W*1/2 18 Hz, C3 α-H), 2.32, 2.18 (s, 3H Ac), 7.42—8.48 (m, 4H, aryl protons), 1.92 (s, 3H, CH_3), 0.76 (s, 3H, 13-CH_3), 0.98 (s, H, 18-CH_3), 1.18 (s, 3H, 10-CH_3), 0.86 (s, 3H, 19-CH_3); ^{13}C NMR ($CDCl_3$) δ: 175.4, 164.8, 144.4, 138.2, 135.8, 128.6, 55.6, 45.8, 29.3, 24.7, 21.5, 20.8; IR (KBr) ν_{max}: 2956 (CH), 1736, 1696 (amide), 1648 (C=N), 1162 (CN), 715 (CCl), 652 (CS) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 668, 653 (M−CH_3), 577 (M−C_7H_7), 625 (M−CH_3CO), 520 (M−$C_9H_{10}NO$), 405 (M−$C_{12}H_{13}N_3SO_2$), 533 (M−Cl). Anal. calcd for $C_{39}H_{58}O_2N_3SCl$: C 70.16, H 8.69, N 6.29; found C 69.98, H 8.45, N 6.08.

5*α*-Cholestan-(6*R*)-spiro-6,4'-acetyl-2'-(acetylaminomethylbenzene)-$\Delta^{2'}$-1',3,4'-thiadiazoline (9) Yield 76%; semi-solid; ^{1}H NMR (400 MHz, CDCl3) δ: 4.58 (m, *W*1/2*h* 18 Hz, C3 α-H), 2.28, 2.12 (s, 3H Ac), 7.28—8.42 (m, 4H, aryl protons), 0.78 (s, 3H, 13-CH_3), 0.96 (s, H, 18-CH_3), 1.14 (s, 3H, 10-CH_3), 0.88 (s, 3H, 19-CH_3); ^{13}C NMR ($CDCl_3$) δ: 175.4, 164.8, 152.2, 134.8, 135.3, 127.2, 54.2, 43.4, 27.5, 24.5, 21.4, 19.8; IR (KBr) ν_{max}: 2954 (CH), 1722, 1694 (amide), 1655 (C=N), 1158 (CN), 638 (CS) cm^{-1}; Mass spectra ($M^{+\cdot}$) at *m/z* 634, 619 (M−CH_3), 543 (M−C_7H_7), 591 (M−CH_3CO), 486 (M−$C_9H_{10}NO$), 371 (M−$C_{12}H_{13}N_3SO_2$); Anal. calcd for $C_{39}H_{59}O_2N_3S$: C 73.93, H 9.30, N 6.63; found C 73.85, H 9.18, N 6.45.

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Organism culture and *in vitro* screening

Antibacterial activity was treated by the disk diffusion method with minor modifications. *S. aureus*, *S. pyogenes*, *S. typhimurium* and *E. coli* were sub cultured in BHI medium and incubated for 18 h at 37 ℃, 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 ℃ and poured onto an agar plate in a laminar flow cabinet. Five paper disks (05 mm diameter) were fixed onto nutrient agar plate. 1 mg of each test compound was dissolved in 100 μL DMSO to prepare stock solution and from stock solution different concentrations 10, 20, 25, 50, and 100 μg/μL of each test compound were prepared. These compounds of different concentration were poured over disk plate on to it. Amoxicillin (30 μg/disk) was used as 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 ℃. 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, and 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 ℃, and the results are presented in Table 2. In the tests, DMSO and amoxicillin were used as negative and positive controls.

Table 1 Antibacterial activities of steroidal thiosemicarbazones (**4**—**6**) and steroidal thiadiazoline derivatives (**7**—**9**)[a]

Compd	Corresponding effect on microganisms			
	S. aureus	*S. pyogenes*	*S. typhimurium*	*E. coli*
4	11.2±0.3	11.2±0.4	10.8±0.5	12.4±0.5
5	11.2±0.5	12.5±0.3	11.0±0.4	10.6±0.4
6	8.8±0.5	9.2±0.2	9.6±0.3	9.2±0.4
7	17.2±0.4	20.5±0.6	17.4±0.4	21.6±0.4
8	20.5±0.4	20.8±0.4	21.9±0.3	22.5±0.6
9	12.8±0.5	15.0±0.5	13.8±0.4	14.2±0.4
Amoxicillin	17.0±0.5	18.2±0.4	17.2±0.8	20.0±0.2
DMSO	—	—	—	—

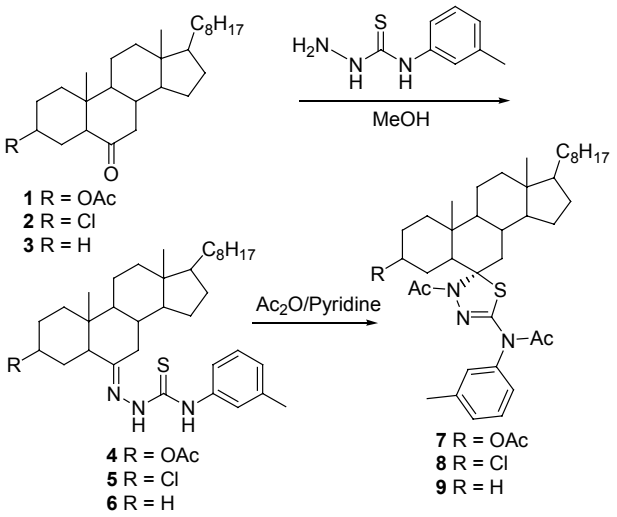
[a] Positive control amoxicillin and negative control (DMSO) measured by the Halo Zone Test (mm).

Results and Discussion

Chemistry

The starting material steroidal thiosemicarbazones (**4**—**6**) were prepared by condensing the steroidal ketones with *m*-toluidinethiosemicarbazide in the presence of catalytic amount of conc. HCl.[13] 3*β*-Acetoxycholest-6-one (**1**),[14] 3*β*-chloro-cholest-6-one (**2**),[15] 5*α*-cholest-6-one (**3**) were prepared according to the published methods.[16] The 1,3,4-thiadiazoline derivatives were synthesized according to the literature procedure by the acetylation of steroidal thiosemicarbazone derivatives as shown in Scheme 1.[17] All the compounds were soluble in DMSO and ethanol. The structures of all the compounds were established on the basis of spectral studies such as IR, ^{1}H NMR, ^{13}C NMR, FAB mass spectra and the elemental analyses were carried out to check the purity of the compounds.

Scheme 1 Schematic diagram showing the synthesis of compounds **7**, **8** and **9**



Assignments of selected characteristic IR band positions provide significant indication for the formation of the cyclized thiadiazoline analogues of thiosemicarbazones. All the compounds showed *ν*(C=N) stretch at 1648—1655 cm^{-1} due to the ring closure. In addition, the absorption band at 1158—1168 cm^{-1} was attributed to the *ν*(C—N) stretch vibrations. The compounds showed intense bands at 638—652 cm^{-1} due to *ν*(C—S) stretch, which also confirm the formation of thiazole ring in all the compounds. Further evidence for the formation of thiadiazoline compounds was obtained from the ^{1}H NMR spectra, which provide diagnostic tools for the positional elucidation of the protons. Assignments of the signals are based on the chemical shifts and intensity patterns. The acetylation proton of thiadiazoline ring of all the compounds is shown as singlet in the range *δ* 2.12—2.32. ^{13}C NMR spectra of the compounds (**7**—**9**) were taken in $CDCl_3$ and the signal obtained further confirmed the proposed structures. All the compounds showed a signal at *δ* 144.4—156.5 due to (N=C—S) and *δ* 43.4—55.6 due to N—C(6)—S, indicating the cyclization of thiocarbamoyl carbon. The characteristic peaks observed within the ^{13}C NMR spectra of thiadia-

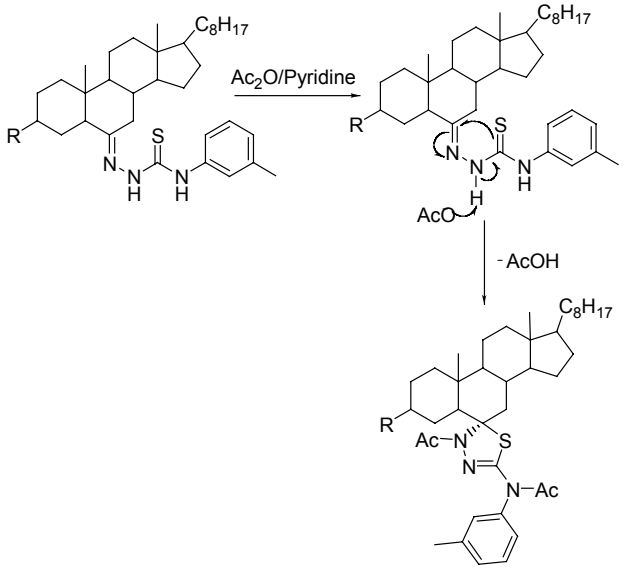
zoline derivatives are given in Experimental section. Characteristic peaks were observed in the mass spectra of compounds **7**—**9**, which followed the similar fragmentation pattern. The spectrum of compound **7** showed a molecular ion peak ($M^{\bullet+}$) at *m*/*z* 691, compound **8** showed a molecular ion peak ($M^{\bullet+}$) at *m*/*z* 668 and compound **9** showed a molecular ion peak ($M^{\bullet+}$) at *m*/*z* 634. Further fragmentation pattern of these compounds has been given in the experimental section.

Stereochemistry of spiro-1,3,4-thiadiazolines

All the 1,3,4-thiadiazolines derivatives (**7**—**9**) have C(6)—S bond axially and C(6)—NAc bond as equatorially oriented. Although sulphur atom is more bulky than the nitrogen but NAc group becomes more bulky than the sulphur atom, so during cyclisation (Scheme 1) the thiadiazoline ring preferably closes at C-6 from the front (*β*, axial) side by the attack of sulphur (C=S) of the thiosemicarbazone moiety and as the bulky group, already attached at C-6, is moved toward back (*α*, equatorial) side to avoid the 1,3-diaxial interactions mainly due to C(10)*β*-Me, and leaving front (*β*, axial) side for the attack of incoming group. So, the compounds (**7**—**9**) of this reaction have *R* geometry at C-6 in which C(6)—S and C(6)—NAc bonds are oriented axially (*β*) and equatorially (*α*), respectively. This arrangement (geometry) provides greater stability to the molecule as they have less 1,3-diaxial interactions.

The mechanism (Scheme 2) for the formation of 1,3,4-thiadiazolines can also be explained on the basis of the hard soft acid and base principle.[18] The harder acetylating reagent reacts with the harder nitrogen atom rather than the softer sulphur atom and this acetylation favours cyclization of thiosemicarbazones to (6*R*)-spiro-1,3,4-thiadiazolines.

Scheme 2 Mechanism of steroidal thiadiazoline derivatives



Anti-bacterial activity

Disc-diffusion and micro dilution assay The compounds (**4**—**9**) were tested for their antibacterial activities by disc-diffusion method using nutrient broth medium [contained (g/L): beef extract 3; peptone 5; pH 7.0].[19] The Gram-positive bacteria and Gram-negative bacteria utilized in this study included *S. aureus*, *S. pyogenes*, *S. typhimurium* and *E. coli.* In the disc-diffusion method, sterile paper discs (5 mm) impregnated with compound dissolved in dimethylsulfoxide (DMSO) at concentration 100 μg/mL were used. Then, the paper discs impregnated with the solution of the compound tested were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 35 ℃ for 24 h. After incubation, the growth inhibition zones are shown in Table 1. The thiadiazoline derivatives were further checked by MIC method. The results are presented in Table 2. The molecular structure of these active compounds showed enhanced activity. The distinct differences in the antibacterial property of these compounds further justify the purpose of this study. The importance of such work lies in the possibility that the new compound might be more efficacious drugs against bacteria for which a thorough investigation regarding the structure-activity relationship, toxicity and in their biological effects could be helpful in designing more potent antibacterial agents for therapeutic use.

Table 2 Minimum inhibition concentration (MIC) of steroidal thiadiazoline derivatives, positive control (Amoxicillin).

Strain	MIC/(μg•mL^{-1})			
	7	**8**	**9**	Positive control
S. aureus	64	32	128	32
S. Pyogenes	32	32	64	32
S. typhimurium	64	32	128	32
E. coli	32	16	64	32

Conclusions

This research involves the synthesis of novel steroidal (6*R*)-spiro-1,3,4-thiadiazoline derivatives by the cyclization of steroidal thiosemicarbazones. The antibacterial activity of these compounds was carried out by disc-diffusion method against culture of bacteria. The biological behavior of these compounds revealed that chloro and acetoxy substituents on the 3*β*-position of the steroidal thiadiazoline ring increased the anti-bacterial activity. Among all the six compounds, compounds **7** and **8** showed better antibacterial activity than the respective drug Amoxicillin. These results identified that steroidal (6*R*)-spiro-1,3,4-thiadiazoline derivatives are new leads in antibacterial chemotherapy. The study suggests the beneficial potential of these leads that need to be further explored in order to discover and develop better and yet safer therapeutic agents for bacterial infections.

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