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ORIGINAL ARTICLE

Synthesis of novel steroidal oxazolo quinoxaline as antibacterial agents

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Abstract Steroidal [oxazolo(4,5-b)quinoxaline-2-yl-hydrazone] derivative **(7a–9a) (7b–9b)** were prepared by the multi-step reactions of steroid. It is prepared via the reaction of steroidal semicarbazones with 2,3-dichloroquinoxaline at 80 °C in ethanol. The structures of the compounds were evident by IR, ^{1}H NMR and mass spectrometry and their purities were confirmed by elemental analyses. The antibacterial activity of these compounds was evaluated 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 compounds **(7a, 7b, 8a, 8b)** are better antibacterial agent as compared with the standard drug amoxicillin.

1. Introduction

Infections such as food poisoning, rheumatic, salmonellosis and diarrhea caused by multidrug-resistant Gram-positive and Gram-negative pathogens such as *Staphylococcus aureus, Streptolococcus Pyogenes, Salmonella typhimurium and Escherichia coli* (Avilffe, 1997). These pathogens are responsible for significant morbidity and mortality in both the hospital (Pfaller et al., 1999) and community settings (Abi-Hanna et al., 2000; Collignon, 1999; Merlino et al., 2000). Million of people in the subtropical regions of the world are infected and 20,000 deaths every year due to these parasitic bacterial infections. Amoxicillin, norfloxacin, ciprofloxacin are the principal drugs of choice in the treatment of bacterial infection since they are effective against extra intestinal and intestinal wall infection (Johnson, 1993), the leading drug, has been shown to be both mutagenic effect in bacteria and carcinogenic to rodents (Alauddin and Smith 1962). These are also showing severe side effects (nausea, metallic taste, dizziness, hypertension, etc.) as well as resistance have been reported (Parihar and Ramana 2004). The ideal treatment for this disease does not, therefore, exist and new agents are required. Oxadiazolines constitute an important class of heterocyclic compounds and widely utilized as a useful synthetic material in drug research (Merlani et al., 2004). The study of quinoxaline and

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oxazolo quinoxaline derivatives have become of much interest in recent years on account of their antibacterial, antiviral, anticancer antifungal, antihelmintic and insecticidal activities (Abid and Azam 2006). In this paper the steroidal oxazoloquinoxaline **(7a–9a) (7b–9b)** has been synthesized by the condensation of the steroidal semicarbazone with 2,3-dichloroquinoxaline in ethanol.

2. Experimental

2.1. Materials and methods

The entire chemicals were purchased from Aldrich Chemical Company (USA) and were used without further purification. The reactions were monitored by precoated aluminium silica gel 60F 254 thin layer plates procured from Merck (Germany). All melting points were measured with a capillary apparatus and are uncorrected. All the compounds were routinely checked by IR, ^{1}H NMR and mass spectrometry. 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-600 MHz spectrophotometer in 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 (100–200 mesh). Anhydrous sodium sulfate was used as a drying agent for the organic phase. All the steroidal ketone derivatives were prepared according to published method (Millurn and Truter, 1956; Anagnostopoulos and Fieser 1954; Backer and Squire 1948).

2.2. General method for the preparation of steroidal semicarbazones

To a solution of steroidal ketones (5.19 mM) in ethanol (50 ml) was added a mixture of semicarbazide hydrochloride (5.19 mM) and sodium acetate (3.0 g) in ethanol (20 ml). The reaction mixture was refluxed for 2 h on a steam bath and cooled. The separated solid was filtered, washed with water and recrystallized from methanol to give compounds steroidal semicarbazones.

2.3. General method for the preparation of oxazolo qunoxalines

A mixture of steroidal semicarbazones (0.01 M) and 2,3-dicholoro quinoxaline (0.01 M) in anhydrous ethanol (15 ml), was refluxed for 24 hr. Progress of reaction was monitored by TLC After completion of the reaction solvent was removed under reduced pressure and residue thus obtained was purified by column chromatography (10:90, diethyl ether:petroleum ether) and further crystallized from the appropriate solvents.

*2.3.1. 3β-Acetoxycholest-5-en-7-[oxazolo (4,5-b) quinoxaline] (**7a**)*

Brown solid (DMSO); yield: 65%; m.p. 168 °C; IR ν_{max} cm^{-1}; 3422 (N–H), 2932 (C–H aliphatic), 1568 (C═N), 1622 (C═C), 1152 (C–N), 1243 (C–O); ^{1}H NMR (DMSO-d_6)/ppm: 8.46 (s, 1H, N–H), 7.20–7.80 (m, 4H, aromatic), 5.24 (s, 1H, C6–H), 4.6 (br m, $w_{1/2}$ = 17 Hz, C3α axial), 2.08 (s, 3H, $OCOCH_3$), 1.14 (C10, CH_3), 0.74 (C13, CH_3), 0.92 and 0.84 for other methyl proton; mass spectra (M^+); at *m*/*z* 626, 579 (M−AcO), 513 (M-side chain), 456 (M−$C_9H_4N_3O$), 441 (M−$C_9H_5N_4O$). Anal. calc. for ($C_{38}H_{51}N_5O_3$); C, 72.96; H, 8.16; N, 11.2. Found: C, 72.95; H, 8.12; N,10.98.

*2.3.2. 3β-Chlorocholest-5-en-7-[oxaazolo (4,5-b) quinoxaline] (**8a**)*

Dark brown solid (DMSO); yield: 85%; m.p. 188 °C; IR ν_{max} cm^{-1}; 3416 (N–H), 2932 (C–H aliphatic), 1558 (C═N), 1172 (C–N), 1254 (C–O); ^{1}H NMR (DMSO-d_6)/ppm: 8.20 (s, 1H, N–H), 7.7–8.4 (m, 4H, aromatic), 5.32 (s, 1H, C6–H), 3.88 (br, m, –1H, $w_{1/2}$ = 15 Hz axial C3α-H), 1.16, (C10–CH_3), 0.76 (C10–CH_3), 0.84, 1.08 (other methyl protons); mass spectra (M^+) at *m*/*z* 604, 568 (M−Cl), 491 (M-side chain), 434 (M−$C_9H_4N_3O$), 419 (M−$C_9H_5N_4O$); Anal. calc. for ($C_{36}H_{48}N_5OCl$); C, 71.76; H, 7.97; N, 11.62. Found: C, 71.65; H, 7.87; N, 11.56.

*2.3.3. 5α-Cholest-5-en-7-[oxazolo (4,5-b) quinoxaline] (**9a**)*

Orange solid (DMSO) yield: 84%; m.p.178 °C; IR (KBr) ν_{max} cm^{-1}: 3442 (N–H), 2926 (C–H aliphatic), 1546 (C═N), 1128 (C–N), 1236 (C–O); ^{1}H NMR (DMSO-d_6)/ppm: 8.12 (s, 1H, NH), 6.40–7.40 (m, 4H, aromatic), 0.68, 0.84, 1.08, 1.26 (CH_3–methylene proton); mass spectra (M^+) at *m*/*z* 568, 455 (M-Side Chain), 398 (M−$C_9H_4N_3O$), 383 (M−$C_9H_5N_4O$); Anal. calc for. ($C_{36}H_{49}N_5O$) C, 76.19; H, 8.64; N, 12.34. Found: C, 76.15; H, 8.58; N, 12.28.

*2.3.4. 3β-Acetoxy-5α-cholestan-6-[oxazolo (4,5-b) quinoxaline] (**7b**)*

Light orange solid. (DMSO); yield: 72%; m.p.264 °C; IR (KBr) ν_{max} cm^{-1}: 3426 (N–H), 2965 (C–H aliphatic), 1565 (C═N), 1155 (C–N), 1252 (C–O). ^{1}H NMR (DMSO-d_6)/ppm: 8.40 (s, 1H, N–H), 7.50–7.90 (m, 4H, aromatic), 4.6 (br m, $w_{1/2}$ = 17 Hz, C3α axial), 2.04 (s, 3H, $OCOCH_3$), 1.20 (C10, CH_3), 0.68 (C13–CH_3), 0.92 and 0.78 for other methyl proton. Mass spectra (M^+) at *m*/*z* 628, 569 (M−AcO), 515 (M-side chain), 457 (M−$C_9H_4N_3O$), 443 (M−$C_9H_5N_4O$); Anal. calc. for ($C_{38}H_{53}N_5O_3$): C, 72.72; H, 8.45; N, 11.16. Found: C, 71.84; H, 8.48; N, 10.65.

*2.3.5. 3β-Chloro-5α-cholestan-6-[oxazolo (4,5-b) quinoxaline] (**8b**)*

Dark brown solid (DMSO); yield: 78%; m.p. 182 °C; IR (KBr) ν_{max} cm^{-1}: 3432 (N–H), 2942 (C–H aliphatic), 1555 (C═N), 1162 (C–N), 1265 (C–O). ^{1}H NMR (DMSO-d_6)/ppm: 8.12 (s, 1H, N–H), 7.6–8.1 (m, 4H, aromatic), 3.88 (br, m, –1H, $w_{1/2}$ = 15 Hz axial C_3α–H), 1.15 (C_{10}–CH_3) 0.76 (C_{13}–CH_3), 0.86, 1.08 (other methyl proton); mass spectra (M^+) at *m*/*z* 606, 570 (M−Cl), 493 (M-side chain), 436 (M−$C_9H_4N_3O$), 421 (M−$C_9H_5N_4O$); Anal. calc. for ($C_{36}H_{50}N_5OCl$) C, 71.52; H, 8.27; N, 11.58. Found: C, 70.45; H, 7.65; N, 10.85.

*2.3.6. 5α-Cholestan-6-[oxazolo(4,5-b)quinoxaline] (**9b**)*

Red orange solid (DMSO); yield: 80%; m.p.242 °C; IR (KBr) ν_{max} cm^{-1}: 3438 (N–H), 2935 (C–H aliphatic), 1542 (C═N), 1144 (C–N), 1238 (C–O). ^{1}H NMR (DMSO-d_6)/ppm: 8.06 (s, 1H, N–H), 6.80–7.50 (m, 4H, aromatic), 0.64, 0.82, 1.04, 1.24 (CH_3–methylene proton). Mass spectra (M^+) at *m*/*z* 570, 457 (M-side chain), 400 (M−$C_9H_4N_3O$), 385

(M−$C_9H_5N_4O$); anal. calc for. ($C_{36}H_{51}N_5O$) C, 75.92, H, 8.96, N, 12.30; found: C, 75.65, H, 8.76, N, 11.98.

2.4. Bioactivity

The in vitro antibacterial activity of the structurally promising steroidal oxazolo quionoxaline derivatives **(7a–9a) (7b–9b)** against two strains of Gram-positive bacteria and two strains of Gram-negative bacteria were investigated using disc-diffusion and micro dilution methods in comparison to the reference drugs amoxicillin. The results of the disc-diffusion methods are shown in Table 1. And that of micro dilution method, for the concentration affords 50% inhibition of bacteria growth (IC_{50}) values, are shown in Table 2. All results clearly revealed that, all tested compounds in the present study were found to have highly statistically significant antibacterial activity against the used strains of Gram-positive and Gram-negative bacteria ($P < 0.05$). In particular, acutely and chloro-derivatives of steroidal oxazolo quinoxalines exhibits the greatest significant activity followed by other derivatives of steroidal oxazolo quinoxalines. On the other hand, compounds **(7a–9a) (7b–9b)** indicate notable activity, with **MIC** values ranging from 0.39 to 0.78 against the both type of bacteria, which is effective in nosocomial infection and often resistant to antibiotic therapy. Moreover, all the tested compounds are significantly more potent than reference drugs as depicted in Tables 1 and 2. This excellent effectiveness makes these substances attractive antibacterial candidates. Studies to establish their in vitro efficacy and safety are being planned for their further development.

3. Results and discussion

3.1. Chemistry

The synthesis of steroidal semicarbazone derivatives are straight forward and the compounds were isolated in good yield. The oxazolo (4,5-b)-quininoxaline derivatives were synthesized by using the literature procedure (Khan et al., 2007)]. The obtained compounds are stable in the solid state as well as in the solution state. The analytical data of these compounds are in good agreement with their composition. The structure of all the compounds presented in Schemes1 and 2 was established by comparing spectral data (IR, ^{1}H NMR and mass). Assignments of selects characteristic IR band positions provide significant indication for the formation of the cyclized oxazolo quinoxaline analogues of semicarbazones. All the compounds showed sharp band in the region (3416–3442) cm^{-1} due to the ν (N–H) stretch. The IR spectra of all the compounds showed ν (C═N) stretch at 1544–1568 cm^{-1}. In addition, the absorption bands at 1128–1172 cm^{-1} were attributed to the ν (C–N) stretch vibrations. The compounds showed intense bands at 1238–1165 cm^{-1} due to ν (C–O) stretch, which also confirm the formation of oxazolo ring in all the compounds. Further evidence for the formation of oxazolo quinoxaline compounds was obtained

Table 1 Antibacterial activity of steroidal derivatives Positive control (amoxycillin) and negative control (DMSO) measured by the halo zone test (mm).

Compounds	Corresponding effect on microorganisms			
	S. aureus	*S. pyogenes*	*S. typhimurium*	*E. coli*
7a	21.5 ± 0.4	22.6 ± 0.7	20.4 ± 0.3	23.2 ± 0.4
8a	23.7 ± 0.6	23.2 ± 0.5	19.5 ± 0.6	21.7 ± 0.6
9a	13.4 ± 0.5	13.2 ± 0.5	14.4 ± 0.4	15.3 ± 0.5
7b	20.4 ± 0.8	22.4 ± 0.5	19.4 ± 0.6	21.8 ± 0.6
8b	21.2 ± 0.6	21.8 ± 0.6	18.6 ± 0.4	19.6 ± 0.5
9b	15.6 ± 0.4	14.6 ± 0.4	12.4 ± 0.5	13.5 ± 0.6
Amoxycillin	17.0 ± 0.5	18.2 ± 0.4	17.2 ± 0.8	20.0 ± 0.2
DMSO	–	–	–	–

Table 2 Antibacterial of activities of the tested compounds and standard drug amoxycillin using the microdilution method expressed as MIC and IC_{50} (μg/mL).

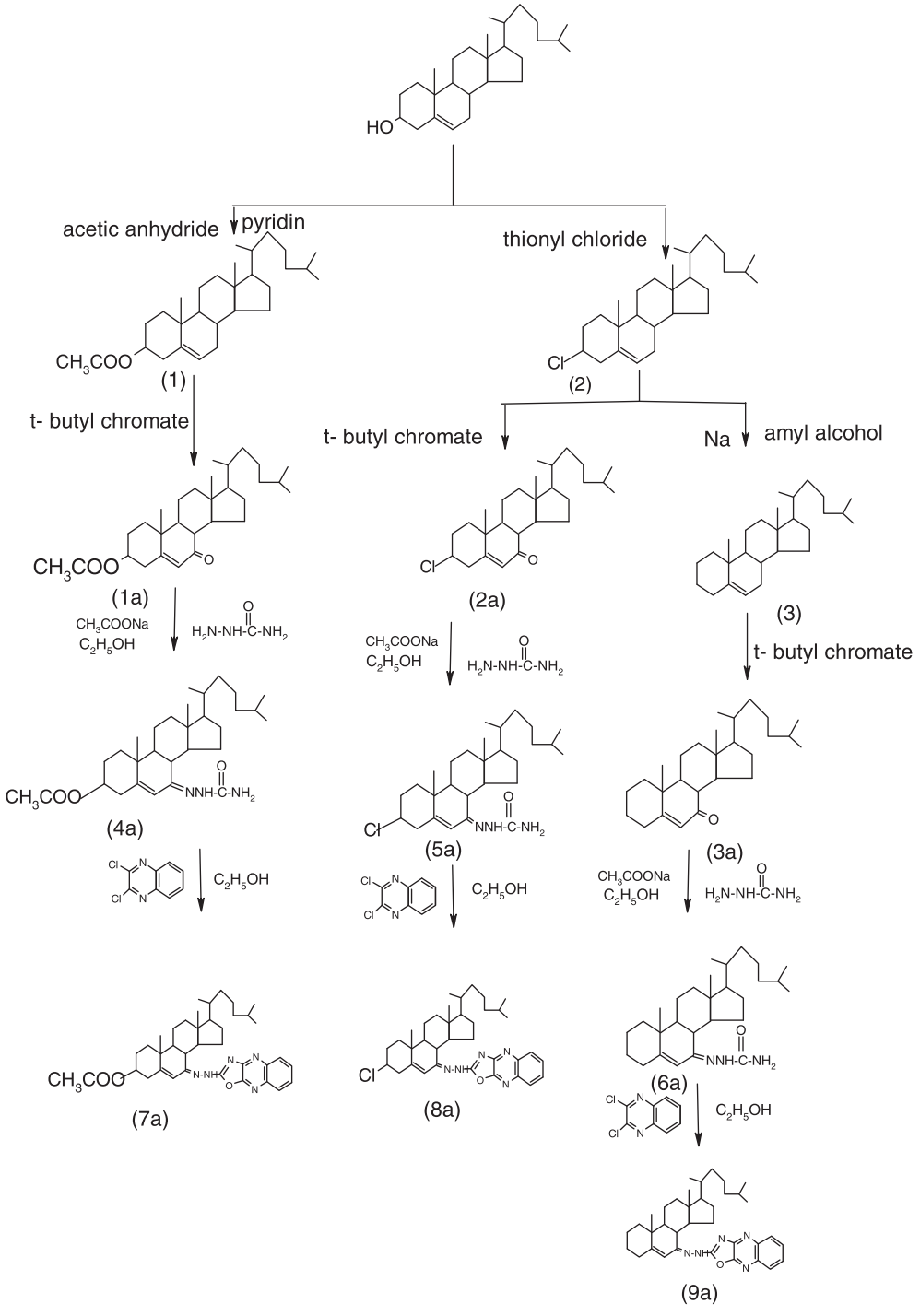
Compounds	Gram-positive bacteria				Gram-negative bacteria			
	B. aureus		*S. pyogenes*		*S. typhimurium*		*E. coli*	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
7a	0.78	0.47	0.78	0.52	0.78	0.47	0.39	0.25
8a	0.78	0.52	0.78	0.48	0.39	0.26	0.39	0.23
9a	3.12	1.80	3.12	1.85	6.25	3.64	6.25	3.68
7b	0.78	0.50	0.39	0.24	0.39	0.22	0.78	0.54
8b	0.39	0.25	0.78	0.47	0.39	0.26	0.39	0.23
9b	6.25	3.62	6.25	3.62	3.12	1.92	3.12	1.94
Amoxycillin	3.12	1.82	3.12	1.80	3.12	1.85	3.12	1.90

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 aromatic protons of oxazolo quinoxaline are shown as multiplet in the range (6.4–8.4) ppm for the compounds (**7a–9a**) **(7b–9b)**. A Singlet due to N–H proton in the compounds (**7a–9a**) **(7b–9b)** was observed at (8.06–8.46) ppm, respectively. Characteristic peak were observed in the mass spectra of compounds (**7a–9a**) **(7b–9b)** which followed the similar fragmentation pattern. The spectrum of the compounds molecular ion peak (M^+). The characteristics peaks observed within the mass spectra of oxazolo quinoxaline compounds are given in experimental section. Compound (**9b)** showed a molecular ion peak (M^+) at m/z 570.

3.2. Pharmacology

3.2.1. Antimicrobial activity

The in vitro antimicrobial activity was performed using the disk diffusion method and the Minimum Inhibitory Concentration (MIC). Amoxicillin was used as positive controls for bacteria.



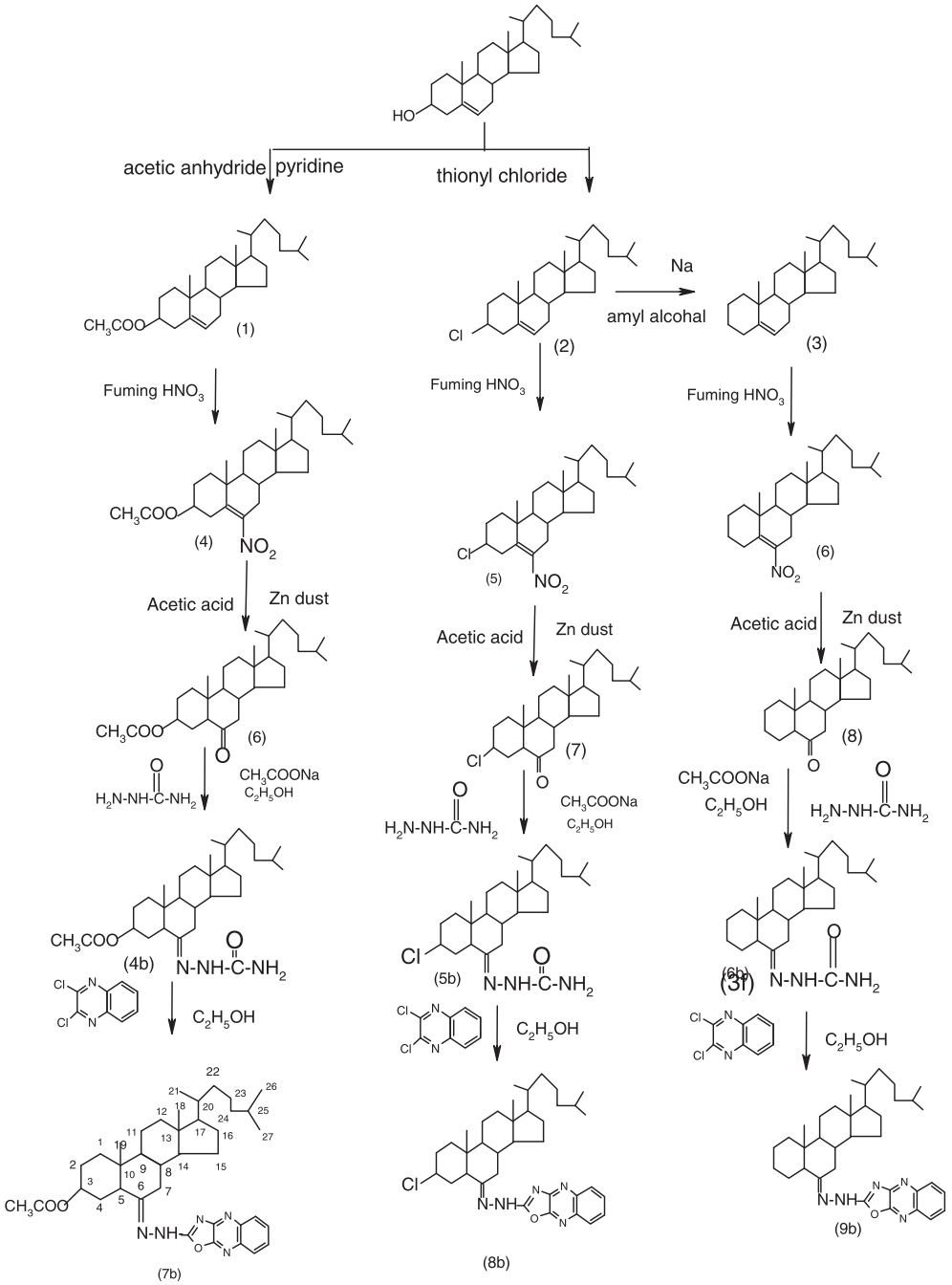
Scheme 1 Schematic diagram showing the synthesis of 5-en-7-[oxazolo(4,5-b)quino-xalines]**(7a–9a)**.

3.2.2. Disc-diffusion assay

The compounds (**7a–9a**) **(7b–9b**) were tested for their antibacterial activities by disc-diffusion method (Wilkins et al., 1972) using nutrient broth medium [contained (g/L): beef extract 3 g; peptone 5 g; pH 7.0]. The Gram-positive bacteria utilized in this study consisted *of S. aureus, S. Pyogenes*. The Gram-negative bacteria included *S. typhimurium, E. coli*. In the disc-diffusion method, sterile paper discs (0.5 mm) impregnated with compound dissolved in dimethylsulfoxide (DMSO) at concentration 200 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 °C for 24 h. After incubation, the growth inhibition zone and are shown in Table 1.

3.2.3. Micro dilution assay

The minimal inhibitory concentration (MIC) values for compounds (**7a–9a**) **(7b–9b)** defined as the lowest concentration of the compound preventing the visible growths were determined by using the micro dilution both methods. The inoculate to 0.5 cFarland standard turbidity. The test compounds dissolved in dimethylsulfoxide (DMSO) were first diluted to the



Scheme 2 Schematic diagram showing the synthesis of 6-[oxazolo(4,5-b)quinoxalines] **(7b–9b)**.

highest concentration (400 μg/mL) to be tested. Then serial twofold dilution was made in concentration range from 0.1 to 400 μg/ml in 10 ml sterile tubes. A prepared suspension of the standard microorganisms was added to each dilution in a 1:1 ratio. Growth (or its lack) of microorganisms was determined visually after incubation for 24 h at 37 °C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC. The concentrations afford 50% inhibition of bacteria growth (IC_{50}) was computed from the dose response curves. MIC and IC_{50} values were studied for the same bacterial strains in the disc-diffusion assay and given in Table 2. Amoxicillin was used as reference drugs. All disc-diffusion and micro dilution experiments were preformed in duplicate and repeated three times.

4. Conclusion

This research examined the antibacterial activities of new cyclized steroidal oxaazolo quinoxaline prepared by the reaction of semicarbazones with 2,3-di-chloroquinoxaline at 80 °C. In vitro antibacterial activities of these compounds were carried out against culture of bacteria the biological behavior of these compounds revealed that chloro and acetoxy substituents on the 3β-position of the steroidal ring increased the antibacterial activity. Among all the six compounds compound **7a, 7b** and **8a, 8b** showed better antibacterial activity than their respective drug.

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