Synthesis, characterization and antimicrobial activity of steroidal (6*R*)-spiro-$\Delta^{2'}$, 1', 3', 4'-oxadiazolines

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Abstract

*A series of new steroidal (6R)-spiro-Δ^{2}`, 1`, 3`, 4`-oxadiazolines (**7-9**) have been synthesized via the reaction of steroidal semicarbazones (**4-6**) with acetic anhydride and pyridine at 80 ^{0}C. The structures of the newly synthesized compounds have been established on the basis of their spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and analytical studies. Antimicrobial activity assessment of the synthesized compounds has been evaluated against different bacterial as well as fungal strains. Difference in the potency of activity against the strains was evaluated on the basis of SAR, and it has been revealed that substitution at 3β-positions in steroidal oxadiazolines enhanced the antimicrobial activities.*

Keywords: semicarbazone, acetic anhydride, pyridine, oxadiazoline, antimicrobial

1. Introduction

Steroids have been the important focus of research throughout the scientific history. But the recent past has seen an exhaustive focus of research being diverted towards these biologically important molecules. This is pertinently true of the rational semi synthetic modifications of steroidal molecules. Probably, it is because of the various advantages associated with steroid based chemotherapeutics. These compounds turn out to be non-toxic, less vulnerable to multi-drug resistance (MDR) and highly bioavailable because of being capable of penetrating the cell wall (Bandey et al., 2011; Savage, 2002). Oxadiazoline derivatives have shown a wide array of pharmacological activities, including antibacterial, anti-fungal, analgesic, anti-inflammatory, anti-hypertension, and muscle-relaxing activities. Consequently, they have attracted increasing attention in the field of drug discovery (Rane et al., 2013; Ergun et al., 2010). As an important group of 1,3,4-oxadiazole derivatives, 3-acetyl-2,3-dihydro-1,3,4-oxadiazoles have also been well documented with biological activities such as anti-cancer and anti-bacterial activities (Polshettiwar and Varma, 2008**;** Brown et al., 2000) .Latest studies on their activities revealed that they also possess potential anticonvulsant and anti HIV activity (Wang et al., 2012; Dogan et al., 1998). Although various modifications of steroids have been tried

but as far the literature precedents are concerned, little efforts have been made towards the efficient synthesis and simultaneous biological analysis of steroid based oxadiazolines. The synthesis of steroidal oxadiazolines (Shamsuzzaman et al., 2004) as also become of interest in recent years because oxadiazolines constitute an important class of heterocyclic compounds which are widely utilized as useful synthetic material in the field of drug research. Intrigued by above observations and in continuation of our previous work (Shamsuzzaman et al., 2014), we here report the convenient and successful synthesis of steroidal oxadiazolines from steroidal semicarbazones under acetylating conditions and study their *in vitro* antimicrobial behavior.

2. Experimental

Melting points were determined on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Pye Unicam SP3-100 spectrophotometer and its values are given in cm^{-1}. ^1H NMR spectra were measured in $CDCl_3$ on a Bruker DRX-300 spectrometer at 400 MHz and ^{13}C NMR spectra at 100 MHz, with TMS as internal standard and its values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer using Argon/Xenon as the FAB gas. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of b.p. 60-80 ^0C. Sodium sulfate (anhydrous) was used as a drying agent.

2.1. General Procedure for the Synthesis of steroidal (6R)-spiro-Δ^2`, 1`, 3`, 4`-oxadiazolines (7-9)

Steroidal semicarbazone (**4-6**) (1.0 g) was dissolved in chloroform (35 mL) and treated with freshly distilled acetic anhydride (2.1 mL) and pyridine (0.4 mL), the mixture was stirred for 9-10 hours over an oil bath at 80 ^0C. Reaction progress was monitored through TLC. After completion of reaction solvents were removed under reduced pressure and the residue was purified by column chromatography over silica gel column (light petroleum-diethyl ether 4:1) to give corresponding oxadiazolines (**7-9**) in good yields.

2.1.1. 3β-Acetoxy-5α-cholestan-(6R)-spiro-Δ^2`, 1`, 3`, 4`-oxadiazoline, 7

Yield: 74 %; Solid; m.p: 119-121 ^0C; Anal. Calc. for $C_{34}H_{55}N_3O_5$: C, 69.71; H, 9.46; N, 7.17; found C, 69.78; H, 9.41; N, 7.24; IR (KBr) ν_{max}, cm^{-1}: 3441 (NH), 1735 (OAc), 1717 (CONH), 1685 (CON), 1640 cm^{-1} (C=N); ^1H NMR (400 MHz, $CDCl_3$): δ 7.27 (s, 1H, NHAc), 4.68 (m, 1H, W½ = 17 Hz axial C_3 α-H), 2.34 (m, 2H, C_7-H_2), 2.29, 2.25, 2.04 (each s, 3H, Ac), 1.18 (s, 3H, C_{10}-CH_3), 0.67 (s, 3H, C_{13}-CH_3), 0.93 (d, 3H,C_{20}-CH_3), 0.86 (d, 6H, C_{25}- 2×CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.5 (NHCO), 171.9 ($OCOCH_3$), 171.2 (NCO), 154.8 (OC=N), 72.8 (C_3), 71.7(C_6), 52.7 (C_5), 42.7 (C_7), 18.62 (CH_3CONH), 16.8 (CH_3CON); MS: *m/z* 585 [$M^{+\cdot}$].

2.1.2. 3β-Chloro-5α-cholestan-(6R)-spiro-Δ^2`, 1`, 3`, 4`-oxadiazoline, 8

Yield: 71 %; Solid; m.p: 138-140 ^0C; Anal. Calc. for $C_{32}H_{52}ClN_3O_3$: C, 68.36; H, 9.32; N, 7.47; found: C, 68.45; H, 9.37; N, 7.58; IR (KBr) ν_{max}, cm^{-1}: 3440 (NH), 1712 (CONH), 1690 (CON), 1645 (C=N) and 753 cm^{-1} (C-Cl); ^1H NMR (400 MHz, $CDCl_3$): δ 7.2 (1H, s, NHAc), 3.81 (1H, m, W½ =14 Hz axial C_3 α-H), 2.35 (m, 2H, C_7-H_2), 2.23, 2.15 (each s, 3H, Ac), 1.17 (s, 3H, C_{10}-CH_3), 0.66 (s, 3H, C_{13}-CH_3), 0.92, (d, 3H,C_{20}-CH_3), 0.78 (d, 6H, C_{25}-2×CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.3 (NH-CO), 171.4 (NCO), 153.9 (OC=N), 71.1 (C_6), 53.8 (C_5), 51.8 (C_3), 43.0 (C_7), 18.16 (CH_3CONH), 18.58 (CH_3CON); MS: *m/z* 561/563 [$M^{+\cdot}$].

2.1.3. 5α-Cholestan-(6R)-spiro- Δ²`, 1`, 3`, 4`-oxadiazoline, 9

Yield: 72 %; Solid; m.p: 125-127 ^{0}C; Anal. Calc. for $C_{32}H_{53}N_3O_3$: C, 72.82; H, 10.12; N, 7.96; found: C, 72.89; H, 10.17; N, 8.02; IR (KBr) ν_{max} cm^{-1}: 3443 (NH), 1715 (CONH), 1680 (CON), 1648 (C=N); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.29 (s, 1H, NHAc), 2.3 (m, 2H, C_7-H2), 2.25, 2.11 (each s, 3H, Ac), 1.12 (s, 3H, C_{10}-CH_3), 0.66 (s, 3H, C_{13}-CH_3), 0.92 (d, 3H, C_{20}-CH_3), 0.78 (d, 6H, C_{25}-2×CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9 (NHCO), 170.7 (NCO), 154.5 (OC=N), 71.8 (C_6), 57.4 (C_5), 42.9 (C_7), 27.93 (C_3), 18.17 (*C*H_3CONH), 18.51 (*C*H_3CON); MS: *m/z* 527 [$M^{+\cdot}$].

2.2. In vitro antibacterial activity

Steroidal oxadiazolines (**7-9**) were screened for the *in vitro* antibacterial activity against the cultures of *S. pyogenes* (ATCC 19615), *S. aureus* (ATCC- 25923), *P. aeruginosa* (ATCC-27853) and *E. coli* (ATCC-25922) by disc diffusion method (Shamsuzzaman et al., 2014). Standard inoculums (1-2) ×10^7 c.f.u. mL^{-1} (0.5 McFarland standards) were introduced onto the surface of sterile agar plates and a sterile glass spreader was used for even distribution of the inoculums. The discs measuring 6 mm in diameter were prepared from Whatman No. 1 filter paper and sterilized by dry heat for 1 h at 140 ^{0}C. The sterile discs previously soaked in a known concentration of the test compounds were placed in nutrient agar medium. Solvent and growth controls were also kept. Chloramphenicol was used as a positive control while the disc poured in DMSO was used as a negative control. The plates were inverted and incubated at 37 ^{0}C for 24 h. The susceptibility was assessed on the basis of diameter of zone of inhibition against different strains of bacteria. Inhibition zones were measured and compared with standard drug. Minimum inhibitory concentration (MIC) which is defined as the lowest concentration of an antibacterial drug that will inhibit the visible growth of a micro-organism after overnight incubation was determined by broth dilution technique. The nutrient broth which contained logarithmic serially two fold diluted amount of test compound and controls were inoculated with approximately 5 ×10^5 c.f.u. mL^{-1} of actively dividing bacterial cells. The cultures were incubated at 37 ^{0}C for 24 h and the growth was monitored visually and spectrophotometrically.

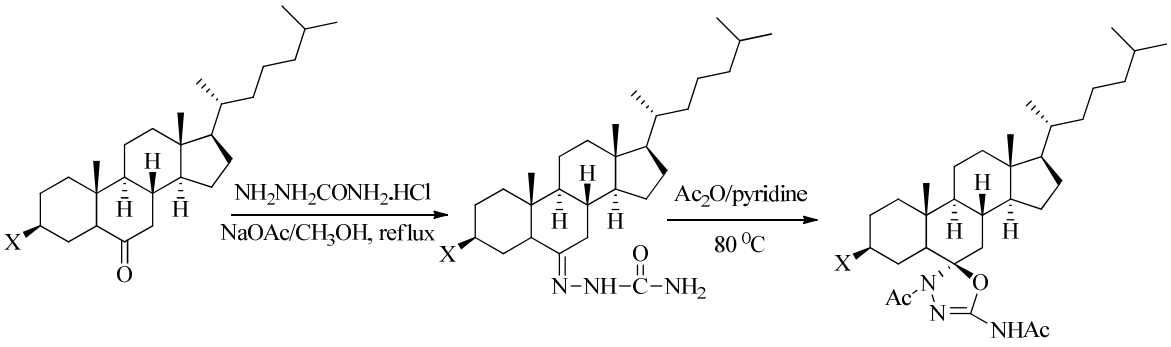
2.3. In vitro antifungal activity

Antifungal screening of steroidal oxadiazolines was done against the cultures of *Candida albicans* (ATCC-10231), *Aspergillus fumigatus* (ATCC-1022), *Trichophyton mentagrophytes* (ATCC-9533) and *Pencillium marneffei* (recultured) in DMSO by agar diffusion method (Shamsuzzaman et al., 2014). Sabouraud agar medium was prepared by dissolving peptone (1 g), D-glucose (4 g) and agar (2 g) in distilled water (100 mL) and adjusting pH to 5.7. Normal saline was used to make a suspension of spore of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 mL saline to get a suspension of corresponding species. 20 mL of agar media was poured into each petri dish. Excess of suspension was decanted and the plates were dried by placing in an incubator at 37 ^{0}C for 1 h. using an agar punch, wells were made and each well was labelled. A control was also prepared in triplicate and maintained at 37 ^{0}C for 3-4 days. Minimum inhibitory concentration (MIC) was determined by broth dilution technique as in antibacterial activity. The Inhibition zones of the synthesized compounds were compared with Nystatin used as standard drug. The nutrient broth which contained logarithmic serially two fold diluted amount of test compound and controls was inoculated with approximately (1.6-6)×10^4 c.f.u. mL^{-1}. The cultures were incubated at 37 ^{0}C for 48 h and the growth was monitored.

3. Results and discussion

3.1. Chemistry

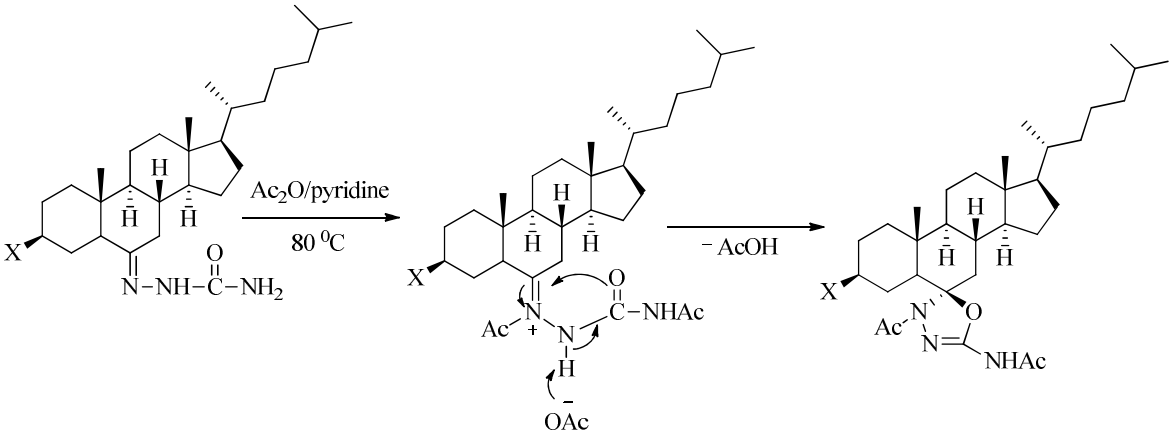
The compounds selected for initial study are 3*β*-acetoxy-5*α*-cholestan-6-one semicarbazone **4**, 3*β*-chloro-5*α*-cholestan-6-one semicarbazone **5** and 5*α*-cholestan-6- one semicarbazone **6** which were synthesized from steroidal ketones (**1-3**), respectively by literature methods (Shamsuzzaman et al., 2004). The convenient synthesis of steroidal (6*R*)-spiro-$\Delta^{2`}$, 1`, 3`, 4`-oxadiazolines (**7-9**) in good yields was carried out by the acetylation of steroidal semicarbazones with acetic anhydride and pyridine at 80 ^{0}C (Alho et al., 2013). The structures of these compounds were characterized by spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and analytical studies. The characterization studies showed good agreement with proposed structures of steroidal oxadiazolines (**7-9**) (**Scheme 1**).



1-3	**4-6**	**7-9**
X	**X**	**X**
OAc	OAc	OAc
Cl	Cl	Cl
H	H	H

Scheme 1. Synthesis of steroidal oxadiazoline derivatives.

In their IR spectra the presence of absorption bands in the range 3340-3343 shows the presence of NH and as the absorption bands at 1680-1690 and 1712-1717 cm^{-1} confirm the presence of CON and CONH groups, respectively in the compounds (**7-9**). The absorption band at 1640-1648 cm^{-1} was ascribed to C=N group. In ^{1}H NMR study the downfield singlet at δ 7.2-7.29 was ascribed to NH of oxadiazoline ring. In ^{13}C NMR study, the signals at δ 172.9-173.5, 170.7-171.9 and 153.9-154.8 confirm the presence of CONH, CON and C=N groups, respectively. Finally the presence of distinct molecular ion peak [$M^{+\cdot}$] at m/z: 585, 561/563 and 527 in the MS also proved the formation of compounds (**7-9**). The tentative mechanism has been proposed for the formation of steroidal oxadiazolines (**7-9**) from steroidal semicarbazones (**4-6**) which starts by N-acylation of the C=N group, with development of a positive charge localized between the C and N atoms ($C=N^+Ac \leftrightarrow C^+ -NAc$). The ring closure is then affected by attack of the oxygen at the cationic center assisted by acetic anhydride leads to the formation of corresponding products (**Scheme 2**).



Scheme 2. A tentative mechanism for the synthesis of steroidal oxadiazolines **7-9**.

It is proposed that there is a considerable amount of steric hindrance to ring-closure from one side of the ring at C-6, which might be explained on the basis that the NAc group is bulkier than oxygen atom, therefore oxadiazoline ring closes at C-6 by the attack of oxygen of the semicarbazone moiety, preferentially from the front (*β*-axial) side so that the bulky NAc group has an equatorial (*α*) orientation to avoid 1,3-diaxial interaction due to the C-10 angular methyl group that results minimum steric hindrance and maximum stability. Thus the only product of this reaction, the oxadiazoline has *R* stereochemistry at C-6. The dreiding models also suggest the attack of oxygen from the *β*-side which pushes the bulkier NAc group to the less hindered *α*-side. Therefore the formulation of the compound as 6*R* is preferred over its isomer (6*S*) (Shamsuzzaman et al., 2004). On the basis of these models it is suggested that the same should also be kinetically favorable. The strategy can also be applied to diverse semicarbazones, in that way oxadiazolines may also allow further modifications on the substituted heterocyclic systems.

3.2. Antimicrobial evaluation

In the context of our studies, steroidal (6*R*)-spiro-$\Delta^{2`}$, 1`, 3`, 4`-oxadiazolines derivatives **7-9** were screened for their *in vitro* antimicrobial activities against Gram-positive, Gram-negative bacterial strains as well as fungus. Chloramphenicol was used as standard drug for bacteria and Nystatin was used as standard drug for fungi. It is clear from the antimicrobial screening data **Tables 1**-**4** that the synthesized compounds showed moderate to good antimicrobial activity. Compound **7** was found to be more potent than the reference drug, Chloramphenicol, in the case of *E. coli* while compound **8** was found almost equally potent antifungal agent against *P. marneffei* in comparison with the reference drug, Nystatin.

Table 1. Showing the zone of inhibition of steroidal oxadiazolines **7-9** with different bacterial strains.

	Zone of inhibition (mm)			
Comp.	*S. aureus*	*S. pyogenes*	*P. aeruginosa*	*E. coli*
7	19.3±0.2	17.6±0.4	20.5 ±0.5	22.1 ±0.1
8	14.1±0.4	12.2±0.8	19.2 ±0.4	12.2 ±0.5
9	17.2±0.2	16.6±0.4	18.7±0.4	17.4 ±0.6
Chloramphenicol	21.6±0.5	22.5±0.4	25.2 ±0.8	20.0 ±0.2
DMSO	-	-	-	-

Table 2. Showing the MIC's of the synthesized compounds with different bacterial strains.

Strains	MIC (µg/mL)			
	7	**8**	**9**	Chloramphenicol
S. aureus	32	32	128	32
S. pyogenes	32	64	64	32
P. aeruginosa	64	64	32	32
E. coli	32	128	128	32

Table 3. Showing the zone of inhibition of steroidal oxadiazolines **7-9** with different fungal strains.

Comp.	Zone of inhibition (mm)			
	C. albicans	*T. mentagrophytes*	*P. marneffei*	*A. fumigatus*
7	25.1± 0.1	19.4± 0.6	16.6± 0.3	15.1±0.4
8	18.1± 0.2	18.2± 0.4	18.4± 0.5	11.4±0.2
9	21.2± 0.2	14.2± 0.5	15.8± 0.2	12.0±0.5
Nystatin	29.0± 0.5	29.0± 0.5	19.5± 0.5	19.5±0.5
DMSO	–	–	–	–

Table 4. Showing the MIC's of the synthesized compounds **7-9** with different fungal strains.

Strains	MIC (µg/mL)			
	7	**8**	**9**	Nystatin
C. albicans	32	64	64	32
T. mentagrophytes	64	64	128	32
P. marneffei	128	32	32	32
A. fumigatus	32	32	64	32

The biological behavior of these compounds revealed that chloro- and acetoxy-substituents on the 3*β*-position of the steroidal oxadiazoline ring increased the antibacterial activity (Shamsuzzaman et al., 2010).This study also supports that oxadiazoline moiety after being attached with steroid nucleus might be one of the factors responsible for enhanced antimicrobial behavior.

4. Conclusions

In summary, the successfully developed, convenient and operationally simple strategy for a better synthesis of steroidal oxadiazolines involves the reaction of steroidal semicarbazone with acetic anhydride and pyridine in chloroform. The reaction completed in 9-10 h and on completion, better yields (71-74%) were obtained. During the antimicrobial screening, Compound **7** showed potential behavior against *E. coli* (being more active than the reference drug, Chloramphenicol) in antibacterial activity while the compound **8** was found almost equally potent as the reference drug, Nystatin in the case of *P. marneffei* strain in antifungal activity.

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References

Alho MAM., Baggio R., D'Accorso NB. **(2013)** Cyclization of 1,2:3,4-di-O-isopropylidene-α-D-galacto-1,6-hexodialdo-1,5-pyranose acylhydrazone and semicarbazone. *ARKIVOC* 129-138.

Bandey AH., Zargar IM and Ganaie, BA. **(2011)** Synthesis and antimicrobial studies of chalconyl pregnenolones. *Steroids* 76:1358-1362.

Brown BJ., Clemens IR and Neesom JK. **(2000)** Diisopropylcarbodiimide: A novel reagent for the synthesis of 1,3,4-oxadiazoles on solid-phase. *Synlett,* 131-133.

Dogan HN., Duran A., Rollas S., Sener G., Armutak Y and Keyer-Uysal, M. **(1998)** Synthesis and structure elucidation of some new hydrazones and oxadiazolines: anticonlsant activitites of 2-(3- acetyloxy-2-naphtyl)-4-acetyl-5-substituted-1,3,4-oxadiazolines. *Med. Sci. Res*. 26:755-758.

Ergun Y., Orhan, FO., Ozer, UG and Gisi G. **(2010)** Synergistic effect of [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one] and antidepressant drugs in the mouse forced swimming test: Possible involvement of serotonergic pathway. *Eur. J. Pharmacol*. 630: 74-78.

Polshettiwar V and Varma RS. **(2008)** Greener and rapid access to bio-active heterocycles: one-pot solvent-free synthesis of 1,3,4-oxadiazoles and 1,3,4-thiadiazoles.*Tetrahedron Lett*. 49:879-883.

Rane AR., Bangalore P., Borhade SD and Khandare, PK. **(2013)** Synthesis and evaluation of novel 4-nitropyrrole-based 1,3,4-oxadiazole derivatives as antimicrobial and anti-tubercular agents. *Eur. J. Med. Chem.* 70:49-58.

Savage PB. **(2002)** Cationic Steroid Antibiotics. *Curr. Med. Chem.* 1:293-304.

Shamsuzzaman, Khan MS., Alam M., Tabassum Z., Ahmad A., Khan AU. **(2010)** Synthesis, antibacterial and antifungal activities of 6, 5 fused steroidal oxazoles in cholestane series. *Eur. J. Med. Chem.* 45:1094-1097.

Shamsuzzaman, Mashrai A., Ahmad A., Dar AM., Khanam H., Danishuddin M., Khan AU. **(2014)** Synthesis, evaluation and docking studies on steroidal pyrazolones as anticancer and antimicrobial agents. *Med. Chem. Res,* 23:348-362.

Shamsuzzaman, Siddique N and Salim A. **(2004)** Stereoselective synthesis of steroidal (3*R*)-spiro-$\Delta^{2'}$, 1', 3', 4'- oxadiazolines *Indian J. Chem.* 43B:410-412.

Wang Z., Wang M., Yao X., Li Y., Qiao W., Geng Y., Liu Y and Wang Q. **(2012)** Hydroxyl may not be indispensable for raltegravir: Design, synthesis and SAR studies of raltegravir derivatives as HIV-1 inhibitors. *Eur. J. Med. Chem.* 50:361-369.