Journal of Saudi Chemical Society (2012) **16**, 7–11

King Saud University

Journal of Saudi Chemical Society

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ORIGINAL ARTICLE

Synthesis and biological evaluation of new oxime-ether derivatives of steroid as anti-bacterial agents ☆

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Received 24 June 2010; accepted 11 October 2010
Available online 20 October 2010

KEYWORDS

Cholesterol;
Oxime-ether;
Anti-bacterial activity;
Chloramphenicol

Abstract Various oxime-ether derivatives of cholesterol have been synthesized by the alkylation of the steroidal oxime with 1-(2-chloroethyl) pyrrolidine hydrogen chloride/chloroethylamine hydrochloride in the presence of sodium methoxide in dry methanol. The structures of these compounds were elucidated by IR ^{1}H NMR, FAB mass spectroscopic methods and elemental analyses. The anti-bacterial activity was first tested *in vitro* by the disk diffusion method against two Gram-positive and two Gram-negative bacteria, and then the minimum inhibitory concentration (MIC) of compounds were determined. The results showed that the chloro derivatives exhibited better anti-bacterial activity than the standard drug chloramphenicol.

1. Introduction

The tissue-lying *Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhimurium* and *Escherichia coli* causes food poisoning, rheumatic, salmonellosis and diarrhea; these are the second leading cause of death from bacterial disease worldwide (Qadri et al., 2005). More than 50 million people worldwide are infected and up to 150,000 die every year due to these bacterial infections (Zhang et al., 2006). Amoxicillin, norfloxacin, chloramphenicol, ciprofloxacin are the most common drugs used for these bacterial infections but are associated with severe side effects (Puertoa et al., 2006). Toxicity and resistance to the drugs also play important role in the treatment failure (Dore and Lacroix, 1987). Therefore; there is an urgent need to screen new compounds for the development of new anti-bacterial agents. The study of oxime-ether derivatives have become of much interest in recent years on account of their antiprotozoan (Brain et al., 1989), anti-bacterial (Chern

☆ A new series of steroidal oxime-ether derivatives were synthesized. The anti-bacterial activity of these compounds was determined with reference to standard drug chloramphenicol.

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Peer review under responsibility of King Saud University.
doi:10.1016/j.jscs.2010.10.013

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et al., 2004), antiretroviral (Emami et al., 2004), antifungal (Jindal et al., 2003), antineoplastic (Karakurt et al., 2001) and antimicrobial activities (Prabhu et al., 1981). Different oxime-ether derivatives have also been reported to possess anticholinergic (Toshio et al., 1980), insecticidal (George et al., 1984) and acaricidal activities (Nolan et al., 1979). In view of these observations and in continuation of our work on synthesis and biological activity of steroidal molecules, it was considered of interest to synthesize some new chemical entities incorporating the active pharmacophores in a single molecular frame work and to evaluate their biological activities. In this paper we have synthesized oxime-ethers by the reaction of steroidal oxime (1, 2, 3) with chloroethylamine hydrochloride/with 1-(2-chloroethyl) pyrrolidine hydrogen chloride. The activities of these compounds were screened *in vitro* against of bacterial strains.

2. Experimental

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 spectrometries. 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 – multiplet. 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). Anhydrous sodium sulfate was used as a drying agent for the organic phase. Compounds **1**, **2a**, **3a**, **1b**, **2b**, **3b**, **1c**, **2c**, **3c**, **1**, 2 and **3** were prepared according to published methods (Khan et al., 2008).

*2.1. General procedure for the syntheses of the steroidal oxime-ether (**4**–**9**)*

To a solution of steroidal oxime **(1–3)** (1.09 mmol) in methanol (10 ml) was added chloroethylamine hydrochloride/1-(2-chloroethyl) pyrrolidine hydrochloride/(1.33 mmol) at room temperature followed by a freshly prepared solution of sodium methoxide (0.18 ml) drop wise. The mixture was refluxed for 1 h. The completion of reaction was detected by TLC. The reaction mixture was cooled, precipitate was collected by filtration, washed with water, air dried and residue obtained was purified by column chromatography (20:80, diethyl ether:petroleum ether). The compounds were recrystallized with methanol.

*2.2. 3β-Acetoxy-6-(2′-aminoethoxyimino)-5α-cholestane (**4**)*

Yields: 90.05; Mp: 183 °C; *Anal.* Calc. for $C_{31}H_{54}N_2O_3$: C, 74.06; H, 10.82; N, 5.57. Found: C, 74.05; H, 10.84; N, 5.58%. IR (KBr) ν_{max}cm^{-1}: 3472 (NH_2), 1736 ($OCOCH_3$), 1661 (C═N), 1389 (N–O), 1298 (C–O), 1225 (C–N); ^{1}H NMR ($CDCl_3$) δ_H: 4.6 (br, m, 1H, J = 18 Hz, C3α-H, axial), 3.82 (t, 2H, J = 6.0 Hz, OCH_2), 3.26 (m, 2H, NCH_2), 2.8 (s, 2H, CH_2NH_2), 2.20 (s, $OCOCH_3$), 0.72 (s, 3H, 13-CH_3), 0.98 (s, 3H, 18-CH_3), 1.12 (s, 3H, 10-CH_3), and 0.84 (s, 3H, 19-CH_3); Mass spectra ($M^{+\cdot}$) at m/z 503, 444 (M–$OCOCH_3$), 487 (M–NH_2), 473 (M–NH_2CH_2), 459 (M–$CH_2CH_2NH_2$), 453 (M–$OCH_2CH_2NH_2$), 390 (M–side chain).

*2.3. 3β-Chloro-6-(2′-aminoethoxyimino)-5α-cholestane (**5**)*

Yields: 70.0%; Mp: 170 °C; *Anal.* Calc. for $C_{29}H_{51}N_2OCl$: C, 72.69; H, 10.73; N, 5.80. Found: C, 73.65; H, 10.72; N, 5.88%. IR (KBr) ν_{max}cm^{-1}: 3465 (NH_2), 1666 (C═N), 1382 (N–O), 1284 (C–O), 1223 (C–N), 724 (C–Cl); ^{1}H NMR ($CDCl_3$) δ_H: 4.3 (br, m, 1H, J = 17 Hz, C3α-H, axial), 3.68 (t, 2H, J = 6.0 Hz, OCH_2), 3.38 (m, 2H, NCH_2), 2.5 (s, 2H, CH_2NH_2), 0.74 (s, 3H, 13-CH_3), 0.96 (s, 3H, 18-CH_3), 1.14(s, 3H, 10-CH_3), and 0.85 (s, 3H, 19-CH_3); Mass spectra ($M^{+\cdot}$) at m/z 480, 464 (M–NH_2), 450 (M–NH_2CH_2), 436 (M–$CH_2CH_2NH_2$), 430 (M–$OCH_2CH_2NH_2$), 367 (M–side chain), 444 (M–Cl).

*2.4. 6-(2′-Aminoethoxyimino)-5α-cholestane (**6**)*

Yields: 79.80%; Mp: 178 °C; *Anal.* Calc. for $C_{29}H_{52}N_2O$: C, 78.32; H, 11.78; N, 6.29. Found: C, 78.30; H, 11.78; N, 6.39%. IR (KBr) ν_{max}cm^{-1}: 3342 (NH_2), 1655 (C═N), 1400 (N–O), 1225 (C–O), 1228 (C–N); ^{1}H NMR ($CDCl_3$) δ_H: 3.95 (t, 2H, J = 6.0 Hz, OCH_2), 3.42 (t, 2H, NCH_2), 2.54 (s, 2H, CH_2NH_2), 0.74 (s, 3H, 13-CH_3), 0.96 (s, 3H, 18-CH_3), 1.14(s, 3H, 10-CH_3), and 0.85 (s, 3H, 19-CH_3); Mass spectra ($M^{+\cdot}$) at m/z 445, 429(M–NH_2), 415 (M–NH_2CH_2), 401 (M–$CH_2CH_2NH_2$), 395 (M–$OCH_2CH_2NH_2$), 332 (M–side chain), 444 (M–Cl).

*2.5. 3β-Acetoxy-6-(2-pyroolidino ethoxy imino)-cholestane (**7**)*

Yields: 72%; Mp: 164–166 °C; *Anal.* Calc. for $C_{35}H_{60}N_2O_3$: C, 75.53; H, 10.79; N, 5.03. Found: C, 75.42; H, 10.72; N, 5.01%. IR (KBr) ν_{max} cm^{-1}: 1735 ($OCOCH_3$), 1660 (C═N), 1375 (N–O), 1322 (C–O), 1262 (C–N). ^{1}H NMR ($CDCl_3$) δ_H: 4.72 (br, m, 1H, J = 18 Hz, C3α axial), 4.21 (t, 2H, J = 6.0 Hz –OCH_2), 3.50 (t, 2H, –NCH_2), 2.89 (m, 8H, N-methylenes of pyrrolidino functionality), 1.07 (s, 3H, 10-CH_3), 0.96 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3) and 0.68 (s, 3H, 13-CH_3); Mass spectra ($M^{+\cdot}$) at m/z 557, 498 (M–AcO), 487 (M–NC_4H_8), 459 (M–$C_6H_{12}N$), 444 (M–side chain), 429 (M–$C_6H_{12}N_2O$).

*2.6. 3β-Chloro-6-(2-pyrrolidino ethoxy imino)-cholestane (**8**)*

Yields: 78%; Mp: 154–156 °C; *Anal.* Calc. for $C_{33}H_{57}N_2OCl$: C, 74.29; H, 10.69; N, 5.25. Found: C, 74.24; H, 10.65; N, 5.23%. IR (KBr) ν_{max} cm^{-1}: 1654 (C═N), 1383 (N–O), 1262 (C–O), 715 (C–Cl). ^{1}H NMR ($CDCl_3$) δ_H: 4.52 (br, m, 1H $w1/2$ = 17 Hz, C3α-H, axial), 4.15 (t, 2H, –OCH_2), 3.84 (t, 2H, –NCH_2), 2.67 (8H, m, N-methylenes of pyrrolidino functionality), 1.12 (s, 3H, 10-CH_3), 0.93 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3) and 0.69 (s, 3H, 13-CH_3); Mass spectra ($M^{+\cdot}$) at 534, 498 (M–Cl), 464 (M–NC_4H_8), 436 (M–NC_6H_{12}), 421 (M–side chain), 406 (M–$N_2C_6H_{12}O$).

2.7. 6-(2′-Pyroolidino ethoxy imino)-5α-cholestane (**9**)

Yields: 78%; Mp: 160 °C; *Anal*. Calc. for $C_{33}H_{58}N_2O$: C, 79.51; H, 11.64; N, 5.62. Found: C, 78.49; H, 5.58; N, 5.61%. IR (KBr) $\nu_{max}cm^{-1}$: 1653 (C═N), 1419 (N–O), 1282 (C–N); ^1H NMR ($CDCl_3$) δ_H: 4.42 (t, 2H, J = 6.2 Hz, $-OCH_2$), 3.62 (t, 2H, $-NCH_2$) 2.58 (m, 8H, N-methylenes of piperidino functionality), 1.10 (s, 3H, 10-CH_3), 0.91 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3) and 0.68 (s, 3H, 13-CH_3); Mass spectra ($M^{+\cdot}$) at m/z 499, 429 (M–C_4H_8N), 401 (M–$C_6H_{12}N$), 386 (M–side chain), 371 (M–$C_6H_{12}N_2O$).

2.8. Organism culture and in vitro screening

Anti-bacterial activity was done 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 °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. About 1 mg of each test compound was dissolved in 100 µL DMSO to prepare stock solution from stock solution different concentration 10, 20, 25, 50, and 100 µg/µL of each test compound were prepared. These compounds of different concentration were poured over disk plate onto it. Chloramphenicol (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 °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 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. Tests using DMSO and chloramphenicol as negative and positive controls (Scheme 1).

3. Results and discussion

3.1. Chemistry

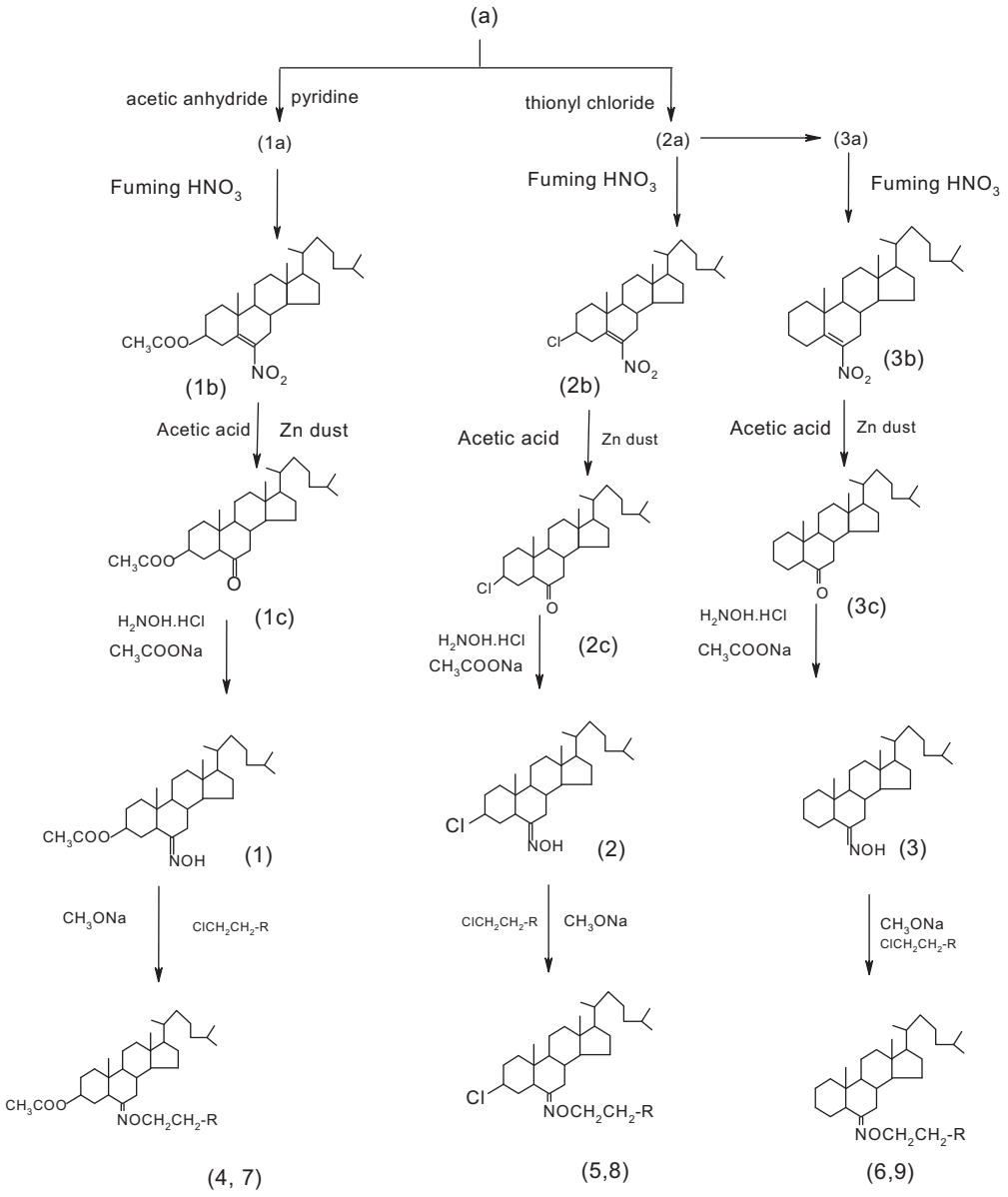
The oxime-ether derivatives were synthesized using literature procedure (Abid and Azam, 2006) and obtained the desired oxime product in 70–90% yields. All the six compounds were prepared by refluxing 1:1.2 M ratio of steroidal oxime, with chloroethylamine hydrochloride/1-(2-chloroethyl) pyrrolidine hydrogen chloride in the presence of sodium methoxide in dry methanol. The obtained compounds are stable in the solid as well as in the solution state. Characteristic IR bands provide significant indications for the formation of steroidal oxime-ether derivatives. The compounds **(4–9)** showed intense band in the region 1653–1666 cm^{-1} due to the ν(C═N) stretch. IR spectra of all the compounds showed ν(N–O) stretch at 1375–1419 cm^{-1}. In addition, the absorption band at 1225–1322 cm^{-1} was attributed to the ν(C–O) stretch vibration, which confirms the formation of desired oxime-ether. The structure of the oxime-ether derivative was further confirmed by ^1H NMR spectra, which proves as a diagnostic tool for the positional elucidation of the proton. Assignments of the

Table 1 Antibacterial activity of steroidal oxime-ether derivatives, positive control chloramphenicol (Chlora.) 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*
4	17.0 ± 0.4	16.5 ± 0.4	17.5 ± 0.2	18.8 ± 0.5
5	18.5 ± 0.4	19.5 ± 0.2	21.5 ± 0.3	22.4 ± 0.4
6	14.5 ± 0.4	13.6 ± 0.2	12.5 ± 0.4	13.5 ± 0.4
7	15.3 ± 0.5	16.5 ± 0.4	14.5 ± 0.4	16.5 ± 0.4
8	19.3 ± 0.2	19.8 ± 0.4	22.5 ± 0.6	21.4 ± 0.4
9	13.4 ± 0.4	14.2 ± 0.5	14.2 ± 0.2	12.4 ± 0.5
(Chlora.)	17.0 ± 0.5	18.2 ± 0.4	17.2 ± 0.8	20.0 ± 0.2
DMSO	–	–	–	–

Table 2 Minimum inhibition concentration (MIC) of steroidal oxime-ether derivatives, positive control: chloramphenicol.

MIC (µg mL^{-1})	Compounds						Positive
Strain	4	5	6	7	8	9	Control
Staphylococcus aureus	32	32	64	64	32	128	32
Streptococcus pyogenes	64	32	128	64	32	128	32
Salmonella typhimurium	64	32	128	32	64	512	32
Escherichia coli	128	32	512	128	32	128	32



Scheme 1.Showing the synthesis of Oxime ether 4-9, compound no. 4-6 R= NH_2

compound no. 7-9 R=

Compound (a): Cholesterol (1a) =3β-Acetoxycholest-5-ene, (2a) = 3β-Chlorocholest-5-ene and (3a) = Cholest-5ene

Scheme 1 Schematic diagram showing the synthesis of steroidal oxime-ether derivatives.

signals are based on chemical shift and intensity pattern. The ^{1}H NMR spectra showed two triplets in the region 3.68–4.42 ppm for (O–CH_2), and 3.26–3.84 ppm for CH_2N proton, respectively, details are given in the Section 2. Characteristic peaks were observed in the mass spectra of all compounds which followed the similar fragmentation pattern. The spectrum of compound **4** showed molecular ion peak ($M^{+\cdot}$) at *m*/*z*-503. The characteristics peaks observed within the mass spectra of oxime-ether derivatives are given in Section 2.

3.2. Pharmacology

3.2.1. Antimicrobial activity

The *in vitro* antimicrobial activity was performed using the disk diffusion method and the Minimum Inhibitory Concen-

tration (MIC) method. Chloramphenicol was used as positive controls for bacteria.

3.3. Disk diffusion and micro dilution assay

The compounds (**4**–**9**) were tested for their anti-bacterial activities by disk-diffusion method [22] using nutrient broth medium [contained (g/L): beef extract 3 g; peptone 5 g; pH 7.0]. The Gram-positive bacteria and Gram-negative bacteria utilized in this study consisted of *S. aureus, S. pyogenes, S. typhimurium* and *E. coli*. In the disk-diffusion method, sterile paper disks (0.5 mm) impregnated with compound dissolved in dimethylsulfoxide (DMSO) at concentration 100 μg/mL were used. Then, the paper disks 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 zones are shown in Table 1. The oxime-ether 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 anti-bacterial 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 a more efficacious drug against bacteria for which a thorough investigation regarding the structure–activity relationship, toxicity and in their biological effects which could be helpful in designing more potent anti-bacterial agents for therapeutic use.

4. Conclusions

This research involves the synthesis of oxime-ether derivatives of cholesterol. The anti-bacterial activity of these compounds was examined using culture of bacteria and results showed that the chloro and acetoxy substituents on the 3β-position of the steroidal oxime-ether increased the anti-bacterial activity. Among the entire six compounds chloro derivative showed better anti-bacterial activity than the respective drug chloramphenicol.

Acknowledgement

The Authors would like to thank the Chemistry Department, Faculty of Science, King Abdul Aziz University for providing the research facilities.

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