



Full paper/Mémoire

Solvent-free synthesis of novel unsymmetric chenodeoxycholic acid bis thiocarbazone derivatives promoted by microwave irradiation and evaluation of their antibacterial activity

Zhichuan Shi, Zhigang Zhao*, Min Liu, Xiaohong Wang

College of Chemistry and Environmental Protection Engineering, Southwest University for Nationalities, Chengdu 610041, PR China

ARTICLE INFO

Article history:

Received 6 March 2013

Accepted after revision 17 May 2013

Available online 5 July 2013

Keywords:

Thiocarhydrazone

Microwave irradiation

Antibacterial activity

Steroid

Thiocarbazon

Solvent-free conditions

ABSTRACT

Eleven new non-symmetric chenodeoxycholic acid bis thiocarbazones were efficiently synthesized via the condensation of steroidal diketones (methyl (5 β)-3,7-dioxocholan-24-oate) and substituted benzaldehyde thiocarbohydrazones by means of microwave irradiation. Compared with the conventional heating method, the yields were increased from 31–58% to 80–89% and the reaction times were reduced from 420–520 min to 5.0–7.5 min. The structures of these novel compounds were characterized by spectral data and elemental analysis. Four compounds (**7a**, **7c**, **7f** and **7g**) possess excellent activity against *Bacillus subtilis* and *Staphylococcus aureus*.

© 2013 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

1. Introduction

The steroid system has been a prime focus of research throughout scientific history and displays a variety of biological functions [1–4]. Steroidal compounds, widely used in traditional medicines, exhibit a wide range of activities, including anticancer and antibacterial [5,6], and have the potential to be developed as drugs for the treatment of a large number of diseases. They have also become the basis of many important discoveries in organic chemistry. Recently, steroidal building blocks have attracted interest with a view to modifying steroidal molecules [7,8]. Their preparation is a stimulating challenge to organic chemists, often demanding the development of new and generally useful reactions. These compounds are often non-toxic, capable of overcoming multi-drug resistance, and highly bioavailable because of

their capacity of penetrating the cell wall. Several steroidal derivatives have been investigated as new curative agents for cancer, and exhibited diverse biological activities, such as antimicrobial, anti-inflammatory, anticancer ones [9–11].

The biological activity of Schiff bases containing thione (C=S) was exploited in the 1960s using *N*-methylisatin- β -thiosemicarbazone, which was effective against smallpox and vaccinia [12,13]. 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone is now being evaluated in clinical trials against several malignancies [14]. Compounds containing thione (C=S) groups occupy an important position among organic reagents. Organic compounds with this structure are known to display a wide range of pharmacological activity, including anticancer, antibacterial and antifungal effects [15–18]. A good deal of research has been carried out on the potential of coordination chemistry of thiocarbohydrazones, and thiocarbohydrazide attached to carbonyl compounds having heterocyclic moiety to give potential drugs [19,20]. Further optimization of this compound is required to improve its antimicrobial activity.

* Corresponding author.

E-mail address: zgz63129@163.com (Z. Zhao).

Although there are many reports based on steroidal units' modification, including derivatization, cyclization [21], there are few reports about steroidal Schiff base. Steroid is known for its abundance and importance in biological systems, which is expected to show relatively low toxicity, augmented biocompatibility [22]. Furthermore, thiourea and its related analogues have become the focus of interest in recent years on account of their pharmacological activities. According to the active principle of superposition. We hoped that thiosemicarbazone derivatives containing steroid unit and thiourea group have potent antibacterial activity. In addition, microwave synthesis is a well-established technique in green chemistry and has many advantages compared with the traditional methods, such as faster reaction times, higher yields; moreover, it is a solvent-free method [23–26]. It was being applied in many organic synthesis. Accordingly, as a further development of our green agenda [27–29], herein we report a microwave-mediated, efficient, high yield and environmentally friendly synthesis of novel steroidal bis thiocarbazon derivatives under solvent-free conditions (Scheme 1) as well as an evaluation of the antibacterial activities of these compounds against four pathogenic strains, *Escherichia coli* (ATCC 35218), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 6538).

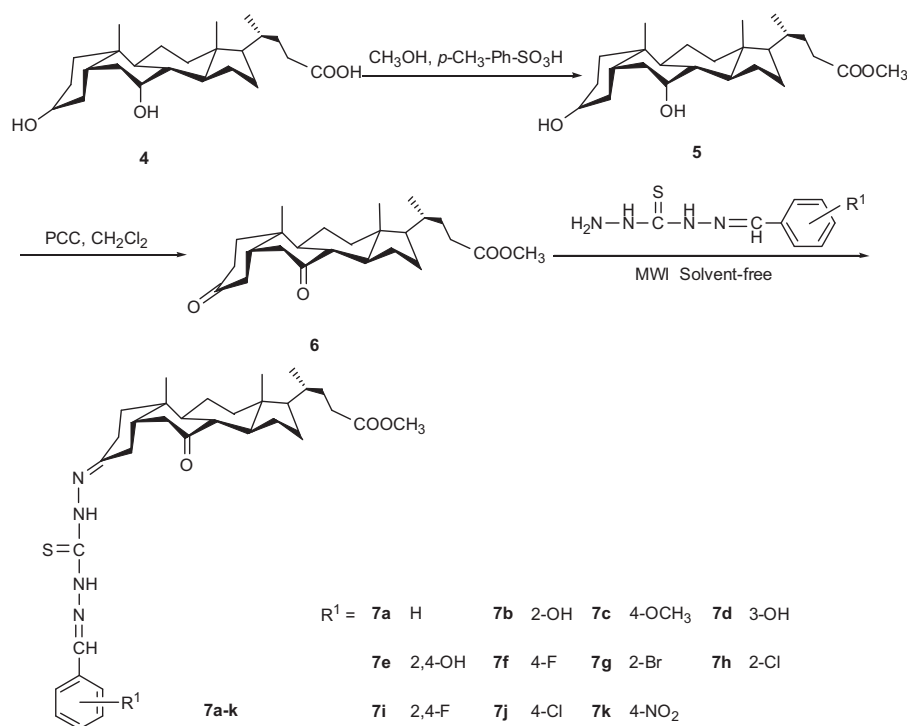
2. Result and discussion

It is known that a carbonyl group at C-3 of steroidal scaffold is more susceptible to nucleophilic addition

reactions than a carbonyl at C-7 [30]. In our quest to find optimal reaction conditions, we studied steroidal diketones with substituted benzaldehyde thiocarbonyhydrazones, employing conventional and microwave-mediated solvent-free thermal procedures. The conventional reaction was conducted with or without catalysts, such as HCl, acetic acid or H_2SO_4 by refluxing in ethanol, acetic acid, methanol, THF, DMF, or DMSO. The result showed that the yield for **7a** was in the range 24–52% (Table 1). But it required a longer reaction time. So, the catalytic properties of HCl have been studied considering the synthesis of compound **7a**.

We then studied the effect of various supports, such as silica gel H, neutral Al_2O_3 and K_2CO_3 with or without HCl catalyst for the synthesis of compound **7a** using the microwave-assisted method. Among the results obtained, the use of HCl in neutral Al_2O_3 received the best yield (84%) (Table 1). Finally, we evidenced a dramatic improvement in the yield and the reaction time (5.5 min) when employing a microwave-assisted method. The microwave-assisted procedure was then applied to the synthesis of compounds **7b–k**.

In order to study the advantages of the microwave-promoted reaction, we compared the synthesis of compounds **7a–k** using conventional heating and the microwave-assisted method under solvent-free conditions. Compared to the conventional thermal heating, the microwave irradiation technique decreased the reaction time from 420–540 min to 5.0–7.5 min and increased the yields from 31–58% to 80–89% (Table 2). Consequently, the use of the microwave technology in conjunction with solvent-free conditions allows



Scheme 1. Synthetic protocol of titled compounds **7a–k**.

Table 1
Effect of the reaction conditions on the synthesis of compound **7a**.

Entry	Solvent	Catalyst	Support	Time (min)	Yield (%)
1	EtOH	–	–	420	24 ^a
2	EtOH	HCl	–	420	52 ^a
3	EtOH	acetic acid	–	420	43 ^a
4	EtOH	H ₂ SO ₄	–	420	39 ^a
5	Acetic acid	–	–	420	40 ^a
6	THF	HCl	–	420	42 ^a
7	MeOH	HCl	–	420	48 ^a
8	DMF	–	–	420	32 ^a
9	DMSO	–	–	420	36 ^a
10	–	–	Silica gel H	5.5	53 ^b
11	–	HCl	Silica gel H	5.5	61 ^b
12	–	–	Neutral Al ₂ O ₃	5.5	32 ^b
13	–	HCl	Neutral Al ₂ O ₃	5.5	84 ^b
14	–	–	K ₂ CO ₃	5.5	12 ^b
15	–	HCl	K ₂ CO ₃	5.5	40 ^b

^a Conventional method.^b Microwave method.

expeditious, environmentally friendly and efficient procedures in organic methodology.

Assignments of selected characteristic IR bands of the steroidal bis thiocarbazon derivatives (**7a–k**) provided useful information about the structure of the compounds. All the compounds exhibited a characteristic strong absorption in the region 3229–3309 cm^{−1} due to the ν (N–H) stretching vibration. Intense absorption bands in the regions 1153–1171 cm^{−1} and 1503–1533 cm^{−1} were attributed to the ν (C=S) and ν (C=N) stretching vibrations, which also confirms the formation of the desired thiocarbazones in all the compounds. All compounds showed a sharp peak at 1731–1735 cm^{−1}, attributable to the ester carbonyl and another sharp peak at 1707–1709 cm^{−1}, due to the steroidal ketones carbonyl (C-7), which also confirms the formation of the mono-substituted compounds. Further evidence of the structure of steroidal bis thiocarbazones was obtained from the ¹H NMR spectra. The signal between δ 8.59 and δ 9.27 ppm can be assigned to the protons of the NHN. The signals due to the other NH protons were observed at δ 8.56–9.45 ppm. In addition, the singlet peaks at 0.64, 1.17–1.21 ppm and the doublet at 0.82–0.89 ppm were characteristics of the methyl group of the steroidal structure.

The singlet at 3.58 ppm was assigned to the protons of COOCH₃. Mass spectra showed the expected molecular peaks at high intensity.

The preliminary results of antibacterial assays indicated that some of the compounds exhibited a moderate to good activity against Gram-negative bacterium (*P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *B. subtilis*). As shown in Table 3, compounds **7b**, **7i–k** exert no potent inhibition activity against any of the bacterial strains tested. Notably, compounds **7d**, **7e** and **7h** were effective against the Gram-positive *S. aureus* and compounds **7a**, **7c**, **7f** and **7g** were significantly more active against *S. aureus* than the standard reference drug, amoxicillin. The minimal inhibitory concentration (MIC) values were 0.5–2 μ g/mL (**7a**, **7c**, **7f** and **7g**) and 16 μ g/mL (**7d**, **7e** and **7h**). The activities of compounds **7a**, **7c**, **7d**, **7f** and **7g** were found similar to that of amoxicillin against *B. subtilis*. Most of the compounds showed poor activity against the Gram-negative *E. coli* and *P. aeruginosa*. A rapid glance at Table 4 shows that the methoxy derivative **7c** was effective at inhibiting Gram-positive *S. aureus* and *B. subtilis*. These results reveal that the electron-donating substituent group in these compounds plays a vital role in the inhibitory activity towards Gram-positive bacteria

Table 2
Synthesis of compounds **7a–k** using microwave irradiation or conventional method.

Compound	R ¹	Conventional heating		Microwave method		T _C /T _{MW}
		T (min)	Yield (%)	T (min)	Yield (%)	
7a	H	420	47	5.5	84	76
7b	2-OH	480	49	6.0	86	80
7c	4-OCH ₃	420	58	5.0	89	84
7d	3-OH	540	37	7.0	82	77
7e	2,4-OH	420	41	5.5	85	76
7f	4-F	420	48	5.0	84	84
7g	2-Br	480	39	6.0	82	80
7h	2-Cl	520	34	7.0	80	84
7i	2,4-F	480	39	5.0	86	96
7j	4-Cl	520	31	7.0	84	74
7k	4-NO ₂	520	35	7.5	85	69

T_C: conventional method needs time; T_{MW}: microwave method needs time.

Table 3

Antibacterial activity of compounds **7a–k** (unit, mm).

Compounds	Zone of inhibition (mm) ^{a,b}			
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. aureus</i>
7a	–	–	20.1 ± 0.2	25.0 ± 0.1
7b	–	–	–	–
7c	–	–	25.5 ± 0.1	25.2 ± 0.2
7d	–	–	20.5 ± 0.1	20.5 ± 0.3
7e	–	–	16.1 ± 0.3	20.5 ± 0.2
7f	15.2 ± 0.2	21.1 ± 0.3	20.1 ± 0.2	25.0 ± 0.3
7g	11.9 ± 0.3	–	20.4 ± 0.3	25.0 ± 0.4
7h	11.9 ± 0.4	–	–	20.2 ± 0.5
7i	–	–	–	–
7j	–	–	–	–
7k	–	–	–	–
Amoxicillin	22.9 ± 0.4	23.5 ± 0.2	25.9 ± 0.4	23.5 ± 0.3
Ciprofloxacin	25.3 ± 0.5	31.4 ± 0.4	30.0 ± 0.3	29.6 ± 0.5
DMSO	–	–	–	–

^a Zones of inhibition less than 10 mm are not shown in the table.^b Reference and compounds (40 µg/mL).

strains. Monosubstituted halogen compounds (except **7j**) showed greater antibacterial activity, whereas disubstituted halogen compounds showed weaker antibacterial activity. With respect to *P. aeruginosa*, only compound **7f** showed strong inhibition. On the other hand, only **7f–h** showed moderate inhibitions for *E. coli*.

Further, the determination of MIC and 50% inhibition of bacteria growth (IC₅₀) of the active compounds suggested that the fluorine-containing derivative **7f** showed the maximum activity against most of the strains tested, with MIC of 32 µg/mL for *E. coli*, 8 µg/mL for *B. subtilis*, 16 µg/mL for *P. aeruginosa*, and 1 µg/mL for *S. aureus*. Compounds **7a**, **7c**, **7f** and **7g** demonstrated notable activity, with MIC values ranging from 0.5 to 2 µg/mL and IC₅₀ from 0.28 to 1.1 µg/mL against *S. aureus*, showing their potential to become a drug against the drug-resistant *S. aureus*. Compound **7c** was as potent as the standard reference drug amoxicillin against *B. subtilis*. The importance of this work lies in the possibility that the new compounds might be more effective against bacteria. A thorough investigation regarding the structure–activity relationship, toxicity and the biological effects may allow

the design of more potent antibacterial agents for therapeutic use.

3. Conclusion

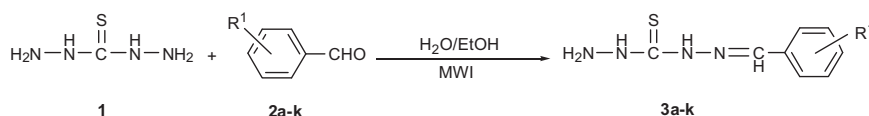
In conclusion, a microwave-assisted solvent-free synthetic procedure in the presence of neutral aluminum oxide was developed for the preparation of asymmetric chenodeoxycholic acid bis thiocarbazones. The present method has many advantages compared to the conventional method, such as shorter reaction times, high yields and compliance with green chemistry protocols. Compound **7c** exhibited promising antibacterial activity similar to that of the standard drug amoxicillin against *B. subtilis*, indicating that this compound is a promising antibacterial compound for further research. Compounds **7d**, **7e** and **7h** exhibited potency similar to that of amoxicillin against Gram-positive *S. aureus*, and compounds **7a**, **7c**, **7f** and **7g** were found to be very promising and almost comparable to amoxicillin, indicating that they can serve as important pharmacophores for the design of new leading antibacterial agents. Studies to

Table 4

Antibacterial activity of compounds **7a–k**.

Compounds	<i>B. subtilis</i>		<i>S. aureus</i>		<i>E. coli</i>		<i>P. aeruginosa</i>	
	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀
7a	16	9	1	0.88	> 128	> 64	> 128	> 64
7b	> 128	> 64	> 128	> 64	> 128	> 64	> 128	> 64
7c	1	0.86	2	1.1	> 128	> 64	> 128	> 64
7d	16	8.1	16	8.2	> 128	> 64	> 128	> 64
7e	64	35.8	16	8.5	> 128	> 64	> 128	> 64
7f	8	4.7	1	0.69	32	19.17	16	8.65
7g	16	8.2	0.5	0.28	128	70.5	128	> 64
7h	> 128	> 64	16	8.3	64	48.5	> 128	> 64
7i	> 128	> 64	> 128	> 64	> 128	> 64	> 128	> 64
7j	> 128	> 64	> 128	> 64	> 128	> 64	> 128	> 64
7k	> 128	> 64	> 128	> 64	> 128	> 64	> 128	> 64
Amoxicillin	2	1	16	6.4	16	7.3	4	2.5
Ciprofloxacin	0.128	0.1	3.12	1.82	0.25	0.18	0.5	0.41

MIC: minimal inhibitory concentration; IC₅₀: 50% inhibition of bacteria growth; MIC and IC₅₀ are given in µg/mL.

Scheme 2. Synthetic protocol of compounds **3a–k**.

establish their in vitro efficacy and safety are being planned for their further development.

4. Experimental

The melting points were measured with a micro-melting point apparatus and are uncorrected. IR spectra were recorded on 1700 PerkinElmer FTIR using KBr disks. ^1H NMR spectra were recorded on a Varian INOVA 400 MHz spectrometer using CDCl_3 or $\text{DMSO}-d_6$ as a solvent and TMS as an internal standard. Mass spectra were determined with a Finnigan LCQ-DECA instrument. Elemental analysis (C, H, and N) was performed with a Carlo Erba EA1106 auto analyzer and the results were within $\pm 0.4\%$ of the theoretical values. All the experiments were monitored by thin layer chromatography (TLC) on pre-coated silica gel plates. Optical rotation was measured on a Wzz-2B polarimeter. All the reactions were performed in a commercial microwave apparatus (XH-100A, 100–1000 W, Beijing Xianghu Science and Technology Development Co. Ltd, Beijing, China). All the solvents were purified before use. Pyridinium chlorochromate (PCC) and methyl (5 β)-3,7-dihydroxycholesterol-24-oate **5** were prepared according to the methods reported [31,32].

4.1. Preparation of substituted benzaldehyde thiocarbohydrazones (**3a–k**)

The aromatic aldehyde (10 mmol) in ethanol (40 mL) was added dropwise to a solution of thiocarbonylhydrazide (15 mmol) in H_2O (50 mL) and acetic acid (1 mL). The mixture was refluxed in the microwave oven at 300 W for 15 min. The reaction was monitored by TLC until completion. The precipitate was collected by filtration and washed with water (3×10 mL). The resulting solid was recrystallised from ethanol to give the product (Scheme 2). The analytical data for **3i** are given below. The melting points of

benzaldehyde thiocarbohydrazones **3a–k** are shown in Table 5.

4.1.1. Compound **3i**

White crystal. Yield 79%. Mp 169–170 °C (MeOH– H_2O). IR (KBr, cm^{-1}): 3409, 3356, 3265, 2930, 2791, 1615, 1564, 1507, 1429, 1283, 1253, 1141, 1077, 1018, 848, 611. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ 11.53 (s, 1H, NH), 9.96 (s, 1H, NH), 8.45–8.39 (m, 1H, ArH), 8.17 (s, 1H, =CH), 7.33–7.27 (m, 1H, ArH), 7.15 (dd, $J = 6.4$ and 8.4 Hz, 1H, ArH), 4.88 (s, 2H, NH_2). ESI-MS m/z (%): 231 ($[\text{M} + 1]^+$, 100). Anal. calcd for $\text{C}_8\text{H}_8\text{F}_2\text{N}_4\text{S}$: C, 41.73; H, 3.50; N, 24.33. Found: C, 41.78; H, 3.48; N, 24.35%.

4.2. Synthesis of methyl (5 β)-3,7-dioxocholan-24-oate (**6**) [37]

Pyridinium chlorochromate (PCC) (3.03 mmol) was added to a solution of methyl (5 β)-3,7-dihydroxycholesterol-24-oate **5** (0.25 g, 0.615 mmol) in dried CH_2Cl_2 (20 mL) at room temperature. The reaction was completed in 12 h. The solvent was removed under reduced pressure, and the crude product was purified by chromatography on silica gel using ethyl acetate to give a white crystal. Yield 86%. Mp 155–156 °C (ethyl acetate) (lit. [38] Mp 154–156 °C), $[\alpha]_D^{25} = -21.5$ (c 0.12, CH_2Cl_2). IR (KBr, cm^{-1}): 2953, 2873, 1709, 1403, 1375, 1214, 1174, 1010, 966. ^1H NMR (CDCl_3 , 400 MHz): δ 3.67 (s, 3H, COOCH_3), 1.31 (s, 3H, 19- CH_3), 0.93 (d, $J = 6.4$ Hz, 3H, 21- CH_3), 0.69 (s, 3H, 18- CH_3). ESI-MS m/z (%): 827 ($[\text{2M} + 23]^+$, 100). Anal. calcd for $\text{C}_{25}\text{H}_{38}\text{O}_4$: C, 74.48; H, 9.54. Found: C, 74.51; H, 9.53%.

4.3. Microwave procedure for the preparation of bis thiocarbazonones (**7a–k**)

The steroidal diketone methyl (5 β)-3,7-dioxocholan-24-oate (**6**) (0.1 mmol) and the substituted benzaldehyde thiocarbohydrazones **3a–k** (0.1 mmol) and natural aluminum oxide (0.3 g) were placed in a porcelain mortar, and concentrated HCl (two drops) was added. After grinding, the mixture was put in a round-bottom flask (25 mL) and irradiated in a microwave oven for 5.0–7.5 min at 350 W. The reaction mixture was cooled to room temperature, dissolved in DMSO and filtered. The product was recrystallised from ethanol in 80–89% yields. The physical and spectral data of the compounds **7a–k** are as follows.

4.3.1. Methyl (5 β)-3-[2-[[[(phenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (**7a**)

White crystal. Yield 84%. Mp 125–126 °C (EtOH), $[\alpha]_D^{25} = -108.7$ (c 0.10, CH_2Cl_2). IR (KBr, cm^{-1}): 3263, 2945, 2871, 1735, 1708, 1630, 1517, 1380, 1235, 1215, 1169, 1099, 1021, 757. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ

Table 5

The melting points of substituted benzaldehyde thiocarbohydrazones **3a–k**.

Product	Formula	R^1	Mp (°C)	Lit. Mp (°C)
3a	$\text{C}_8\text{H}_{10}\text{N}_4\text{S}$	H	197–198	198 [33]
3b	$\text{C}_8\text{H}_{10}\text{N}_4\text{OS}$	2-OH	218–219	218 [33]
3c	$\text{C}_9\text{H}_{12}\text{N}_4\text{OS}$	4- OCH_3	198–199	198–200 [34]
3d	$\text{C}_8\text{H}_{10}\text{N}_4\text{OS}$	3-OH	201–202	200–201 [28]
3e	$\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2\text{S}$	2,4-OH	214–215	215–216 [35]
3f	$\text{C}_8\text{H}_9\text{FN}_4\text{S}$	4-F	208–209	208–209 [28]
3g	$\text{C}_8\text{H}_9\text{BrN}_4\text{S}$	2-Br	200–201	200–201 [35]
3h	$\text{C}_8\text{H}_9\text{ClN}_4\text{S}$	2-Cl	209–210	210 [33]
3i	$\text{C}_8\text{H}_8\text{F}_2\text{N}_4\text{S}$	2,4-F	169–170	–
3j	$\text{C}_8\text{H}_9\text{ClN}_4\text{S}$	4-Cl	211–212	211–212 [28]
3k	$\text{C}_8\text{H}_9\text{N}_5\text{O}_2\text{S}$	4- NO_2	204–206	204–206 [36]

11.72 (s, 1H, NH), 10.60 (s, 1H, NH), 8.10 (s, 1H, =CH), 7.72 (s, 2H, ArH), 7.43 (t, $J = 5.6$ Hz, 3H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 1157 ($[2M + 1]^+$, 100). Anal. calcd for C₃₃H₄₆N₄O₃S: C, 68.48; H, 8.01; N, 9.68. Found. C, 68.45; H, 8.04; N, 9.69%.

4.3.2. Methyl (5 β)-3-[2-[[2-(hydroxyphenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7b)

White crystal. Yield 86%. Mp 173–174 °C (EtOH), $[\alpha]_D = -58.9$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3265, 3128, 2949, 1735, 1707, 1615, 1533, 1488, 1438, 1379, 1272, 1226, 1199, 1153, 1038, 958. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.74 (s, 1H, NH), 11.64 (s, 1H, OH), 10.89 (d, $J = 9.2$ Hz, 1H, NH), 8.69 (s, 1H, =CH), 7.35 (s, 1H, ArH), 7.29 (t, $J = 8.0$ Hz, 2H, ArH), 6.91 (t, $J = 4.4$ Hz, 1H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 1189 ($[2M + 1]^+$, 100). Anal. calcd for C₃₃H₄₆N₄O₄S: calcd. C, 66.64; H, 7.79; N, 9.42. Found. C, 66.59; H, 7.81; N, 9.43%.

4.3.3. Methyl (5 β)-3-[2-[[4-(methoxyphenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7c)

White crystal. Yield 89%. Mp 120–121 °C (EtOH), $[\alpha]_D = -154.8$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3253, 2944, 1735, 1708, 1605, 1505, 1379, 1301, 1250, 1169, 1103, 1027, 931. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.63 (s, 1H, NH), 10.51 (s, 1H, NH), 8.05 (s, 1H, =CH), 7.65 (t, $J = 4.4$ Hz, 2H, ArH), 7.01 (d, $J = 8.4$, 2H, ArH), 3.80 (s, 3H, Ar-OCH₃), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 631 ($[M + 23]^+$, 100). Anal. calcd for C₃₄H₄₈N₄O₄S: C, 67.07; H, 7.95; N, 9.20. Found. C, 67.00; H, 6.97; N, 9.26%.

4.3.4. Methyl (5 β)-3-[2-[[3-(hydroxyphenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7d)

White crystal. Yield 82%. Mp 144–145 °C (EtOH), $[\alpha]_D = -100.7$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3272, 2946, 2870, 1707, 1581, 1522, 1451, 1380, 1225, 1170, 955, 784. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.36 (s, 1H, NH), 10.57 (s, 1H, NH), 9.62 (d, $J = 5.2$ Hz, 1H, OH), 8.02 (s, 1H, =CH), 7.28–7.18 (m, 1H, ArH), 7.14–7.07 (m, 2H, ArH), 6.82 (d, $J = 7.6$ Hz, 1H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 1189 ($[2M + 1]^+$, 100). Anal. calcd for C₃₃H₄₆N₄O₄S: C, 66.64; H, 7.79; N, 9.42. Found. C, 66.68; H, 7.75; N, 9.45%.

4.3.5. Methyl (5 β)-3-[2-[[2,4-dihydroxyphenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7e)

White crystal. Yield 85%. Mp 222–223 °C (EtOH), $[\alpha]_D = -97.4$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3309, 3233, 3149, 2950, 1732, 1709, 1624, 1515, 1378, 1330, 1208, 1171, 1111, 979, 885. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.76 (s, 1H, NH), 11.55 (s, 1H, OH), 10.75 (s, 1H, NH), 9.92 (s, 1H, OH), 8.56 (s, 1H, =CH), 7.12 (s, 1H, ArH), 6.34 (d, $J = 7.2$ Hz, 1H, ArH), 6.28 (d, $J = 2.0$ Hz, 1H, ArH), 3.58 (s, 3H,

COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 611 ($[M + 1]^+$, 100). Anal. calcd for C₃₃H₄₆N₄O₅S: C, 64.89; H, 7.59; N, 9.17. Found. C, 64.92; H, 7.57; N, 9.15%.

4.3.6. Methyl (5 β)-3-[2-[[4-(fluorophenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7f)

White crystal. Yield 84%. Mp 124–125 °C (EtOH), $[\alpha]_D = -97.9$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3229, 2945, 2870, 1735, 1708, 1601, 1503, 1380, 1232, 1156, 1097, 1017, 896, 835. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.72 (s, 1H, NH), 10.61 (s, 1H, NH), 8.08 (s, 1H, =CH), 7.77 (t, $J = 12$ and 5.2 Hz, 2H, ArH), 7.29 (t, $J = 8.8$ and 8.8 Hz, 2H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 1193 ($[2M + 1]^+$, 100). Anal. calcd for C₃₃H₄₅FN₄O₃S: C, 66.41; H, 7.60; N, 9.39. Found. C, 66.48; H, 7.58; N, 9.37%.

4.3.7. Methyl (5 β)-3-[2-[[2-(bromophenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7g)

White crystal. Yield 82%. Mp 129–130 °C (EtOH), $[\alpha]_D = -79.4$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3236, 2944, 2869, 1734, 1708, 1589, 1516, 1466, 1435, 1378, 1228, 1203, 1168, 1100, 1021, 821, 757. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.80 (s, 1H, NH), 10.68 (s, 1H, NH), 8.47 (s, 1H, =CH), 8.12 (s, 1H, ArH), 7.67 (d, $J = 8.0$ Hz, 1H, ArH), 7.47–7.41 (m, 1H, ArH), 7.37–7.33 (m, 1H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 657 ($[M + 1]^+$, 100), 658 ($[M + 2]^+$, 93). Anal. calcd for C₃₃H₄₅BrN₄O₃S: C, 60.26; H, 6.90; N, 8.52. Found. C, 60.30; H, 6.87; N, 8.50%.

4.3.8. Methyl (5 β)-3-[2-[[2-(chlorophenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7h)

White crystal. Yield 80%. Mp 127–128 °C (EtOH), $[\alpha]_D = -86.7$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3239, 2945, 2870, 1735, 1708, 1594, 1516, 1468, 1437, 1379, 1228, 1204, 1169, 1049, 893. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.79 (s, 1H, NH), 10.68 (s, 1H, NH), 8.51 (s, 1H, =CH), 8.19 (s, 1H, ArH), 7.58–7.48 (m, 1H, ArH), 7.45–7.42 (m, 2H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.89 (d, $J = 6.4$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 1225 ($[2M + 1]^+$, 100), 1226 ($[2M + 2]^+$, 42). Anal. calcd for C₃₃H₄₅ClN₄O₃S: C, 64.63; H, 7.40; N, 9.14. Found. C, 64.60; H, 7.41; N, 9.17%.

4.3.9. Methyl (5 β)-3-[2-[[2,4-difluorophenylideneamino)thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7i)

White crystal. Yield 86%. Mp 126–127 °C (EtOH), $[\alpha]_D = -102.7$ (c 0.10, CH₂Cl₂). IR (KBr, cm⁻¹): 3253, 2948, 2872, 1734, 1708, 1614, 1523, 1499, 1431, 1381, 1279, 1230, 1171, 1140, 1100, 965, 850. ¹H NMR (DMSO-*d*₆, 400 MHz): δ 11.80 (s, 1H, NH), 10.68 (s, 1H, NH), 8.72 (s, 1H, =CH), 8.19 (d, $J = 10.4$ Hz, 1H, ArH), 7.41–7.33 (m, 1H, ArH), 7.22 (d, $J = 8.0$ Hz, 1H, ArH), 3.58 (s, 3H, COOCH₃), 1.21 (s, 3H, 19-CH₃), 0.82 (d, $J = 6.0$ Hz, 3H, 21-CH₃), 0.64 (s, 3H, 18-CH₃). ESI-MS m/z (%): 615 ($[M + 1]^+$, 100); Anal.

calcd for $C_{33}H_{44}F_2N_4O_3S$: C, 64.47; H, 7.21; N, 9.11. Found. C, 64.50; H, 7.20; N, 9.10%.

4.3.10. Methyl (5 β)-3-[2-[(4-chlorophenylideneamino)-amino]thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7j)

White crystal. Yield 84%. Mp 131–132 °C (EtOH), $[\alpha]_D = -84.2$ (c 0.10, CH_2Cl_2). IR (KBr, cm^{-1}): 3250, 2948, 2864, 1731, 1708, 1633, 1591, 1521, 1489, 1241, 1209, 1168, 1086, 1016, 828. 1H NMR (DMSO- d_6 , 400 MHz): δ 11.75 (s, 1H, NH), 10.49 (s, 1H, NH), 8.49 (s, 1H, =CH), 7.71 (dd, $J = 10.4$ and 8.8 Hz, 2H, ArH), 7.56–7.44 (m, 2H, ArH), 3.58 (s, 3H, $COOCH_3$), 1.17 (s, 3H, 19- CH_3), 0.82 (d, $J = 6.0$ Hz, 3H, 21- CH_3), 0.64 (s, 3H, 18- CH_3). ESI-MS m/z (%): 613 ($[M+1]^+$, 100), 614 ($[M+2]^+$, 43). Anal. calcd for $C_{33}H_{45}ClN_4O_3S$: C, 64.63; H, 7.40; N, 9.14. Found. C, 64.68; H, 7.42; N, 9.12%.

4.3.11. Methyl (5 β)-3-[2-[(4-nitrophenylideneamino)-amino]thioxomethyl]hydrazinylidene]-7-oxocholan-24-oate (7k)

White crystal. Yield 85%. Mp 129–130 °C (EtOH), $[\alpha]_D = -135.7$ (c 0.11, CH_2Cl_2). IR (KBr, cm^{-1}): 3238, 2947, 2871, 1735, 1708, 1589, 1519, 1484, 1439, 1380, 1241, 1207, 1171, 1009, 822. 1H NMR (DMSO- d_6 , 400 MHz): δ 11.76 (s, 1H, NH), 10.65 (s, 1H, NH), 8.48 (s, 1H, =CH), 7.83–7.61 (m, 4H, ArH), 3.58 (s, 3H, $COOCH_3$), 1.17 (s, 3H, 19- CH_3), 0.82 (d, $J = 6.4$ Hz, 3H, 21- CH_3), 0.64 (s, 3H, 18- CH_3). ESI-MS m/z (%): 1247 ($[2M+1]^+$, 100). Anal. calcd for $C_{33}H_{45}N_5O_5S$: C, 64.54; H, 7.27; N, 11.23. Found. C, 64.59; H, 7.25; N, 7.29%.

4.4. Conventional procedure for the preparation of bis thiocarbazones (7a–k)

The steroidal diketone methyl (5 β)-3,7-dioxocholan-24-oate (**6**) (0.1 mmol) and the substituted benzaldehyde thiocarbohydrazones **3a–k** (0.1 mmol) were dissolved in ethanol (15 mL). After complete dissolution, two drops of HCl were added. The mixture was stirred for 420–540 min at 80 °C. After cooling, the products were filtered and recrystallized from ethanol in 31–58% yields.

4.5. Bioactivity

The in vitro antimicrobial activity of the structurally promising steroidal bis thiocarbazones **7a–k** against two strains of Gram-positive bacteria (*B. subtilis* and *S. aureus*) and two strains of Gram-negative (*E. coli* and *P. aeruginosa*) were investigated using disc-diffusion method and microdilution method in comparison to the reference drugs amoxicillin and ciprofloxacin. Dimethylformamide was used as the negative control.

4.5.1. Disc-diffusion assay

The compounds were tested for their antibacterial activities by disc-diffusion method using a nutrient agar medium [39,40]. In the disc-diffusion method, serial dilutions of the test compounds were prepared to final concentrations of 40, 20, 10, 5 and 0 $\mu g/mL$ in DMSO. Sterile paper discs (6 mm) were then placed onto the

nutrient agar medium and 20 μL (40, 20, 10, 5 and 0 mg/mL) of the sample were added per disc in five replications. Then, the paper discs impregnated with the solutions of the tested compounds in DMSO were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 37 °C for 16–18 h (Table 3). The IC_{50} was computed from the diameter values of growth inhibition zones.

4.5.2. Microdilution assay

The MIC were determined by microdilution, both methods [41] using standard inoculums of 2×10^6 CFU/mL. Serial dilutions of the test compounds were prepared to final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 $\mu g/mL$ in DMSO. To each tube was added 1 mL of a bacterial fluid (0.5 McFarland standard). The MIC that inhibits the visible growth after 18 h was determined visually after incubation for 18 h at 37 °C (Table 4).

Acknowledgments

This research was financially supported by the Science and Technology Department of Sichuan Province (No. 2011JY0035), the Fundamental Research Funds of the Central University, Southwest University for Nationalities (No. 13NZYQN12) and the Postgraduate Degree Construction Project of Southwest University for Nationalities of P.R. China (No. 2013XWD-S0703).

References

- [1] D.G. Rivera, O. Pando, F. Coll, Tetrahedron 62 (2006) 8327.
- [2] R.M. Mohareb, W.W. Wardakhan, G.A. Elmegeed, R.M.S. Ashour, Steroids 77 (2012) 1560.
- [3] C.F. Gan, J.G. Cui, Y.M. Huang, L.Y. Jia, W.X. Wei, Steroids 77 (2012) 255.
- [4] M.G. Barthakur, A. Hasib, J. Gogoi, R.C. Boruah, Steroids 75 (2010) 445.
- [5] M. Ibrahim-Ouali, K. Hamze, L. Rocheblave, Steroids 76 (2011) 324.
- [6] E. Virtanen, E. Kolehmainen, Eur. J. Org. Chem. 2004 (2004) 3385.
- [7] J.R. Hanson, Nat. Prod. Rep. 22 (2005) 104.
- [8] D.B. Salunke, B.G. Hazra, V.S. Pore, M.K. Bhat, P.B. Nahar, M.V. Deshpande, J. Med. Chem. 47 (2004) 1591.
- [9] M. El-Far, G.A. Elmegeed, E.F. Eskander, H.M. Rady, M.A. Tantawy, Eur. J. Med. Chem. 44 (2009) 3936.
- [10] S.A. Khan, M. Yusuf, Eur. J. Med. Chem. 44 (2009) 2597.
- [11] P.B. Savage, Eur. J. Org. Chem. 2002 (2002) 759.
- [12] R.W. Brockman, J.R. Thomson, M.J. Bell, H.E. Skipper, Cancer Res. 16 (1956) 167.
- [13] D.J. Bauer, Brit. Med. Bull. 41 (1985) 309.
- [14] Y. Yen, K. Margolin, J. Doroshow, M. Fishman, B. Johnson, C. Clairmont, D. Sullivan, M. Sznol, Cancer Chemother. Pharmacol. 54 (2004) 331.
- [15] S.A. Khan, P. Kumar, R. Joshi, P.F. Iqbal, K. Saleem, Eur. J. Med. Chem. 43 (2008) 2029.
- [16] Y.K. Qin, R.G. Xing, S. Liu, K.C. Li, X.T. Meng, R.F. Li, J.H. Cui, B. Li, P.C. Li, Carbohydr. Polym. 87 (2012) 2664.
- [17] S. Singh, F. Athar, M.R. Maurya, A. Azam, Eur. J. Med. Chem. 41 (2006) 592.
- [18] H. Beraldo, D. Gambino, Mini-Rev. Med. Chem. 4 (2004) 31.
- [19] D. Dragancea, A.W. Addison, M. Zeller, M.E. Foster, M.J. Prushan, L.K. Thompson, M.D. Revenco, A.D. Hunter, Inorg. Chim. Acta 363 (2010) 2065.
- [20] M.P. Sathisha, U.N. Shetti, V.K. Revankar, K.S.R. Pai, Eur. J. Med. Chem. 43 (2008) 2338.
- [21] B.S. Chhikara, R. Chandra, V. Tandon, Tetrahedron Lett. 45 (2004) 7585.
- [22] S. Dutta, T. Kar, D. Mandal, P.K. Das, Langmuir 29 (2013) 316.
- [23] V. Polshettiwar, R.S. Varma, Acc. Chem. Res. 41 (2008) 629.
- [24] P. Anastas, J. Warner, Green Chemistry: Theory and Practice, Oxford University Press, New York, 1998.
- [25] C.O. Kappe, Angew. Chem. Int. Ed. 43 (2004) 6242.
- [26] A. Loupy, C. R. Chimie 7 (2004) 103.
- [27] Z.C. Shi, Z.G. Zhao, X.L. Liu, Y. Chen, Chin. Chem. Lett. 22 (2011) 405.

- [28] Z.C. Shi, Z.G. Zhao, X.L. Liu, L.L. Wu, J. Chem. Res. 35 (2011) 198.
- [29] G.H. Li, Z.C. Shi, X.R. Li, Z.G. Zhao, J. Chem. Res. 35 (2011) 278.
- [30] R.P. Bonar-Law, A.P. Davis, Tetrahedron 49 (1993) 9829.
- [31] E.J. Corey, M.G. Bock, Tetrahedron Lett. 16 (1975) 2643.
- [32] Z.G. Zhao, P.Y. Zhang, Z.X. Yang, S.H. Chen, Chin. J. Org. Chem. 25 (2005) 679, In Chinese.
- [33] V.V. Dabholkar, T.D. Ravi, Indian J. Chem. 49B (2010) 593.
- [34] Z. Li, X. Feng, Y.L. Zhao, J. Heterocycl. Chem. 45 (2008) 1489.
- [35] X.J. Yang, Z.C. Shi, Z.G. Zhao, Chin. J. Synth. Chem. 19 (2011) 352, In Chinese.
- [36] F. Han, Y.H. Bao, Z.G. Yang, T.M. Fyles, J.Z. Zhao, X.J. Peng, J.L. Fan, Y.K. Wu, S.G. Sun, Chem. Eur. J. 13 (2007) 2880.
- [37] L.Y. Qiu, Z.C. Shi, Q.G. Mei, Z.G. Zhao, J. Chem. Res. 35 (2011) 456.
- [38] Z.Q. Wang, H.Q. Que, L.Z. Jiang, W.S. Zhou, Chin. J. Org. Chem. 9 (1989) 83, In Chinese.
- [39] Performance standard for antimicrobial susceptibility testing: 19th informational supplement. Clinical and Laboratory Standard Institute, Wayne, PA, 2009, M100-S19.
- [40] S.A. Khan, K. Saleem, Z. Khan, Eur. J. Med. Chem. 42 (2007) 103.
- [41] F.F. Zhang, L.L. Gan, C.H. Zhou, Bioorg. Med. Chem. Lett. 20 (2010) 1881.