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Synthesis and antiproliferative activity of some steroidal thiosemicarbazones, semicarbazones and hydrozones

Chunfang Gan, Jianguo Cui *, Shaoyang Su, Qifu Lin, Linyi Jia, Lianghua Fan, Yanmin Huang *

College of Chemistry and Life Science, Guangxi Teachers Education University, Nanning 530001, China

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ABSTRACT

Steroidal thiosemicarbazones, semicarbazones and hydrazones have received extensive attention of scientists recently because they exhibit some biological activities such as antibacterial, antiviral and anticancer. Using different steroids as starting materials, through different chemical methods, 24 steroidal compounds with thiosemicarbazone, semicarbazone or hydrazone groups in their structures, were synthesized, characterized by IR, NMR and MS. The antiproliferative activity of the compounds was evaluated against human gastric cancer (SGC-7901) and human liver cancer (Bel-7404) cells. The structure–activity relationship of these compounds was discussed. The results showed that compound **3** and **12a**–**12c** exhibited significant inhibitory activity to Bel-7404 cells, and IC_{50} values of them were 4.2, 11.0, 7.4 and 15.0 μM respectively (Cisplatin, IC_{50}: 11.6 μM).

1. Introduction

Recently, numerous compounds with biological activities were separated from marine organism [1–6]. Many compounds with steroidal skeletons showed significant antibacterial, antiviral, antitumor or other biological activities [7–11]. Hydrazone compounds and their derivatives are a class of substances displaying the above [12,13] activities. A variety of steroidal hydrazone derivatives with unique structures had been synthesized with their bioactivities assayed [14–17]. Especially, steroids with thiosemicarbazone structure were known to possess antiamoebic, antinociceptive, antiangiogeni, antibacterial, antifungal and anti-inflammatory properties [18–22]. However, steroidal thiosemicarbazone with antiproliferative activity was rarely reported.

In previous researches, a series of steroidal oxime compounds had been synthesized by our group, and the in vitro antiproliferative activity of these compounds had been investigated [23–25]. The results indicated that some steroidal compounds with 3,6-dioxime groups in the steroidal nucleus showed a good cytotoxicity against some cancer cells. In this paper, some steroidal hydrazone derivatives with 3,6-disubstituted structure and different side chains were synthesized. In these compounds, a semicarbazone or thiosemicarbazone group was introduced into the 3-, 6- or 22-position of steroidal nucleus respectively, and other potency groups such as hydroxyl, oxime or carbonyl groups were introduced into another position. Using some natural sterols with different side chains, such as cholesterol and stigmasterol as starting materials, a total of 24 steroidal hydrazone derivatives with different structural characteristics were designed and synthesized through different methods. The antiproliferative activity of synthesized compounds was evaluated.

2. Results and discussion

2.1. Chemistry

Compounds **1**, **4a**–**4c** and **10** were prepared according to the method of literature [25].

First, compounds **2** and **3** were obtained by the reaction of compound **1** with semicarbazide or thiosemicarbazide (Scheme 1). The structures of **2** and **3** were confirmed by NMR spectra. In the ^{13}C NMR of **2** and **3**, the chemical shift of 6-carbonyl in the compound **1** disappeared, and resonances of C-6 that appeared at 155.5 ppm for compound **2** and at 158.6 ppm for compound **3** showed that the 6-carbonyl of **1** had been transformed into the 6-C=N group of **2** or **3**. Moreover, in the ^{1}H NMR, the chemical shift of **2** at 5.433 (NH_2), 6.116 (NH_2), 8.356 (—NH—) and **3** at 6.326 (NH_2), 7.249 (NH_2), 8.749 (—NH—) ppm demonstrated further formation of 6-semicarbazone and 6-thiosemicarbazone groups.

* Corresponding authors. Tel.: +86 771 3908065; fax: +86 771 3908308 (J. Cui). Tel.: +86 13977159868 (Y. Huang).

E-mail addresses: cuijg1954@126.com (J. Cui), huangyanmin828@163.com (Y. Huang).

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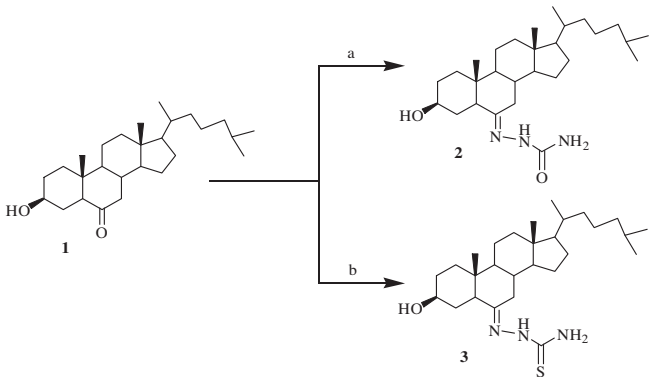
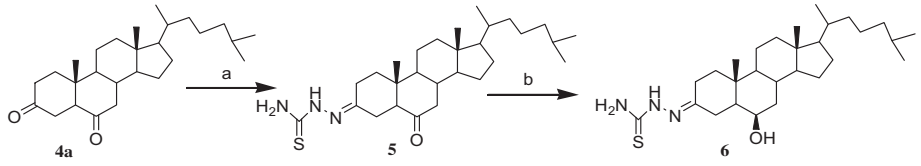


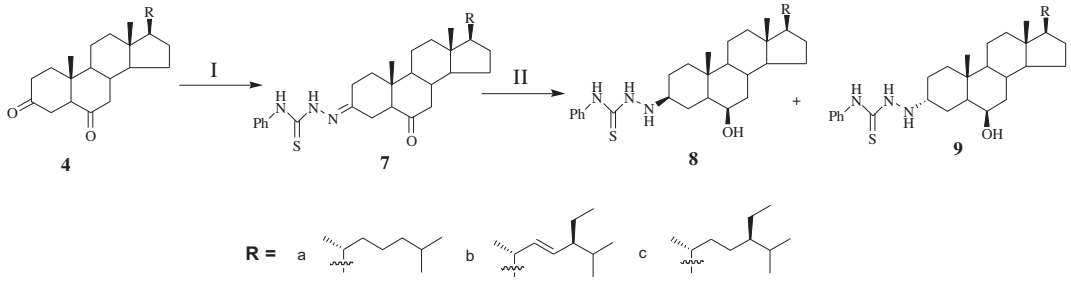
Scheme 1. Reagents and conditions: (a) $NH_2NHCONH_2$/95%EtOH, $CH_3COONa{\cdot}3H_2O$, 70 °C; and (b) $NH_2NHCSNH_2$/EtOH, 70 °C.

Next, starting from compound **4a**, compound **5** with 3-thiosemicarbazone group was yielded by controlling an appropriate molar ratio of **4a** and thiosemicarbazide because 3-carbonyl group was more active than 6-carbonyl, and thiosemicarbazide was selectively reacted with 3-carbonyl (see Scheme 2). Compound **5** was a mixture of *(3E)*- and *(3Z)*-isomer (1:1, from the ^{1}H NMR). In ^{13}C NMR spectrum of compound **5**, the characteristic peak at 154.9 ppm indicated that the presence of 3-thiosemicarbazone group and resonances which showed at 179.0 and at 178.9 ppm were belonged to the C=S chemical shift of (*E*)- and (*Z*)-**5** respectively. The chemical shift of 6-carbonyl at 209.9 and 209.5 ppm demonstrated further that compound **5** was the mixture of (*E*)- and (*Z*)-configuration isomer. Next, the 6-carbonyl of **5** was converted to 6-hydroxyl of compound **6** by the reduction of $NaBH_4$, but the 3-thiosemicarbazone group was still kept in compound **6**. From the ^{13}C NMR spectra of compound **6**, the characteristic signal of the 6-carbonyl of **5** was disappeared at 209 ppm, instead of at 70.9 ppm which was the chemical shift of 6-hydroxyl's carbon. The presence of multiple peaks at 3.88 ppm to 3.80 ppm in C_6—H of **6** illustrated further that the 6-carbonyl group of **5** had been converted to 6-hydroxyl, where product **6** was generated.

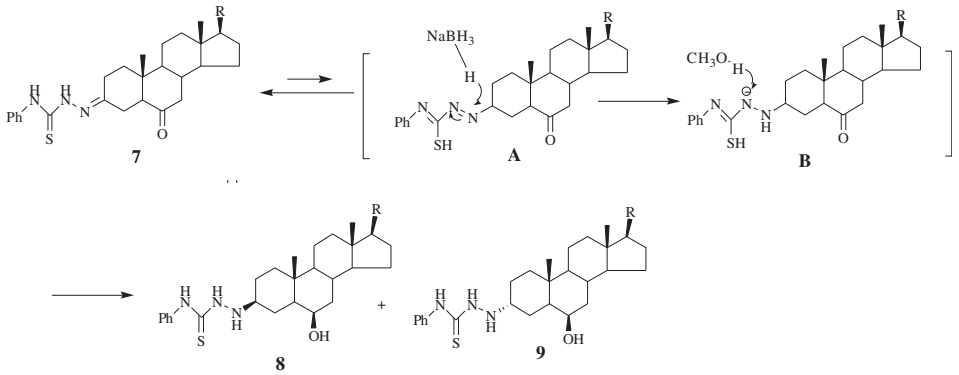
To investigate the effect of substituted group to biological activity in steroidal thiosemicarbazones, several new phenylthiosemicarbazonesteroid derivatives were prepared and evaluated for their cytotoxic activity (**Scheme 3**). The reaction of compounds **4a–4c** with phenylthiosemicarbazide gave 3-(4′-phenyl)-thiosemicarbazonesteroids **7a–7c** with different branched chain. **7a–7c** were also composed of the mixture of *(3E)*- and *(3Z)*-isomer with 1:1 proportion, and their structures were confirmed by their spectral data. Interestingly, when **7a–7c** were reduced by sodium borohydride, differing from compound **5**, compounds **8a–8c** and **9a–9c** were obtained in which both 6-carbonyl and 3-(4′-phenyl)thiosemicarbazone groups were reduced simultaneously. From the NMR spectrum of **8a** and **9a**, it was obviously seen that ^{13}C chemical shift of



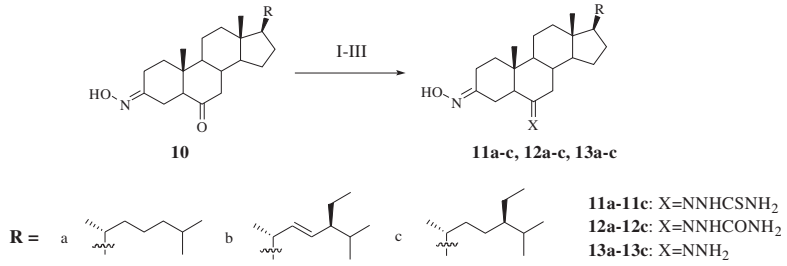
Scheme 2. Reagents and conditions: (a) $NH_2CSNHNH_2$/CH_3CH_2OH, 80 °C; and (b) $NaBH_4$, CH_3OH.



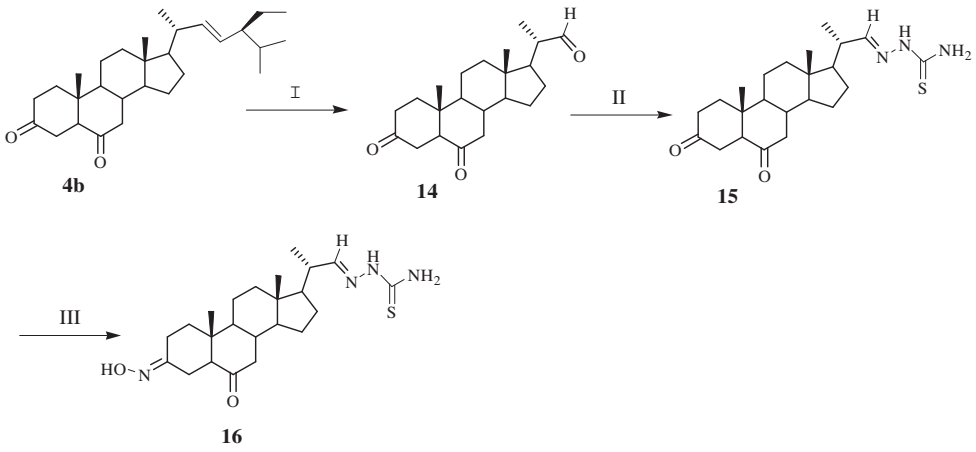
Scheme 3. Reagents and conditions: (I) $PhNHCSNHNH_2$/CH_3CH_2OH, 80 °C; and (II) $NaBH_4$, CH_3OH.



Scheme 4. The formation mechanism of compounds **8a–8c** and **9a–9c**.



Scheme 5. Reagents and conditions: (I) $NH_2NHCSNH_2/CH_3COOH/CH_3CH_2OH$; (II) $NH_2NHCONH_2{\cdot}HCl$/95%EtOH; and (III): $N_2H_4{\cdot}H_2O$ (80%)/CH_3COOH/CH_3CH_2OH.



Scheme 6. Reagents and conditions: (I) O_3, CH_2Cl_2, Me_2S, −78 °C; (II) $NH_2NHCSNH_2/C_2H_5OH/CH_3COOH$, pH = 4; and (III) $NH_2OH.HCl$/95%EtOH/$NaOAc{\cdot}3H_2O$.

6-carbonyl at 210 ppm disappeared, replaced by ^{13}C chemical shift of 6-hydroxyl at 71.8 ppm. In addition, the characteristic signal of 3-C=N at 154.7 ppm for **7a** disappeared and was replaced by the signals of 3-C—NH— at 60.4 for **8a** and 55.7 ppm for **9a**. It showed clearly that 3-(4′-phenyl)thiosemicarbazone group of **7a** had been reduced. In their ^1H NMR, the chemical shift of C_3—H at 2.95–2.80 ppm for **8a** (3α-configuration, C—H connected to amino-group) and 3.34–3.27 ppm for **9a** (3β-configuration) illustrated further that the C=N double bond of 3-thiosemicabazone had been transformed into C—N single bond. Here, the chemical shift at 2.95–2.80 ppm in upfield for **8a** was assigned to 3-αH, and 3.34–3.27 ppm in downfield for **9a** assigned to 3-βH, respectively. Compound **8a** (**8a**:**9a** ≈ 5:1) was a main product because 3-(4'-phenylthiosemicarbamide) group located at *e*-bond is more stable than that at *a*-bond.

Under the same reaction conditions, why only 6-carbonyl in compound **5** was reduced, but both 6-carbonyl and 3-(4′-phenyl)thiosemicarbazone group in compound **7** were reduced? A proposed mechanism for the formation of **8** and **9** is shown in Scheme 4.

In the reaction, an intermediate **A** with enol-configuration in compound **7** was formed due to the conjugated effect of 4′-phenyl. Sodium borohydride attacking the N=N double bond of **A** produced an intermediate **B**. The **B** obtaining a proton from CH_3OH produced compounds **8** and **9**. Within this reaction, compound **8** with 3β-substituent was the main product.

In order to study the effect of various nitrogenous substituent on the substrate to the antiproliferative activity, compounds **11a**–**11c**, **12a**–**12c** and **13a**–**13c** possessing 3-oxime group and different side chains in their structures were synthesized by compounds **10a**–**10c** reacted with different nitrogenous reagents (Scheme 5). Their structures were confirmed by spectrum analysis. Comparing the ^1H NMR of compound **11a** with **10a**, the proton chemical shift of NH_2 for **11a** appears in 6.758, 7.232–7.248 ppm. A resonance signal is found within 3.273–3.322 ppm, which is chemical shift of C_2—βH and moved to the downfield due to the deshielding influence of the hydroxyl in (3*E*)-hydroximino group. In the ^{13}C NMR of **11a**, the chemical shift of carbonyl carbon is not found, the ^{13}C chemical shift of two C=N appear at 159.5 and 155.0 ppm respectively. The chemical shift at 179.0 ppm is indicative of carbon in C=S bond of 6-thiosemicarbazone group.

Using **4b** as starting material, another nitrogenous steroidal compound **16** with 3-hydroximino and 22-thiosemicarbazone groups in its structure was synthesized (Scheme 6). The ozonolysis of **4b** was performed in a mixture of CH_2Cl_2 and MeOH (CH_2Cl_2:MeOH = 4:1) at −78 °C. After bubbling O_2 to expel the excess O_3 and adding Me_2S to decompose the produced ozonide, compound **14** was obtained. Utilizing the difference between the activity of 22-aldehyde and 3,6-dicarbonyl group in **14**, compound **15** with 22-thiosemicarbarzone group in the structure was obtained by a selective reaction of 22-aldehyde with thiosemicarbarzide. Furthermore, a selective oximation of 3-carbonyl group in compound **15** produced compound

Table 1
In vitro antiproliferative activities of target compounds (μM).

Compounds	**2**	**3**	**6**	**7a**	**7b**	**7c**	**8a**	**8b**
SGC-7901	47.8	76.3	>100	30.8	67.4	>100	>100	>100
Bel-7404	28.3	4.2	>100	>100	32.8	89.5	>100	>100
	8c	**9a**	**9b**	**9c**	**11a**	**11b**	**11c**	**12a**
SGC-7901	>100	>100	>100	>100	17.2	>100	15.9	13.2
Bel-7404	>100	32.4	>100	>100	27.8	>100	11.0	11.0
	12b	**12c**	**13a**	**13b**	**13c**	**15**	**16**	**Cisplatin**
SGC-7901	32.3	26.2	>100	>100	>100	19.1	>100	6.7
Bel-7404	7.4	15.0	>100	>100	>100	43.1	78.7	23.2

16. The structures of all synthesized compounds were confirmed by their spectral data. Compounds **15** and **16** have similar atom rank with the side chain of cholesterol, except that the carbon atoms in branch chain of **15** and **16** were replaced by nitrogen atoms and a sulfur atom.

2.2. In vitro evaluation of the antiproliferative activity

All synthesized compounds were evaluated for their antiproliferative activities in vitro against SGC 7901 (human gastric carcinoma) and Bel 7404 (human liver carcinoma) cell lines using a MTT assay. The results were summarized as IC_{50} values in μM in Table 1.

Comparing compound **3** with **2**, compound **3** with 3-hydroxyl and 6-thiosemicarbazone groups had better antiproliferative activity than compound **2** with 3-hydroxyl and 6-semicarbazone groups against Bel 7404 cells. Compound **3** showed significant increase in its cytotoxicity against these cells in comparison of compound **6** with same 3,6-disubstituted groups. However, the structure of **6** possesses an opposite arrangement of the two functional groups. The results showed that thiosemicarbazone group in 6-position favored the increase of the compound's cytotoxicity.

In addition, substituting hydrogen atom in thiosemicarbazone group with phenyl group increased the antiproliferative activity of these compounds (compare **7a**–**7c** with **6**). However, compounds **8a**–**8c** and **9a**–**9c** showed an obvious decrease in their cytotoxicity against these cancer cells after 3-(4′-phenyl)thiosemicarbazone group of **7a**–**7c** was reduced.

The results showed that the compounds' cytotoxicity increased when 6-substituted group was thiosemicarbazone or semicarbazone for the compounds with 3-hydroximino group (see **11a**–**11c** and **12a**–**12c**), but it was almost inactive when 6-substituted group was hydrazone (**13a**–**13c**). For compounds possessing 6-thiosemicarbazone group in the structure, the presence of double bond in the side chain resulted in a remarkable decrease of the cytotoxicity of compounds (comparing **11b** and **11a**, **11c**). Though no obvious difference was found in their cytotoxicity for compounds **12a**–**12c** bearing 6-semicarbazone group which displayed a better antiproliferative activity than cisplatin did (a positive control) against Bel-7404 cells. Among them, compound **12b** with 22,23-double bond in its structure showed an excellent antiproliferative activity with IC_{50} value of 7.4 μM.

Apparently, for compounds possessing 22-thiosemicarbazone, compound **15** with 3-carbonyl had better cytotoxicity than compound **16** with 3-hydroximino group.

3. Conclusion

Using various steroids as starting materials, through different chemical methods, 24 steroidal compounds with the characteristic thiosemicarbazone, semicarbazone or hydrazone groups in their structures were synthesized, and their structures were characterized by IR, NMR and MS. The in vitro antitumor activity of these compounds was assayed against human gastric cancer (SGC-7901) and human liver cancer (Bel-7404) cells. The results showed that compounds with the 6-thiosemicarbazone or 6-semicarbazone group in the structures exhibited significant antiproliferative activity in vitro. Moreover, compounds **3** and **12a–12c**, with IC_{50} value of 4.2, 11.0, 7.4 and 15.0 μM, displayed better antiproliferative activity than cisplatin did (a positive control) against Bel-7404 cells. The presence of 22-double bond in the side chain resulted in a remarkable decrease of the cytotoxicity for compounds possessing 6-thiosemicarbazone group.

4. Experimental

4.1. Chemistry

4.1.1. Reagent and Instrument

The synthetic reagents were analytically pure, and the solvents were purified by general methods before being used. Infrared spectra were measured with a Nicolet FT-360 Spectrophotometer (Thermo Scientific, America); Melting points were determined by X_6 microscopic melting point meter and was uncorrected; The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AV-600 spectrometer at working frequencies 600 and 150 MHz and a Bruker AV-300 spectrometer at working frequencies 300 and 75 MHz, respectively. Chemical shifts are expressed in parts per million (δ) values and coupling constants (J) in Hertz. LRESIMS were recorded on a Thermo-DSQ instrument, while HRESIMS were measured on a Agilent 6210 TOFMS instrument.

4.1.2. 3β-hydroxycholestan-6-semicarbazone (*2*)

$CH_3COONa{\cdot}3H_2O$ (34 mg, 0.25 mmol) and semicarbazide hydrochloride (34 mg, 0.3 mmol) were added to the solution of 100 mg (0.25 mmol) compound **1** in 20 mL 95% ethanol. After the solution was heated to 70 °C, the mixture was stirred at the temperature for 10 h (the progress of the reaction was monitored by TLC, $V_{dichloromethane}$:$V_{methanol}$ = 20:1). Then, the reaction was terminated and majority of solvent was evaporated under reduced pressure. Suitable amount of water was added to the reaction mixture, and the product was extracted with CH_2Cl_2 (15 mL × 3). The combined extract was washed with saturated $NaHCO_3$ solution, water and saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The crude product was separated by the column chromatography using CH_2Cl_2/CH_3OH (eluent:$V_{dichloromethane}$:$V_{methanol}$ = 40:1) to give 65 mg of **2** (56%) as white solid. m.p. 234–236 °C. IR (KBr) ν/cm^{-1}: 3468, 2929, 2864, 1687, 1662, 1580, 1466, 1384, 1066; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.645 (s, 3H, 18-CH_3), 0.703 (s, 3H, 19-CH_3), 0.867 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.871 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.907 (d, 3H, J = 6.3, 21-CH_3), 2.687 (dd, 1H, J = 13.2, 3.0, C5—H), 3.67–3.52 (m, 1H, C3—αH), 5.433 (br s, 1H, —NH_2), 6.116 (br s, 1H, —NH_2), 8.356 (br s, 1H, —NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 158.6 (6-C), 152.7 (—C═O), 71.0 (3-C), 56.5 (14-C), 56.2 (17-C), 54.3 (9-C), 50.9 (5-C), 42.9 (13-C), 39.6 (10-C), 39.5 (24-C), 39.2 (12-C), 36.3 (1-C), 36.1 (22-C), 35.7 (20-C), 31.7 (4-C), 31.4 (7-C), 30.9 (8-C), 29.7 (2-C), 28.2 (16-C), 28.0 (25-C), 24.1 (19-C), 23.8 (15-C), 22.8 (26-C), 22.6 (27-C), 21.4 (23-C), 18.6 (11-C), 12.7 (21-C), 12.1 (18-C); HRESI-MS, m/z: 460.3919 $[M+H]^+$, (calcd for $C_{28}H_{50}N_3O_2$, 460.3903).

4.1.3. 3β-hydroxycholestan-6-thiosemicarbazone (*3*)

Compound **1** (100 mg, 0.25 mmol) was dissolved in 20 mL of CH_3CH_2OH, and some glacial acetic acid was dripped into the solution to adjust pH value of solution to 3–5 after the solid was completely dissolved. The mixture was heated to 70 °C, and 27 mg (0.3 mmol) thiosemicarbazide was added into the solution after 10 min. The mixture was stirred for 6 h until no starting material was observed (the progress of the reaction was monitored by TLC, petroleum ether: ethyl acetate = 1:1). The reaction was terminated and majority of solvent was evaporated under reduced pressure. The mixture was extracted with ethyl acetate (10 mL × 3). The combined extract was washed with saturated $NaHCO_3$ solution, water and saturated brine, dried with anhydrous sodium sulfate. After solvent was removed under reduced pressure, the residue was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (2:1) as the eluent. Compound **3** was obtained as white solid (78 mg, 65%), m.p. 259–261 °C.

IR (KBr) ν/cm^{-1}: 3432, 2929, 2864, 1662, 1580, 1466, 1384, 1066; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.669 (s, 3H, 18-CH_3), 0.717 (s, 3H, 19-CH_3), 0.892 (d, 6H, J = 6.3, 26-CH_3 or 27-CH_3), 1.151 (d, 3H, J = 6.3, 21-CH_3), 2.646 (br d, 1H, J = 12.9, C5—H), 3.73–3.56 (m, 1H, C3—αH), 6.326 (br s, 1H, —NH_2), 7.249 (br s, 1H, —NH_2), 8.749 (s, 1H, —NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 179.1 (C=S), 155.5 (6-C), 71.0 (3-C), 56.4 (14-C), 56.1 (17-C), 54.3 (9-C), 51.1 (5-C), 43.1 (13-C), 39.6 (24-C), 39.5 (12-C), 39.4 (10-C), 36.5 (1-C), 36.3 (22-C), 36.1 (20-C), 35.7 (8-C), 31.6 (4-C), 31.5 (7-C), 31.0 (2-C), 28.1 (16-C), 28.0 (25-C), 24.2 (19-C), 23.8 (15-C), 22.8 (26-C), 22.6 (27-C), 21.5 (23-C), 18.6 (11-C), 12.7 (21-C), 12.1 (18-C); HRESI-MS: m/z: 476.3670 $[M+H]^+$ (calcd for $C_{28}H_{50}N_3OS$, 476.3675).

4.1.4. Cholestan-3,6-dione 3-thiosemicarbazone (**5**)

Compound **5** was prepared similarly as the procedure for the synthesis of **3**, but from compound **4a**.

White solid, yield: 60%. m.p. 189–191 °C. Compound **5** was the mixture of (3*E*)- and (3*Z*)-isomer (ratio: *E*:*Z* = 1:1, ^{1}H NMR integral area determined). IR (KBr) ν/cm^{-1}: 3403, 3256, 2929, 2851, 1699, 1593, 1409, 1364, 1245; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.683 (s, 3H, 18-CH_3), 0.861 (s, 3H, 19-CH_3), 0.872 (d, 6H, J = 6.3, 26-CH_3 and 27-CH_3), 0.922 (d, 3H, J = 6.3, 21-CH_3), 2.43–2.34 (m, 4H, C4—H and C7—H), 2.686 (d, 1H, J = 12.3, 3.6, C5—H), 6.414 (br s, 1H, —NH_2), 7.234 (br s, 1H, —NH_2), 8.823 (s, 0.5H, —NH—, *Z*-), 8.881 (s, 0.5H, —NH—, *E*-); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 209.9 (6-C, *E*-), 209.5 (6-C, *Z*-), 179.0 (C=S, *E*-), 178.9 (C=S, *Z*-), 154.9 (3-C), 57.8 (5-C), 56.6 (14-C), 56.1 (17-C), 53.6 (13-C, *E*-), 53.4 (13-C, *Z*-), 46.6 (10-C, *E*-), 46.5 (10-C, *Z*-), 42.9 (9-C), 41.7 (7-C, *E*-), 41.6 (7-C, *Z*-), 39.5 (12-C), 39.3 (24-C), 38.1 (22-C), 37.8 (8-C), 37.1 (20-C), 36.1 (2-C), 35.6 (16-C), 30.0 (25-C), 28.0 (4-C), 24.0 (1-C), 23.8 (23-C), 22.8 (26-C), 22.6 (27-C), 22.5 (15-C), 21.5 (11-C), 18.6 (21-C), 12.5 (19-C), 12.0 (18-C); HRESI-MS: 474.3532 $[M+H]^+$ (calcd for $C_{28}H_{48}N_3OS$, 474.3518).

4.1.5. 6-Hydroxycholestan-3-thiosemicarbazone (**6**)

$NaBH_4$ (20 mg, 0.5 mmol) was added in batches to a solution of **5** (120 mg, 0.25 mmol) in anhydrous methanol (20 mL) at room temperature in 10 min. The mixture was stirred at room temperature until no raw material was observed (by TLC, eluent:$V_{\text{petroleum ether}}$:$V_{\text{ethyl acetate}}$ = 1:1). Then the solution was neutralized with 1 mol.L^{-1} HCl. After evaporation of the majority of MeOH under reduced pressure, a small amount of water was added and extracted with ethyl acetate (15 mL × 3). The combined extract was washed with cold water, saturated $NaHCO_3$ and brine. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure and the resulting crude product was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (1:1) as the eluent to give 60 mg of white solid **6**, yield: 50%. m.p. 221–223 °C. Product **6** was the mixture of (3*E*)- and (3*Z*)-isomer (ratio: *E*:*Z* = 1:1). IR (KBr) ν/cm^{-1}: 3460, 1686, 1666, 1564, 1466, 1384; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.723 (s, 3H, 18-CH_3), 0.880 (d, 6H, J = 6.3, 26-CH_3 and 27-CH_3), 0.924 (d, 3H, J = 6.3, 21-CH_3), 1.125 (s, 3H, 19-CH_3), 2.46–2.34 (m, 3H, C4—H and C7—H), 3.88–3.80 (m, 1H, C6—H), 6.24 (br s, 1H, —NH_2), 7.25 (br s, 1H, —NH_2), 8.694 (s, 0.5H, —NH—, *Z*-), 8.937 (s, 0.5H, —NH—, *E*-); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 178.9 (C=S), 157.1 (3-C, *E*-), 156.6 (3-C, *Z*-), 70.9 (6-C), 56.3 (14-C), 55.9 (17-C), 53.8 (9-C), 49.1 (5-C), 47.9 (13-C), 42.7 (10-C), 39.8 (7-C), 39.5 (12-C), 36.1 (24-C), 36.0 (22-C), 35.7 (20-C), 30.2 (8-C), 29.7 (2-C), 28.2 (4-C), 28.0 (16-C), 27.4 (25-C), 24.2 (1-C), 23.8 (23-C), 22.8 (26-C), 22.5 (27-C), 21.1 (15-C), 21.0 (11-C), 18.7 (21-C), 15.0 (19-C), 12.5 (18-C); HRESI-MS: 476.3667 $[M+H]^+$ (calcd for $C_{28}H_{50}N_3OS$, 476.3675).

Compounds **7a–7c** were prepared similarly as the procedure for the synthesis of **5**, but 4-phenyl-3-thiosemicarbazide was used as an attack reagent instead of thiosemicarbazide.

4.1.6. 6-Oxo-cholestan-3-(4'-phenyl)thiosemicarbazone (**7a**)

Light yellow solid, yield 49.3%, m.p. 203–205 °C. **7a** was the mixture of (3*E*)- and (3*Z*)-isomer (ratio: *E*:*Z* = 1:1). IR (KBr) ν/cm^{-1}: 3415, 3260, 2949, 2868, 1711, 1597, 1527, 1466, 1442, 1380, 1278, 1176; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.668 (s, 3H, 18-CH_3), 0.855 (s, 3H, 19-CH_3), 0.866 (d, 6H, J = 6.9, 26-CH_3 and 27-CH_3), 0.915 (d, 3H, J = 6.0, 21-CH_3), 2.002–2.091 (m, 2H, 7-CH_2), 2.747 (d, 1H, J = 14.7, C5—H), 7.189 (t, 1H, J = 7.2, p-Ph—H), 7.351 (t, 2H, J = 7.5, m-Ph—H), 7.633 (d, 2H, J = 7.8, o-Ph—H), 9.017 (s, 0.5H, —CNH—N, *Z*-), 9.089 (s, 0.5H, —CNH—N, *E*-), 9.284 (s, 0.5H, —Ph—NH—C, *Z*-), 9.344 (s, 0.5H, —Ph—NH—C, *E*-); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 210.0 (6-C, *E*), 209.8 (6-C, *Z*), 176.1 (C=S, *E*), 176.0 (C=S, *Z*-), 154.7 (3-C), [138.1,128.7,128.7,125.8,124.1,124.1] (C_6H_5), 57.8 (14-C), 56.7 (5-C, *Z*-), 56.6 (5-C, *E*-), 56.1 (17-C), 53.4 (13-C, *Z*-), 53.3 (13-C, *E*-), 46.6 (10-C, *E*-), 46.5 (10-C, *Z*-), 42.9 (7-C), 41.7 (9-C), 41.6 (12-C), 39.5 (24-C), 39.3 (22-C), 38.2 (4-C, *Z*-), 37.8 (20-C), 37.1 (4-C, *E*-), 36.1 (8-C), 35.7 (25-C), 30.5 (2-C, *E*-), 30.1 (2-C, *Z*-), 28.0 (16-C), 23.9 (1-C), 23.8 (15-C), 22.9 (27-C), 22.6 (26-C), 21.5 (23-C), 18.7 (11-C), 12.6 (21-C), 12.4 (19-C), 12.0 (18-C); HRESI-MS: 550.3827 $[M+H]^+$ (calcd for $C_{34}H_{52}N_3OS$, 550.3831).

4.1.7. 6-oxo-stigmastan-3-(4′-phenyl)thiosemicarbazone (**7b**)

From compound **4b**. Light yellow solid, yield: 67.7%. m.p. 193–195 °C. **7b** was the mixture of (3*E*)- and (3*Z*)-isomer (ratio: *E*:*Z* = 1:1). IR (KBr) ν/cm^{-1}: 3411, 3284, 2945, 2864, 1711, 1593, 1531, 1437, 1388, 1270, 1184, 1074; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.708 (s, 3H, 18-CH_3), 0.804 (d, 3H, J = 6.6, 26-CH_3), 0.815 (t, 3H, J = 6.3, 29-CH_3), 0.856 (d, 3H, J = 6.6, 27-CH_3), 0.878 (s, 3H, 19-CH_3), 1.037 (d, 3H, J = 6.6, 21-CH_3), 2.52–2.34 (m, 4H, C4—H, C5—H and C7—H), 2.730 (dd, 1H, J = 12.9, 2.4, C7—H), 5.033 (dd, 1H, J = 15.0, 8.4, C22—H), 5.155 (dd, 1H, J = 15.3, 8.4, C23—H), 7.218 (t, 1H, J = 7.2, p-Ph—H), 7.379 (t, 2H, J = 7.8, m-Ph—H), 7.652 (d, 2H, J = 8.1, o-Ph—H), 8.878 (s, 0.48H, —CNH—N, *Z*-), 8.917 (s, 0.5H, —CNH—N, *E*-), 9.280 (s, 0.48H, —Ph—NH—C, *Z*-), 9.326 (s, 0.48H, —Ph—NH—C, *E*-); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 210.0 (6-C, *E*-), 209.6 (6-C, *Z*-), 176.2 (C=S, *Z*-), 176.1 (C=S, *E*-), 154.2 (3-C), [138.0, 128.7, 128.7, 125.9, 124.1, 124.1] (C_6H_5), 137.9 (22-C), 129.6 (23-C), 57.8 (14-C), 56.7 (5-C), 55.8 (17-C), 53.5 (24-C), 53.4 (13-C), 51.2 (9-C), 46.6 (10-C, *E*-), 46.5 (10-C, *Z*-), 42.8 (20-C), 41.8 (7-C, *Z*-), 41.6 (7-C, *E*-), 40.5 (9-C), 39.2 (12-C), 38.1 (8-C, *E*-), 37.9 (25-C), 37.1 (8-C, *Z*-), 31.9 (16-C), 30.5 (4-C, *E*-), 30.1 (4-C, *Z*-), 28.7 (2-C), 25.4 (1-C), 24.0 (15-C), 22.7 (28-C), 22.5 (11-C), 21.2 (26-C), 21.1 (27-C), 21.2 (21-C), 19.0 (C-19), 12.5 (18-C), 12.2 (29-C); HRESI-MS: 576.3989 $[M+H]^+$ (calcd for $C_{36}H_{54}N_3OS$, 576.3988).

4.1.8. 6-Oxo-sitostan-3-(4′-phenyl)thiosemicarbazone (**7c**)

From compound **4c**. Light yellow solid, yield: 40.9%. m.p. 203–206 °C. **7c** was the mixture of (3*E*)- and (3*Z*)-isomer (ratio: *E*:*Z* = 1:1). IR (KBr) ν/cm^{-1}: 3427, 3246, 2941, 2868, 1703, 1589, 1527, 1442, 1380, 1266, 1188; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.695 (s, 3H, 18-CH_3), 0.793 (d, 3H, J = 6.9, 26-CH_3), 0.826 (d, 3H, J = 6.9, 27-CH_3), 0.860 (t, 3H, J = 6.6, 29-CH_3), 0.883 (s, 3H, 19-CH_3), 0.943 (d, 3H, J = 4.8, 21-CH_3), 2.53–2.37 (m, 4H, C4—H, C5—H and C7—H), 2.722 (br d, 1H, J = 9.3, C7—H), 7.225 (t, 1H, J = 7.2, p-Ph—H), 7.386 (t, 2H, J = 7.2, m-Ph—H), 7.657 (d, 2H, J = 7.5, o-Ph—H), 8.802 (br s, 1H, —CSNH—N), 9.280 (s, 0.5H, —Ph—NH—C, *Z*-), 9.321 (s, 0.5H, —Ph—NH—C, *E*-); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 209.9 (6-C, *E*-), 209.6 (6-C, *Z*-), 176.2 (C=S, *E*-), 176.1 (C=S, *Z*-), 154.2 (3-C), [138.0, 128.7, 128.7, 125.8, 124.1, 124.1] (C_6H_5), 57.8 (14-C), 56.7 (5-C, *Z*-), 56.6 (5-C, *E*-), 56.0 (17-C), 53.5 (24-C), 53.4 (9-C), 46.6 (13-C, *E*-), 46.5 (13-C, *Z*-), 45.8 (7-C), 43.0 (12-C), 41.7 (10-C, *E*-), 41.6 (10-C, *Z*-), 39.4 (20-C), 39.0 (8-C, *Z*-), 38.8 (8-C, *Z*-), 38.1 (4-C, *Z*-), 37.8 (4-C, *E*-), 37.1 (22-C), 36.0 (16-C), 35.8 (23-C), 33.8 (2-C, *E*-), 33.6 (2-C, *Z*-), 32.4 (1-C, *E*-), 31.4 (1-C, *Z*-), 30.5 (25-C), 24.0 (15-C), 23.0 (11-C), 21.5 (26-C), 21.4

(27-C), 20.2 (21-C), 19.0 (19-C), 18.8 (28-C), 12.4 (18-C), 12.0 (29-C); HRESI-MS: 578.4139 $[M+H]^+$ (calcd for $C_{36}H_{56}N_3OS$, 578.4144).

4.1.9. 6β-Hydroxycholestan-3-(4′-phenyl)thiosemicarbamides (**8a, 9a**)

$NaBH_4$ (28 mg, 0.72 mmol) was added in batches to a solution of **7a** (200 mg, 0.36 mmol) in 10 mL of methanol (add 2 mL of CH_2Cl_2 to help dissolving) at room temperature in 10 min. The mixture was stirred for 20 min at room temperature until no raw material. (by TLC, elution:$V_{petroleum}$:$V_{ethyl\ acetat}$ = 4:1). Then the solution was neutralized with 1 mol L^{-1} HCl. After most of methanol was evaporated under reduced pressure, a small amount of water was added and extracted with ethyl acetate (20 mL × 3). The extract was washed with cold water, saturated $NaHCO_3$ and brine. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (4:1) as the eluent to give 59 mg of **8a** (light yellow solid), yield 30%. m.p. 200–203 °C. IR (KBr) ν/cm^{-1}: 3432, 2921, 2851, 1589, 1544, 1495, 1454, 1384, 1270, 1200, 1082, 1041, 739, 686; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.688 (s, 3H, 18-CH_3), 0.876 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.881 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.917 (d, 3H, J = 6.3, 21-CH_3), 1.010 (s, 3H, 19-CH_3), 1.998 (br d, 1H, J = 12.6, C4—βH), 2.95–2.80 (m, 1H, C3—αH), 3.782 (br s, 1H, 3-C—NH—), 3.948 (br s, 1H, C6—βH), 7.212 (t, 1H, J = 7.5, p-Ph—H), 7.381 (t, 2H, J = 7.8, m-Ph—H), 7.637 (d, 2H, J = 7.8, o-Ph—H), 8.122 (br s, 1H, CSNH), 9.349 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.4 (C═S), [138.0, 128.7, 128.7, 125.7, 123.9, 123.9] (—C_6H_5), 71.8 (6-C), 60.4 (3-C), 56.3 (14-C), 56.2 (17-C), 54.2 (9-C), 47.4 (5-C), 42.7 (13-C), 39.9 (7-C), 39.7 (12-C), 39.5 (24-C), 38.4 (10-C), 36.2 (1-C), 35.8 (22-C), 35.7 (20-C), 30.8 (8-C), 30.3 (4-C), 28.2 (16-C), 28.0 (25-C), 26.9 (2-C), 24.2 (15-C), 23.9 (23-C), 22.8 (26-C), 22.6 (27-C), 21.0 (11-C), 18.7 (21-C), 15.9 (19-C), 12.1 (18-C); ESI-MS, m/z: 554.4 $(M+H)^+$; HRESI-MS: 554.4152 $[M+H]^+$(calcd for $C_{34}H_{56}N_3OS$, 554.4144).

Isomer **9a** of **8a**, 6β-hydroxycholestan-3α-(4′-phenyl)thiosemicarbamide, was obtained as a byproduct (13 mg, yield 6.5%). m.p. 107–108 °C. IR (KBr) ν/cm^{-1}: 3440, 2937, 2365, 2336, 1642, 1540, 1380, 1078; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.699 (s, 3H, 18-CH_3), 0.878 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.882 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.921 (d, 3H, J = 6.3, 21-CH_3), 1.037 (s, 3H, 19-CH_3), 2.016 (br d, 2H, J = 12.3, C4—H), 3.34–3.27 (m, 1H, C3—βH), 3.713 (br s, 1H, C6—βH), 3.751 (br s, 1H, 3-C—NH—), 7.234 (t, 1H, J = 7.5, p-Ph—H), 7.404 (t, 2H, J = 7.5, m-Ph—H), 7.485 (br s, 1H, CSNH), 7.614 (d, 2H, J = 7.8, o-Ph—H), 9.237 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.5 (C═S), [138.0, 128.8, 128.8, 125.7, 123.6, 123.6] (—C_6H_5), 71.7 (6-C), 56.3 (14-C), 56.2 (17-C), 55.7 (3-C), 54.6 (9-C), 43.4 (5-C), 42.7 (13-C), 39.9 (12-C), 39.5 (24-C), 36.3 (1-C), 36.2 (7-C), 35.8 (22-C), 35.1 (20-C), 30.3 (4-C), 30.0 (8-C), 28.2 (16-C), 28.0 (25-C), 24.4 (2-C), 24.2 (15-C), 23.8 (23-C), 22.8 (26-C), 22.7 (27-C), 20.6 (11-C), 18.7 (21-C), 15.1 (19-C), 12.1 (18-C); ESI-MS, m/z: 554.4 $(M+H)^+$; HRESI-MS: 554.4151 $[M+H]^+$(calcd for $C_{34}H_{56}N_3OS$, 554.4144).

4.1.10. 6β-Hydroxystigmastan-3-(4′-phenyl)thiosemicarbamides (**8b, 9b**)

Compounds **8b** and **9b** were prepared similarly as the procedure for the synthesis of **8a** and **9a**, but from compound **7b**.

6β-Hydroxystigmastan-3β-(4′-phenyl)thiosemicarbamide (**8b**): light yellow solid, yield: 37%. m.p. 145–146 °C. IR (KBr) ν/cm^{-1}: 3411, 2925, 2868, 1613, 1544, 1503, 1446, 1380, 1266, 1078, 972; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.708 (s, 3H, 18-CH_3), 0.809 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.818 (t, 3H, J = 7.2, 29-CH_3), 0.861 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 1.013 (s, 3H, 19-CH_3), 1.025 (d, 3H, J = 6.9, 21-CH_3), 2.08–1.96 (m, 2H, C4—H), 2.95–2.80 (m, 1H, C3—αH), 3.780 (br s, 1H, 3-C—NH—), 3.960 (br d, 1H, J = 3.3, C6—βH), 5.019 (dd, 1H, J = 15.3, 8.3, C22—H), 5.157 (dd, 1H, J = 15.3, 8.3, C23—H), 7.213 (t, 1H, J = 7.5, p-Ph—H), 7.380 (t, 2H, J = 7.5, m-Ph—H), 7.633 (d, 2H, J = 7.8, o-Ph—H), 8.131 (br s, 1H, CSNH), 9.351 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.2 (C = S), 138.3 (22-C), [138.0, 128.7, 128.7, 125.7, 123.9, 123.9] (—C_6H_5), 129.3 (23-C), 71.8 (6-C), 60.3 (3-C), 56.3 (14-C), 56.0 (17-C), 54.2 (9-C), 51.3 (24-C), 47.4 (5-C), 42.5 (20-C), 40.6 (13-C), 39.8 (12-C), 39.7 (7-C), 38.4 (10-C), 35.8 (1-C), 31.9 (8-C), 30.8 (25-C), 30.3 (4-C), 28.9 (16-C), 26.9 (2-C), 25.4 (28-C), 24.3 (15-C), 21.2 (11-C), 21.1 (26-C), 20.9 (27-C), 19.0 (21-C), 15.9 (18-C), 12.3 (19-C), 12.2 (29-C); ESI-MS, m/z: 580.5 $(M+H)^+$; HRES-I-MS: 580.4316 $[M+H]^+$ (calcd for $C_{36}H_{56}N_3OS$, 580.4301).

6β-Hydroxystigmastan-3α-(4′-phenyl)thiosemicarbamide (**9b**): light yellow solid, yield: 12.0%. m.p. 165–167 °C. IR (KBr) ν/cm^{-1}: 3432, 2941, 2868, 1625, 1544, 1442, 1376, 1266, 1192, 1086; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.715 (s, 3H, 18-CH_3), 0.810 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.819 (t, 3H, J = 7.2, 29-CH_3), 0.862 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 1.028 (d, 3H, J = 6.3 Hz, 21-CH_3), 1.029 (s, 3H, 19-CH_3), 2.06–1.97 (m, 2H, C4—H), 3.37–3.29 (m, 1H, C3—βH), 3.702 (br s, 1H, C6—βH), 3.798 (br s, 1H, 3-C—NH—), 5.023 (dd, 1H, J = 15.0, 8.4, C22—H), 5.158 (dd, 1H, J = 15.3, 8.4, C23—H), 7.231 (t, 1H, J = 7.5, p-Ph—H), 7.400 (t, 2H, J = 7.5, m-Ph—H), 7.608 (d, 2H, J = 7.8, o-Ph—H), 7.858 (br s, 1H, CSNH), 9.248 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.3 (C═S), 138.3 (22-C), [138.0, 128.8, 128.8, 125.7, 123.7, 123.7] (—C_6H_5), 129.3 (23-C), 71.6 (6-C), 56.3 (14-C), 56.0 (17-C), 55.6 (3-C), 54.6 (9-C), 51.3 (24-C), 43.4 (5-C), 42.5 (13-C), 40.6 (20-C), 39.8 (12-C), 39.7 (7-C), 36.3 (1-C), 35.0 (10-C), 31.9 (8-C), 30.3 (25-C), 29.7 (4-C), 28.9 (16-C), 28.3 (2-C), 25.4 (28-C), 24.4 (15-C), 24.2 (21-C), 21.2 (26-C), 21.1 (27-C), 20.6 (11-C), 19.0 (18-C), 15.1 (19-C), 12.3 (29-C); ESI-MS, m/z: 580.5 $(M+H)^+$; HRESI-MS: 580.4266 $[M+H]^+$ (calcd for $C_{36}H_{56}N_3OS$, 580.4301).

4.1.11. 6β-hydroxysitostan-3-(4′-phenyl)thiosemicarbamides (**8c, 9c**)

Compounds **8c** and **9c** were prepared similarly as the procedure for the synthesis of **8a** and **9a**, but from compound **7c**.

6β-hydroxysitostan-3β-(4′-phenyl)thiosemicarbamide (**8c**): light yellow solid, yield: 33.3%. m.p. 214–215 °C. IR (KBr) ν/cm^{-1}: 3436, 2953, 2361, 2328, 1634, 1552, 1503, 1413; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.696 (s, 3H, 18-CH_3), 0.789 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.817 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.860 (t, 3H, J = 6.6, 29-CH_3), 0.926 (d, 3H, J = 6.3, 21-CH_3), 1.022 (s, 3H, 19-CH_3), 2.004 (br d, 1H, J = 12.6, C4—βH), 2.94–2.82 (m, 1H, C3—αH), 3.800 (br s, 1H, 3-C—NH—), 3.886 (d, 1H, J = 3.6, C6—βH), 7.219 (t, 1H, J = 7.5, p-Ph—H), 7.388 (t, 2H, J = 7.5, m-Ph—H), 7.643 (d, 2H, J = 7.8, o-Ph—H), 7.853 (br s, 1H, CSNH—), 9.340 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.3 (C═S), [138.0, 128.7, 128.7, 125.7, 123.9, 123.9] (—C_6H_5), 71.8 (6-C), 60.4 (3-C), 56.3 (14-C), 56.1 (17-C), 54.1 (9-C), 47.4 (5-C), 45.8 (24-C), 42.6 (13-C), 39.8 (7-C), 39.7 (12-C), 38.8 (10-C), 38.4 (1-C), 36.2 (20-C), 35.8 (8-C), 33.8 (22-C), 30.8 (4-C), 30.3 (25-C), 29.1 (2-C), 28.2 (16-C), 26.9 (23-C), 24.2 (15-C), 23.0 (28-C), 20.9 (11-C), 20.2 (26-C), 19.9 (27-C), 18.7 (21-C), 15.9 (19-C), 12.1 (18-C), 12.0 (29-C); HRESI-MS: 582.4451 $[M+H]^+$ (calcd for $C_{36}H_{56}N_3OS$, 582.4457).

6β-Hydroxysitostan-3α-(4′-phenyl)thiosemicarbamide (**9c**): light yellow solid, yield: 11.7%. m.p. 148–150 °C. IR (KBr) ν/cm^{-1}: 3452, 2953, 2353, 2332, 1629, 1556, 1503, 1413; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.698 (s, 3H, 18-CH_3), 0.788 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.817 (d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.838 (t, 3H, J = 6.6, 29-CH_3), 0.928 (d, 3H, J = 6.6, 21-CH_3), 1.030 (s, 3H, 19-CH_3), 2.06–1.96 (m, 2H, C4—H), 3.35–3.29 (m, 1H, C3—βH), 3.707 (br s, 1H, C6—βH), 3.781 (br s, 1H, 3-C—NH—), 7.232 (t, 1H, J = 7.5, p-Ph—H), 7.401 (t, 2H, J = 7.5, m-Ph—H), 7.607 (d, 2H, J = 7.8, o-Ph—H), 7.729 (br s, 1H, CSNH), 9.243 (br s, 1H, Ph—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 180.4 (C═S), [138.0, 128.8, 128.8,

125.7, 123.6, 123.6] (—C_6H_5), 71.7 (6-C), 56.2 (14-C), 55.7 (17-C), 55.6 (3-C), 54.6 (9-C), 45.8 (24-C), 43.4 (5-C), 42.6 (13-C), 39.9 (7-C), 39.8 (12-C), 38.8 (10-C), 36.3 (1-C), 36.1 (20-C), 35.1 (8-C), 33.9 (22-C), 30.3 (4-C), 29.7 (25-C), 29.1 (2-C), 28.2 (16-C), 26.1 (23-C), 24.2 (15-C), 23.1 (28-C), 20.6 (11-C), 20.2 (26-C), 19.8 (27-C), 18.7 (21-C), 15.1 (19-C), 12.1 (18-C), 12.0 (29-C); ESI-MS, *m/z*: 582.7 $(M+H)^+$; HRESI-MS: 582.4453 $[M+H]^+$(calcd for $C_{36}H_{56}N_3OS$, 582.4457).

Compounds **11a–11c** were prepared similarly as the procedure for the synthesis of **3**, but from **10a–10c**.

4.1.12. (3E)-hydroximinocholestan-6-thiosemicarbazone (**11a**)

White solid, yield: 35.4%. m.p. 165–168 °C. IR (KBr) ν/cm^{-1}: 3390, 3308, 3223, 2924, 2851,1727, 1600, 1510, 1465, 1384, 1281, 1069, 954; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.683 (s, 3H, 18-CH_3), 0.820 (s, 3H, 19-CH_3), 0.872 (d, 3H, J = 6.6, 26-CH_3), 0.893 (d, 3H, J = 6.6, 27-CH_3), 0.924 (d, 3H, J = 6.3, 21-CH_3), 2.391 (dd, 1H, J = 8.1, 2.4, C7—βH), 2.689 (dd, 1H, J = 11.7, 2.4, C7—αH), 3.323 (td, 1H, J = 12.9, 5.2, C2—βH), 6.754 (br s, 1H, —NH_2), 7.194 (d, 1H, J = 4.8, —NH_2), 8.843 (s, 1H, —NH); ^{13}C NMR($CDCl_3$, 75 MHz) δ: 179.0 (C=S), 159.5 (3-C), 155.0 (6-C), 56.4 (14-C), 56.1 (17-C), 53.9 (9-C), 52.3 (5-C), 43.0 (13-C), 40.5 (10-C), 40.3 (24-C), 39.5 (12-C), 36.4 (22-C), 36.3 (20-C), 36.1 (8-C), 35.7 (7-C), 31.7 (16-C), 29.7 (25-C), 28.6 (2-C), 28.0 (1-C), 24.2 (4-C), 23.8 (15-C), 22.8 (27-C), 22.6 (26-C), 21.4 (23-C), 19.9 (11-C), 18.6 (21-C), 12.0 (19-C), 11.9 (18-C); HRESI-MS, *m/z*: 489.3601 $[M+H]^+$ (calcd for $C_{28}H_{49}N_4OS$, 489.3627).

4.1.13. (3E)-hydroximinostigmastan-6-thiosemicarbazone (**11b**)

White solid, yield: 72%. m.p. 203–205 °C; IR (KBr) ν/cm^{-1}: 3423, 3374, 3260, 2953, 2868, 1593, 1486, 1384, 1282, 1074, 968; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.703 (s, 3H, 18-CH_3), 0.825 (d, 3H, J = 6.3, 26-CH_3), 0.821 (s, 3H, 19-CH_3), 0.823 (t, 3H, J = 7.2, 29-CH_3), 0.873 (d, 3H, J = 6.3, 27-CH_3), 1.033 (d, 3H, J = 6.5, 21-CH_3), 2.392 (dd, 1H, J = 8.1, 3.0, C7—βH), 2.677 (dd, 1H, J = 13.1, 3.0, C7—αH), 3.306 (dd, 1H, J = 14.5, 3.9, C2—βH), 5.049 (dd, 1H, J = 15.2, 8.3, C22—H), 5.163 (dd, 1H, J = 15.2, 8.2, C23—H), 6.736 (br d, 1H, J = 4.2, —NH_2), 7.194 (br d, 1H, J = 4.2, —NH_2), 8.130 (br s, 1H, —NOH), 8.829 (s, 1H, —NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 179.1 (C=S), 159.5 (3-C), 154.9 (6-C), 137.9 (22-C), 129.7 (23-C), 56.5 (14-C), 55.9 (17-C), 53.9 (9-C), 52.3 (24-C), 51.2 (5-C), 42.9 (13-C), 40.5 (10-C), 40.4 (20-C), 40.3 (12-C), 39.3 (8-C), 36.5 (25-C), 36.3 (7-C), 31.9 (16-C), 31.7 (2-C), 28.7 (28-C), 28.6 (1-C), 25.3 (4-C), 24.3 (15-C), 21.4 (11-C), 21.2 (26-C), 21.1 (27-C), 19.0 (21-C), 12.3 (19-C), 12.2 (18-C), 11.9 (29-C); HRESI-MS, *m/z*: 513.3623 $[M-H]^-$ (calcd for $C_{30}H_{49}N_4OS$, 513.3627).

4.1.14. (3E)-hydroximinositostan-6-thiosemicarbazone (**11c**)

White solid, yield: 69%. m.p 197–199 °C. IR (KBr) ν/cm^{-1}: 3407, 2916, 2851, 1711, 1461, 1384, 1277, 1122, 938; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.688 (s, 3H, 18-CH_3), 0.821–0.938 (m, 15H, 5CH_3), 2.706 (d, 1H, J = 12.9, C7—αH), 3.281∼3.368 (m, 1H, C2—βH), 6.934 (br s, 1H, —NH_2), 7.209 (br s, 1H, —NH_2), 8.899 (br s, 1H, —NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 178.9 (C=S), 159.5 (3-C), 155.2 (6-C), 56.4 (14-C), 56.0 (17-C), 53.8 (9-C), 52.3 (5-C), 51.1 (24-C), 45.8 (13-C), 43.0 (10-C), 40.4 (12-C), 39.5 (20-C), 37.7 (8-C), 36.5 (22-C), 36.0 (7-C), 33.9 (25-C), 31.8 (16-C), 29.2 (2-C), 28.6 (23-C), 28.1 (1-C), 26.1 (4-C), 24.2 (15-C), 23.1 (28-C), 21.4 (11-C), 19.8 (26-C), 19.0 (27-C), 18.7 (21-C), 12.1 (19-C), 12.0 (18-C), 11.9 (29-C); HRESI-MS, *m/z*: 517.3951 $[M+H]^+$ (calcd for $C_{30}H_{53}N_4OS$, 517.3940).

Compounds **12a–12c**, **13a–13c** were prepared similarly as the procedure for the synthesis of **2**, but from **10a–10c**, and semicarbazide hydrochloride and $N_2H_4{\cdot}H_2O$ were used as attack reagents.

4.1.15. (3E)-hydroximinocholestan-6-semicarbazone (**12a**)

White solid, yield: 42.4%. m.p. 258–260 °C. IR (KBr) ν/cm^{-1}: 3447, 3370, 3219, 3149, 2941, 2863, 1653, 1580, 1474, 1380, 1286, 954; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.679 (s, 3H, 18-CH_3), 0.832 (s, 3H, 19-CH_3), 0.882 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.886 (d, 3H, J = 6.6, 26-CH_3 or 27-CH_3), 0.925 (d, 3H, J = 6.3, 21-CH_3), 2.41–2.37 (m, 1H, C7—βH), 2.639 (dd, 1H, J = 13.5, 3.6, C7—αH), 3.38–3.27 (m, 1H, C2—βH), 5.044 (br s, 1H, —NH_2), 6.063 (br s, 1H, —NH_2), 7.935 (s, 1H, —NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 160.1 (3-C), 158.0 (6-C), 152.0 (C=O), 56.1 (14-C), 54.0 (17-C), 52.1 (9-C), 51.0 (5-C), 42.9 (10-C), 40.1 (13-C), 39.9 (24-C), 39.5 (12-C), 36.5 (22-C), 36.1 (20-C), 35.7 (7-C), 31.2 (8-C), 29.7 (16-C), 28.6 (25-C), 28.1 (2-C), 28.0 (1-C), 24.1 (4-C), 23.8 (15-C), 22.8 (26-C), 22.6 (27-C), 21.4 (23-C), 19.9 (11-C), 18.6 (21-C), 12.0 (19-C), 11.9 (18-C); HRESI-MS, *m/z*: 473.3837 $[M+H]^+$ (calcd for $C_{28}H_{49}N_4O_2$, 473.3856).

4.1.16. (3E)-hydroximinostigmastan-6-semicarbazone (**12b**)

White solid, yield: 76%. m.p 290–291 °C. IR (KBr) ν/cm^{-1}: 3472, 3244, 2953, 2868, 1691, 1572, 1458, 1380, 972; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.695 (s, 3H, 18-CH_3), 0.807 (s, 3H, 19-CH_3), 0.823 (t, 3H, J = 6.3, 29-CH_3), 0.859 (d, 3H, J = 6.3, 26-CH_3), 0.869 (d, 3H, J = 6.3, 27-CH_3), 1.031 (d, 3H, J = 6.3, 21-CH_3), 2.81–2.70 (m, 1H, C7—αH), 2.996 (br d, 1H, J = 13.4, C2—βH), 5.039 (dd, 1H, J = 15.0, 8.4, C22—H), 5.166 (dd, 1H, J = 15.3, 8.4, C23—H), 6.096 (br s, 1H, —NH_2), 8.838 (br s, 1H, —NH_2), 9.831 (s, 1H, —NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 159.2 (3-C), 153.5 (6-C), 151.3 (C=O), 138.0 (22-C), 129.5 (23-C), 56.6 (14-C), 55.9 (17-C), 53.8 (9-C), 51.2 (24-C), 50.6 (5-C), 42.8 (10-C), 40.4 (13-C), 39.7 (20-C), 39.2 (12-C), 37.3 (25-C), 35.6 (7-C), 31.9 (8-C), 31.2 (16-C), 30.3 (2-C), 29.7 (28-C), 25.4 (1-C), 24.3 (4-C), 22.7 (15-C), 21.3 (11-C), 21.2 (26-C), 21.1 (27-C), 19.1 (21-C), 12.3 (19-C), 11.9 (18-C), 11.7 (29-C); HRESI-MS, *m/z*: 499.3994 $[M+H]^+$ (calcd for $C_{30}H_{51}N_4O_2$, 499.4012).

4.1.17. (3E)-hydroximinositostan-6-semicarbazone (**12c**)

White solid, yield: 72%. m.p. 246–248 °C. IR (KBr) ν/cm^{-1}: 3447, 3370, 3210, 2953, 2867, 1674, 1584, 1461, 1380, 1077; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.678 (s, 3H, 18-CH_3), 0.807 (s, 3H, 19-CH_3), 0.832 (d, 3H, J = 6.6, 26-CH_3), 0.854 (d, 3H, J = 6.6, 27-CH_3), 0.865 (t, 3H, J = 6.6, 29-CH_3), 0.930 (d, 3H, J = 6.0, 21-CH_3), 2.84–2.79 (m, 1H, C7—αH), 2.964 (br d, 1H, J = 14.1, C2—βH), 6.031 (br s, 1H, —NH_2), 8.924 (br s, 1H, —NH_2), 9.733 (s, 1H, —NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 159.0 (3-C), 153.6 (6-C), 151.3 (C=O), 56.5 (14-C), 56.1 (17-C), 53.4 (9-C), 50.6 (5-C), 45.8 (24-C), 42.9 (10-C), 39.6 (13-C), 39.3 (12-C), 37.3 (20-C), 36.1 (22-C), 35.6 (7-C), 33.9 (8-C), 31.3 (25-C), 30.3 (16-C), 29.7 (2-C), 29.1 (23-C), 28.2 (1-C), 26.1 (4-C), 24.1 (15-C), 23.1 (28-C), 21.3 (11-C), 19.8 (26-C), 19.0 (27-C), 18.7 (21-C), 12.1 (19-C), 12.0 (18-C), 11.9 (29-C); HRESI-MS, *m/z*: 501.4151 $[M+H]^+$ (calcd for $C_{30}H_{53}N_4O_2$, 501.4169).

4.1.18. (3E)-hydroximinocholestan-6-hydrozone (**13a**)

Light yellow solid, yield: 73.5%. m.p. 173–175 °C. IR (KBr) ν/cm^{-1}: 3452, 2945, 2863, 1641, 1465, 1380, 1241, 1171, 1020; ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.658 (s, 3H, 18-CH_3), 0.878 (d, 3H, J = 6.6, 26-CH_3), 0.881 (d, 3H, J = 6.6, 27-CH_3), 0.924 (d, 3H, J = 6.6, 21-CH_3), 0.938 (s, 3H, 19-CH_3), 2.258 (t, 1H, J = 13.8, C7—βH), 2.416 (dd, 1H, J = 12.3, 5.4, C2—αH), 3.311 (dd, 1H, J = 13.8, 4.2, C7—αH), 3.392 (br d, 1H, J = 13.2, C2—βH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.7 (6-C), 165.7 (3-C), 57.0 (14-C), 56.2 (17-C), 54.5 (9-C), 52.6 (5-C), 43.0 (10-C), 40.0 (13-C), 39.8 (24-C), 39.5 (12-C), 39.0 (22-C), 36.1 (20-C), 35.7 (8-C), 35.6 (7-C), 32.8 (16-C), 30.7 (25-C), 28.1 (2-C), 28.0 (1-C), 25.6 (4-C), 24.1 (23-C), 23.9 (15-C), 22.8 (26-C), 22.6 (27-C), 21.6 (11-C), 18.7 (21-C), 12.2 (19-C), 12.1 (18-C); HRESI-MS, *m/z*: 430.3796 $[M+H]^+$ (calcd for $C_{27}H_{48}N_3O$, 430.3797).

*4.1.19. (3E)-hydroximinostigmastan-6-hydrozone (**13b**)*

Light yellow solid, yield: 83%. m.p. 197–198 °C; IR (KBr) ν/cm^{-1}: 3448, 2953, 2868, 1642, 1462, 1380, 975; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.693 (s, 3H, 18-CH_3), 0.821 (t, 3H, J = 6.0, 29-CH_3), 0.833 (d, 3H, J = 6.6, 26 or 27-CH_3), 0.861 (d, 3H, J = 6.6, 26 or 27-CH_3), 0.934 (s, 3H, 19-CH_3), 1.031 (d, 3H, J = 6.3, 21-CH_3), 2.246 (t, 1H, J = 14.7, C5-H), 2.40 (br s, 2H, —NH_2), 3.42–3.29 (m, 2H, C7—αH and C2—αH), 5.036 (dd, 1H, J = 15.0, 8.4, C22—H), 5.158 (dd, 1H, J = 15.0, 8.4, C23—H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.3 (6-C), 165.4 (3-C), 138.1 (22-C), 129.5 (23-C), 57.1 (14-C), 56.0 (17-C), 54.5 (9-C), 52.6 (24-C), 51.3 (5-C), 42.8 (10-C), 40.4 (13-C), 40.0 (20-C), 39.7 (12-C), 39.0 (8-C), 35.7 (25-C), 32.7 (7-C), 31.9 (16-C), 30.8 (2-C), 28.9 (28-C), 25.5 (1-C), 25.4 (4-C), 24.1 (15-C), 21.5 (11-C), 21.2 (26-C), 21.1 (27-C), 19.0 (21-C), 12.2 (19-C), 12.1 (18-C), 11.9 (29-C); HRESI-MS, m/z: 456.3952 $[M+H]^+$ (calcd for $C_{29}H_{50}N_3O$, 456.3954).

*4.1.20. (3E)-hydroximinositostan-6-hydrozone (**13c**)*

Light yellow solid, yield: 81%. m.p 189–191 °C; IR (KBr) ν/cm^{-1}: 3423, 2953, 2867, 1637, 1461, 1380, 1249; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.672 (s, 3H, 18-CH_3), 0.825 (d, 3H, J = 6.6, 26 or 27-CH_3), 0.847 (d, 3H, J = 6.6, 26 or 27-CH_3), 0.858 (t, 3H, J = 6.9, 29-CH_3),, 0.924 (d, 3H, J = 6.3, 21-CH_3), 0.932 (s, 3H, 19-CH_3), 2.252 (t, 1H, J = 14.7, C5—H), 2.419 (br s, 2H, —NH_2), 3.299 (dd, 1H, J = 14.1, 4.2, C7—αH), 3.381 (br d, 1H, J = 13.2, C2—αH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.7 (6-C), 165.7 (3-C), 57.0 (14-C), 56.2 (17-C), 54.5 (9-C), 52.6 (5-C), 45.8 (24-C), 43.0 (10-C), 40.0 (13-C), 39.8 (12-C), 39.0 (20-C), 36.1 (22-C), 35.7 (8-C), 33.9 (7-C), 32.8 (25-C), 30.7 (16-C), 29.1 (2-C), 28.2 (23-C), 26.1 (1-C), 25.6 (15-C), 24.1 (4-C), 23.1 (28-C), 21.6 (11-C), 19.8 (26-C), 19.0 (27-C), 18.7 (21-C), 12.2 (19-C), 12.1 (18-C), 11.9 (29-C); HRESI-MS, m/z: 458.4124 $[M + H]^+$ (calcd for $C_{29}H_{50}N_3O$, 458.4110).

*4.1.21. 3,6-Dioxo-22,23-secostigmastan-22-aldehyde (**14**)*

A flow of O_3 in O_2 was bubbled through a solution of **4b** (500 mg, 1.17 mmol) in a mixture of CH_2Cl_2 (16 mL) and MeOH (4 mL) at −78 °C until the solution turned pale blue. The reaction was monitored by TLC ($V_{\text{ethyl acetate}}$:$V_{\text{petroleum ether}}$ = 4:1). Then the mixture was purged with O_2 for 20 min, and Me_2S (2 mL) was added. The mixture was allowed to warm to room temperature and was stirred overnight. After removing solvent and dimethylsulfide under reduced pressure, the residue was dissolved with 60 mL CH_2Cl_2. The organic layer was washed with distilled water and saturated salt water, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was separated by column chromatography ($V_{\text{petroleum ether}}$:$V_{\text{ethyl acetate}}$ = 4:1) to afford compound **14** (242 mg, 70%) as white solid. m.p. 168–169 °C. IR (KBr) ν/cm^{-1}: 2931, 2871, 2734, 1709, 1463, 1386, 1265, 1227, 920; ^{1}H NMR (DMSO, 600 MHz) δ: 0.689 (3H, s, 18-CH_3), 0.869 (3H, s, 19-CH_3), 1.045 (3H, d, J = 6.5, 21-CH_3), 2.782 (1H, dd, J = 13.5, 4.0, C5—H), 9.538 (1H, d, J = 6.5, C22—H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 209.4 (3-C), 209.3 (6-C), 205.7 (22-C), 56.5 (14-C), 55.5 (5-C), 52.4 (17-C), 51.0 (20-C), 49.0 (13-C), 46.2 (9-C), 43.5 (7-C), 41.0 (10-C), 39.0 (4-C), 37.7 (12-C), 37.6 (2-C), 37.4 (1-C), 37.2 (8-C), 26.9 (16-C), 24.4 (15-C), 21.6 (11-C), 13.6 (19-C), 12.6 (18-C), 12.5 (21-C).

*4.1.22. 3,6-Dioxo-22,23-secostigmastan-22-thiosemicarbazone (**15**)*

Compound **15** was prepared similarly as the procedure for the synthesis of **3**, but from compound **14**. White solid, yield: 20.0%. m.p. 210–212 °C. IR (KBr) ν/cm^{-1}: 3436, 3256, 3141, 2937, 2847, 1711, 1605, 1540, 1375, 1236, 1102; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 0.745 (s, 3H, 18-CH_3), 0.971 (s, 3H, 19-CH_3), 1.148 (d, 3H, J = 6.6, 21-CH_3), 2.65–2.54 (m, 2H, C4—H and C5—H), 6.401 (br s, 1H, —NH), 7.054 (br s, 1H, —NH), 7.170 (d, 1H, J = 6.6, C_{22}—H), 9.702 (s, 1H, =N—NH—); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 211.2 (3-C), 208.8 (6-C), 178.2 (C=S), 152.5 (22-C), 57.5 (17-C), 56.2 (14-C), 53.7 (5-C), 53.4 (13-C), 46.5 (9-C), 43.4 (7-C), 41.2 (10-C), 39.5 (4-C), 39.1 (12-C), 38.0 (2-C), 37.9 (1-C), 37.4 (8-C), 37.0 (20-C), 27.5 (16-C), 24.1 (15-C), 21.6 (11-C), 17.4 (21-C), 12.6 (19-C), 12.3 (18-C).

*4.1.23. 6-Oxo-3-hydroximino-22,23-secostigmastan-22-thiosemicarbazone (**16**)*

Compound **15** (150 mg, 0.36 mmol) and $CH_3COONa{\cdot}3H_2O$ (49 mg, 0.36 mmol) were dissolved in 25 mL of 95% CH_3CH_2OH. After the mixture was heated to 60 °C, $NH_2OH{\cdot}HCl$ (26 mg, 0.37 mmol) was added. The mixture was stirred for 1 h at 65 °C and monitored by TLC ($V_{\text{ethyl acetate}}$:$V_{\text{petroleum ether}}$ = 1:1). Then the reaction was terminated and majority of solvent was evaporated under reduced pressure. Distilled water was added and white solid generated, and the product was extracted with ethyl acetate (20 × 3 mL). The combined extract was washed with water and saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash chromatography ($V_{\text{ethyl acetate}}$:$V_{\text{petroleum ether}}$ = 1:1) to give 48 mg of **16** as light yellow solid. Yield: 30.9%. m.p. 182–184 °C. IR (KBr) ν/cm^{-1}: 3432, 3256, 3150, 2945, 2859, 1707, 1597, 1540, 1376, 1111; ^{1}H NMR (DMSO, 300 MHz) δ: 0.663 (s, 3H, 18-CH_3), 0.750 (s, 3H, 19-CH_3), 1.057 (d, 3H, J = 6.6, 21-CH_3), 2.51–2.38 (m, 4H, C4 and C7—H), 3,10–2.96 (m, 1H, C20—H), 7.279 (d, 1H, J = 6.6, C22—H), 7.424 (br s, 1H, —NH), 7.944 (br s, 1H, —NH), 10.26 (br s, N—OH), 10.949 (br s, 1H, =NNH—); ^{13}C NMR (DMSO, 75 MHz) δ: 210.4 (6-C), 178.0 (C=S), 156.7 (3-C), 152.1 (22-C), 57.1 (5-C), 56.0 (17-C), 53.8 (14-C), 52.6 (13-C), 46.2 (9-C), 43.2 (7-C), 41.7 (10-C), 37.6 (12-C), 36.7 (8-C), 30.0 (20-C), 27.5 (4-C), 27.1 (2-C), 26.9 (1-C), 24.1 (15-C), 21.4 (11-C), 19.7 (21-C), 17.8 (19-C), 12.4 (18-C); ESI-MS, m/z: 433.4 $[M + H]^+$ (calcd for $C_{23}H_{37}N_4O_2S$, 433.3).

4.2. Antiproliferative activity

4.2.1. Materials

Stock solutions of the compounds were prepared in sterile dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 10 mg/mL and afterward diluted with complete nutrient medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate.

4.2.2. Cell culture

SGC 7901 and Bel 7404 cancer cells were grown in the medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C.

4.2.3. Assay for cell viability

The cell proliferation assay was undertaken by a MTT method using 96-well plates. Using cisplatin as a positive control, the antiproliferative activity of the compounds was determined. Briefly, cells (3×10^4 cells per well) were seeded in 96-wells plates. One day after seeding, cells in the wells were respectively treated with target compounds at various concentrations. An equal amount of DMSO was added to the cells used as negative controls. All were treated in triplicate. After reincubated for 72 h, 20 μL of the tetrazolium dye (MTT) (5 mg/mL) solution were added to each well, and the cells were incubated for an additional 4 h. After the supernatant was discarded, 200 μL of DMSO were added to dissolve the purple formazan crystals formed. The absorbance values (A) at 492 nm were determined using a MLLTISKAN MK3 analysis spectrometer (Thermo Scientific Co.). The IC_{50} values were calculated as the concentration of drug yielding 50% cell survival.

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