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Short communication

Isolation and identification of novel impurities in spironolactone

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Abstract

Three known and five new steroidal compounds as impurities in spironolactone were isolated from the enriched mother liquor by using various chromatographic methods. Their structures were elucidated by spectrometric analysis. New compounds were characterized as 3-(3,3-dimethoxy-5α,7α-epidithio-17β-hydroxy-4-androstan-17α-yl) propionic acid γ-lactone (**6**); 3-(3-oxo-7α-acetylthio-6β,17β-dihydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**7**); 7α-acetylthio-17β-20-isopropylidendioxy-21-nor-17α-pregn-4-en-3-one (**8**); 7α-acetylthio-3-oxo-pregna-4,17(20)i-dien-22-oic acid methyl ester (**9**) and 7α-acetylthio-17-methyl-18-nor-androsta-4-en-3-one (**10**).

Keywords: Steroids; Spironolactone; Chromatographic methods; Spectrometric analysis

1. Introduction

Spironolactone [3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone, **1**] [1,2] is widely used as an aldosterone-antagonist diuretic in the clinic. As a potassium-sparing diuretic, it is safe and efficacious in treatment of refractory oedema associated with heart failure (below), cirrhosis of liver (with or without ascites), or the nephritic syndrome, and in ascites associated with malignancy. The production of spironolactone [3,4] is always accompanied by side reactions leading to various unwanted impurities. As per the stringent regulatory requirements, the impurity profile study has to be carried out for any final product to characterize the impurities present at a lever of 0.1%. In an attempt to clarify the impurities existed in spironolactone produced by the scheme shown in Fig. 1 (4-AD(**2**) as starting material) [5], six novel spironolactone-analogs were isolated, identified and reported in our preceding paper [6]. During the course of our further investigation, another three known compounds (**3**–**5**) together with five new analogs (**6**–**10**) (Fig. 2) were obtained from the enriched mother liquor by general column chromatography and analytical HPLC. The study may be helpful to identify and characterize all the impurities that are present at a level of 0.1% and be useful in the quality control of the production of spironolactone. This paper describes the isolation and structural elucidation of the new impurities.

2. Experimental

2.1. Samples

The enriched mother liquor from repeated crystallization of spironolactone was provided by Xianju Zhiyao Erchang, Zhe-Jiang Province, China.

2.2. General chromatography and high performance liquid chromatography

General column chromatography was carried out with silica gel 60H from Qingdao Haiyang Chemical Group Co., China. HPLC was performed on HP-1100, with UV detection at 240 nm at a flow rate of 1.0 ml/min on Lichrosorb RP-18 (10 μm, 300 mm × 4 mm) column with methanol–water (75:25) as mobile phase.

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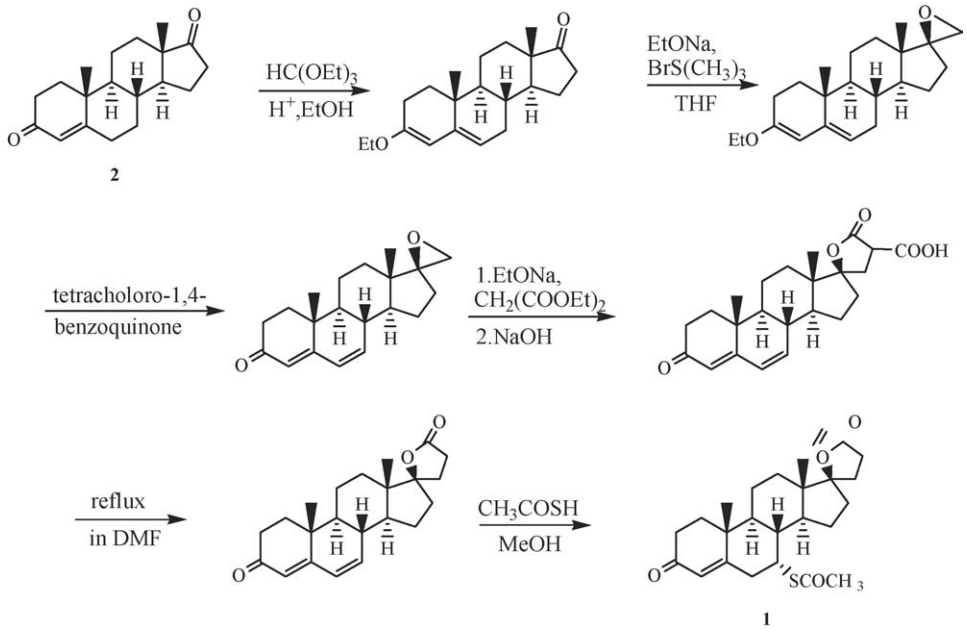

Fig. 1. Scheme for the synthesis of spironolactone (**1**).

2.3. NMR spectroscopy

^{1}H, ^{13}C, and 2D NMR spectra were recorded on Bruker-AM-400 spectrometer using TMS as internal standard.

2.4. MS spectrometry

EI-MS and ESI-MS spectra were recorded on MAT-711 and Quayyro spectrometer, respectively.

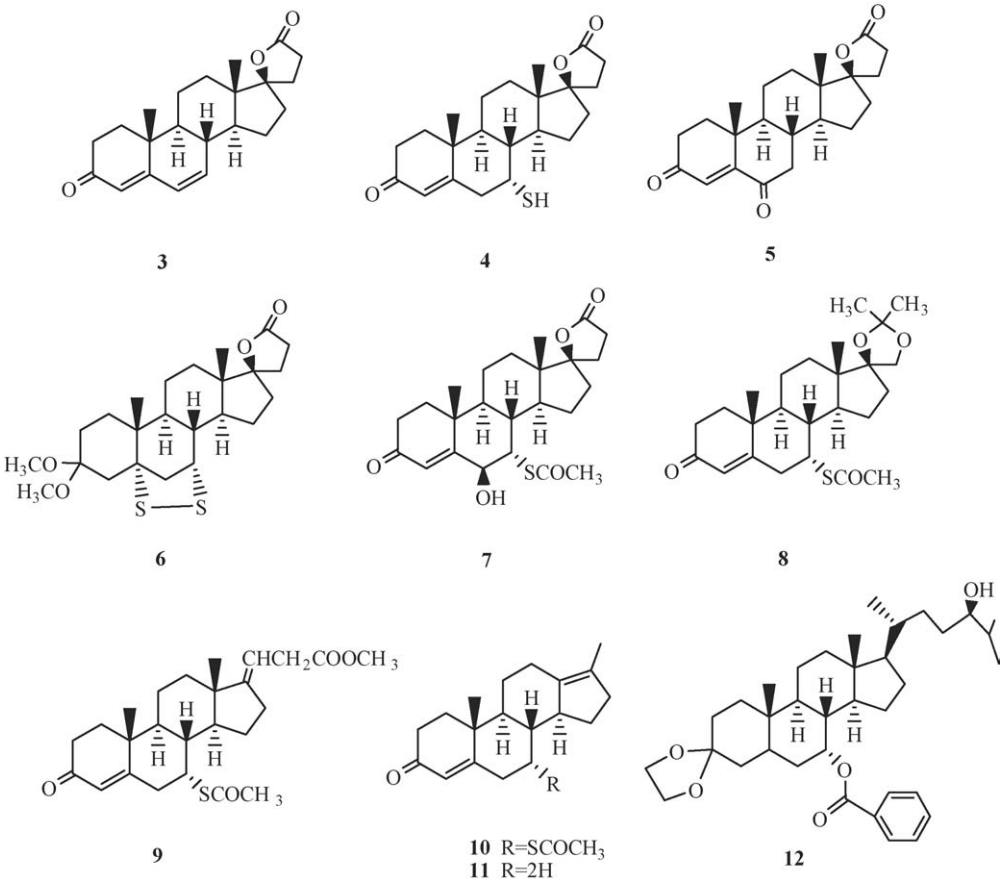

Fig. 2. The structures of **3**–**12**.

Table 1
The ^{1}H NMR data of compounds (**3**–**10**) [$CDCl_3$, δ (ppm) (J in Hz)]

Atom ^{1}H	**1**	**3**	**4**[a]	**5**	**6**[a]	**7**	**8**	**9**[a]	**10**
4	5.70 brs	5.68 s	5.70 brs	6.18 s		5.82 s	5.68 s	5.61 brs	5.73 d J = (1.69)
6		6.04 d J = (9.62)				4.18 d J = (3.02)			
7	3.95 AB J = (6.83) (2.63)	6.04 dd (9.62) (1.38)	3.58 AB (6.59) (3.29)		3.77 AB (6.80) (2.84)	3.88 t (3.16)	3.94 AB (6.60) (2.93)	4.02 AB (6.60) (2.93)	4.11 A B (6.71) (3.69)
18	0.94 s	1.02 s	0.97 s	0.98 s	0.92 s	1.03 s	0.91 s	0.86 s	1.60 brs
19	1.19 s	1.12 s	1.29 s	1.18 s	1.13 s	1.39 s	1.22 s	1.32 s	1.13 s
20								5.27 t J = (7.50)	
3-OCH_3					3.11 s, 3.13 s				
7-$SCOCH_3$2.35 s						2.38 s	2.33 s	2.33 s	2.32 s
21-CH_3							1.32 s, 1.37 s		
22-OCH_3								3.62 s	

[a] Measured in Acetone-d6.

2.5. FT-IR spectroscopy

FT-IR spectra were recorded on Perkin-Elmer-599B FT-IR as KBr pellet.

2.6. Polarimeter

Optical rotation was recorded on Jasco-DIP-181 polarimeter using acetone as solvent.

3. Synthesis of spironolactone

The scheme for the synthesis of spironolactone [5] is shown in Fig. 1.

4. Results and discussion

4.1. Isolation of the impurities

The enriched mother liquor (250 ml, 4-AD (10 kg) was used for the synthesis) was silica gel 60H (2 kg) chromatographed, using a gradient elution system of cyclohexane–acetone (10:1, 8:1, 6:1, 5:1, 3:1, 2:1, 1:1). The fractions 5–10 were subjected repeatedly to silica gel flash chromatography with cyclohexane–acetone (10:1) as solvent to yield compounds **4**, **6** and **10**, and compound **9** was obtained by analytic RP-HPLC using methanol–H_2O (75:25) as mobile phase. The fractions 11–24 were also subjected repeatedly to silica gel 60H flash chromatography with cyclohexane–acetone (5:1) as solvent to yield compounds **5**, **7** and **8**. The fraction 25–35 were further fractioned into four parts by silica gel 60H with cyclohexane–acetone (5:1). Among them, the third fractions were subjected repeatedly to silica gel flash chromatography with cyclohexane–acetone (3:1) as solvent to yield compound **3**.

4.2. Structural elucidation

4.2.1. Compounds *3*–*5*

The structures of the known compounds canrenone (**3**) [7,8], 7α-thio-spironolactone (**4**) [7–9] and 3-(3.6-dioxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**5**) [8,10] were confirmed by comparing their spectral data (MS, ^{1}H and ^{13}C NMR) with those in the literatures.

4.2.2. Compound *6* [3-(3,3-dimethoxy-5α,7α-epidithio-17β-hydroxy-4-androstan-17α-yl) propionic acid γ-lactone]

Compound **6** was obtained as amorphous powder. Using HREIMS, its molecular formula was deduced to be $C_{24}H_{36}O_4S_2$. Comparing with compound **1**, the NMR data (Tables 1 and 2) of **6** showed that the 7-acetylthio group and the 4,5-unsaturated bond were missed, due to the absence of the corresponding protons signals. Two methoxy (δ_H 3.11, 3H, s, δ_C 47.6 and δ_H 3.13, 3H, s, δ_C 47.6) on C-3 (δ_C 99.3) was deduced from the correlations between C-3 and 3-OCH_3 in HMBC spectrum (Fig. 3). Considering the existing of only one proton at C-7 (δ_C 52.8) and the chemical shift of 7-H (δ_H 3.77, 1H, m), C-7 and C-5 (δ_C 70.1) (a quaternary carbon) in low field, a ring joined from C-5 to C-7 through a S link bridge was constructed to satisfy the seven unsaturation degrees of **6**. The fragment ion peaks in HREIMS spectrum in Table 3 shows the atom S was decomposed from the molecule successively, and the five-membered ring with two S atoms was confirmed. The HMBC correlations between C-5/7-H, C-9/7-H, C-5/6-H and C-5/4-H also supported the structure.

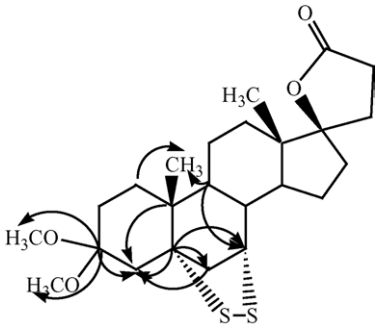

Fig. 3. The key HMBC correlations of **6**.

Table 2
The ^{13}C NMR data of compounds (**3**–**10**) [$CDCl_3$, δ (ppm)]

Atom ^{13}C	**1**	**3**	**4**[a]	**5**	**6**[a]	**7**	**8**	**9**[a]	**10**
1	35.6	33.8	35.6	35.0	31.0	37.1	35.5	36.0	35.5
2	33.9	33.8	33.9	33.6	28.9	34.1	33.9	34.1	33.9
3	198.4	199.3	198.5	199.0	99.4	198.5	198.8	197.5	199.1
4	126.7	123.8	127.6	125.5	39.3	129.0	126.8	126.8	126.6
5	165.5	163.0	165.2	160.0	70.1	164.5	166.2	166.5	166.8
6	39.9	128.2	42.0	201.0	48.0	76.6	39.9	40.2	39.8
7	45.1	139.4	40.1	45.8	52.8	49.1	45.1	46.4	45.3
8	39.0	37.7	39.5	34.1	41.9	32.3	38.4	37.3	48.0
9	49.5	46.9	46.0	50.2	45.3	49.1	50.0	51.0	48.6
10	38.5	35.9	38.5	39.4	42.0	37.9	38.5	39.2	38.6
11	20.5	20.0	20.1	20.1	20.8	20.5	20.5	21.1	25.0
12	31.1	31.5	31.2	31.8	31.9	31.1	31.2	34.3	28.2
13	45.5	46.3	45.3	45.3	46.1	45.5	43.9	47.0	129.0
14	46.0	50.3	45.3	49.7	46.1	45.3	47.0	53.8	49.2
15	22.3	22.4	22.1	22.5	22.3	22.2	22.1	22.5	25.9
16	35.2	35.4	35.3	35.3	35.6	35.2	34.2	30.6	37.0
17	95.4	95.3	95.6	95.1	95.3	95.7	91.0	121.7	134.4
18	14.6	14.3	14.7	14.3	15.2	14.6	14.2	15.6	13.5
19	17.8	16.2	17.7	17.3	17.9	20.3	17.8	17.3	18.3
20	31.1	31.1	30.8	31.0	31.4	31.2	71.6	154.5	
21	29.2	29.2	29.0	28.9	29.5	29.2	108.0	32.2	
22	176.3	176.5	176.2	176.6	175.8	176.7		173.4	
7-SCO	194.1					194.2	194.6	194.2	194.7
7-$SCOCH_3$	31.3					31.3	31.3	30.9	31.7
3-OCH_3					47.6, 47.6				
22-OCH_3								51.2	
21-CH_3							26.0, 26.9		

[a] Measured in Acetone-d6.

The β position of 7-H was determined by comparing the closely similar chemical shift of the characteristic signal 7-H to that in 7α-spironolactone, whereas, it differed distinctly from that in 7β-spironolactone (7α-H δ_H 3.28, td, J = 12.22; 5.35 Hz). The configuration of C-5 is determined by the movement of the chemical shift of 19-CH_3 and C-10. The signals were usually downfield shifted due to the deshielding effect of 5α substituent group [11,12]. Comparing with the reference compound **12** [13] ($\delta_{19\text{-H}}$ 0.89 and $\delta_{C\text{-}19}$ 12.2), the signals of 19-CH_3 (δ_H 1.13 and δ_C 17.9) in **6** were significantly downfield shifted by 0.24 and 5.5 ppm. The configuration of 5α-S was also favored by the markedly downfield shift of C-10 ($\delta_{C\text{-}10}$ 42.0 in **6** while $\delta_{C\text{-}10}$ 35.9 in **12**) whereas the upfield shift would be observed in 5β substituent [11].

For NMR data see Tables 1 and 2, $[\alpha]_D^{20} = -188$ (c = 1.37, $(CH_3)_2CO$). HREIMS: m/z = 452.2058 (calcd m/z 452.2057, Δ −0.3 ppm).

Table 3
The fragment ion peaks of compound **6** in the HREIMS spectrum

Mass (m/z)	% Relative abundance	Delta (ppm)	Composition
355.2266	100.00	0.8	$C_{23}H_{31}O_3$
387.1990	15.89	0.4	$C_{23}H_{31}O_3S$
388.2050	5.01	2.3	$C_{23}H_{32}O_3S$
420.1797	12.30	−0.4	$C_{23}H_{32}O_3S_2$
452.2058	14.34	−0.3	$C_{24}H_{36}O_4S_2$

*4.2.3. Compound **7** [3-(3-oxo-7α-acetylthio-6β,17β-dihydroxy-4-androsten-17α-yl) propionic acid γ-lactone]*

Compound **7**, amorphous powder, had the molecular formula $C_{24}H_{32}O_5S$, as determined by positive ESIMS (m/z 455.3 $[M + Na]^+$ and 887.1 $[2M + Na]^+$). A strong absorption band in IR spectrum at 3411 cm^{-1} showed that the compound was hydroxylated. The band at 1772 cm^{-1} indicted that the γ-lactone group of spironolactone was still intact [7]. In ^1H NMR, the doublet at δ_H 4.18 (1H, J = 3.02 Hz) was assigned to the C-6 (δ_C 76.6) proton which was evidenced by HMBC correlations between C-10 (δ_C 37.9)/6-H (δ_H 4.18) and C-4 (δ_C 129.0)/6-H. The triplet at δ_H 3.88 (1H, J = 3.16 Hz) was assigned to the C-7 (δ_C 49.1). The configuration of C-6 can be determined by the C-19 methyl signal, which was downfield shifted by 80 Hz as compared to that in reference compound **1**. The deshielding effect could be attributed to the 1,3-diaxial interaction with the 6β-hydroxy group [14]. The small coupling constant suggested β-configuration of the hydroxy group at C-6 and α-configuration of the substituent at C-7 [15]. The 6β–OH was also proved by the cross-peak between 6-H and 4-H in ROESY.

For NMR data see Tables 1 and 2, $[\alpha]_D^{20} = -26$ (c = 1.63, $(CH_3)_2CO$). IR: 3411, 1772. ESIMS: m/z = 455.3 $[M + Na]^+$ and 887.1 $[2M + Na]^+$.

*4.2.4. Compound **8** [7α-acetylthio-17β-20-isopropylidendioxy-21-nor-17α-pregn-4-en-3-one]*

Compound **8**, oil, had the molecular formula $C_{25}H_{36}O_4S$, as determined by ESI MS (m/z 455.3 $[M + Na]^+$) in combination

with the ^{1}H and ^{13}C NMR data (Tables 1 and 2). The absence of ^{13}C signal at 176 ppm (C-22 in **1**) and C-17 shifted from 95.4 ppm (in **1**) to 91.0 ppm (in **8**) displayed the difference between **8** and **1** in the γ-lactone. The two doublet δ_H 3.59 and δ_H 4.05 ($J = 8.98$ Hz) were assigned to C-20, which were proved by the cross-peaks in HMQC and the correlations with the C-13 (δ_C 43.9) and C-16 (δ_C 34.2) in HMBC. The strong HMBC correlations between the two-methyl signals (δ_H 1.32 and 1.37) and the δ_C 108.0 (C-21) indicated that the both methyls were joined to the C-21. The Overhauser effects in ROESY between 20-H and 14-H confirmed the C-20 was on the α orientation with regard to the α position of 14-H.

For NMR data see Tables 1 and 2, $[\alpha]_D^{20} = -45$ ($c = 1.65$, $(CH_3)_2CO$). ESI MS: $m/z = 455.3$ $[M + Na]^+$.

4.2.5. Compound **9**

[7α-acetylthio-17-methyl-18-nor-androsta-4-en-3-one]

Compond **9** was obtained as an oil by HPLC, which had the molecular formula $C_{25}H_{34}O_4S$, as determined by ESI MS (m/z 453.3 $[M + Na]^+$ and 882.9 $[2M + Na]^+$). Comparison of the NMR data (Tables 1 and 2) with that of **1** showed that γ-lactone should be opened and a methyl ester (δ_H 3.62, 3H, s; δ_C 51.2, 22-OCH_3 and δ_C 173.4, C-22) was formed. After assignment of ^{1}H and ^{13}C by HMQC and HMBC, the trisubstituted olefin Δ 17.20 ($\delta_{20\text{-}H}$ 5.27, 1H, m; $\delta_{C\text{-}17}$ 154.5 and $\delta_{C\text{-}20}$ 121.7) formed via elimination reaction was proved by the HMBC correlations between C-14, C-15/16-H and C-16/14-H. The geometry of the *trans*-17(20)-ene was determined by 18-CH_3 (δ_H 0.86, 3H, s) which was upfield shifted by 0.07 ppm compared to that (δ_H 0.93, 3H, s) in reference compound **2** [16], because NMR studies [17–19] have shown that, in 17(20)-ene with a *cis*-oriented ethylidene side chain, the deshielding effect of C-18 angular methyl group occurs in contrast to the trans ethylidene side chain relative to the corresponding C-17 ketone in NMR.

For NMR data see Tables 1 and 2, $[\alpha]_D^{20} = -44$ ($c = 1.15$, $(CH_3)_2CO$). ESI MS: $m/z = 453.3$ $[M + Na]^+$ and 882.9 $[2M + Na]^+$.

4.2.6. Compound **10**

[7α-acetylthio-3-oxo-pregna-4,17(20)i-dien-22-oic acid methyl ester]

Compound **10**, oil, had the molecular formula $C_{21}H_{28}O_2S$, as determined by EIMS (m/z 344 $[M]^+$ (0.02%) and m/z 268 $[M - HSCOCH_3]^+$ (100%)). The ^{1}H and ^{13}C NMR (Tables 1 and 2), HMBC and HMQC, and their comparison with spironolactone and compound **11** (δ_C 128.4, C-13 and δ_C 135.3, C-17) [20], allowed the assignment of the structure shown in Fig. 2. The olefin 13(17) was evidenced by the significantly downfield shift of 18-CH_3 (δ_H 1.60, markedly downfield shifted by 0.66 ppm compared to **1**) and was further established by the strong correlations between 18-H and C-13, C-17 and C-16 in HMBC. The 18-CH_3 migrated from C-13 to C-17 possibly by Wagner–Meerwein rearrangement of a Diaxial 13β-methyl-17α-ol [21].

For NMR data see Tables 1 and 2, $[\alpha]_D^{20} = +5$ ($c = 0.90$, $(CH_3)_2CO$). EIMS: $m/z = 344$ $[M]^+$ (0.02%) and m/z 268 $[M - HSCOCH_3]^+$ (100%).

5. Conclusion

In summary, three known and five new impurities have been identified in spironolactone. The known compounds had been reported as metabolites or the intermediate compounds in the biotransformation of spironolactone [8,9], so the structural elucidation of the impurities may be helpful to identify those very minor metabolites or the correlated intermediates which were difficult to characterize by LC-MS or GC-MS analysis. The discovery of the new compounds which have similar chemical and biological characters to spironolactone would provide the opportunities to study their bioactivities and the possibilities to develop potent new drugs for clinical use. Further biological tests on the new compounds are in progress.

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