Isolation and Structural Elucidation of Related Impurities in Canrenone

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Ten steroidal compounds as impurities in canrenone were isolated from the enriched mother liquor by using various chromatographic methods. Their structures were elucidated by spectrometric analysis, among which three new compounds were characterized as 3-(3-oxo-7α-(ethoxycarbonyl)methyl-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**1**), 3-(3-oxo-7α-ethoxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**2**) and 3-(3-oxo-5β-propionic acid-γ-lactone-6β,17β-hydroxy-4-androstan-17α-yl) propionic acid γ-lactone (**3**).

Key Words: Canrenone, Imputities, Chromatographic methods, Spectrometric analysis

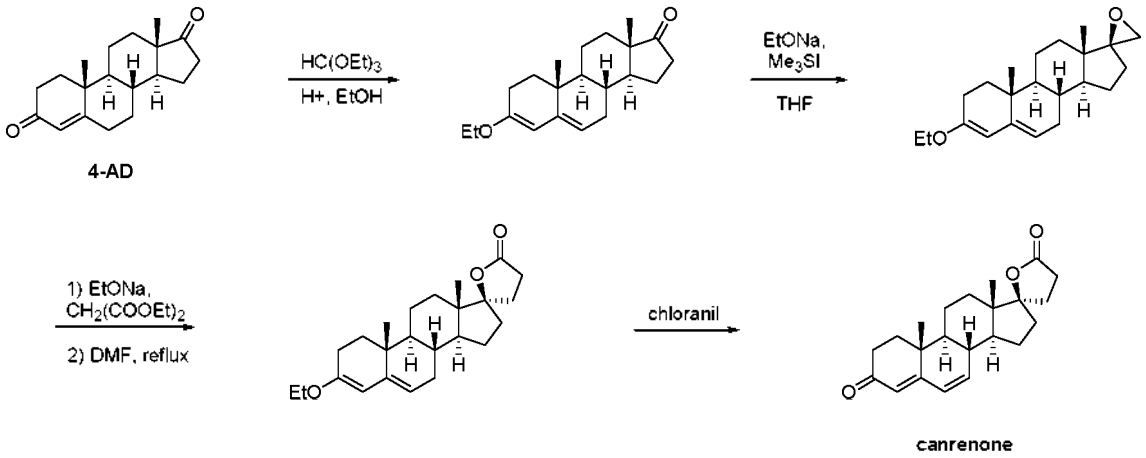
Introduction

Canrenone, [3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl) propionic acid γ-lactone], is useful in the treatment of primary hyperaldosteronism and refractory edema with secondary hyperaldosteronism such as is seen in patients with cardiac failure, hepatic cirrhosis, nephrotic syndrome, and severe ascites.[1,2] On the other hand, canrenone is the key intermediate in the synthesis of spironolactone, a widely used aldosterone-antagonist diuretic in clinic[3,4] and eplerenone, the first selective aldosterone receptor antagonist which was approved by the FDA for the treatment of hypertension.[5,6] It is known that the production of canrenone[7,8] is always accompanied by side reactions leading to various unwanted impurities, so the impurity profile study should be carried out for any final product to characterize the impurities present at a level of 0.1% with regard to the stringent regulatory requirements.

In an attempt to clarify the impurities existed in canrenone produced by the process shown in Scheme 1 starting from androsta-4-ene-3,17-dione (4-AD for short), a series of impurities were obtained from the enriched mother liquor by general column chromatography (Figure 1). Three new compounds were characterized as 3-(3-oxo-7α-(ethoxycarbonyl)methyl-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**1**), 3-(3-oxo-7α-ethoxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**2**) and 3-(3-oxo-5β-propionic acid-γ-lactone-6β,17β-hydroxy-4-androstan-17α-yl) propionic acid γ-lactone (**3**), combined with seven known compounds: 18-norandrostra-4,6,13-(17)-trien-3-one (**4**),[9] 3-(3,6-dioxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**5**),[10] androsta-4,6-diene-3,17-dione (**6**),[11] 3-(3-oxo-6α,7α-epoxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**7**),[12] 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**8**),[14] 3-(3-oxo-6β,7α-dihydroxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**9**),[12,14] and 3-(3-oxo-6α,7β-dihydroxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone (**10**).[12,14] The structure of these compounds were further elucidated by means of 1D and 2D (HSQC, HMBC and ROESY) NMR data. This study would then presumably be advantageous to the identification and characterization of all impurities that are present at a level of 0.1% and as well be favorable in the quality control of canrenone production.

Experimental Section

Reagents and Instruments. All solvents were of analytical



Scheme 1. the synthesis of canrennone.

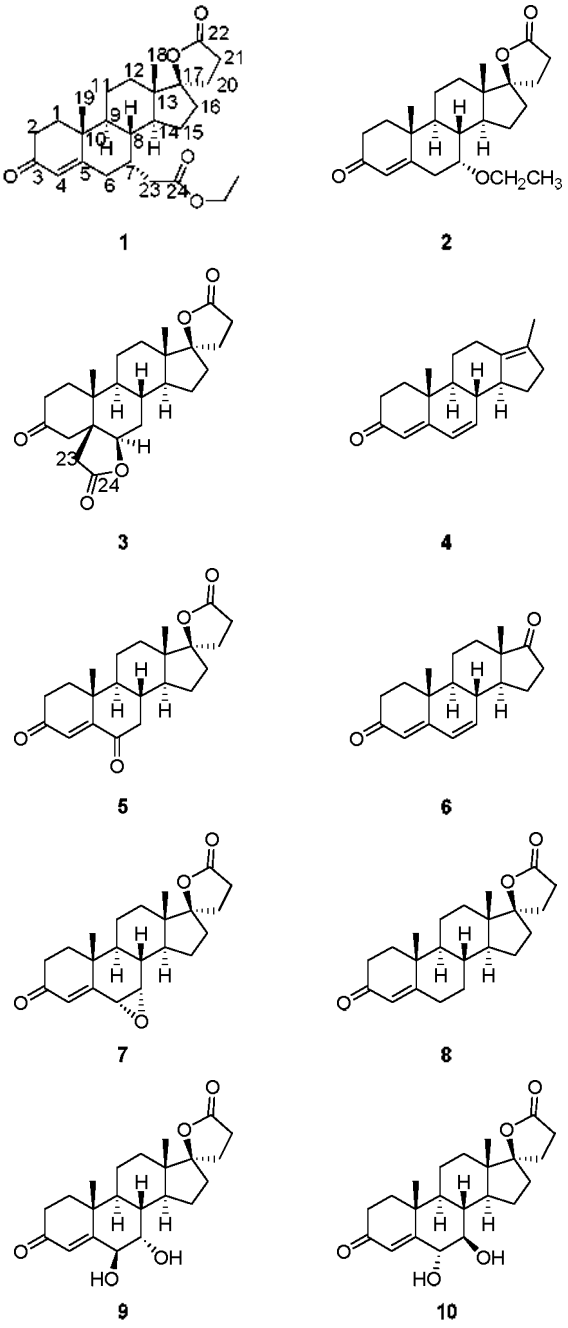


Figure 1. Structures of compounds **1** ~ **10**.

grade. ^{1}H, ^{13}C, and 2D NMR spectra were recorded on Bruker-AM-400 spectrometer using TMS as internal standard. EIMS spectra were recorded on MAT-711. FT-IR spectra were recorded on Perkin-Elmer-599B as KBr pellet. UV spectra was measured on a Varian CARY 300 Bio Spectrometer using menthol as solvent. Optical rotation was recorded on Jasco-DIP-181 polarimeter using acetone as solvent. The chemical shift values are reported in ppm relative to the solvent used. Silica gel for column chromatography and silica gel GF254 plates (0.10 mm) for TLC were purchased from Qingdao Marine Chemical Group Co., P. R. China. Reversed-phase chromatography was with RP-18 (LiChroprep, 40 ~ 63 μm, Merck, Darmstadt, Germany).

Raw Material. The enriched mother liquor from repeated crystallization of canrenonne was provided by Desano Pharma, Shanghai, China. The method used for the synthesis of canrenone was shown in Scheme 1.

Extraction and Isolation. The mother liquor was concentrated in methanol and pyridine at 80 °C for about 8 h, and then, the residue (100 g) was dissolved in dichloromethane. Dichloromethane extracts were chromatographed on silica gel 60H (3 kg) using a gradient elution system of petroleum ether-acetone (5:1 to 1:1) to get seven fractions A-G. Fraction A was repeatedly chromatographed (silica gel H, cyclohexane-ethyl acetate 4:1) to yield compound **4**. Fractions B-E were also repeatedly subjected to silica gel flash chromatography with cyclohexane-ethyl acetate (4:1 to 1:1) as solvent to yield compounds **1**, **2** and **5-8**, while compound **6** which was isolated in crystalline form could be recrystallized from acetone. The last two fractions F and G were further purified by RP-C_{18} silica gel (methanol-H_2O 70:30) to yield dilactone compound **3** and dihydroxyl compounds **9**, **10**.

Compound 1: $C_{26}H_{36}O_5$, amorphous powder; $[\alpha]_D^{20}$+6.6 (*c* 1.4, acetone); IR (KBr) ν_{max}: 2953, 1770, 1727, 1674, 1619 cm^{-1}; UV (MeOH) λ_{max} (log ε): 241 nm; HR-EIMS: *m/z* 428.2571 $[M]^+$ (calcd. for $C_{26}H_{36}O_5$, 428.2563); EIMS: *m/z* (%) 428 $[M]^+$ (12), 341 $[M\text{-}CH_2COOC_2H_5^+]$ (100); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 1.

Compound 2: $C_{24}H_{34}O_4$, amorphous powder; $[\alpha]_D^{20}$ -1.2 (*c* 0.9, acetone); IR (KBr) ν_{max}: 2966, 2900, 1772, 1664, 1619 cm^{-1}; UV (MeOH) λ_{max} (log ε): 243 nm; HR-EIMS: *m/z* 386.2453 $[M]^+$ (calcd. for $C_{24}H_{34}O_4$, 386.2457); EIMS: *m/z* (%) 386 $[M]^+$ (100), 341 $[M\text{-}OCH_2CH_3^-]$ (80); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 1.

Compound 3: $C_{24}H_{32}O_5$, white solid; $[\alpha]_D^{20}$+30 (*c* 0.1, acetone); IR (KBr) ν_{max}: 2921, 2848, 1770, 1650, 1403 cm^{-1}; HR-EIMS: *m/z* 400.2256 $[M]^+$ (calcd. for $C_{24}H_{32}O_5$, 400.2250); EIMS: *m/z* (%) 400 $[M]^-$ (45), 111 (100); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 1.

Compound 4: $C_{19}H_{24}O$, yellow oil; EIMS: *m/z* (%) 286 $[M]^-$ (48), 174 (100); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.12 (1 H, s, H-6), 6.12 (1 H, s, H-7), 5.68 (1 H, s, H-4), 1.62 (3 H, s, H-18), 1.05 (3 H, s, H-19); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 199.6 (C-3), 163.6 (C-5), 140.8 (C-7), 135.1 (C-17), 129.7 (C-13), 127.4 (C-6), 123.6 (C-4), 50.8 (C-14), 48.9 (C-8), 46.9 (C-9), 36.9 (C-16), 36.1 (C-10), 33.9 (C-1), 33.8 (C-2), 27.3 (C-12), 25.5 (C-15), 24.5 (C-11), 16.5 (C-19), 13.4 (C-18).

Compound 5: $C_{22}H_{28}O_4$, amorphous powder; EIMS: *m/z* (%) 356 $[M]^+$ (84), 341 $[M\text{-}CH_3^+]$ (20), 328 $[M\text{-}CO^-]$ (28); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.15 (1 H, s, H-4), 1.16 (3 H, s, H-19), 0.97 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 201.1 (C-6), 199.1 (C-3), 176.4 (C-22), 160.1 (C-5), 125.6 (C-4), 95.2 (C-17), 50.3 (C-9), 49.8 (C-14), 45.9 (C-7), 45.5 (C-13), 39.5 (C-10), 35.4 (C-16), 35.2 (C-1), 34.2 (C-8), 33.8 (C-2), 31.1 (C-12), 31.1 (C-20), 29.1 (C-21), 22.6 (C-15), 20.2 (C-11), 17.4 (C-19), 14.4 (C-18).

Compound 6: $C_{19}H_{24}O_2$, white solid; EIMS: *m/z* (%) 284 $[M]^-$ (100), 136 (100); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.16 (1 H, s, H-6), 6.16 (1 H, s, H-7), 5.67 (1 H, s, H-4), 1.12 (3 H, s, H-19), 0.94 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 219.4 (C-17), 199.2 (C-3), 162.9 (C-5), 138.3(C-7), 128.7 (C-6), 124.0 (C-4), 50.6 (C-14), 48.6 (C-9), 48.2 (C-13), 36.9 (C-8),

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **1** ~ **3**.

compound	**1**		**2**		**3**	
position	δ_H (int, mult, J in Hz)	δ_C	δ_H (int, mult, J in Hz)	δ_C	δ_H (int, mult, J in Hz)	δ_C
1		35.7		35.4		35.4
2		33.9		35.4		36.3
3		199.1		198.9		208.1
4	5.68 (1 H, s)	123.4	5.71 (1 H, s)	126.2	2.11, 2.82 (2 H, d, 15.1)	46.9
5		168.9		168.2		49.5
6		39.3		36.2	4.11 (1 H, d, 3.8)	83.2
7		39.6	3.47 (1 H, m)	74.4		31.6
8		39.9		40.2		30.9
9		53.4		45.5		40.8
10		38.2		38.2		37.3
11		20.6		20.3		20.4
12		31.2		31.1		29.4
13		46.7		45.4		45.5
14		48.9		43.3		50.2
15		26.2		22.4		22.8
16		35.5		33.9		33.1
17		94.7		95.9		95.8
18	0.96 (3 H, s)	14.8	0.94 (3 H, s)	14.2	0.97 (3 H, s)	14.6
19	1.18 (3 H, s)	17.3	1.17 (3 H, s)	17.2	1.09 (3 H, s)	18.8
20		31.1		31.2		31.1
21		29.2		29.3		21.2
22		176.6		176.8		176.4
23	2.65, 2.28 (2 H, dd, 15.9, 2.1)	40.1			2.34, 2.69 (2 H, d, 17.1)	41.4
24		172.3				175.6
OCH_2CH_3	4.12 (2 H, q, 7.3)	60.4	3.24, 3.54 (2 H, dq, 9.3, 7.0)	63.6		
OCH_2CH_3	1.24 (3 H, t, 7.3)	14.2	1.10 (3 H, t, 7.0)	15.2		

36.0 (C-10), 35.5 (C-16), 33.8 (C-1), 33.8 (C-2), 31.1 (C-12), 21.3 (C-15), 19.9 (C-11), 16.2 (C-19), 13.6 (C-18).

Compound 7: $C_{22}H_{28}O_4$, amorphous powder; EIMS: *m/z* (%) 356 $[M]^+$ (100), 138 (90); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.15 (1 H, s, H-4), 3.37 (1 H, dd, J = 18.6, 3.7 Hz, H-6), 3.31 (1 H, dd, J = 18.6, 3.7 Hz, H-7), 1.22 (3 H, s, H-19), 1.01 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 198.1 (C-3), 176.4 (C-22), 162.3 (C-5), 129.5 (C-4), 95.1 (C-17), 58.3(C-7), 55.5 (C-6), 51.4 (C-14), 46.3 (C-9), 46.2 (C-13), 36.2 (C-16), 35.7 (C-8), 35.6 (C-10), 35.3 (C-1), 34.0 (C-2), 31.4 (C-12), 31.1 (C-20), 29.1 (C-21), 22.6 (C-15), 20.7 (C-11), 16.9 (C-19), 14.4 (C-18).

Compound 8: $C_{22}H_{30}O_3$, white powder; EIMS: *m/z* (%) 342 $[M]^+$ (100), 300 (32); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.71 (1 H, s, H-4), 1.19 (3 H, s, H-19), 0.96 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 199.4 (C-3), 176.7 (C-22), 170.5 (C-5), 123.9 (C-4), 95.7 (C-17), 53.4 (C-14), 49.2 (C-9), 45.5 (C-13), 38.5 (C-10), 35.8 (C-8), 35.7 (C-2), 35.4 (C-1), 33.9 (C-16), 32.6 (C-6), 31.6 (C-7), 31.4 (C-12), 31.2 (C-20), 29.2 (C-21), 22.4 (C-15), 20.4 (C-11), 17.4 (C-19), 14.5 (C-18).

Compound 9: $C_{22}H_{30}O_5$, white solid; EIMS: *m/z* (%) 374 $[M]^+$ (100), 345 (85); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.19 (1 H, s, H-4), 4.18 (1 H, d, J = 7.8 Hz, H-6), 3.22 (1 H, t, J = 9.5 Hz, H-7), 1.23 (3 H, s, H-19), 0.99 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 199.2 (C-3), 176.8 (C-22), 166.8 (C-5), 120.8 (C-4), 95.2 (C-17), 79.7(C-7), 73.7 (C-6), 50.4 (C-9), 48.6 (C-14), 46.1 (C-13), 40.6 (C-8), 38.5 (C-10), 36.3 (C-1), 35.6 (C-12), 33.6 (C-2), 31.3 (C-16), 31.1 (C-20), 29.3 (C-21), 25.6 (C-15), 20.3 (C-11), 18.4 (C-19), 14.7 (C-18).

Compound 10: $C_{22}H_{30}O_5$, white solid; EIMS: *m/z* (%) 374 $[M]^+$ (40), 345 (100); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.87 (1 H, s, H-4), 4.15 (1 H, d, J = 2.9 Hz, H-6), 3.87 (1 H, t, J = 2.4 Hz, H-7), 1.37 (3 H, s, H-19), 1.01 (3 H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 200.0 (C-3), 176.9 (C-22), 166.6 (C-5), 129.3 (C-4), 95.9 (C-17), 77.0 (C-7), 70.7 (C-6), 45.6 (C-13), 44.2 (C-9), 43.2 (C-14), 37.8 (C-10), 36.9 (C-1), 35.5 (C-12), 34.7 (C-8), 34.1 (C-2), 31.4 (C-16), 31.3 (C-20), 29.3 (C-21), 22.3 (C-15), 20.3 (C-11), 19.9 (C-19), 14.4 (C-18).

Results and Discussion

Compound **1**, amorphous powder. Its molecular formula $C_{26}H_{36}O_5$ was determined by HR-EIMS (*m/z* = 428.2571 $[M]^+$, calcd. for $C_{26}H_{36}O_5$ 428.2563). The NMR spectrum indicated a typical canrenone skeleton.[10] The DEPT spectrum of compound **1** revealed that it had one ethoxy, two methyl, ten methylene, five methine groups and seven quaternary carbons. Comparison of the NMR data with that of canrenone showed that the 17β-lactonic ring and 4,5-unsaturated keto were intact, but the 6,7-unsaturated bond was missed due to the absence of the corresponding protons signals. The ethoxycarbonyl group was deduced from the triplet at δ_H 1.24 (3H, t, J = 7.3 Hz) and the quartet at δ_H 4.12 (2H, q, J = 7.3 Hz) in ^{1}H NMR together with the signal

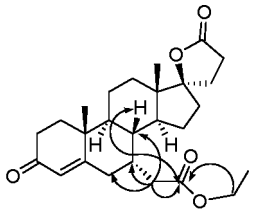


Figure 2. Select HMBC (H ⟶ C) and ROESY (⟷) correlations of **1**.

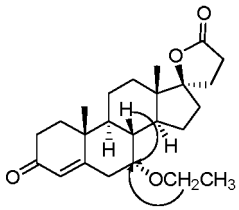


Figure 3. Select HMBC (H ⟶ C) and ROESY (⟷) correlations of **2**.

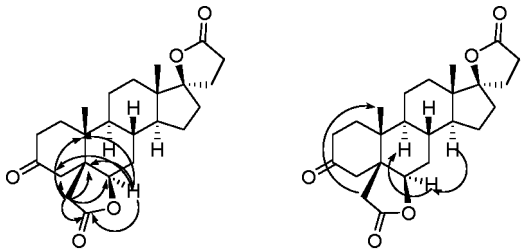


Figure 4. Select HMBC (H ⟶ C) and ROESY (⟷) correlations of **3**.

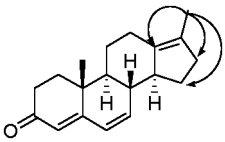


Figure 5. The key HMBC (H ⟶ C) correlations of **4**.

at δ_C 172.3 in ^{13}C NMR. In HMBC spectrum, the correlations of H-23 (δ_H 2.65, 2.28)/C-6 (δ_C 39.3), C-7 (δ_C 39.6), C-8 (δ_C 39.9) confirmed that the C-23 was attached to C-7. The crosspeaks between H-23/C-24 and H-7/C-24 indicated that the ethoxycarbonyl group was linked to the methylene (Figure 2). The stereostructure mainly depended on analyzing the ROESY spectrum. From the ROESY, the β orientation of H-7 was determined by the crosspeak between H-7 and H-8 and the absence of NOE effects between H-7 and H-9 (Figure 2). Therefore, compound **1** was established to be 3-(3-oxo-7α-(ethoxycarbonyl) methyl-17β-hydroxyl-4-androsten-17α-yl) propionic acid γ-lactone.

Compound **2** was obtained as amorphous powder, which had the molecular formula $C_{24}H_{34}O_4$, as determined by HR-EIMS (*m/z* = 386.2453 $[M]^+$, calcd. for $C_{24}H_{34}O_4$ 386.2457). The ^{1}H and ^{13}C NMR spectra were very similar to those of compound **1** except that the substituent at C-7 was replaced by an ethoxy group. In ^{1}H NMR, the triplet at δ_H 1.10 (3H, t, J = 7.0 Hz) and the double quartet at δ_H 3.24 (1H, dq, J = 9.3, 7.0 Hz), 3.54 (1 H, dq, J = 9.3, 7.0 Hz) were assigned to the ethoxy protons. In HMBC spectrum, the methylene protons at δ_H 3.24, 3.54 were correlated with carbon at δ_C 74.4 (C-7) which indicated that the ethoxy group was joined to the C-7 (Figure 3). The Overhauser effects in ROESY between H-7 and H-8 confirmed the ethoxy group was on the α orientation (Figure 3). Thus, the structure of **2** was elucidated as 3-(3-oxo-7α-ethoxy-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone.

Compound **3** was isolated in a very trace amount as a white solid. It had the molecular formula $C_{24}H_{32}O_5$, determined *via* the molecular ion peak at *m/z* 400.2256 $[M^-]$ (calcd. for $C_{24}H_{32}O_5$ 400.2250) in the HR-EIMS. Analysis of the NMR data and comparison with that of canrenone indicated that compound **3** retained the 17β-lactonic ring but olefinic bond in C-4 and C-5 was missed. The weak absorption in UV spectrum also proved the absence of the conjugated system. In the DEPT spectrum, 24 carbon signals including two methyl, eleven methylene, four methine groups and seven quaternary carbons were detected. It was reasonable to assume that compound **3** had two ester carbonyl group (δ_C 176.4 and δ_C 175.6) and one carbonyl carbon at δ_C 176.4 was assigned to the ester carbonyl in 17β-lactonic ring by its HMBC crosspeak. Considering the existence of methylene signals (δ_H 2.11, 2.82, 2H, d, J = 15.1) at C-4 (δ_C 46.9) and only one proton (δ_H 4.11, 1H, d, J = 3.8) at C-6 (δ_C 83.2) together with the chemical shift of C-5 (a quaternary carbon) at δ_C 49.5 in high field, a lactone ring joined from C-5 to C-6 was constructed. Further important evidence came from HMBC correlations in H-6/C-4, C-5, C-10, C-24 and H-23/C-4, C-5, C-6, C-10, C-24 (Figure 4). The relative configurations of the stereogenic centers were studied by means of ROESY experiment. The correlation between one proton (δ_H 2.69) at C-23 (δ_C 41.4) and H-19 indicated that the methylene was joined to C-5 as β position. Furthermore, α orientation of H-6 was determined by the ROESY crosspeaks between H-6/H-9 and H-6/H-14, respectively (Figure 4). The rigid skeleton of lactonic ring also determined that the bonds joined to C-5 and C-6 should stand in the same face. From the above spectral data, compound **3** was determined as 3-(3-oxo-5β-propionic acid-γ-lactone-6β,17β-hydroxyl-4-androstan-17α-yl) propionic acid γ-lactone.

Among the known compounds we isolated, compounds **4** and **10** have just been mentioned without any spectral data in the present study.[9,12,14] Here, we first report the relative spectra data of the two compounds: Compound **4** was obtained as yellow oil. Its molecular formula was assigned as $C_{19}H_{24}O$ from the EIMS (*m/z* 268 $[M]^+$). The ^{1}H and ^{13}C NMR, HSQC and HMBC, and their comparison with canrenone allowed the assignment of the structure shown in Figure 1. The olefin 13(17) was evidenced by the significantly downfield shift of 18-CH_3 (δ_H 1.62, markedly downfield shifted by 0.59 ppm compared to canrenone) and was further established by the strong correlations between H-18/C-13, C-16, C-17 in HMBC (Figure 5). The 18-CH_3 migrated from C-13 to C-17 possibly by Wagner-Meerwein rearrangement of a diaxial 13β-methyl-17α-ol.[15] Compound **10**, a white solid, had the same molecular weight with compound **9** (*m/z* 374 $[M]^+$). The close similarity of the ^{1}H and ^{13}C chemical shifts of compound **9** and **10**, together with their HMBC and ROESY spectrum clearly showed that **10** was an isomer of **9**. The difference between **9** and **10** may be only the stereochemistry of 6-OH and 7-OH. The overhauser effects between H-7 and

H-9 showed the α position of H-7 with regard to the α position of H-9, while the β position of H-6 followed from the ROESY crosspeak between H-6 and H-8. The NOE effects between H-6 and H-19 further demonstrated the β position of H-6. The other five known compounds were identified on the basis of spectroscopic analysis and comparing spectra data with literature.

Conclusion

In summary, three new and seven known impurities were identified in canrenone *via* chromatographic and spectrometric methods. These known compounds have been reported as metabolites or intermediate compounds in canrenone biotransformation.[10-14] Consequently, structural elucidation of the impurities may be helpful to identify minor metabolites or the correlated intermediates which are difficult to characterize by LC-MS or GC-MS analysis. Discovery of these new compounds bearing similar chemical structures may also be useful in the quality control of canrenone production.

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