

Article

Structural Modifications of Deoxycholic Acid to Obtain Three Known Brassinosteroid Analogues and Full NMR Spectroscopic Characterization

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Abstract: An improved synthesis route for obtaining known brassinosteroid analogues, i.e., methyl 2 α ,3 α -dihydroxy-6-oxo-5 α -cholan-24-oate (**11**), methyl 3 α -hydroxy-6-oxo-7-oxa-5 α -cholan-24-oate (**15**) and methyl 3 α -hydroxy-6-oxa-7-oxo-5 α -cholan-24-oate (**16**), from hyodeoxycholic acid (**4**) maintaining the native side chain is described. In the alternative procedure, the di-oxidized product **6**, obtained in the oxidation of methyl hyodeoxycholate **5**, was converted almost quantitatively into the target monoketone **7** by stereoselective reduction with NaBH₄, increasing the overall yield of this synthetic route to 96.8%. The complete ¹H- and ¹³C-NMR assignments for all compounds synthesized in this work have been made by 1D and 2D heteronuclear correlation *gs*-HSQC and *gs*-HMBC techniques. Thus, it was possible to update the spectroscopic information of ¹H-NMR and to accomplish a complete assignment of all ¹³C-NMR signals for analogues **5–16**, which were previously reported only in partial form.

Keywords: synthesis; deoxycholic acid derivatives; brassinosteroid analogues; full NMR spectroscopic characterization

1. Introduction

Bile acids, such as deoxycholic (**1**), chenodeoxycholic (**2**), cholic (**3**) and hyodeoxycholic (**4**) (Figure 1), have been used as substrates for the synthesis of a large number of brassinosteroid analogues, keeping the methyl ester or carboxylic function in the side chain [1–7]. In particular, hyodeoxycholic acid (**4**) has been used because it contains the modifiable organic functions at suitable positions, satisfying the structural requirements on the A and/or B and A/B *trans* fusion ring and side chain of active brassinosteroids [8–26]. Hyodeoxycholic acid (HDA) extracted from hog bile was initially used as a precursor for steroid synthesis [27]. It has also been shown that HDA prevents cholesterol-induced gallstones in animals fed with a lithogenic diet [28], whereas oral administration of HDA leads to a decrease in the LDL-cholesterol concentration, a strong stimulation of hepatic cholesterol biosynthesis and an excessive loss of cholesterol in feces [29].

In this work, we report an improved synthesis route to obtain known brassinosteroid analogues, starting from hyodeoxycholic acid (**4**), and maintaining the native side chain. Additionally, the full NMR spectroscopic characterization of these derivatives is performed.

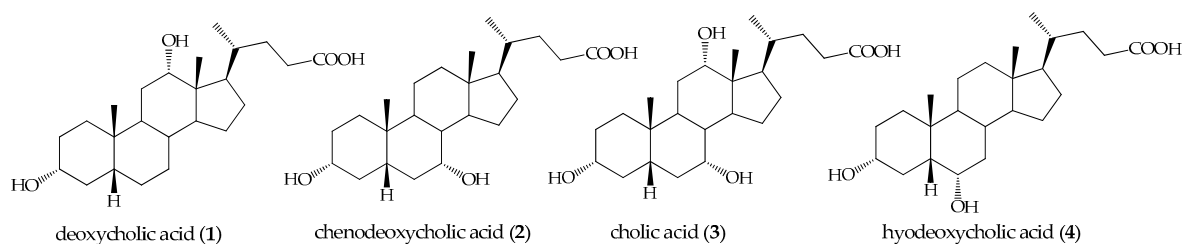
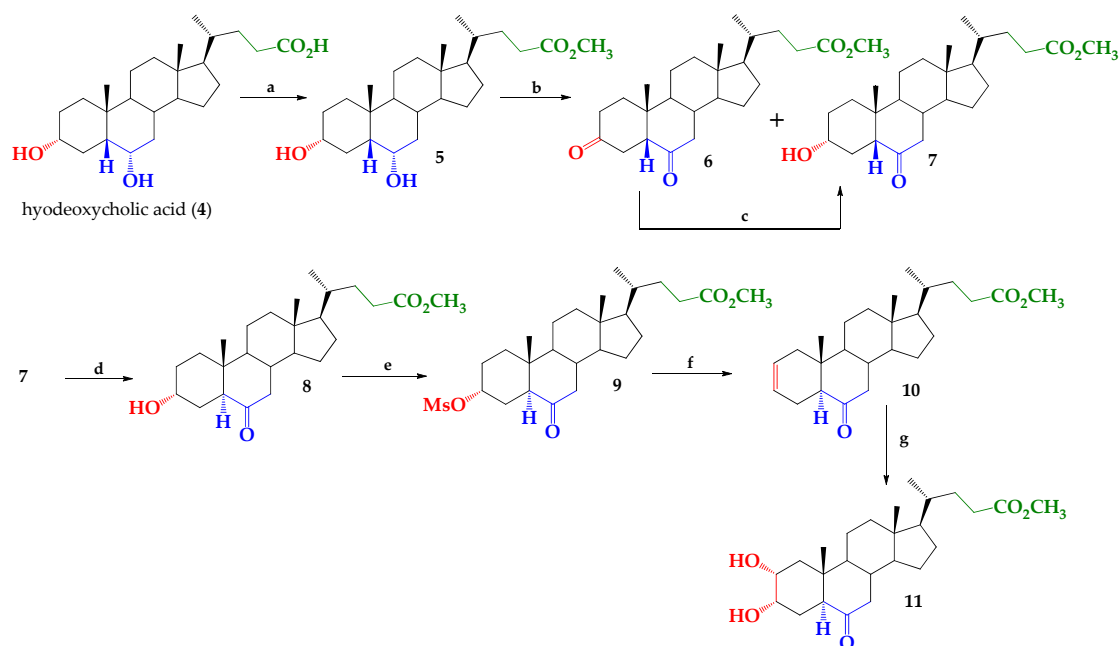


Figure 1. Structure of bile acids used for the synthesis of brassinolide and its analogues.

2. Results and Discussion

Fischer esterification of hyodeoxycholic acid (4) with the H_2SO_4 -MeOH system gave the methyl hyodeoxycholate (5) in almost quantitative yield (98%). Subsequently, selective oxidation of the C-6 hydroxyl group with PCC/ CH_2Cl_2 afforded the di- and mono-oxidized compounds 6 and 7 with 30% and 68% yield, respectively. These yields are similar to those reported when PDC/ CH_2Cl_2 was the oxidizing agent [13]. However, the yield of compound 7 was 67%–70% when K_2CrO_4 was used as an oxidizing agent [30,31]. Thus, in order to increase the yield of this reaction, 6 was conveniently converted to the desired monoketone 7 by selective reduction with NaBH_4 /MeOH at a low temperature (0–5 °C) with 96.8% yield (Scheme 1). This reaction has been used to modify steroids with similar structures [24]. The overall yield of 7 is increased from 68% to 96.8%. The physical properties and spectroscopic data (IR and $^1\text{H-NMR}$) of compounds 6 and 7 were consistent with those previously reported [8,30–32]. The $^{13}\text{C-NMR}$ spectroscopic data for compound 7 has been informed but not assigned [32].



Scheme 1. Synthesis of hyodeoxycholic acid derivatives 5–10 and brassinosteroid analogue 11. *Reagents and conditions:* (a) $\text{CH}_3\text{OH}/\text{H}_2\text{SO}_4$, reflux, 2 h; (b) PCC/DCM, 48 h, r.t., C.C. separation; (c) NaBH_4 /MeOH, 0–5 °C, 1 h; (d) $\text{HCl}/\text{CH}_3\text{OH}$ 2.5%, r.t., 24 h; (e) $\text{CH}_3\text{SO}_2\text{Cl}/\text{DCM}$, DMAP, 0 °C \geq r.t. 1 h; (f) $\text{Li}_2\text{CO}_3/\text{LiBr}$, DMF, 80 °C, 1.5 h; (g) OsO_4 4%/NMO, acetone, r.t., 72 h.

Compound 7 was readily isomerized (94.4% yield) under acid conditions (2.5% HCl/MeOH) to give the derivative 8 possessing 5α -cholestan-6-one skeleton [8,13,32]. This isomerization reaction can be performed under alkaline conditions as well, but under these conditions the obtained compound

was isomerized and hydrolyzed (carboxylic acid) with 84% yield [31]. Thus, to get **8**, an additional esterification reaction was carried out with 94% yield. The physical properties, IR, and ^1H -NMR spectroscopic data of compound **8** have been previously reported, but the signals of ^{13}C -NMR were not assigned [32]. The change of the 5β -cholestan to 5α -cholestan skeleton (A/B ring *cis* for *trans* fusion) implies a very important structural modification, and therefore a detailed analysis of NMR spectroscopic data for compounds **7** and **8** must be performed. The main differences found in the ^1H - and ^{13}C -NMR data are shown in Figure 2. The signals corresponding to H-3 β and H-5 α ($\delta_{\text{H}} = 4.16$ and $\delta_{\text{H}} = 2.70$ ppm, respectively) in compound **8**, are displaced to downfield as compared to compound **7** ($\delta_{\text{H}} = 3.62$ and $\delta_{\text{H-5}\beta} = 2.33$ ppm, respectively). On the other hand, the signal of CH₃-19 in the ^{13}C -NMR of compound **8** appears at a higher field ($\delta_{\text{C}} = 12.29$ ppm) than in the spectrum of compound **7** ($\delta_{\text{C}} = 23.16$ ppm).

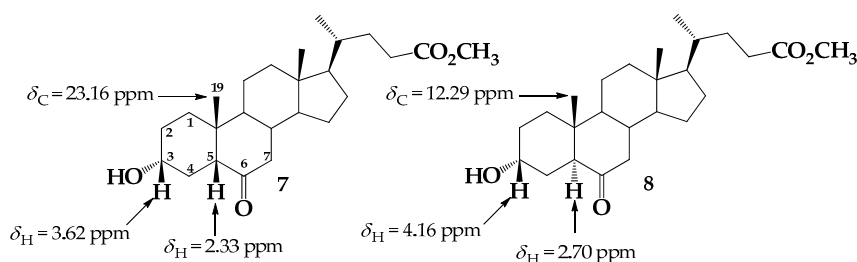
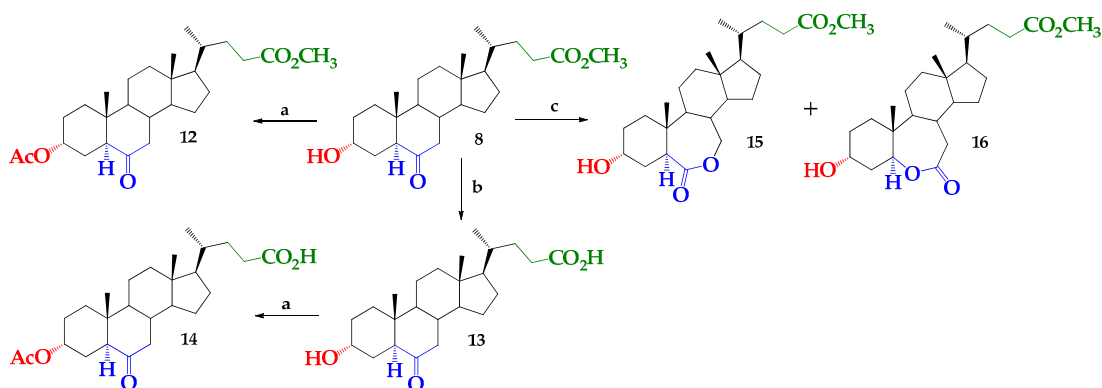


Figure 2. Major differences observed in the ^1H - and ^{13}C -NMR data of compounds **7** and **8**, associated with the change from the 5β -cholestan to 5α -cholestan skeleton (A/B ring *cis* for *trans* fusion).

The reaction of compound **8** with methanesulfonyl chloride (mesyl chloride) in CH_2Cl_2 /DMAP produces the mesylated derivative **9** with 61% yield [28]. The major ^1H -NMR spectroscopic evidence for formation of this derivative is the presence of signals at $\delta_{\text{H}} = 5.02$ and 2.97 ppm assigned to hydrogen H-3 β (b.s. 1H) and $\text{CH}_3\text{-SO}_2\text{-}$ (s, 3H), respectively, whereas in the ^{13}C -NMR spectrum, a signal corresponding to $\text{CH}_3\text{-SO}_2$ was observed at $\delta_{\text{C}} = 38.38$ ppm. Subsequent treatment of compound **9** with the $\text{Li}_2\text{CO}_3/\text{LiBr}$ system at 80 °C in dimethylformamide (DMF) produces the alquene **10** with 74.9% yield [33] (Scheme 2). The ^1H -NMR spectroscopic data of alquene **10** was consistent with that previously reported [8,32]. Additionally, in the ^{13}C -NMR spectrum the signals at $\delta_{\text{C}} = 124.49$ (C-2) and 124.95 (C-3) ppm are observed. Both signals were assigned from the 2D HMBC spectrum, where the H-3 ($\delta_{\text{H}} = 5.69\text{--}5.66$ ppm, m, 1H) showed $^3J_{\text{H-C}}$ correlation with C-1 ($\delta_{\text{C}} = 39.34$ ppm) and $^2J_{\text{H-C}}$ with C-4 ($\delta_{\text{C}} = 27.88$ ppm), while H-2 ($\delta_{\text{H}} = 5.57\text{--}5.55$ ppm, m, 1H) showed $^3J_{\text{H-C}}$ correlation with C-4 ($\delta_{\text{C}} = 27.88$ ppm) and $^2J_{\text{H-C}}$ with C-1 ($\delta_{\text{C}} = 39.34$ ppm).



Scheme 2. Synthesis of hyodeoxycholic acid derivatives **8**, **12–14** and known 7-oxalactone **15** and 6-oxalactone **16**. Reagents and conditions: (a) $\text{Ac}_2\text{O}/\text{DMAP}$, $\text{CH}_2\text{Cl}_2/\text{py}$, r.t., 30 min; (b) $\text{K}_2\text{CO}_3/\text{CH}_3\text{OH}$, reflux, 1 h; (c) *m*-CPBA/ CH_2Cl_2 , NaHCO_3 r.t., 24 h.

Compound **11** was obtained with 51% yield by stereospecific α -face hydroxylation of olefin **10** using a catalytic amount of osmium tetroxide (4.0%) in a mixture of $(\text{CH}_3)_2\text{CO-H}_2\text{O}$ (3:1) and 2.0 mL of pyridine, and in the presence of *N*-methylmorpholine *N*-oxide [33]. The ^1H - and ^{13}C -NMR spectroscopic data of diol **11** were consistent with those reported [8,32]. In addition, the spatial orientations of α -OH in C-2 and α -OH in C-3 were determined by selective-excitation 1D NOESY experiments, where H-2 β (3.77 ppm, b.d, $J = 11.1$ Hz, 1H) showed a long-range spatial correlation with CH₃-19 (0.75 ppm, s, 3H), while the observed signals at $\delta_{\text{C}} = 68.29$ and 68.39 ppm in the ^{13}C -NMR spectrum were assigned to C-2 and C-3, respectively.

In order to get additional deoxycholic acid derivatives, which may be of interest, the chemical transformations described in Scheme 2 were performed. Thus, compound **8** was acetylated under standard conditions ($\text{Ac}_2\text{O/DMAP/CH}_2\text{Cl}_2$), and commercial compound **12** was obtained with 95% yield. The synthesis of this derivative has been previously reported, but no NMR spectroscopic data was included [25]. The ^1H -NMR spectrum of the acetylated derivative **12** shows a singlet at $\delta_{\text{H}} = 2.04$ ppm (3H, CH_3CO) and a multiplet at $\delta_{\text{H}} = 5.12$ ppm corresponding to H-3 β (m, 1H). The latter is shifted to downfield as compared with H-3 β ($\delta_{\text{H}} = 4.16$ ppm, m 1H) in compound **8**. Additionally, in the ^{13}C -NMR spectrum the signals appearing at $\delta_{\text{C}} = 170.31$ (C=O) and 21.39 (CH_3CO) ppm (Table 1) confirmed the presence of acetylated derivative **12**.

Table 1. Chemical shifts, δ , of ^{13}C -NMR (CDCl_3 , 100.6 MHz) for compounds **12**–**16**.

C	12	13 *	14	15	16
1	32.38	32.87	32.36	32.88	27.46
2	27.89	28.96	27.88	22.13	30.75
3	68.87	66.01	68.85	64.88	66.31
4	25.26	28.70	25.28	30.81	35.63
5	52.58	52.83	52.56	41.78	79.65
6	211.86	215.65	211.84	176.76	175.35
7	46.71	47.59	46.73	70.44	38.07
8	37.91	39.41	37.93	39.45	35.27
9	53.73	55.03	53.72	58.35	57.97
10	41.26	43.71	41.24	36.29	39.85
11	21.06	22.17	21.09	24.80	22.17
12	39.44	40.78	39.47	39.68	39.66
13	43.02	44.16	43.07	42.66	42.71
14	55.79	57.18	55.76	55.72	56.00
15	25.00	28.42	25.00	28.23	25.24
16	23.88	24.92	23.87	27.82	27.93
17	56.74	57.89	56.75	51.45	55.48
18	12.01	12.41	12.01	11.79	11.78
19	12.39	12.68	12.40	14.51	11.53
20	35.24	36.60	35.25	35.31	34.83
21	18.19	18.72	18.17	18.14	18.12
22	30.67	32.01	30.65	31.01	30.99
23	30.84	32.24	30.83	32.49	31.15
24	174.67	178.19	179.20	174.60	174.64
CH ₃ O	51.45	-	-	51.50	51.52
CH ₃ C=O-	170.31	-	170.33	-	-
C=O-	21.39	-	21.41	-	-

* The ^{13}C -NMR spectrum of compound **13** was recorded in CD_3OD solution.

The synthesis of compound **13** by saponification of **8** with NaOH/dioxane under reflux conditions has been previously informed [31]. Saponification of compound **8** under mild conditions ($\text{K}_2\text{CO}_3/\text{MeOH}$, reflux) produces the carboxylic acid **13** with similar yields (82.2% yield) (Scheme 2). The physical property data, IR and ^1H -NMR were consistent with those reported [31]. The ^{13}C -NMR spectrum shows a signal at $\delta_{\text{C}} = 178.19$ ppm (C=O) (Table 1), confirming the presence of the carboxylic

function. Standard acetylation ($\text{Ac}_2\text{O}/\text{DMAP}/\text{CH}_2\text{Cl}_2$) of **13** produces compound **14** with 72.8% yield. Compound **14** showed signals at 3373–2495 (OH), 1736 (C=O) and 1708 (C=O) cm^{-1} in the IR spectrum, while in $^1\text{H-NMR}$ the signals observed at $\delta_{\text{H}} = 5.12$ ppm (m, 1H) and 2.03 ppm (s, 3H) were assigned to the H-3 β and CH_3CO , respectively. In the $^{13}\text{C-NMR}$ spectrum the observed signals at $\delta_{\text{C}} = 170.33$ and 21.41 ppm (Table 1) were assigned to the acetyl group, while the signal at $\delta_{\text{C}} = 179.20$ ppm (Table 1) was assigned to the carboxylic function. These spectroscopic data confirmed the structure of compound **14**.

The Baeyer-Villiger oxidation of 3 α -hydroxy-6-oxo-5 α -cholanate **8** with *m*-CPBA/ CH_2Cl_2 gave a mixture of known 7-oxalactone **15** and 6-oxalactone **16** with 10.8% and 14.9% yields, respectively. The physical and IR, $^1\text{H-NMR}$ spectroscopic data of **15** and **16** were consistent with those reported for these compounds [14,25]. Considering that the original $^1\text{H-NMR}$ spectra were recorded with a 60 MHz spectrometer, and no $^{13}\text{C-NMR}$ data was reported, we believe that it is worth updating the analysis of NMR data for these compounds.

The full structural assignment of compound **15** and in particular the 7-oxalactone position was mainly assigned by ^1H , ^{13}C , 2D HSQC and 2D HMBC NMR spectroscopy. In the $^1\text{H-NMR}$ spectrum of compound **15** appears a signal at $\delta_{\text{H}} = 4.08$ – 4.06 ppm (m, 2H), which was assigned to the two hydrogens H-7 and correlated by 2D $^1\text{H-}^{13}\text{C}$ HSQC with the signal $\delta_{\text{C}} = 70.44$ ppm (C-7) with pair multiplicity (from DEPT-135 analysis). Additionally, from the 2D $^1\text{H-}^{13}\text{C}$ HMBC spectrum important heteronuclear correlations were observed, i.e., $^2J_{\text{HC}}$ correlations between H-5 α ($\delta_{\text{H}} = 3.17$ ppm, dd, $J = 4.4$ and 12.2 Hz) with the signals at $\delta_{\text{C}} = 176.76$ (C=O of lactone function), $\delta_{\text{C}} = 36.29$ and $\delta_{\text{C}} = 30.81$ ppm which were assigned to the carbons C-6, quaternary C-10 and C-4, respectively. Additionally, H-5 α showed $^3J_{\text{HC}}$ correlation with signals at $\delta_{\text{C}} = 30.81$, $\delta_{\text{C}} = 14.52$, $\delta_{\text{C}} = 32.88$ and $\delta_{\text{C}} = 58.35$ ppm, which were assigned to the carbons C-4, CH_3 -19, C-1 and C-9, respectively (Figure 4a). These 2D HMBC observations unequivocally confirm the 7-oxalactone position for compound **15**.

A similar analysis was performed for the structure determination of 6-oxolactone **16**; thus, in the $^1\text{H-NMR}$ spectrum for **16** a signal at $\delta_{\text{H}} = 4.60$ ppm (dd, 1H, $J = 5.3$ and 11.3 Hz) was observed, correlated by 2D $^1\text{H-}^{13}\text{C}$ HSQC with the signal at $\delta_{\text{C}} = 79.65$ ppm (C-7) with impair multiplicity (from DEPT-135 analysis) and assigned to H-5 α . Additionally, H-5 α showed $^2J_{\text{HC}}$ correlation with signals at $\delta_{\text{C}} = 35.63$ ppm and $^3J_{\text{HC}}$ correlation with signals at $\delta_{\text{C}} = 11.53$, $\delta_{\text{C}} = 57.97$ and $\delta_{\text{C}} = 175.35$ ppm, which were assigned to the carbons C-4, CH_3 -19, C-9 and C-6 (C=O, of lactone function), respectively (Figure 4b).

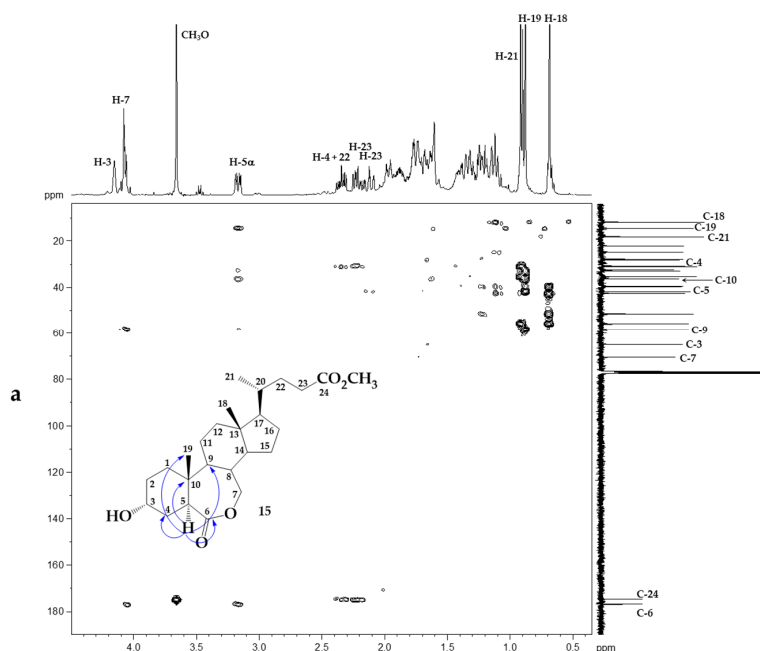


Figure 3. Cont.

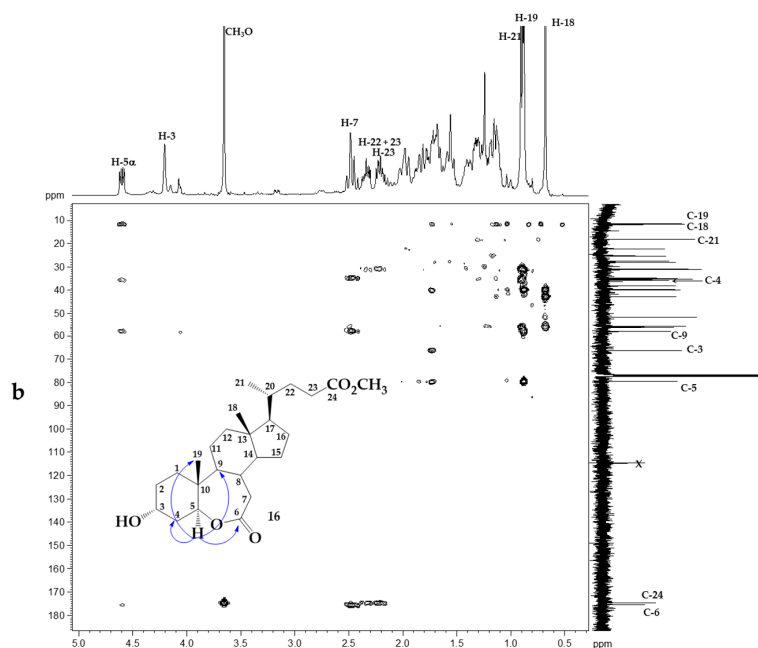


Figure 4. (a) Inverse detection heteronuclear-correlated 2D ^1H - ^{13}C HMBC contour plot and major observed $^2J_{\text{HC}}$ and $^3J_{\text{HC}}$ correlations for H-5 α in 7-oxalactone **15** and (b) for 6-oxalactone **16**.

3. Materials and Methods

3.1. General

All reagents were purchased from commercial suppliers (Merck, Darmstadt, Germany or Aldrich, St. Louis, MO, USA), and used without further purification. Melting points were measured on a Stuart-Scientific SMP3 apparatus (Staffordshire, UK) and are uncorrected. ^1H , ^{13}C , ^{13}C DEPT-135, sel. gs1D ^1H NOESY, gs 2D HSQC and gs 2D HMBC NMR spectra were recorded in CDCl_3 or MeOD solutions, and are referenced to the residual peaks of CHCl_3 at $\delta = 7.26$ ppm and $\delta = 77.00$ ppm for ^1H and ^{13}C , respectively and CD_3OD at $\delta = 3.30$ ppm and $\delta = 49.00$ ppm for ^1H and ^{13}C , respectively, on a Bruker Avance 400 Digital NMR spectrometer (Bruker, Rheinstetten, Germany), operating at 400.1 MHz for ^1H and 100.6 MHz for ^{13}C . Chemical shifts are reported in δ ppm and coupling constants (J) are given in Hz. IR spectra were recorded as KBr disks in a FT-IR Nicolet 6700 spectrometer (Thermo Scientific, San Jose, CA, USA) and frequencies are reported in cm^{-1} . For analytical TLC, silica gel 60 (Merck) in 0.25 mm layer was used and TLC spots were detected by heating after spraying with 25% H_2SO_4 in H_2O . Chromatographic separations were carried out by conventional column on silica gel 60 (230–400 mesh, Merck) using EtOAc-hexane gradients of increasing polarity. All organic extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure, below 40 °C.

3.2. Synthesis

Methyl 3 α , 6 α -dihydroxy-5 β -cholan-24-oate (5). A solution of **4** (20.0 g, 50.95 mmol) in MeOH (150 mL) and 1.0 mL of H_2SO_4 was refluxed for 2.0 h. The end of reaction was verified by TLC. Then the solvent was removed (until a 40 mL approximate volume) and diluted with EtOAc (90 mL). The organic layer was washed with saturated solution of NaHCO_3 (40 mL) and water (2×30 mL), dried over Na_2SO_4 , and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH_2Cl_2 (15 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 \rightarrow 7.6:2.4). Compound **5** (19.50 g 98% yield) was a colorless solid (m.p. = 60–65 °C, MeOH/Et $_2$ O); IR (cm^{-1}): 3385 (O-H); 2938 (C-CH $_3$); 2858 (C-CH $_2$ -C); 1743 (C=O); 1452 (CH $_2$); 1376 (CH $_3$); 1169 (C-O). ^1H -NMR: 4.05 (ddd, $J = 4.8, 4.8$ and 11.9 Hz, 1 H, H-6); 3.66 (s, 3H, CH $_3$ O); 3.62 (m,

1H, H-3); 2.35 (ddd, $J = 5.1, 10.1$ and 15.0 Hz, 1H, H-23); 2.21 (ddd, $J = 6.6, 9.6$ and 15 Hz, 1H, H-23); 0.91 (d, $J = 5.1$ Hz, 3H, H-21); 0.90 (s, 3H, H-19); 0.63 (s, 3H, H-18). ^{13}C -NMR: See Table 2.

Table 2. Chemical shifts, δ , of ^{13}C -RMN (CDCl_3 , 100.6 MHz) for compounds 5–11.

C	5	6	7	8	9	10	11
1	35.56	35.71	34.37	31.65	27.80	39.34	40.18
2	29.20	36.42	34.86	28.16	31.64	124.49	68.29
3	71.48	208.58	70.15	65.41	78.71	124.95	68.39
4	34.86	39.83	29.85	27.90	26.56	27.88	26.28
5	48.40	59.67	59.40	51.65	51.78	53.35	50.69
6	67.98	210.75	213.88	212.70	211.08	211.96	214.15
7	30.11	42.08	37.97	46.83	46.52	46.95	46.73
8	34.79	36.61	37.06	37.94	37.79	37.67	35.65
9	39.81	40.83	39.60	53.77	53.35	53.82	53.66
10	35.90	38.22	39.99	41.53	40.99	40.00	42.98
11	20.72	21.23	20.83	21.03	20.94	21.08	21.17
12	39.92	39.41	42.91	39.45	39.25	39.44	39.35
13	42.80	43.02	43.09	42.89	42.90	42.84	42.55
14	55.89	55.74	55.79	55.71	56.58	56.69	56.61
15	28.10	27.88	27.98	27.67	51.79	53.35	23.91
16	24.17	23.85	23.96	23.88	23.79	21.70	27.91
17	56.14	56.70	56.82	56.73	55.62	55.75	55.69
18	11.98	11.92	11.96	12.00	11.92	11.92	12.01
19	23.48	22.40	23.16	12.29	12.39	13.48	13.55
20	35.32	35.19	35.28	35.29	35.19	35.29	35.30
21	18.21	18.18	18.23	18.21	18.13	18.23	18.22
22	30.91	30.80	30.90	30.89	30.79	30.90	31.04
23	31.02	30.95	31.05	31.03	30.94	31.03	30.89
24	174.71	174.50	174.67	174.67	174.56	174.64	174.72
CH ₃ O-	51.48	51.44	51.52	51.50	51.42	51.49	51.54
MsO-	-	-	-	-	38.38	-	-

Methyl 3, 6-dioxo-5 β -cholan-24-oate (6) and *Methyl 3 α -hydroxy-6-oxo-5 β -cholan-24-oate (7)*. To a solution of 5 (16.50 g, 40.58 mmol) in DCM (100 mL), 8.75 g, (40.58 mmol) of PCC in 60 mL of DCM, were added by slow dripping. The reaction mixture was slowly stirred for 48 h at room temperature and the end of reaction was verified by TLC, filtered on silica (pore size 60 Å, 220–440 mesh) with DCM 30 mL. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH_2Cl_2 (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 \rightarrow 5.8:4.2). Two fractions were obtained: Fraction I, 4.95 g (30.0% yield) of compound 6; Fraction II, 11.2 g (68% yield) of compound 7. Compound 6 was a colorless solid (m.p. = 106–107 °C, MeOH/Et₂O). IR (cm^{-1}): 2947 (C-CH₃); 2874 (C-CH₃); 1743 (C=O); 1717 (C=O); 1689 (C=O); 1469 (CH₂); 1383 (CH₃); 1164 (C-O). ^1H -NMR: 3.64 (s, 3H, CH₃O); 2.63 (dd, $J = 13.4$ and 14.7 Hz, 1H, H-12); 0.93 (s, 3H, H-19); 0.91 (d, $J = 6.5$ Hz, 3H, H-21); 0.67 (s, 3H, H-18). ^{13}C -NMR: See Table 2. Compound 7 was a colorless solid (m.p. = 95–100 °C, MeOH/Et₂O). IR (cm^{-1}): 3301 (O-H); 2943 (C-CH₃); 2867 (C-CH₂-C); 2849 (C-CH₂-C); 2828 (CH₃O); 1740 (C=O); 1709 (C=O); 1436 (CH₂); 1326 (CH₃); 1171 (C-O). ^1H -NMR: 3.66 (s, 3H, CH₃O); 3.62 (m, 1H, H-3); 2.33 (ddd, $J = 5, 10$ and 15.2 Hz, 1H, H-23); 0.92 (d, $J = 6.4$ Hz, 3H, H-21); 0.83 (s, 3H, H-19); 0.64 (s, 3H, H-18). ^{13}C -NMR: See Table 2. ^1H -NMR and ^{13}C -NMR are shown in Supplementary Materials.

Methyl 3 α -hydroxy-6-oxo-5 β -cholan-24-oate (7). A solution of compound 6 (4.95 g, 12.3 mmol) was prepared in 100 mL of MeOH. This solution was placed in a bath of ice-water between 0–5 °C. Subsequently 0.47 g (12.3 mmol) of NaBH₄ were added in four portions (approximately 0.118 g each) maintaining the temperature and with slow stirring. The end of reaction was verified by TLC, so 20 mL of acetone and 5 mL of HCl 2.5% were added, maintaining the reaction temperature. The reaction mixture was concentrated by evaporation under reduced pressure to a volume of about 15 mL, and

then AcOEt (50 mL) was added. The organic layer was washed with saturated solution of NaHCO₃ (20 mL) and water (2 × 30 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 5.8:4.2). Two fractions were obtained: Fraction I, 2.88 g of unreacted compound **6**; Fraction II, 2.04 g (41.2%) of compound **7**. Later reduction with NaHB₄/CH₃OH at 0–5 °C of recovered compound **6** (2.88 g), produced 2.75 g of **7** with 95.5% yield. The total yield in both reductions for compound **7** was 96.8%. The physical and spectroscopic properties of compound **7** were identical to those reported above for oxidation of compound **5**. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 3α-hydroxy-6-oxo-5α-cholan-24-oate (8). Compound **7** (4.79 g, 11.8 mmol) was dissolved in 100 mL of 2.5% v/v HCl-MeOH, at room temperature and constant agitation for 24 h. The end of reaction was verified by TLC. The solvent was evaporated under reduced pressure and the crude was re-dissolved in 40 mL of AcOEt. The organic layer was washed with saturated solution of NaHCO₃ (30 mL) and water (2 × 30 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 6.0:4.0). Compound **8** (4.79 g, 94.4% yield) was a colorless solid (m.p. = 128–132 °C, MeOH/Et₂O) IR (cm⁻¹): 3302 (O-H); 2943 (C-CH₃); 2867 (C-CH₂-C); 2849 (C-CH₂-C); 2828 (CH₃O); 1740 (C=O); 1709 (C=O); 1436 (CH₂); 1375 (CH₃); 1257 (C-O); 1172 (C-O). ¹H-NMR: 4.16 (m, 1H, H-3); 3.66 (s, 3H, CH₃O); 2.70 (t, J = 7.9 Hz, 1H, H-5); 2.33 (ddd, J = 5.3, 10.3 and 15.5 Hz, 1H, H-23); 0.91 (d, J = 6.4 Hz, 3H, H-21); 0.72 (s, 3H, H-19); 0.65 (s, 3H, H-18). ¹³C-NMR: See Table 2. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 3α-methanesulfonyl-6-oxo-5α-cholan-24-oate (9). Compound **8** (1.00 g, 2.47 mmol) was dissolved in 20 mL of DCM, 5 mg of DMAP and 1 mL of pyridine were added, then the mixture was cooled to 0–5 °C. Later 1.0 mL (12.9 mmol) of CH₃SO₂Cl slowly was added with gentle agitation. The mixture was kept at 0 °C for 30 min and subsequently left at room temperature. The end of reaction was verified by TLC. The solvent was evaporated under reduced pressure and the crude was re-dissolved in 20 mL of AcOEt. The organic layer was washed with saturated solution of NaHCO₃ (40 mL) and water (2 × 30 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 5.8:4.2). Two fractions were obtained: Fraction I, 726 mg (61.0% yield) of compound **9**, and Fraction II, 230 mg of unreacted compound **8**. Compound **9** was a colorless solid (m.p. = 129–131 °C, MeOH/Et₂O) IR (cm⁻¹): 2945 (C-CH₃); 2867 (C-CH₂-C); 1736 (C=O); 1709 (C=O); 1435 (CH₂); 1351 (CH₃); 1255 (C-O); 1171 (C-O). ¹H-NMR: 5.02 (b.s, 1H, H-3); 3.64 (s, 3H, CH₃O); 2.97 (s, 3H, CH₃SO₃); 2.60 (dd, J = 2.3 and 12.4 Hz, 1H, H-5); 0.901 (d, J = 6.4 Hz, 3H, H-21); 0.713 (s, 3H, H-19); 0.639 (s, 3H, H-18). ¹³C-NMR: See Table 2. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 2-en-6-oxo-5α-cholan-24-oate (10). To a solution of compound **9** (1.30 g, 0.269 mmol) in 40 mL of DMF, 200 mg Li₂CO₃ (2.71 mmol) and LiBr (235 mg, 2.71 mmol) were added. Then the reaction mixture was stirred and refluxed at 80 °C for 1.5 h. The end of reaction was verified by TLC, the mixture was then filtered and the mother liquor concentrated to a volume of approximately 15 mL under reduced pressure. Then AcOEt (30 mL) were added. The organic layer was washed with water (2 × 20 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 2.4:7.6). Compound **10** (0.900 g, 74.9% yield) was a colorless solid (m.p. = 67–69 °C, hexane/Et₂O) IR (cm⁻¹): 3020 (CH=); 2968 (CH₃-); 2939 (CH₃-); 2899 (C-CH₂-C); 2867 (C-CH₂-C); 1737 (C=O); 1703 (C=O); 1635 (C=C); 1435 (CH₂); 1383 (CH₃); 1250, (C-O); 1168 (C-O). ¹H-NMR: 5.69–5.66 (m, 1H, H-3); 5.57–5.55 (m, 1H, H-2); 3.66 (s, 3H, CH₃O); 0.924 (d, J = 6.4 Hz, 3H, H-21); 0.701 (s, 3H, H-19); 0.665 (s, 3H, H-18). ¹³C-NMR: See Table 2. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 2 α ,3 α -dihydroxy-6-oxo-5 α -cholan-24-oate (11). A solution of compound **10** (500 mg, 1.29 mmol) in 30 mL of acetone and 10 mL water was prepared. Later 10 mg of NMMNO, 2 mL 4.0% (0.314 mmol) OsO₄ solution and 2 mL of pyridine were added. The mixture was kept under constant stirring at room temperature for 72 h. The end of reaction was verified by TLC, and then 25 mL of Na₂S₂O₃ saturated solution were added and allowed to stir for 30 min. The reaction was concentrated to a volume of approximately 15 mL under reduced pressure. Then AcOEt (40 mL) were added. The organic layer was washed with water (2 × 25 mL), dried over anhydrous Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 8.2:1.8). Two fractions were obtained: Fraction I, 218 mg of unreacted compound **10** and Fraction II, 277 mg (51.0% yield) of compound **11**. Compound **11** was a colorless solid (m.p. = 178–179 °C, MeOH/Et₂O). IR (cm⁻¹): 3392 (OH); 2944 (CH₃-); 2867 (C-CH₂-C); 1738 (C=O); 1709 (C=O); 1435 (CH₂); 1376 (CH₃); 1255, C-O); 1170 (C-O). ¹H-NMR: 4.05 (b.s, 1H, H-3); 3.77 (b.d, J = 11.1 Hz, 1H, H-2); 3.66 (s, 3H, CH₃O); 2.67 (dd, J = 3.0 and 12.6 Hz, 1H, H-5); 0.926 (d, J = 6.4 Hz, 3H, H-21); 0.752 (s, 3H, H-19); 0.659 (s, 3H, H-18). ¹³C-NMR: See Table 2. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 3 α -acetoxy-6-oxo-5 α -cholan-24-oate (12). To a solution of compound **8** (2.00g, 4.94 mmol) in 20 mL of DCM, 5 mg of DMAP, 1 mL of pyridine and 0.5 mL (5.3 mmol) of Ac₂O were added. The reaction mixture was kept under constant stirring and room temperature for 30 min. The end of reaction was verified by TLC, the mixture was then concentrated to a volume approximately 5 mL under reduced pressure. Then AcOEt (30 mL) were added. The organic layer was washed with water (2 × 15 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 4.7:5.3). Compound **12** (1.9 g, 95% yield) was a colorless solid (m.p. = 174–176 °C, MeOH/Et₂O) IR (cm⁻¹): 2943 (CH₃); 2869 (CH₂); 1737 (C=O); 1708 (C=O); 1435 (CH₂); 1376 (CH₃); 1262 (C-O); 1221 (C-O); 1172 (C-O). ¹H-NMR: 5.12 (m, 1H, H-3); 3.66 (s, 3H, CH₃O); 2.56 (dd, J = 3.3 and 12.0 Hz, 1H, H-5); 2.38 (ddd, J = 5.4, 10.3 and 15.4 Hz, 1H, H-23); 2.04 (s, 3H, CH₃CO); 0.94 (d, J = 6.4 Hz, 3H, H-21); 0.74 (s, 3H, H-19); 0.67 (s, 3H, H-18). ¹³C-NMR: See Table 1. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Acid 3 α -hydroxy-6-oxo-5 α -cholan-24-oic (13). A solution of compound **8** (5.0 g, 12.36 mmol) in 60 mL of MeOH was prepared. Later 20 mL of 15% P/V K₂CO₃ solution were added, and the reaction mixture was refluxed for 1 h. The end of reaction was verified by TLC, the mixture was then concentrated to a volume approximately 15 mL under reduced pressure. This was acidified with 20 mL of 10% HCl solution. Then AcOEt (40 mL) were added. The organic layer was washed with water (2 × 15 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and a colorless solid (4.11 g, 82.2% yield) was obtained. Compound **13** (m.p. = 102–107 °C, MeOH/Et₂O) IR (cm⁻¹): 3403–2500 (O-H); 2945 (CH₃); 2869 (CH₂); 1712 (C=O); 1689 (C=O); 1442 (CH₂); 1380 (CH₃). ¹H-NMR (CD₃OD): 4.04 (m, 1H, H-C3); 2.75 (t, J = 8.1 Hz, 1H, H-5); 2.31 (ddd, J = 5.3, 9.9 and 15.2 Hz, 1H, H-23); 0.96 (d, J = 6.5 Hz, 3H, H-21); 0.730 (s, 3H, H-19); 0.71 (s, 3H, H-18). ¹³C-NMR: See Table 1. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Acid 3 α -acetoxy-6-oxo-5 α -cholan-24-oic (14). To a solution of compound **13** (5.0 g, 12.8 mmol) in 60 mL of DCM, 10 mg of DMAP, 1 mL of pyridine and 1.2 mL (12.8 mmol) of Ac₂O were added. The reaction mixture was kept under constant stirring and room temperature for 1 h. The end of reaction was verified by TLC, the mixture was then concentrated to a volume approximately 10 mL under reduced pressure. Then AcOEt (40 mL) were added. The organic layer was washed with 5% HCl (1 × 10 mL), water (2 × 15 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 → 8.8:11.2). Compound **14** (3.64 g, 72.8% yield) was a colorless solid (m.p. = 171–177 °C, MeOH/Et₂O) IR (cm⁻¹): 3373–2495 (O-H); 2947 (CH₃); 2869 (CH₂); 1736 (C=O); 1708 (C=O); 1444 (CH₂); 1376 (CH₃). ¹H-NMR: 5.12 (m, 1H, H-3); 2.56 (dd, J = 3.3

and 12.0 Hz, 1H, H-5); 2.38 (ddd, $J = 5.4, 10.3$ and 15.4 Hz, 1H, H-23); 2.03 (s, 3H, CH₃CO); 0.94 (d, $J = 6.4$ Hz, 3H, H-21); 0.74 (s, 3H, H-19); 0.67 (s, 3H, H-18). ¹³C-NMR: See Table 1. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials.

Methyl 3 α -hydroxy-6-oxo-7-oxa-5 α -cholan-24-oate (15) and *methyl 3 α -hydroxy-6-oxa-7-oxo-5 α -cholan-24-oate (16)*. To a solution of **8** (100 mg, 0.247 mmol) in 60 mL of DCM, 55.3 mg (0.320 mmol) of *m*-CPBA (77%) and 269 mg (0.320 mmol) of NaHCO₃ were added and the mixture was stirred at room temperature by 24 h. The end of reaction was verified by TLC, the mixture was filtered then concentrated to a volume approximately 5 mL under reduced pressure. Then AcOEt (40 mL) were added. The organic layer was washed with saturated NaHCO₃ solution (2 \times 20 mL), water (2 \times 15 mL), dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure and the crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with EtOAc/hexane mixtures of increasing polarity (0.2:9.8 \rightarrow 6.0:4.0) Three fractions were obtained. Fraction I: 37 mg of unreacted compound **8**. Fraction II: 11.2 mg (10.8% yield) of compound **15** and Fraction III: 15.5 mg (14.9% yield) of compound **16**. Compound **15** was a colorless solid (m.p. = 140–142 °C, MeOH/Et₂O) IR (cm⁻¹): 3446 (O-H); 2947 (C-CH₃); 2871 (C-CH₂-C); 2849 (C-CH₂-C); 1732 (C=O); 1436 (CH₂); 1373 (CH₃); 1251 (C-O); 1168 (C-O). ¹H-NMR: 4.15 (b.s. 1H, H-3); 4.08–4.06 (m, 2H, H-7); 3.66 (s, 3H, CH₃O); 3.17 (dd, $J = 4.4$ and 12.2 Hz, 1H, H-5); 0.907 (d, $J = 6.5$ Hz, 3H, H-21); 0.878 (s, 3H, H-19); 0.700 (s, 3H, H-18). ¹³C-NMR: See Table 1. Compound **16** was a colorless solid (m.p. = 155–157 °C). IR (cm⁻¹): 3446 (O-H); 2946 (C-CH₃); 1732 (C=O); 1444 (CH₂); 1377 (CH₃); 1250 (C-O); 1166 (C-O). ¹H-NMR: 4.60 (dd, $J = 5.3$ and 11.3 Hz, 1H, H-5); 4.20 (b.s. 1H, H-3); 3.65 (s, 3H, CH₃O); 0.897 (d, $J = 6.6$ Hz, 3H, H-21); 0.878 (s, 3H, H-19); 0.678 (s, 3H, H-18). ¹³C-NMR: See Table 1. ¹H-NMR and ¹³C-NMR are shown in Supplementary Materials

4. Conclusions

An improved synthesis of known brassinosteroid analogues 7-oxalactone **15**, 6-oxalactone **16** and the 2 α ,3 α -diol **11** from hydoxycholeic acid has been described (**4**). These compounds and intermediates were obtained through modification of hydoxycholeic acid as described previously. In the alternative procedure described here, the co-product **6** obtained in the oxidation of methyl hydoxycholeate **5** was converted into the target monoketone **7** by stereoselective reduction with NaBH₄, increasing the overall yield of this synthetic route to 96.8%.

Additionally, using mono- and bi-dimensional NMR techniques, we have updated the ¹H-NMR spectroscopic information for derivatives of hydoxycholeic acid **5–16**, and a complete assignment of all ¹³C-NMR signals has also been made.

Derivatives **8**, **13** and **14** will be used in the synthesis of new brassinosteroid analogues, while derivatives **8**, **11**, **15** and **16** will be evaluated as plant-growth regulators in the Rice Lamina Inclination Assay and for antifungal activity in plants. These results will be reported later.

Supplementary Materials: Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/21/9/1139/s1>.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

TLA Three letter acronym

LD	linear dichroism
DCM	Dichloromethane
PCC	Pyridinium ChloroChromate
PDC	Pyridinium Dichromate
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
NMO	N-Methylmorpholine-N-Oxide
<i>m</i> -CPBA	3-Chloro or <i>meta</i> -chloroperoxybenzoic acid
NOESY	Nuclear Overhauser Spectroscopy
DEPT-135	Distortionless Enhancement by Polarization Transfer with flip angle of 135°
gs	gradient selected
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation

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Sample Availability: Samples of compounds 5–16 are available from the authors.



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