Enzymatic α/β Inversion of the C-7-Hydroxyl of Steroids

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Introduction

Selective modification of the functional groups of the steroid skeleton is a longstanding target of synthetic chemistry.[1] Enzymes have been widely used to achieve this goal, both in natural and unnatural media.[2] In recent years, we have reported on the regioselective acylation of steroid hydroxyls catalyzed by lipases and proteases in organic solvents,[3] as well as on their selective oxidation (or reduction) catalyzed by specific hydroxysteroid dehydrogenases in water or biphasic media.[4] More specifically, in one of these works[4a] we studied the α/β inversion of the C-3 OH of different steroid derivatives. In this note we will discuss the results obtained for a similar transformation at the C-7 OH position.

Results and Discussion

Steroids hydroxylated at C-7 are not so commonly found in nature and are mainly related to the bile acids family. Nevertheless, they do have important pharmaceutical applications due to their ability to dissolve cholesterol gallstones avoiding surgery.[5] This property seems to be displayed with a greater extent by ursodeoxycholic acid ($3\alpha,7\beta$-dihydroxy-5β-cholanoic acid, **2b**), which is industrially prepared—on a ton scale—from cholic acid ($3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoic acid, **1a**) by a sequence of chemical reactions whose last two steps involve the selective α/β inversion of the C-7 OH (Scheme I),[6] the overall yield of this inversion being about 70%.[6b] More recent papers have described better reductive conditions;[7] however, the stereochemical control is never absolute and ursodeoxycholic acid (**2b**) has to be purified by the byproduct chenodeoxycholic acid ($3\alpha,7\alpha$-dihydroxy-5β-cholanoic acid, **2c**). We thought that an enzymatic approach involving the regioselective oxidation of 7α-OH and the subsequent stereoselective reduction of the 7-keto might afford higher yields, and also be safer (Scheme II).[8] Following a literature procedure,[9] we purified the 7β-hydroxysteroid dehydrogenase (7β-HSDH) from *Clostridium absonum* and subsequently submitted compound **2c** as well as other steroids to the enzymatic transformation of Scheme II.

Considering free bile acids first, the four compounds **1a–4a** were quantitatively oxidized to the corresponding 7-cheto derivatives **1c–4c** by action of the commercially available NAD-dependent 7α-HSDH. The cofactor was used in catalytic amounts and regenerated in situ by a coupled enzymatic process (lactate–lactate dehydrogenase). The reductions catalyzed by the NADP-dependent 7β-HSDH (cofactor regenerated by the glucose–glucose dehydrogenase system) were also quantitative, affording the 7β-hydroxy derivatives **1b**, **2b**, and **4b** with >99% purities. However compound **3c** (7-ketoiocholic acid) was completely unreactive. As a possible explanation, we can hypothesize that the 6α-OH of **3c** offers a serious steric hindrance to the approach of the reducing cofactor from the α-side or prevents the steroid from properly fitting the enzyme binding site. This was not the case with the corresponding 6β-OH of **4c**, which in fact was reduced nicely by 7β-HSDH (Figure 1).

All the above-described transformations were performed in water buffers, as the carboxyl moiety of **1a–4a** allows the solubilization of these steroids under our reaction conditions (pH between 6.5 and 8). However, their solubilities were not particularly high (4%, i.e., 0.1 M, or less). In order to overcome this limitation, we considered their corresponding amino acid conjugates, specifically the glyco and tauro conjugates of cholic acid (**5a** and **6a**) and taurochenodeoxycholic acid (**7a**). With these compounds too, the oxidations of **5a–7a** catalyzed by 7α-HSDH were very efficient, affording the corresponding ketones **5c–7c**. The 7-keto cholic acid derivatives **5c** and **6c** were then reduced by 7β-HSDH. Quite unexpectedly, compound **7c** was not a substrate for this enzyme under our experimental conditions. Further experiments showed that both **7c** and the expected product **7b** become inhibitors of 7β-HSDH above a concentration of 2 mM (about 1 mg/mL), and at 10 mM (5 mg/mL) practically no residual enzymatic activity was detected. This is most likely due to the formation of micelles, because the critical micellar concentrations of these two compounds are very low, as suggested by the data reported by Stevens et al.[10]

Finally, we turned our attention to neutral steroids and, as a representative model, we chose cholic methyl ester **8a**. The initial attempt to oxidize **8a** in a biphasic system aqueous buffer–AcOEt[4b] by action of the usual NAD-dependent 7α-HSDH was unsuccessful. As the K_M of this enzyme for the water-soluble cholic acid is quite high (around 10^{-3} M), it is probable that the solubility of **8a** in water is far below its K_M for this enzyme, thus preventing the biocatalytic oxidation. To overcome this problem, we considered a new 7α-HSDH, namely the NADP-dependent 7α-HSDH from *C. absonum*.[9] This enzyme was also

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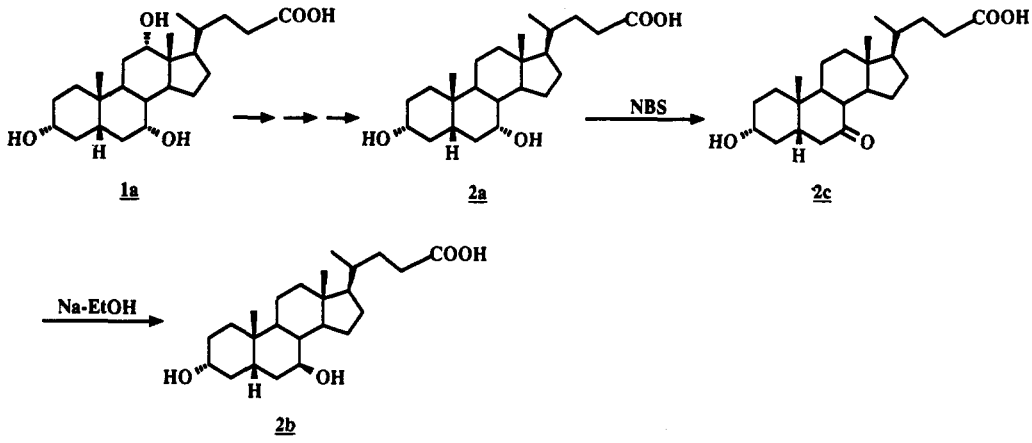
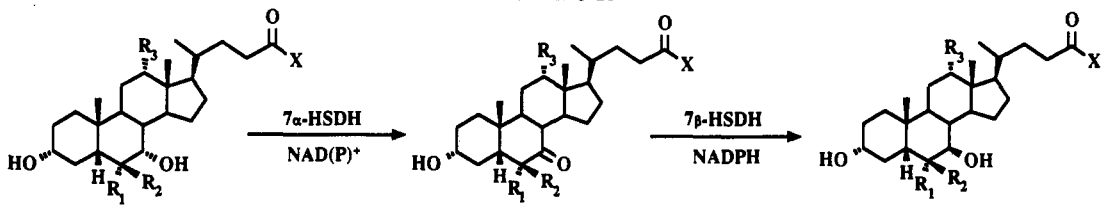
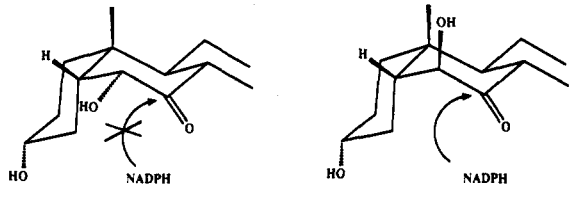
Scheme I



Scheme II



R_1	R_2	R_3	X	
H	H	OH	OH	1
H	H	H	OH	2
H	OH	H	OH	3
OH	H	H	OH	4
H	H	OH	$NHCH_2COOH$	5
H	H	OH	$NHCH_2CH_2SO_3^-$	6
H	H	H	$NHCH_2CH_2SO_3^-$	7
H	H	OH	OCH_3	8



3c 4c

Figure 1.

purified by standard procedures[9] and showed a lower K_M for cholic acid (about 10^{-5} M). A new oxidative attempt of **8a** in biphasic system with this enzyme was successful, and the 7-ketocholate methyl ester **8c** was obtained quantitatively. Unfortunately, the subsequent reduction of **8c** occurred with a low degree of conversion (<20%), probably due to enzyme inactivation under these reaction conditions. The stability of 7β-HSDH was not improved by immobilization on solid supports such as Eupergit C or activated Sepharose.[4c]

In conclusion, we have shown that the α/β inversion of the 7-hydroxyl of water-soluble bile acids can be efficiently obtained by the sequential action of the two specific enzymes 7α- and 7β-HSDH. In order to extend this transformation to neutral steroids it will be necessary to isolate a 7β-HSDH more stable in biphasic systems.

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Experimental Section

Materials and Methods. 7α-Hydroxysteroid dehydrogenase from *Escherichia coli* (EC 1.1.1.159), lactate dehydrogenase from rabbit muscle (EC 1.1.1.27), glucose dehydrogenase from *Bacillus megaterium* (EC 1.1.1.47), glutamate dehydrogenase from *Proteus* (EC 1.4.1.4), NAD, NADP, Eupergit C, cyanogen bromide-activated Sepharose, and Reactive Red Agarose (type 3000 CL) were obtained from Sigma. 7β-Hydroxysteroid dehydrogenase and 7α-hydroxysteroid dehydrogenase from *C. absonum* were purified as described by Macdonald et al.[9] Steroids were purchased from Steraloids. All other reagents and solvents were analytical grade. ^{1}H-NMR were recorded in d_6-DMSO or $CDCl_3$. TLC were carried out on commercial silica gel GF_{254} plates with chloroform–methanol–acetic acid (10:1:0.5, system 1; 15:1:0.5, system 2; 10:4:0.3, system 3; 10:3:0.3, system 4). Compounds were detected with the Komarowsky's reagent.[11] Melting points were uncorrected and determined in open-ended capillaries.

7α-Hydroxysteroid Dehydrogenase Catalyzed Oxidation of 7α-Hydroxy Bile Acids 1a–7a to 7-Keto Derivatives 1c–7c. The following procedure for the synthesis of 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (**1c**) is representative. A solution (40 mL) containing 0.05 M potassium phosphate, 0.15 M pyruvate, 1 mM dithiothreitol, 0.19 mM (5 mg) NAD, 12.5 mM (200 mg) 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (**1a**), 10 U of 7α-HSDH, and 1 mg (≃800 U) of lactic dehydrogenase was titrated to pH 8.5 and left to stand at room temperature in the dark. The course of the reaction was monitored by TLC and HPLC. After 24 h the reaction was complete. The solution was acidified to pH 3 and extracted with AcOEt. The organic layer

was anhydrified and evaporated giving 172 mg (86%) of **1c**.[12] The product was crystallized from ethyl acetate: mp 194–5 °C (lit.[4c] mp 194–5 °C, from AcOEt); TLC R_f 0.42 (system 1); ^{1}H-NMR δ 3.80 (1 H, t, J = 2.8 Hz, H-12β), 3.34 (1 H, sept, J = 4.6 Hz, H-3β), 1.11 (3 H, s, C-19 Me), 0.92 (3 H, d, J = 6.3 Hz, C-21 Me), 0.59 (3 H, s, C-18 Me).

Oxidation of compounds **2a–4a** was carried out in similar way (reaction times 24–48 h). The products were recovered (yields between 85% and 94%), crystallized and characterized. **2c**:[12] mp 202–204 °C (from AcOEt; lit.:[13] 199–201 °C); TLC R_f 0.44 (system 2); ^{1}H-NMR δ 3.42 (1 H, sept, J = 4.5 Hz, H-3β), 1.50 (3 H, s C-19 Me), 0.79 (3 H, d, J = 6 Hz, C-21 Me), 0.52 (3 H, s, C-18 Me). **3c**:[12] mp 188–190 °C (from AcOEt; Lit.[13] 185–187 °C); TLC R_f 0.56 (system 1); ^{1}H-NMR δ 4.39 (1 H, t, J = 4.39 Hz, H-6β), 3.39 (1 H, sept, J = 4.5 Hz, H-3β), 1.10 (3 H, s, C-19 Me), 0.79 (3 H, d, J = 6.2 H, C-21), 0.53 (3 H, s, C-18 Me). **4c**: mp 224–227 °C (from AcOEt–MeOH); TLC R_f 0.36 (system 1); ^{1}H-NMR δ 3.50 (1 H, d, J = 3 Hz, H-6α), 3.40 (1 H, sept, J = 5 Hz, H-3β), 1.30 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 7 Hz, C-21 Me), 0.71 (3 H, s, C-18 Me).

Oxidation of compounds **5a–7a** was carried out in similar way (reaction times 24–48 h). Due to the lower solubilities of these compounds in organic solvents, the acidified reaction mixtures were freeze-dried and lyophylized. The residues were extracted with EtOH and filtered, and the solvent was evaporated to give the products **5c–7c**. As these intermediates contained also some amounts of organic and inorganic salts, they were not characterized before reduction with 7β-HSDH. **5c**: TLC 0.56 (system 3). **6c**: 0.51 (system 3). **7c**: 0.49 (system 4).

7β-Hydroxysteroid Dehydrogenase Catalyzed Reduction of 7-Keto Bile Acids to 7β-Hydroxy Bile Acids. The following procedure for the synthesis of 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid (**1b**) is representative. A solution (40 mL) containing 0.05 M potassium phosphate, 0.05 M glucose, 1 mM dithiothreitol, 0.16 mM (5 mg) NADP, 12.5 mM (200 mg) 3α,12α-dihydroxy-7-keto-5β-cholan-24-oic acid (**1c**), 2 U of 7β-HSDH, and 25 U of glucose dehydrogenase was titrated to pH 6.8. The reaction monitored by TLC and HPLC was complete after 24 h. The compound was recovered as described above (86% yield) and crystallized from AcOEt: mp 144–147 °C (lit.[14] mp 127–129 °C, from AcOEt–hexane); $[\alpha]_D$ + 69.0° (c 0.5, EtOH); TLC R_f 0.31 (system 1); ^{1}H-NMR δ 3.80 (1 H, t, J = 2.8 Hz, H-12β), 3.33 (2 H, m, H-3β and H-7α), 0.94 (3 H, d, J = 7 Hz, C-21 Me), 0.86 (3 H, s, C-19 Me), 0.63 (3 H, s, C-18 Me).

Reduction of compounds **2c**, **4c**, and **5c** were carried out in similar ways (reaction times 48–72 h). The products were crystallized from AcOEt. **2b**:[12] mp 202–203 °C (lit.[13] mp 203–204 °C); TLC R_f 0.30 (system 2); ^{1}H-NMR δ 3.30 (2 H, m, H-3β and H-7α), 0.88 (3 H, d, J = 7 Hz, C-21 Me), 0.86 (3 H, s, C-19 Me), 0.60 (3 H, s, C-18 Me). **4b**:[12] mp 227–228 °C (lit.[13] mp 226–228 °C); TLC R_f 0.31 (system 1); ^{1}H-NMR δ 3.47 (1 H, t, J = 3 H, H-6α), 3.38 (1 H, sept, J = 5 Hz, H-3β), 3.34 (1 H, dd, J_1 = 9.5 Hz, J_2 = 5.5 Hz, H-7α), 1.06 (3 H, s, C-19 Me), 0.91 (3 H, d, J = 7 Hz, C-21 Me), 0.70 (3 H, s, C-18 Me). **5b**:[15] mp 245–247 °C; $[\alpha]_D$ +75.2° (c 0.5, EtOH); TLC R_f 0.44 (system 3); ^{1}H-NMR δ 8.08 (1 H, t, J = 5 Hz, NH), 3.77 (1 H, t, J = 2.6 Hz, H-12β), 3.62 (2 H, d, J = 5 Hz, CH_2COO), 3.30 (2 H, m, H-3β and H-7α), 0.93 (3 H, d, J = 7 Hz, C-21 Me), 0.84 (3 H, s, C-19 Me), 0.60 (3 H, s, C-18 Me).

Reduction of compound **6c** was carried out similarly. However, due to the lower solubility of **6b** in organic solvent, the acidified water reaction mixture was freeze-dried and then lyophilized. The solid residue was extracted with EtOH, the solvent evaporated, and the crude solid purified from organic and inorganic contaminants by cromatography on XAD8. Following washing with H_2O, the steroid was eluted with MeOH and then crystallized from AcOEt-MeOH. **6b**:[15] mp 205–208 °C; $[\alpha]_D$ +32.0° (c 0.47, EtOH): TLC 0.40 (system 3); ^{1}H-NMR δ 7.58 (1 H, t, J = 5 Hz, NH), 3.79 (1 H, t, J = 2.6 Hz, H-12β), 3.30 (4 H, m, H-3β, H-7α and HN-CH_2), 2.58 (2 H, t, J = 7 Hz, CH_2-SO_3), 0.93 (3 H, d, J = 7 Hz, C-21 Me), 0.85 (3 H, s, C-19 Me), 0.61 (3 H, s, C-18 Me).

Methyl 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oate (8c). The conditions for the oxidation of **8a** with a two-phase system in a shaken vessel (150 strokes min^{-1}) were as follows.[4b] Aqueous phase: 10 mL of 0.05 M phosphate buffer pH 8.5 containing 0.06 M α-ketoglutarate, 0.1 M ammonium acetate, 1 mM dithiothreitol, 1 mg of NADP, 5 U of 7α-HSDH from *C. absonum* (NADP dependent), 40 U of glutamate dehydrogenase. Organic phase: 10 mL of AcOEt containing 0.25 mmol of **8a** (100 mg). The steroid was completely transformed after 48 h. The organic phase was recovered and the solvent evaporated to give pure **8c**:[12] ^{1}H-NMR δ ($CDCl_3$) 4.00 (1 H, br t, H-12β), 3.67 (3 H, s, COO*Me*), 3.58 (1 H, sept, J = 4.5 Hz, H-3β), 1.18 (3 H, s, C-19 Me), 0.96 (3 H, d, J = 7 Hz, C-21 Me), 0.67 (3 H, s, C-18 Me).

As described in the text, the attempted reduction of **8c** in a similar two-phase system was unsuccessful. Conditions: aqueous phase, 15 mL of 0.05 M phosphate buffer pH 6.8 containing 0.05 M glucose, 1 mM dithiothreitol, 2 mg of NADP, 1 U of 7β-HSDH, 20 U of glucose dehydrogenase; organic phase, 10 mL of AcOEt containing 100 mg of **8c**.

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(13) Steraloids catalogue.

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(15) Analytycal and spectroscopic properties of this product were identical to those of the compound obtained by condensation of **1b** with the appropriate amino acid.[16]

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