and concentrated to give 143 g (98%) of oil which was used without further purification: NMR ($CDCl_3$) 8.2–7.4 (m, 10 H, Ar H), 6.7 (d, 1 H, $J_{H1,F}$ = 11.9 Hz, C_1), 5.6 (d, 1 H, $J_{H2,F}$ = 50 Hz, C_2), 5.5 (dd, 1 H, J = 22 Hz, 3 Hz, C_3), 4.75 (m, 3 H, C_4, C_5).

1-(3′,5′-Di-*O*-benzoyl-2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (8c). A solution of 2,4-bis-*O*-(trimethylsilyl)-5-ethyluracil (**11c**) (0.408 mol), 3,5-di-*O*-benzoyl-2-deoxy-2-fluoro-α-D-arabinofuranosyl bromide (**7**) (0.343 mol), and 1.7 L of $CHCl_3$ (alcohol free) was stirred at reflux for 20 h.[12a] The cooled reaction mixture was washed (H_2O, 2 × 2 L) and dried (Na_2SO_4), and the solvent was removed at reduced pressure. The solid product was recrystallized from 1.5 L of hot, absolute ethyl alcohol to give 126 g of **8c** (76.2%): mp 155–157 °C; NMR ($CDCl_3$) 8.7 (s, 1 H, C=CH), 8.2–7.2 (m, 10 H, Ar H), 6.34 (dd, 1 H, J = 3 Hz, $J_{H1',F}$ = 22 Hz, $C_{1'}$), 5.63 (dd, 1 H, J = 3 Hz, $J_{H3',F}$ = 17.7 Hz, $C_{3'}$), 5.31 (dd, 1 H, J = 3 Hz, $J_{H2',F}$ = 50 Hz, $C_{2'}$), 4.8 (m, 2 H, $C_{5'}$), 4.7 (dd, 1 H, $C_{4'}$), 2.19 (q, 2 H, J = 7 Hz, CH_2), 0.95 (t, 3 H, J = 7 Hz, CH_3). Anal. ($C_{25}H_{23}N_2O_7F$) C, H, N.

1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil, FEAU (1c). A solution of 275 mL (12.75 mol) of liquified ammonia and 1.72 L of methyl alcohol was stirred at ca. 0 °C. The solid 1-(3′,5′-di-*O*-benzoyl-2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (**8c**), 172 g (0.356 mol), was added portionwise over 5 to 10 min. The ice bath was removed and the slurry stirred for 65 h (HPLC monitoring indicated only a trace of the incomplete hydrolysis product, 5-benzoyl[12b] remained). The solvent was removed and the crude product recrystallized from 700 mL of boiling CH_3CN. After crystallization at 0 °C, 4 h, the product was removed by filtration, washed with cold CH_3CN (2 × 100 mL), and dried to give **1c**, 90.8 g (93%): mp 171–173 °C; NMR ($NaOD/D_2O$) 7.5 (s, 1 H, C=CH), 6.3 (dd, 1 H, $J_{H,F}$ = 16 Hz, $C_{1'}$), 5.16 (m, 1 H, $J_{H2',F}$ = 52 Hz, $C_{2'}$), 4.4 (m, 1 H, $J_{H3',F}$ = 19.9 Hz, $C_{3'}$), 4.0–3.77 (m, 3 H, $C_{4'}$, $C_{5'}$), 2.27 (q, 2 H, J = 7.5 Hz, CH_2), 1.05 (t, 3 H, J = 7.5 Hz, CH_3). Anal. ($C_{11}H_{15}N_2O_5F$) C, H, N.

2-Fluoro-2-deoxy-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl Bromide (7) Stock Solution. A solution of 9.29 g (20.0 mmol) of **6** and 42 mL of dry CH_2Cl_2 was stirred at ca. 20 °C and 11.7 mL (43.4 mmol) of 30% hydrogen bromide in acetic acid added. The solution was stirred for 18 h in a stoppered flask, then washed (H_2O, 2 × 60 mL, and saturated $NaHCO_3$ solution, 2 × 60 mL), and dried ($MgSO_4$), and the solvent was evaporated at reduced pressure to give 8.48 g of a light yellow oil. The material was found to be pure **8a** (NMR). The oil was diluted with CH_2Cl_2 to exactly 50 mL in a volumetric flask. The concentration of **7** was calculated as 0.4 M and portions of this solution were used for the general coupling procedure as described below.

2,4-Bis-*O*-(trimethylsilyl)pyrimidine 11a–d Stock Solution. A stock solution of each of the four uracils was prepared in this way; a mixture of 75 mL of CH_3CN, 4.75 mL of hexamethyldisilazane, 0.25 g of ammonium sulfate, and 2.24 g (20 mmol) of uracil was stirred at reflux 18 h. The solution of the bis-silylated pyrimidine was calculated to be 0.252 M and used as described below.

General Procedure for the Coupling Reaction. A 4.4-mL portion of the silylated uracil solution (1.1 mmol) was evaporated to dryness and dissolved in 2 mL of the appropriate, dry, solvent (CH_3CN, CH_2Cl_2, $CHCl_3$ or CCl_4). A 2.5-mL portion of the 0.4 M solution of **7** (1.0 mmol) was evaporated to dryness and dissolved in 2 mL of the same solvent as above. The solution of **7** was then added to the solution of the silylated pyrimidine **11a–d**. The flask that had contained **7** was rinsed with 1 mL of the same solvent and added to the reaction (total volume 5 mL). The reaction was stirred at reflux and samples were taken at intervals to determine when the reaction was complete. When HPLC[12a] indicated that **7** was consumed, the HPLC area count of each isomer was used to calculate the anomer ratios listed in Table I.

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Registry No. **1c**, 83546-42-3; **2**, 6974-32-9; α-**3**, 79439-67-1; β-**3**, 67525-66-0; **4**, 22224-41-5; **6**, 97614-43-2; **7**, 97614-44-3; **8c**, 95740-18-4; **11c**, 31167-05-2; 5-ethyluracil, 4212-49-1.

Enzymatic α/β Inversion of C-3 Hydroxyl of Bile Acids and Study of the Effects of Organic Solvents on Reaction Rates

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Enzymatic α/β inversion of the C-3 hydroxyl of numerous bile acids containing different numbers of hydroxyl groups in the skeleton and side chains of different lengths has been carried out. Inversion was obtained in two steps through the sequential use of the commercial enzymes 3α- and 3β-hydroxysteroid dehydrogenase, employed in the free form or immobilized on Eupergit C. The transformations were practically quantitative and the products more than 98% pure. NAD was regenerated in situ with the pyruvate/lactic dehydrogenase system and NADH with the formate/formate dehydrogenase system. The effects of product inhibition on reaction rates and the favorable effects produced by low concentrations (7–10%, v/v) of ethyl acetate and ethanol were also examined.

Introduction

The NAD(P)-dependent oxidoreductases[2] have been successfully used for the regio-, stereo-, and (or) enantiospecific oxidoreduction of the hydroxyl keto groups of a variety of compounds.[3] In particular, hydroxysteroid dehydrogenases have been employed for preparative-scale transformations of bile acids and neutral steroids in aqueous and in organic media.[4] The potential use of these

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(2) Abbreviations: NAD, β-nicotinamide adenine dinucleotide; K_m, Michaelis constant; K_i, product inhibition constant; V_{max}, maximal enzymatic velocity; DTT, dithiothreitol.

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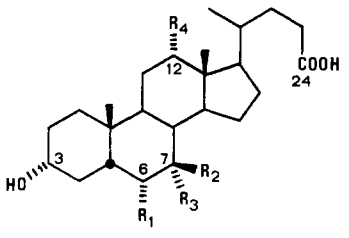
Scheme I



	R_1	R_2	R_3	R_4
1a	H	H	OH	OH
1b	OH	H	OH	H
1c	H	H	OH	H
1d	H	OH	H	H
1e	H	H	H	OH

enzymes for the synthesis of standards, enzyme substrates, metabolites, and pharmaceuticals has increased with the development of effective methods for nicotinamide cofactor regeneration[3-5] and enzyme immobilization.[6]

This paper reports the α/β inversion of the C-3 hydroxyl of numerous bile acids with different numbers and positions of the hydroxyls in the skeleton and different lengths of the side chain. Inversion was obtained in two steps through the sequential use of the commercial enzymes 3α- and 3β-hydroxysteroid dehydrogenase, employed in the free form or immobilized on Eupergit C. The effects of product inhibition on reaction rates and the favorable effects produced by organic solvents dissolved in the aqueous buffer were also examined.

Results and Discussion

α/β Inversions. Several 3α-hydroxyl bile acids were transformed into their corresponding 3β-hydroxyl derivatives with two commercial hydroxysteroid dehydrogenases. The approach involved the preparation of the intermediate 3-keto derivatives through the regiospecific oxidation of the 3α-hydroxyls, catalyzed by 3α-hydroxysteroid dehydrogenase (3α-HSDH) (reaction 1). The pyruvate/lactic dehydrogenase (LDH) system was used to regenerate NAD (reaction 2).

3α-hydroxyl bile acid + $NAD^+ \xrightleftharpoons{3\alpha\text{-HSDH}}$ 3-keto bile acid + NADH + H^+ (1)

pyruvate + NADH + $H^+ \xrightleftharpoons{LDH}$ lactate + NAD^+ (2)

The 3-keto bile acids obtained were then stereospecifically reduced to the 3β-hydroxyl derivatives with 3β-hydroxysteroid dehydrogenase (3β-HSDH) (reaction 3). NADH was regenerated with the formate/formate dehydrogenase (FDH) system (reaction 4).

3-keto bile acid + NADH + $H^+ \xrightleftharpoons{3\beta\text{-HSDH}}$ 3β-hydroxyl bile acid + NAD^+ (3)

$HCOOH + NAD^+ \xrightleftharpoons{FDH} CO_2 + NADH + H^+$ (4)

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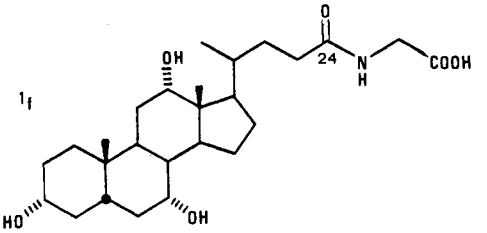
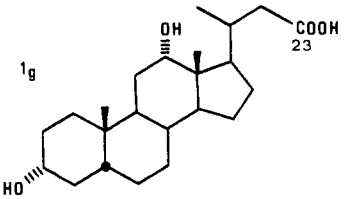
Table I. Relative Rates of Oxidation of 3α-Hydroxyl Bile Acids by 3α-Hydroxysteroid Dehydrogenase

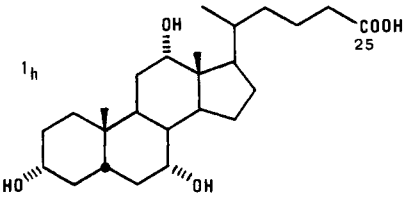
substrate	relative rate[a] buffer	buffer containing 7% (v/v) ethyl acetate
1a	80	70
1b	100	98
1c	83	85
1d	100	91
1e	28	56
1f	96	91
1g	30	59
1h	31	30

[a] The initial rate determinations were carried out in 0.1 M potassium phosphate buffer, pH 8.5, 25 °C, in the presence and absence of 7% (v/v) ethyl acetate. Saturating concentrations of NAD (1 mM) and bile acid (5 mM) were used. The oxidation rate of **1b** in buffer was taken as 100.

The 3α-hydroxyl bile acids (**1a–e**) studied are shown in Scheme I. Furthermore, α/β inversion of glycocholic acid [3α,7α,12α-trihydroxy-5β-cholan-24-oic acid *N*-(carboxymethyl)amide] (**1f**), nordeoxycholic acid (3α,12α-dihydroxy-23-nor-5β-cholan-23-oic acid) (**1g**), and homocholic acid (3α,7α,12α-trihydroxy-25-homo-5β-cholan-25-oic acid) (**1h**) was also done.







The oxidation of 3α-hydroxyl compounds **1a–h** was practically quantitative, and the crude products were 98–99% pure, as demonstrated by HPLC, TLC, and NMR. This confirmed the very high specificity of 3α-hydroxysteroid dehydrogenase, which ignored the presence of the 6α-, 7α-, 7β-, and 12α-hydroxyls in the bile acids. The rates of preparative conversion of the various bile acids reflected initial reaction rates. Table I shows that deoxycholic acid (**1e**), nordeoxycholic acid (**1g**), and homocholic acid (**1h**) were the poorest substrates (slowest rates) and also shows that although the shortening of the side chain of bile acid did not affect activity (see **1g** and the parent compound **1e**), the lengthening of the aliphatic part of the side chain markedly slowed enzymatic activity (see **1h** and the parent compound **1a**). It is interesting that in two cases (**1e** and **1g**) the initial reaction rates were higher in the presence of ethyl acetate than in buffer alone (Table I). The 3β-hydroxysteroid dehydrogenase catalyzed reductions of the

3-keto bile acids **2a–h** to the corresponding 3β-hydroxyl derivatives were also obtained with quantitative yields and practically absolute stereospecificity, as demonstrated by NMR data. In this case, all the various substrates except **2b** were transformed by the enzyme with similar rates. The rate for **2b** was about 50% slower. Compared to the chemical methods employed for α/β inversion of C-3 hydroxyls of bile acids,[7] the enzymatic method is more facile, does not require chromatographic separation, and gives pure products simply by acid precipitation and crystallization. The intermediate keto derivatives can be processed without crystallization if chemical characterization is not needed.

For repeated transformations, the reusable and more stable immobilized enzymes can be employed. Eupergit C was selected as the matrix because these oxirane-activated acrylic beads were found to be very handy and to have high binding capacity for the enzymes. The stability of the enzymes and the yields of enzyme activity after immobilization (40–50%) were very close to those obtained[4e] for Sepharose CL-4B immobilized enzymes. Unlike Sepharose CL-4B, Eupergit C adsorbed a certain amount of bile acids, and therefore the same preparation could not be used to transform a different substrate unless the matrix was extensively washed with a buffer containing 10% ethanol.

Pyruvate/lactic dehydrogenase was chosen as the NAD-regenerating system because of its greater simplicity in comparison with the more stable α-ketoglutarate/glutamate dehydrogenase system,[4e,5b] which also needs ammonia as substrate and adenosine diphosphate as enzyme activator. In addition, we have observed that there is less pyruvate degradation[5b] when the reaction is carried out in the dark. Formate/formate dehydrogenase[3b,8] was preferred to glucose/glucose dehydrogenase[3a,3c,4e] as the NADH-regenerating system because of the poor stability of glucose dehydrogenase under our experimental conditions. In fact, while glucose dehydrogenase is satisfactorily stable at pH 6.5,[3c] we found it to be markedly unstable (particularly in the presence of bile acids) at the pH value (7–7.2) we had to use to keep the bile acids in solution.

Effects of Organic Solvents on Reaction Rates. Enzymatic oxidations and reductions were carried out in the presence of ethyl acetate (7%, v/v) or ethanol (10%, v/v). Initially, the organic solvent (ethanol) was added to the aqueous buffer only in the reduction reactions, with the aim of increasing the solubility of the 3-keto bile acids (particularly compound **2c**) at pH 7, at which the activity of 3β-hydroxysteroid dehydrogenase is close to its optimum. Afterwards, the organic solvent (ethyl acetate) was also added to the oxidation reactions, in spite of the good solubility of bile acids at pH 8.5, because, quite surprisingly, we observed that the presence of the organic solvent reduced the time needed to achieve the complete conversion of substrates by about 30%. This prompted us to study the mechanism of this favorable effect.

Table II. 7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic Acid (2a) as Product Inhibitor of 3α-Hydroxysteroid Dehydrogenase with 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid (1a) as Substrate[a]

inhibitor concn, μM	buffer			buffer containing 7% (v/v) ethyl acetate	
	K_m, μM	V_{max}, rel	K_i, μM	K_m, μM	V_{max}, rel
0	4.8	100		34	100[b]
8.2	4.7	52	8.9		
20.5	5.0	30	8.8		
82.0				58	45
164.0				82	38

[a] Details of assay conditions are given in the Experimental Section. [b] In the presence of 7% ethyl acetate, the V_{max} value is 87% of that in buffer.

3α-Hydroxysteroid dehydrogenase[9] and 3β-hydroxysteroid dehydrogenase[10] follow ordered mechanisms, with the cofactor binding first, which means that bile acid products should behave as noncompetitive inhibitors for bile acid substrates. In fact, Tables II and III show that in buffer alone the products reduced the V_{max} values without affecting the K_m values of the hydroxysteroid dehydrogenases. As clearly pointed out by Lee and Whitesides,[5b,11] noncompetitive inhibition, which has the effect of lowering the apparent concentration of the enzyme, becomes a serious drawback in large-scale synthesis when the ratio $K_i/K_m < 1$ or when K_i is low. With both 3α-hydroxysteroid dehydrogenase (Table II) and 3β-hydroxysteroid dehydrogenase (Table III), the ratio $K_i/K_m > 1$ (about 2 and 5, respectively), but the K_i value for 3α-hydroxysteroid dehydrogenase in buffer is about $^1/_{50}$ that for 3β-hydroxysteroid dehydrogenase. The much higher affinity of the product for 3α-hydroxysteroid dehydrogenase should explain why the bile acid oxidations required about 10 times more enzyme units than the reductions.

Organic solvents behaved mainly as competitive inhibitors for bile acid substrates, i.e., increased the K_m values (7–8 times) without affecting (3β-hydroxysteroid dehydrogenase) or barely affecting (3α-hydroxysteroid dehydrogenase) the V_{max} values (Tables II and III).[12] However, it should be stressed that competitive inhibition can be overcome in preparative synthesis by high substrate concentrations. In fact, under our conditions, even after 90% of the substrate was transformed, its concentration (1.25 mM) was still higher than the K_m values of the enzymes and therefore high enough to assure good activity. In the presence of organic solvents, bile acid products gave mixed inhibition (Table II) or noncompetitive inhibition (Table III), and in both cases, the affinity of the products for the enzymes was lower than in buffer alone. With 3β-hydroxysteroid dehydrogenase, for instance, the K_i value in 10% ethanol is 4 times higher, which means that the concentration of product that will reduce the apparent concentration of the enzyme to 50% will be 4 times higher in the presence of the organic solvent than in buffer alone. Therefore, in spite of the negative effects on substrate K_m values and enzyme stabilities,[13] the organic solvents in-

(7) So far, three chemical methods have mainly been used for the α/β inversion of C-3 hydroxyl of bile acids. (a) The first method (Danielsson, H.; Eneroth, P.; Hellström, K.; Sjövall, J. *J. Biol. Chem.* **1962**, *237*, 3657) is based on the selective oxidation of the 3α-hydroxyl group followed by catalytic reduction (four steps plus two chromatographic separations). (b) The second method (Bose, A. K.; Lal, B.; Hoffman, W. A.; Manhas, M. S. *Tetrahedron Lett.* **1973**, 1619) is based on the Mitsunobu reaction, which utilizes azodicarboxylate and triphenylphosphine (three steps plus one chromatographic separation). (c) The third method (Chang, F. C. *J. Org. Chem.* **1979**, *44*, 4567) is based on the tosylation of the 3α-hydroxyl group followed by DMF treatment (four steps plus one chromatographic separation or six steps with no chromatographic separation). Chang compared the three methods and found that the yields were 5% for the first method, 15% for the second method, and 35–50% for the third method.

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(12) The solvents did not substantially influence the K_m values of the hydroxysteroid dehydrogenases for NAD(H), nor did they affect the activity of the enzymes used for the regeneration of the nicotinamide cofactors.

Table III. 3β,7α,12α-Trihydroxy-5β-cholan-24-oic Acid (3a) as Product Inhibitor of 3β-Hydroxysteroid Dehydrogenase with 7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic Acid (2a) as Substrate[a]

	buffer			buffer containing 10% (v/v) ethanol		
inhibitor concn, μM	K_m, μM	V_{max}, rel	K_i, μM	K_m, μM	V_{max}, rel	K_i, μM
0	90	100		830	100[b]	
160	90	74	455			
480	95	49	461			
570				850	77	1908
1600				880	55	1955

[a] Details of assay conditions are given in the Experimental Section. [b] In the presence of 10% ethanol, the V_{max} value is 98% of that in buffer.

creased the rates of transformation by decreasing the affinity of the products for the enzymes. These results may also be relevant to other dehydrogenases (e.g., horse liver alcohol dehydrogenase,[3f] alcohol dehydrogenase from *Thermoanaerobium brockij*,[3g] and glycerol dehydrogenase[11] used in organic synthesis and suggest that a more systematic investigation of the influence of these and of other solvents on enzymatic transformations might reveal conditions that will increase their favorable effects.

Experimental Section

Materials. 3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50, 42 units mg^{-1} of protein), 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51, 26 units mg^{-1} of protein), lactic dehydrogenase (EC 1.1.1.27, 800 units mg^{-1} of lyophilized powder), and glucose dehydrogenase (EC 1.1.1.47, 135 units mg^{-1} of protein) were purchased from Sigma. Formate dehydrogenase (EC 1.2.1.2, 3 units mg^{-1} of protein), NAD, NADH, and DTT were obtained from Boehringer. Eupergit C was bought from Rohm Pharm, and bile acid substrates were bought from Steraloids, except for homocholic acid (**1h**), which was synthesized by the procedure of Kuramoto et al.[14] All other reagents and compounds were of analytical grade.

General Methods. Melting points were uncorrected and were determined in open-ended capillaries. ^1H NMR spectra were obtained on a Bruker 270 (270 MHz) instrument in deuteriated dimethyl sulfoxide with Me_4Si as internal standard. Optical rotations were measured in a Perkin-Elmer 141 polarimeter, in methanol. HPLC was carried out with a JASCO Trirotar pump equipped with a GP-A30 solvent delivery system, a Uvidec 100 III detector, and a Finepak Sil C_{18} column (250 mm × 4.6 mm internal diameter). A 60-min Concave 2 gradient from 30% to 60% acetonitrile in 50 mM sodium phosphate, pH 3 (system 1), or a 30-min linear gradient from 30% to 50% acetonitrile in 50 mM sodium phosphate, pH 3 (system 2), was employed. The flow rate was 1 mL min^{-1}, and readings were made at 220 nm. The data were computed by a HP-3390 A reporting integrator (Hewlett-Packard). HPTLC was done on precoated silica gel 60 F_{254} plates (Merck) with chloroform–methanol (10:3, system 1), chloroform–methanol–acetic acid (40:3:2, system 2), chloroform–methanol–acetic acid (20:3:1, system 3), or chloroform–methanol–acetic acid (3:1:1, system 4). Compounds were detected with Komarowsky's reagent.[15] The enzymatic assays were performed by the procedures in the literature[16] and units of activity expressed as μmol min^{-1}.

Enzyme Immobilization on Eupergit C. (a) 3α-Hydroxysteroid dehydrogenase (400 units) and lactic dehydrogenase (3 mg) were dissolved in 12 mL of 1 M potassium phosphate buffer, pH 7.5, containing 0.5 mM NAD, and mixed with 3 g of Eupergit C. The mixture was left to stand at room temperature overnight, and then the swollen matrix (about 15 mL) was thoroughly washed with 0.1 M potassium phosphate buffer, pH 7, before use. (b) 3β-Hydroxysteroid dehydrogenase (28 units) and formate dehydrogenase (30 units) were dissolved in 8 mL of 1 M potassium phosphate buffer, pH 7.5, containing 0.5 mM NAD^+, mixed with 2 g of Eupergit C, and processed as described above. In all cases, negligible activity was found in the washings, and this indicates that the enzymes were completely linked to the matrix.

3α-Hydroxysteroid Dehydrogenase Catalyzed Oxidation of 3α-Hydroxyl Bile Acids (1b–h) to 3-Keto Derivatives (2b–h).[17] The following procedure for the synthesis of 7α-hydroxy-3-oxo-5β-cholan-24-oic acid (**2c**) is representative. A solution (150 mL) containing 0.05 M potassium phosphate, 7% (v/v) ethyl acetate, 0.075 M pyruvate, 1 mM DTT, 75 mg of serum albumin, 0.2 mM (20 mg) NAD, 13 mM (0.8 g) sodium 3α,7α-dihydroxy-5β-cholanoate, 260 units of 3α-hydroxysteroid dehydrogenase, and 2 mg 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 40 h, the reaction was complete. The solution was acidified to pH 3 and the precipitated solid collected by filtration, washed with cold water, and dried. The product was then dissolved in ethanol, the solution was filtered and evaporated, and the residue was dried. The yield was 0.65 g (86%). The product was crystallized from ethyl acetate: mp 88–90 °C (lit.[18] mp 85–87 °C, from aqueous methanol); TLC R_f 0.54 (system 1); HPLC t_R 29 min (system 2); $[\alpha]_D$ +20°; ^1H NMR δ 0.64 (3 H, s, C-18 Me), 0.89 (3 H, d, J = 6.3 Hz, C-21 Me), 0.94 (3 H, s, C-19 Me), 3.69 (1 H, q, J = 3.0 Hz, H-7β).

The syntheses of most of the other keto derivatives were carried out in a similar way. For **2e**, **2g**, and **2h**, twice the amount of 3α-hydroxysteroid dehydrogenase was used due to the slower oxidation rates of the substrates (see Table I). The products were recovered (yields between 79% and 91%), crystallized from ethyl acetate, and characterized. **2b**: mp 195–197 °C; TLC R_f 0.58 (system 1); HPLC t_R 30 min (system 2); $[\alpha]_D$ +12°; ^1H NMR δ 0.64 (3 H, s, C-18 Me), 0.89 (3 H, d, J = 6.3 Hz, C-21 Me), 0.94 (3 H, s, C-19 Me), 3.61 (2 H, m, H-6β and H-7β). Anal. Calcd for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 70.73; H, 9.54. **2d**: mp 73–75 °C; TLC R_f 0.53 (system 1); HPLC t_R 32 min (system 2); $[\alpha]_D$ +67°; ^1H NMR δ 0.65 (3 H, s, C-18 Me), 0.89 (3 H, d, J = 6.3 Hz, C-21 Me), 0.96 (3 H, s, C-19 Me), 3.28 (1 H, dt, J_1 = 10.6 Hz, J_2 = 4.5 Hz, H-7α). Anal. Calcd for $C_{24}H_{38}O_4$: C, 73.81; H, 9.81. Found: C, 74.08; H, 9.68. **2e**: mp 103–105 °C (lit.[19] mp 105 °C, from dilute acetic acid; TLC R_f 0.59 (system 1); HPLC t_R 35 min (system 2); $[\alpha]_D$ +52° (lit.[19] +52°, in ethanol); ^1H NMR δ 0.63 (3 H, s, C-18 Me), 0.92 (3 H, d, J = 6.3 Hz, C-21 Me), 0.94 (3 H, s, C-19 Me), 3.82 (1 H, t, J = 2.8 Hz, H-12β). **2f**: mp 218–220 °C; TLC R_f 0.69 (system 4); HPLC t_R 28 min (system 2); $[\alpha]_D$ +34°; ^1H NMR δ 0.62 (3 H, s, C-18 Me), 0.92 (3 H, s, C-19 Me), 0.94 (3 H, d, J = 6.3 Hz, C-21 Me), 3.68 (1 H, q, J = 3.0 Hz, H-7β), 3.70 (2 H, d, J = 6.0 Hz, CH_2 glycine moiety), 3.82 (1 H, t, J = 2.8 Hz, H-12β), 8.09 (1 H, t, J = 6.0 Hz, NH). Anal. Calcd for $C_{26}H_{41}NO_6$: C, 67.36; H, 8.91; N, 3.02. Found: C, 67.68; H, 8.70; N, 2.88. **2g**: mp 216–218 °C; TLC R_f 0.57 (system 1); HPLC t_R 29 min (system 2); $[\alpha]_D$ + 56°; ^1H NMR δ 0.66 (3 H, s, C-18 Me),

(13) The residual activities of the enzymes incubated for 3 days in the presence of 7% ethyl acetate or 10% ethanol were 70–85% of those of the enzymes incubated in buffer alone.

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0.94 (3 H, s, C-19 Me), 0.97 (3 H, d, J = 6.7 Hz, C-21 Me), 3.81 (1 H, t, J = 2.8 Hz, H-12β). Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.37; H, 9.63. Found: C, 73.22; H, 9.84. **2h**: mp 105–107 °C; TLC R_f 0.44 (system 1); HPLC t_R 25 min (system 2); $[\alpha]_D$ +43°; ^{1}H NMR δ 0.63 (3 H, s, C-18 Me), 0.92 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 6.3 Hz, C-21 Me), 3.68 (1 H, q, J = 3.0 Hz, H-7β), 3.82 (1 H, t, J = 2.8 Hz, H-12β). Anal. Calcd for $C_{25}H_{40}O_5$: C, 71.39; H, 9.59. Found: C, 71.11; H, 9.50.

7α-Hydroxy-3-oxo-5β-cholan-24-oic acid (**2c**) was also prepared with Eupergit C immobilized enzymes (15 mL of swollen matrix), under conditions identical with those employed with the free enzymes and with mechanical stirring. The transformation was complete after 48 h. The immobilized enzymes were used for two other runs, and the residual activities of 3α-hydroxysteroid dehydrogenase and lactic dehydrogenase were 70% and 74%.

3β-Hydroxysteroid Dehydrogenase Catalyzed Reduction of 3-Keto Bile Acids (2a–h) to 3β-Hydroxyl Bile Acids (3a–h). The following procedure for the synthesis of 3β,7α-dihydroxy-5β-cholan-24-oic acid (**3c**) is representative. A solution (100 mL) containing 0.05 M potassium phosphate, 10% (v/v) ethanol, 0.1 M formate, 1 mM DTT, 0.2 mM (14 mg) NADH, 6.5 mM (0.27 g) sodium 7α-hydroxy-3-oxo-5β-cholanoate, 16 units of 3β-hydroxysteroid dehydrogenase, and 15 units of formate dehydrogenase was titrated to pH 7.2. After 6 h, the bile acid substrate (0.27 g in 3 mL of H_2O) was added again. The reaction, monitored by TLC and HPLC, was complete after 30 h. The compound was recovered as described above (81% yield) and crystallized from ethyl acetate: mp 196–198 °C (lit.[7a] mp 193 °C, from acetone–petroleum ether); TLC R_f 0.45 (system 2); HPLC t_r 23 min (system 2); $[\alpha]_D$ +7° (lit.[7a] $[\alpha]_D$ +8°, in ethanol); ^{1}H NMR δ 0.61 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 0.88 (3 H, d, J = 6.3 Hz, C-21 Me), 3.63 (1 H, q, J = 3.0 Hz, H-7β), 3.80 (1 H, t, J = 3.0 Hz, H-3α).

The syntheses of the other 3β-hydroxy bile acids were carried out in similar ways. The products were recovered (yields between 78% and 89%), crystallized from ethyl acetate, and characterized. **3a**: mp 207–209 °C (lit.[20] mp 200–202 °C, from ethyl acetate); TLC R_f 0.27 (system 3); HPLC t_R 48 min (system 1); $[\alpha]_D$ +31° (lit.[7a] $[\alpha]_D$ +30°, in ethanol); ^{1}H NMR δ 0.59 (3 H, s, C-18 Me), 0.83 (3 H, s, C-19 Me), 0.92 (3 H, d, J = 6.3 Hz, C-21 Me), 3.61 (1 H, q, J = 3.0 Hz, H-7β), 3.78 (2 H, m, H-3α and H-12β). **3b**: mp 181–183 °C; TLC R_f 0.39 (system 3); HPLC t_R 24 min (system 2); $[\alpha]_D$ +5°; ^{1}H NMR δ 0.61 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 0.87 (3 H, d, J = 6.3 Hz, C-21 Me), 3.56 (1 H, t, J = 3.0 Hz, H-6β or 7β), 3.64 (1 H, dd, J_1 = 3.3 Hz, J_2 = 5.3 Hz, H-6β or 7β), 3.83 (1 H, m, J = 3 Hz, H-3α). Anal. Calcd for $C_{24}H_{40}O_5$: C, 70.55; H, 9.87. Found: C, 70.29; H, 10.04. **3d**: mp 166–168 °C (lit.[21] mp 166–168 °C, from ethyl acetate–hexane); TLC R_f 0.45 (system 2); HPLC t_R 30 min (system 2); $[\alpha]_D$ +56°; ^{1}H NMR δ 0.63 (3 H, s, C-18 Me), 0.87 (3 H, d, J = 6.3 Hz, C-21 Me), 0.89 (3 H, s, C-19 Me), 3.26 (1 H, dt, J_1 = 10.6 Hz, J_2 = 4.5 Hz, H-7α), 3.82 (1 H, t, J = 3.0 Hz, H-3α). **3e**: mp 176–178 °C (lit.[22] mp 174–176 °C, from chloroform); TLC R_f 0.46 (system 2); HPLC t_R 27 min (system 2); $[\alpha]_D$ +45° (lit.[22] $[\alpha]_D$ +46°, in ethanol); ^{1}H NMR δ 0.60 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 0.91 (3 H, d, J = 6.3 Hz, C-21 Me), 3.78 (1 H, t, J = 2.8 Hz, H-12β), 3.88 (1 H, t, J = 3.0 Hz, H-3α). **3f**: mp 224–226 °C; TLC R_f 0.48 (system 4); HPLC t_R 24 min (system 2); $[\alpha]_D$ +25°; ^{1}H NMR δ 0.59 (3 H, s, C-18 Me), 0.83 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 6.3 Hz, C-21 Me), 3.61 (1 H, q, J = 3.0 Hz, H-7β), 3.70 (2 H, d, J = 6.0 Hz, CH_2 glycine moiety), 2.78 (2 H, m, H-3α and H-12β), 8.08 (1 H, t, J = 6.0 Hz, NH). Anal. Calcd for $C_{26}H_{43}NO_6$: C, 67.07; H, 9.31; N, 3.01. Found: C, 67.01; H, 9.42; N, 2.88. **3g**: mp 175–177 °C; TLC R_f 0.45 (system 2); HPLC t_R 24 min (system 2); $[\alpha]_D$ +44°; ^{1}H NMR δ 0.63 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 0.96 (3 H, d, J = 6.7 Hz, C-21 Me), 3.77 (1 H, t, J = 2.8 Hz, H-12β), 3.87 (1 H, t, J = 3.0 Hz, H-3α). Anal. Calcd for $C_{23}H_{38}O_4$: C, 72.97; H, 10.12. Found: C, 73.26; H, 10.06. **3h**: mp 196–198 °C; TLC R_f 0.28 (system 3); HPLC t_R 23 min (system 2); $[\alpha]_D$ +33°; ^{1}H NMR δ 0.59 (3 H, s, C-18 Me), 0.83 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 6.3 Hz, C-21 Me), 3.61 (1 H, q, J = 3.0 Hz, H-7β), 3.78 (2 H, m, H-3α and H-12β). Anal. Calcd for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 70.87; H, 9.88.

3β,7α-Dihydroxy-5β-cholan-24-oic acid (**3c**) was also prepared with Eupergit C immobilized enzymes (10 mL of swollen matrix) under conditions identical with those employed with the free enzymes and with mechanical stirring. The transformation was complete after 3 days. The immobilized enzymes were used twice, and the residual activities of 3β-hydroxysteroid dehydrogenase and formate dehydrogenase were 65% and 75%.

Enzyme Assays. The effects of organic solvents (ethanol and ethyl acetate) and of bile acid products on the kinetic parameters (K_m and V_{max}) of 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase were investigated. 3α-Hydroxysteroid dehydrogenase activity was assayed in 0.1 M potassium phosphate buffer (with or without organic solvent), pH 8.5, containing 1 mM NAD^+ and variable concentrations (2–300 μM) of cholic acid (**1a**). The concentration of the product, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid (**2a**), was varied from 0 to 164 μM. 3β-Hydroxysteroid dehydrogenase activity was assayed in 0.1 M potassium phosphate buffer (with or without organic solvent), pH 7, containing 0.15 mM NADH and variable concentrations (10–2500 μM) of the substrate 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid (**2a**). The concentration of the product, 3β,7α,12α-trihydroxy-5β-cholan-24-oic acid (**3a**), was varied from 0 to 1.6 mM. K_m and V_{max} values were obtained from the initial rate measurements, by using the equations of Wilkinson[23] programmed on an Apple IIc computer.[24]

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Registry No. **1a**, 81-25-4; **1b**, 547-75-1; **1c**, 474-25-9; **1d**, 128-13-2; **1e**, 83-44-3; **1f**, 475-31-0; **1g**, 53608-86-9; **1h**, 7170-94-7; **2a**, 2304-89-4; **2b**, 88725-35-3; **2c**, 4185-00-6; **2d**, 77060-26-5; **2e**, 4185-01-7; **2f**, 2304-89-4; **2g**, 111239-24-8; **2h**, 111239-25-9; **3a**, 3338-16-7; **3b**, 111320-73-1; **3c**, 566-24-5; **3d**, 78919-26-3; **3e**, 570-63-8; **3f**, 107589-98-0; **3g**, 111239-26-0; **3h**, 111239-27-1; 3α-hydroxysteroid dehydrogenase, 9028-56-2; 3β-hydroxysteroid dehydrogenase, 9015-81-0; ethyl acetate, 141-78-6; ethanol, 64-17-5.

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