Preparative-Scale Regio- and Stereospecific Oxidoreduction of Cholic Acid and Dehydrocholic Acid Catalyzed by Hydroxysteroid Dehydrogenases

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NAD(P)-dependent hydroxysteroid dehydrogenases were used as catalysts for the oxidoreduction of the hydroxyl-keto groups of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and dehydrocholic acid (3,7,12-trioxo-5β-cholan-24-oic acid). Cholic acid was regiospecifically oxidized, on a preparative scale, at each of the three possible positions, and dehydrocholic acid regio- and stereospecifically reduced at each of the three positions. The compounds were quantitatively transformed and the products were 97–99% pure. The assignment of product structure was made by NMR. The nicotinamide cofactors were enzymatically regenerated, in situ, with the α-ketoglutarate/glutamate dehydrogenase, formate/formate dehydrogenase or glucose/glucose dehydrogenase systems. The enzymes were employed in the free form or immobilized on Sepharose CL-4B.

The NAD(P)-dependent oxidoreductases[1] have been attracting increasing interest from organic chemists because of their high specificity as catalysts for the oxidoreduction of a variety of hydroxyl and carbonyl compounds.[2-13] For use in syntheses, the oxidoreductases can be roughly divided into two groups. The enzymes of the first group, which includes, for instance, horse liver alcohol dehydrogenase and some oxidoreductases of bakers' yeast, have good stereospecificity but accept substrates with remarkable structural differences.[5-8] The enzymes of the second group, mainly obtained from microbial sources, accept only substrates with limited structural differences.[9-13] Among the enzymes of the second group are the hydroxysteroid dehydrogenases which catalyze the reversible oxidoreduction of the hydroxyl-keto groups of steroids. Because of their high regio- and stereospecificity, these oxidoreductases can be used for the synthesis of neutral steroids[11] and bile acids[12,13] and also for the analysis.[14-17]

We have systematically investigated the usefulness of the hydroxysteroid dehydrogenases for bile acid synthesis, with cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and dehydrocholic acid (3,7,12-trioxo-5β-cholan-24-oic acid) as model substrates. Cholic acid has been regiospecifically oxidized, on a preparative scale, at each of the three possible positions (Scheme I) and dehydrocholic acid regio- and stereospecifically reduced at each of the three positions (Scheme II). The bile acid derivatives are of interest for use as standards, enzyme substrates, and potential metabolites.[18-23] The nicotinamide cofactors were enzymatically regenerated, in situ, with α-ketoglutarate/glutamate dehydrogenase (to regenerate NAD and NADP), formate/formate dehydrogenase (NADH), or glucose/glucose dehydrogenase (NADH and NADPH). The enzymes were employed in the free form or immobilized on Sepharose CL-4B.

Results and Discussion

The enzymatic approach made it possible to oxidize or reduce with very high regio- and stereospecificity the hydroxyl or keto groups of cholic acid and dehydrocholic acid. The substrates were quantitatively transformed into the products in a single step.

The purity of the prepared bile acids was demonstrated by chromatographic analyses and NMR. HPLC, whose high-resolution capacity is well-known, showed the presence of only one compound in each of the crystallized products. In the crude materials, the amount of steroid byproducts was also less than 1%, except for 7β-hydroxy-3,12-dioxo-5β-cholan-24-oic acid (**9**), which contained about 3% of the 7α-epimer **8**, ascribable to contamination with 7α-hydroxysteroid dehydrogenase activity in the 7β-hydroxysteroid dehydrogenase.

NMR spectra confirmed the purity of the products and also served for the assignment of structure. As expected, the chemical shifts of the methyl singlets at C-18 and C-19 and of the methyl doublet at C-21 of the side chain were markedly influenced by the introduction of oxo groups in the steroid skeleton (for data see the Experimental Section).

The data in Table I show the chemical shifts and the splitting patterns of the signals of the protons geminal to the hydroxyl groups in the various compounds. Comparison of the data of products **2**, **3**, and **4** with those of their precursor **1** shows that each product selectively lacks one proton signal—3β, 7β or 12β, respectively—and this proves the regiospecificity of the oxidation reactions. Similarly, the data of products **6–10** demonstrate the regiospecificity of the reduction reactions. Furthermore, the

(1) Abbreviations: NAD, β-nicotinamide adenine dinucleotide; NADP, β-nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; DTT, dithiothreitol.

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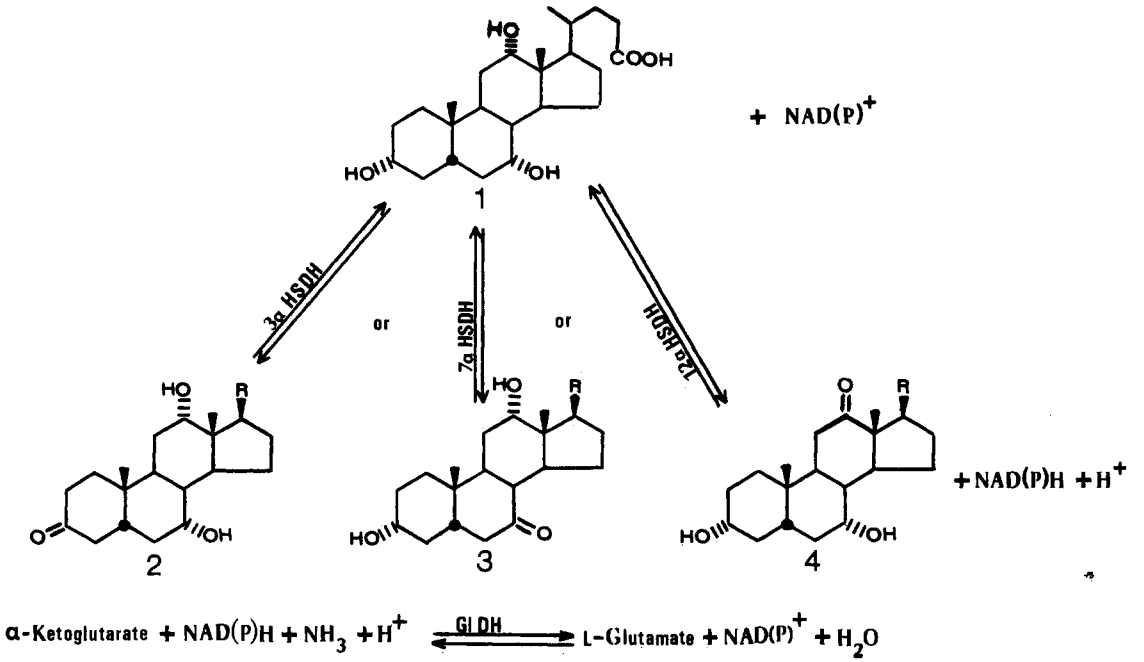
Scheme I. Enzymatic Oxidation of Cholic Acid (Compound 1)[a]



[a] NAD was used with 3α-hydroxysteroid dehydrogenase (3α- HSDH) and 7α-hydroxysteroid dehydrogenase (7α-HSDH) and NADP with 12α-hydroxysteroid dehydrogenase (12α-HSDH). Coenzyme regeneration was carried out with glutamate dehydrogenase (GlDH).

Table I. ^{1}H NMR Data of Protons Geminal to OH at C-3, C-7, and C-12 (in Me_2SO)

		chemical shift, δ (J, Hz)				
	5β-cholan-24-oic acid	$3\beta_a$	$3\alpha_e$	$7\beta_e$	$7\alpha_a$	$12\beta_e$
(1)	3α,7α,12α-trihydroxy	3.18, sept (10.7, 4.6)		3.61, q (3.0)		3.78, t (2.8)
(2)	7α,12α-dihydroxy-3-oxo			3.68, q (3.0)		3.82, t (2.8)
(3)	3α,12α-dihydroxy-7-oxo	3.34, sept (10.7, 4.6)				3.80, t (2.8)
(4)	3α,7α-dihydroxy-12-oxo	3.18, sept (10.7, 4.6)		3.71, q (3.0)		
(5)	3,7,12-trioxo					
(6)	3α-hydroxy-7,12-dioxo	3.33, sept (10.7, 4.6)				
(7)	3β-hydroxy-7,12-dioxo		3.79, t (3.0)			
(8)	7α-hydroxy-3,12-dioxo			3.78, q (3.3)		
(9)	7β-hydroxy-3,12-dioxo				3.40, dt (10.6, 4.5)	
(10)	12α-hydroxy-3,7-dioxo					3.83, t (3.2)

chemical shifts and the splitting patterns of the signals (easily explainable from perspective models) also prove the stereochemistry of the formed hydroxyl groups.

In the enzymatic process, the coupling of a second enzymatic reaction gave two advantages. First, the costly coenzymes were regenerated at the expense of much cheaper compounds (α-ketoglutarate, formate, glucose) that acted as ultimate reducing–oxidizing agents. This enabled us to use catalytic amounts of coenzymes which were, however, at concentrations high enough to ensure almost maximum enzymatic rates. Second, the transformation of the substrates was complete, due to the high value of the overall equilibrium constant for the coupled reactions.[24]

Immobilization onto insoluble matrices stabilized the enzymes and also made it possible to recover them at the end of the process. The enzymes therefore were usable for repeated transformations, thus decreasing the incidence of the enzyme cost on the synthesis. However, if economical reasons can be discarded, the free enzymes are perfectly suitable and easier to handle, particularly for people not skilled in enzyme technology. Free enzymes are also preferable for occasional use.

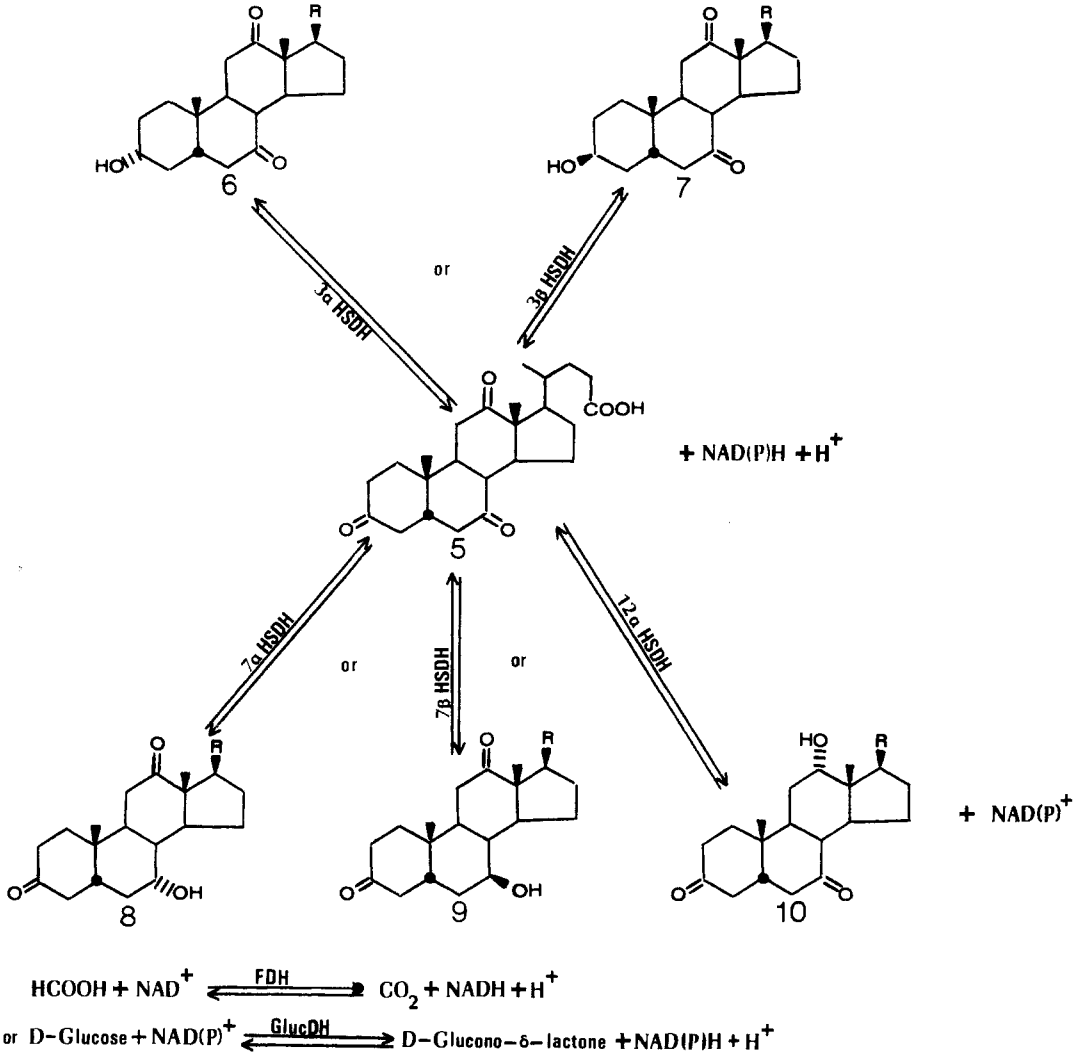
The products were simply isolated from the reaction medium by acid precipitation, and the yields were between 78% and 91%. The enzymes employed for coenzyme regeneration and the majority of the hydroxysteroid dehydrogenases are commercially available. 12α-Hydroxysteroid dehydrogenase was extracted from the bacterial cells and used without purification, whereas 7β-hydroxysteroid dehydrogenase had to be partially purified.

The products were synthesized more simply and in higher yields with the enzymatic method than with the chemical method, which requires several steps and gives rise to contaminating byproducts (see notes 32–38 in the Experimental Section). The present method, based on the use of specific isolated enzymes, also gave better results than those obtained by Sawada et al. who prepared compound 4 using immobilized living cells.[25] It should be

(24) The overall equilibrium constant of the oxidation of cholic acid coupled to the reduction of α-ketoglutarate is higher than 10^6 (lit.[12,14] and: Smith, E. L.; Austen, B. M.; Blumenthal, K. M.; Nyc, J. F. In *The Enzmes*; Boyer, P. D., Ed.; Academic: New York, 1974; p 425). The overall equilibrium constant of the reduction of dehydrocholic acid coupled to the oxidation of formate or glucose is higher than 10^{15} (lit.[2,8,10,14]).

(25) Sawada, H.; Kinoshita, S.; Yoshida, T.; Taguchi, H. *J. Ferment. Technol.* 1981, 59, 111. The authors transformed dehydrocholic acid to 12-ketochenodeoxycholic acid (compound 4) with an 80% yield using immobilized living cells of *Brevibacterium fuscum*. The product was contaminated with untransformed substrate, with an intermediate and with cholic acid (10%). The bile acid concentration in the reaction medium was much lower (45 times) than that used by us in the preparation of compound 4.

Scheme II. Enzymatic Reduction of Dehydrocholic Acid (Compound 5)[a]



[a] NADH was used with 3α-hydroxysteroid dehydrogenase (3α-HSDH), 3β-hydroxysteroid dehydrogenase (3β-HSDH) and 7α-hydroxysteroid dehydrogenase (7α-HSDH), and NADP with 7β-hydroxysteroid dehydrogenase (7β-HSDH) and 12α-hydroxysteroid dehydrogenase (12α-HSDH). Coenzyme regeneration was carried out with formate dehydrogenase (FDH) in the case of products 6 and 7, or with glucose dehydrogenase (GlucDH) in the case of products 8, 9 and 10.

mentioned that 7β-hydroxy-3,12-dioxo-5β-cholan-24-oic acid is a compound previously undescribed in the literature.

The preparations, which can be easily scaled up, are suitable for laboratory-scale synthesis of standards, enzyme substrates, potential metabolites, and pharmaceuticals. With the available hydroxysteroid dehydrogenases[26] a great variety of free or conjugated bile acid derivatives can be synthesized, and work is in progress to carry out α/β inversions and to assess the usefulness of these enzymes for the reversible "protection" of hydroxyl–keto groups during the chemical synthesis of steroids.

Experimental Section

Materials. 3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50, 15 U mg^{-1} of protein), 7α-hydroxysteroid dehydrogenase (EC 1.1.1.159, 7 U mg^{-1} of protein), 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51, 26 U mg^{-1} of protein), glutamate dehydrogenase from *Proteus* species (EC 1.4.1.4, 300 U mg^{-1} of protein), and glucose dehydrogenase (EC 1.1.1.47, 200 U mg^{-1} of protein) were obtained from Sigma. 12α-Hydroxysteroid dehydrogenase (EC 1.1.1.176, 2.4 U mg^{-1} of protein) was extracted from *Clostridium* group P as described by Macdonald et al.[27] 7β-Hydroxysteroid dehydrogenase (1.5 U mg^{-1} of protein) was extracted from *Clostridium absonum* and partially purified by using Procion Red affinity chromatography.[28,29] Glutamate dehydrogenase from beef liver (EC 1.4.1.3, 120 U mg^{-1} of protein) and formate dehydrogenase (EC 1.2.1.2, 3 U mg^{-1} of protein), NAD, NADP, NADH, NADPH, and DTT were purchased from Boehringer. Sepharose CL-4B was from Pharmacia and 2,2,2-trifluoroethanesulfonyl chloride from Fluka. All other reagents and compounds were of analytical grade.

General Methods. Melting points are uncorrected and were determined in open-ended capillaries. Optical rotations were measured in a Perkin-Elmer 141 polarimeter, in methanol. ^1H NMR spectra were recorded on a Brucker 270 (270 MHz) instrument in deuterated dimethyl sulfoxide with Me_4Si as internal standard. Mass spectra were recorded on a VG Analytical 70-70 EQ-HF, high-resolution spectrometer equipped with its own standard FAB source. The fast bombardment gun was operated with a 6.5-kV xenon beam. The sample was dissolved in glycerin as matrix. HPTLC was developed on precoated silica gel 60 F_{254} plates (Merck) with chloroform–methanol–acetic acid (20:2:1,

(26) The presence of 12β-hydroxysteroid dehydrogenase activity in *Clostridium paraputrificum* has been reported recently (Edenharder, R.; Schneider, J. *Appl. Environ. Microbiol.* **1985**, *49*, 964).

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system 1) or chloroform–methanol (40:5, system 2). Compounds were detected with Komarowsky's reagent.[30] 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 i.d.). A 60-min Concave 2 gradient from 10 mM sodium phosphate, pH 3, 30% acetonitrile, to 10 mM sodium phosphate, pH 3, 60% acetonitrile, 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).

Enzyme Assays. Assays were carried out in 3-mL cuvettes, with spectrophotometric monitoring at 340 nm of the formation or consumption of NAD(P)H. The conditions for the commercial enzymes (3α-hydroxysteroid dehydrogenase, 7α-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase, glutamate dehydrogenase, glucose dehydrogenase, and formate dehydrogenase) were those indicated by the manufacturers. The activity of 12α-hydroxysteroid dehydrogenase was measured in 0.1 M potassium phosphate buffer, pH 8, containing 0.5 mM NADP and 1 mM cholic acid, and the activity of 7β-hydroxysteroid dehydrogenase in 0.2 M glycine–NaOH buffer, pH 9.5, containing 0.5 mM NADP and 1 mM 3α,7β-dihydroxy-5β-cholan-24-oic acid. For the immobilized enzymes, the gel was suspended in the test solution with continuous magnetic stirring.

Enzyme Immobilization. Sepharose CL-4B was activated following the method described by Nilsson and Mosbach,[31] using 0.22 mmol of 2,2,2-trifluoroethanesulfonyl chloride per milliliter of settled gel. The enzymes were then immobilized as follows: (a) Coimmobilization of 3α-hydroxysteroid dehydrogenase and glutamate dehydrogenase from beef liver was carried out as described below. The 3α-hydroxysteroid dehydrogenase (240 U) and glutamate dehydrogenase (360 U) were coupled to the activated matrix (24 mL of settled gel) in 0.1 M potassium phosphate buffer, pH 8, containing 0.5 mM NAD, with gentle stirring of the coupling mixture (40 mL) at 4 °C overnight. The unreacted groups on the matrix were blocked by treating the gel with 0.1 M ethanolamine, pH 8, for 3 h. Then the supernatant was withdrawn, and the immobilized enzymes were thoroughly washed with 0.1 M potassium phosphate buffer, pH 7, before assaying for enzymatic activity. The yields of immobilization were 55% for 3α-hydroxysteroid dehydrogenase and 53% for glutamate dehydrogenase. (b) The coimmobilization of 3α-hydroxysteroid dehydrogenase (135 U) and formate dehydrogenase (35 U) on activated Sepharose CL-4B (12 mL) was carried out as described above. The yields were 46% for 3α-hydroxysteroid dehydrogenase and 41% for formate dehydrogenase. (c) The coimmobilization of 12α-hydroxysteroid dehydrogenase (39 U) and glutamate dehydrogenase from *Proteus* (155 U, previously dialyzed at 4 °C against 0.1 M potassium phosphate buffer, pH 8) on the activated matrix (15 mL) was carried out similarly, using 0.5 mM NADP instead of NAD in the coupling solution. The yields were 51% for 12α-hydroxysteroid dehydrogenase and 56% for glutamate dehydrogenase. (d) The coimmobilization of 12α-hydroxysteroid dehydrogenase (37 U) and glucose dehydrogenase (40 U) on the activated matrix (15 mL) was carried out as above. The yields were 49% for 12α-hydroxysteroid dehydrogenase and 30% for glucose dehydrogenase.

Synthesis of 7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic Acid (2).[32] A solution (200 mL) containing 0.05 M potassium phosphate, 0.1 M ammonium acetate, 1 mM adenosine diphosphate, 1 mM DTT, 0.05 M α-ketoglutarate, 0.1 mM (13 mg) NAD, 14 mM (1.2 g) sodium cholate, and 110 U 3α-hydroxysteroid dehydrogenase coimmobilized with 158 U glutamate dehydrogenase was titrated to pH 8.5 and gently stirred at room temperature. The course of the oxidation was monitored by TLC and HPLC. After 36 h the reaction was complete. The turnover number (moles of product generated per mole of NAD) for the coenzyme was 140. The supernatant was withdrawn, and the immobilized enzymes were washed with water (2 × 30 ml). The immobilized enzymes were reused and the residual activities of 3α-hydroxysteroid dehydrogenase and glutamate dehydrogenase were 91% and 85%, after three consecutive runs. To recover the product, the pooled supernatant and washings were acidified to pH 3 with 6 M HCl and kept at 2 °C for 3 h. The precipitated solid was collected by filtration, washed with cold water, and dried. The yield was 0.88 g (78% of theoretical). The product was crystallized from ethyl acetate: mp 179–182 °C (lit.[23] mp 180–181 °C, from ethyl acetate); R_f 0.55 (system 1); HPLC t_r 51 min; $[\alpha]_D$ +41°; ^1H NMR δ 0.62 (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).

Synthesis of 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic Acid (3).[33] A solution (200 mL) containing 0.05 M potassium phosphate, 0.1 M ammonium acetate, 1 mM adenosine diphosphate, 1 mM DTT, 0.05 M α-ketoglutarate, 0.15 mM (20 mg) NAD, 28 mM (2.4 g) sodium cholate, 48 U 7α-hydroxysteroid dehydrogenase, and 300 U glutamate dehydrogenase from beef liver was titrated to pH 8.5. The course of the reaction was monitored by TLC and HPLC. After 20 h the reaction was complete. The turnover number for the coenzyme was 187. 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, filtered, rotaevaporated, and dried. The yield was 1.86 g (82%). The product was crystallized from ethyl acetate: mp 194–195 °C (lit.[18] mp 199–200 °C, from ethyl acetate); R_f 0.47 (system 1); HPLC t_r 33 min; $[\alpha]_D$ −6°; ^1H NMR δ 0.59 (3 H, s, C-18 Me), 1.11 (3 H, s, C-19 Me), 0.92 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 3α,7α-Dihydroxy-12-oxo-5β-cholan-24-oic Acid (4).[34] The method was a slight modification of a previously published procedure.[13] A solution (50 mL) containing 0.1 M potassium phosphate, 0.1 M ammonium acetate, 1 mM DTT, 3 mM NaN_3; 0.1 M α-ketoglutarate, 0.1 mM (4 mg) NADP, 56 mM (1.2 g) sodium cholate, and 16 U 12α-hydroxysteroid dehydrogenase coimmobilized with 70 U glutamate dehydrogenase was titrated to pH 8 and gently stirred at room temperature. The reaction, monitored by TLC and HPLC, was complete after 48 h. The turnover number for the coenzyme was 560. The product was recovered in the same way as product **2**, and the yield was 0.97 g (85%). The residual activities of 12α-hydroxysteroid dehydrogenase and glutamate dehydrogenase were about 75% after nine consecutive runs. The product was crystallized from ethyl acetate: mp 222–224 °C; R_f 0.46 (system 1); HPLC t_r 39 min; $[\alpha]_D$ +78°; ^1H NMR δ 0.96 (3 H, s, C-18 Me), 0.94 (3 H, s, C-19 Me), 0.76 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 3α-Hydroxy-7,12-dioxo-5β-cholan-24-oic Acid (6).[35] A solution (100 mL) containing 0.05 M potassium phosphate, 10% ethanol, 0.1 M formate, 1 mM DTT, 0.1 mM (7 mg) NADH, 7 mM (0.3 g) sodium dehydrocholate, and 52 U 3α-hydroxysteroid dehydrogenase coimmobilized with 12 U formate dehydrogenase was titrated to pH 6.8 and gently stirred at room temperature. After 5 h, sodium dehydrocholate (0.3 g in 4 mL of H_2O) was added again. The reaction, monitored by TLC and HPLC, was complete after 12 h. The turnover number for the coenzyme was 140. The product was recovered in the same way as product **2**, and the yield was 0.51 g (89%). The residual activities of 3α-hydroxysteroid dehydrogenase and formate dehydrogenase were 87% and 93%, respectively, after two consecutive runs. The product was crystallized from ethyl acetate: mp 190–191 °C; R_f 0.49 (system 2); HPLC t_r 21 min; $[\alpha]_D$ +32°; ^1H NMR δ 0.98 (3 H, s, C-18 Me), 1.24 (3 H, s, C-19 Me), 0.74 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 3β-Hydroxy-7,12-dioxo-5β-cholan-24-oic Acid (7).[36] Sodium dehydrocholate (0.6 g) was reduced in the same

(30) Macdonald, I. A. *J. Chromatogr.* **1977**, *136*, 348.
(31) Nilsson, K.; Mosbach, K. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 449.
(32) Compound **2** was previously prepared from cholic acid by a five-step (lit.[19]) or four-step (lit.[23]) chemical sysnthesis with a 40% or 75% yield.
(33) Compound **3** was previously prepared from cholic acid by different methods (lit.[18,20,21] and: Haslewood, G. A. R. *Biochem. J.* **1944**, *38*, 108. Heusser, H.; Wuthier, H. *Helv. Chim. Acta* **1947**, *30*, 2165) which had several shortcomings and gave the pure product in poor yields. For instance, the most recent of these methods (lit.[21]) involved a four-step synthesis that gave an impure product which had to be purified by column chromatography.
(34) Compound **4** was previously prepared from cholic acid by a four-step synthesis with an approximate 40% yield (lit.[20] and: Hofmann, A. F. *Acta Chem. Scand.* **1963**, *17*, 173).
(35) Compound **6** was previously prepared by Fieser and Rajagopalan (lit.[20]) from cholic acid by a four-step synthesis. The yield was not given.

way as product **6**, by using 17 U 3β-hydroxysteroid dehydrogenase and 20 U formate dehydrogenase. The reaction was complete after 25 h. The turnover number for the coenzyme was 140. The product was recovered as was product **3**, and the yield was 0.49 g (86%). The product was crystallized from ethyl acetate–acetone: mp 270–272 °C; R_f 0.51 (system 2); HPLC t_r 18 min; $[\alpha]_D$ +22°; ^{1}H NMR δ 0.98 (3 H, s, C-18 Me), 1.26 (3 H, s, C-19 Me), 0.74 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 7α-Hydroxy-3,12-dioxo-5β-cholan-24-oic Acid (8).[37] Sodium dehydrocholate (0.7 g) was reduced in the same way as product **6**, by using 0.1 M glucose instead of 0.1 M formate, 5 U 7α-hydroxysteroid dehydrogenase, and 10 U glucose dehydrogenase. The reaction was complete after 9 h. The turnover number for the coenzyme was 160. The product was recovered in the same way as product **3**, and the yield was 0.61 g (91%). The product was crystallized from ethyl acetate: mp 193–195 °C; R_f 0.48 (system 2); HPLC t_r 36 min; $[\alpha]_D$ +73°; ^{1}H NMR δ 1.00 (3 H, s, C-18 Me), 1.05 (3 H, s, C-19 Me), 0.77 (3 H, d, J = 6.3 Hz, C-21 Me).

Synthesis of 7β-Hydroxy-3,12-dioxo-5β-cholan-24-oic Acid (9). Sodium dehydrocholate (0.6 g) was reduced in the same way as product **8**, by using NADPH instead of NADH, 8 U 7β-hydroxysteroid dehydrogenase, and 15 U glucose dehydrogenase. The reaction was complete after 30 h. The turnover number for the coenzyme was 140. The product was recovered in the same way as product **3**, and the yield was 0.49 g (86%). The product was crystallized from ethyl acetate: mp 187–189°C; R_f 0.47 (system 2); HPLC t_r 14 min; $[\alpha]_D$ +90°; ^{1}H NMR δ 1.01 (3 H, s, C-18 Me), 1.05 (3 H, s, C-19 Me), 0.77 (3 H, d, J = 6.3 Hz, C-21 Me); mass spectrum (positive FAB), m/z (relative intensity) 405 ($(M + H)^+$, 100), 387 (98), 369 (30), 353 (14), 351 (17), 341 (21), 323 (12), 263 (12), 219 (9), 145 (18), 121 (23), 105 (35). Anal. Calcd for $C_{24}H_{36}O_5$: C, 71.26; H, 8.97. Found: C, 70.97; H, 8.78.

Synthesis of 12α-Hydroxy-3,7-dioxo-5β-cholan-24-oic Acid (10).[38] Sodium dehydrocholate (0.6 g) was reduced in the same way as product **9**, by using 12 U 12α-hydroxysteroid dehydrogenase coimmobilized with 8 U glucose dehydrogenase. The reaction was complete after 26 h. The turnover number for the coenzyme was 140. The product was recovered as was product **2**, and the yield was 0.50 g (87%). The product was crystallized from ethyl acetate: mp 168–170 °C (lit.[18] mp 168–169 °C, from ethyl acetate); R_f 0.44 (system 2); HPLC t_r 31 min; $[\alpha]_D$ −9°; ^{1}H NMR δ 0.63 (3 H, s, C-18 Me), 1.22 (3 H, s, C-19 Me), 0.93 (3 H, d, J = 6.3 Hz, C-21 Me).

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Registry No. **1**, 81-25-4; **2**, 2304-89-4; **3**, 911-40-0; **4**, 2458-08-4; **5**, 81-23-2; **6**, 517-33-9; **7**, 3615-35-8; **8**, 2304-91-8; **9**, 102649-81-0; **10**, 2304-92-9; EC 1.1.1.50, 9028-56-2; EC 1.1.1.159, 39361-64-3; EC 1.1.1.51, 9015-81-0; EC 1.4.1.4, 9029-11-2; EC 1.1.1.47, 9028-53-9; EC 1.1.1.176, 61642-40-8; EC 1.4.1.3, 9029-12-3; EC 1.2.1.2, 9028-85-7.

(36) Compound **7** was previously prepared from cholic acid by a five-step synthesis, which also involved a chromatographic purification of an intermediate, with a yield lower than 30% (lit.[21] and: Chang, F. C. *J. Org. Chem.* **1979**, *44*, 4567. Blickenstaff, R. T.; Atkinson, K.; Breaux, D.; Foster, E.; Kim, Y.; Wolf, G. C. *J. Org. Chem.* **1971**, *36*, 1271).

(37) Compound **8** was previously prepared from cholic acid by a four-step synthesis (lit.[20] and: Nicotra, F.; Ranzi, B. M. *Ann. Microbiol.* **1978**, *28*, 11). The yield was not given.

(38) Compound **10** was previously prepared from cholic acid by a six-step synthesis (lit.[18]). The yield was not given.

Roritoxins, New Macrocyclic Trichothecenes from *Myrothecium roridum*

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Four new macrocyclic trichothecenes (roritoxins A–D) which contain a tetrahydropyranyl ring fused to a γ-lactone or lactol ring in the macrolide side chain were isolated from a culture of *Myrothecium roridum* ATCC 20605 grown on rice substrate. Roritoxin C is unique in that it is the first reported fungal-produced trichothecene which possesses a 9,10-epoxide group. In liquid media this fungal culture produces a new antibiotic, (+)-(1*R*,6*R*)-5-(*S*)-hydroxy-4-methyl-7-oxabicyclo[4.1.0]hept-3-en-2-one.

During the past few years we have been involved in screening *Myrothecium verrucaria* and *M. roridum* isolates for the production of trichothecenes, which are a family of sesquiterpenes important as mycotoxins[1] and anticancer agents.[2]

The research group at Warner-Lambert has reported on an interesting isolate of *M. roridum*, CL-514 (ATCC 20605), which in a liquid medium produced a novel array of trichoverroids (roridin L-2[3] and trichoverritone[4]) as well as two unusual macrocyclic trichothecenes, 12′-hydroxyisoverrucarin J and isosatratoxin H.[5] We have studied a variety of fermentation conditions for this isolate and now report the isolation of a series of new antibiotics whose production depends strongly on the type of media used to grow this culture.

Results and Discussion

Initially, when *M. roridum* isolate CL-514 was grown in a sucrose/glycerol based liquid medium, conditions we

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