Efficient Preparative-scale Enzymatic Synthesis of Specifically Deuteriated Bile Acids

Sergio Riva, Gianluca Ottolina, and Giacomo Carrea*
Istituto di chimica degli Ormoni, C.N.R. Via Mario Bianco 9, 20131 Milano, Italy
Bruno Danieli
Dipartimento di Chimica Organica e Industriale, Centro C.N.R. di Studio delle Sostanze Organiche Naturali, Via Venezian 21, 20133 Milano, Italy

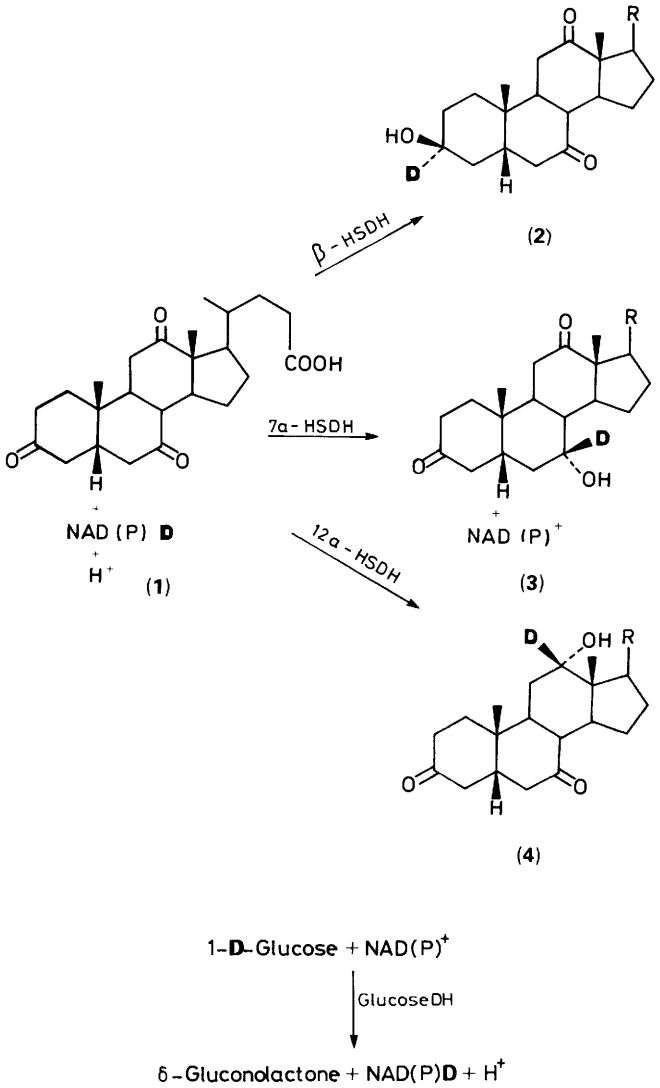
The regio- and stereo-specific syntheses of [3α-^{2}H]-3β-hydroxy-7,12-dioxo-5β-cholan-24-oic acid, [7β-^{2}H]-7α-hydroxy-3,12-dioxo-5β-cholan-24-oic acid, and [12β-^{2}H]-12α-hydroxy-3,7-dioxo-5β-cholan-24-oic acid have been carried out on preparative scale by coupling the reduction of 3,7,12-trioxo-5β-cholan-24-oic acid, catalyzed by 3β,17β-, 7α-or 12α-hydroxysteroid dehydrogenase, to the oxidation of [1-^{2}H]glucose, catalyzed by glucose dehydrogenase. The transfer of deuterium from glucose to bile acid was mediated by catalytic amounts of coenzyme continuously recycled *in situ*. The isotopic purity of deuteriated bile acids, determined by ^{1}H n.m.r. spectrometry, was ≥94%.

Deuteriated compounds can be synthesized enzymatically on a small scale (0.1 mmol) with procedures involving the stoicheiometric consumption of labelled NAD(P)H (see review article in ref. 1). These procedures, of course, are not economically convenient, especially for large-scale preparations. To overcome this limitation, Wong and Whitesides[2] developed a coupled-enzymes system in which the coenzyme is regenerated *in situ* by formate dehydrogenase and deuteriated formic acid as the ^{2}H source. With this method gram-quantities of labelled compounds can be prepared. The system, however, is only usable with NAD-dependent enzymes, since formate dehydrogenase does not accept NADP. Here we report the preparative-scale enzymatic synthesis of deuteriated bile acids by a coupled-enzymes system suitable for both NAD- and NADP-dependent enzymes. The system consisted of specific hydroxysteroid dehydrogenases, that, as previously reported,[3–5] catalyze the regio- and stereo-specific oxidoreduction of the hydroxy-keto groups of a variety of steroids, and glucose dehydrogenase, that regenerates the coenzymes.

Results and Discussion

The synthesis of [3α-^{2}H]-3β-hydroxy-7,12-dioxo-5β-cholan-24-oic acid (**2**), [7β-^{2}H]-7α-hydroxy-3,12-dioxo-5β-cholan-24-oic acid (**3**), and [12β-^{2}H]-12α-hydroxy-3,7-dioxo-5β-cholan-24-oic acid (**4**) were carried out by coupling the reduction of 3,7,12-trioxo-5β-cholan-24-oic acid (**1**) catalyzed by specific hydroxysteroid dehydrogenases to the oxidation of [1-^{2}H]glucose catalyzed by glucose dehydrogenase (Scheme). The transfer of deuterium from glucose to bile acid was mediated by catalytic amounts of coenzyme continuously recycled *in situ*.

The coenzyme regenerating system was suitable for both NAD-dependent (3β,17β-and 7α-hydroxysteroid dehydrogenase) and NADP-dependent (12α-hydroxysteroid dehydrogenase) enzymes, because glucose dehydrogenase works with both NAD and NADP. The turnover number (mol of product generated per mol of coenzyme) was *ca.* 125 and the bile acid conversion was quantitative because of the high overall equilibrium constant of the coupled reactions.[3] It should be noted that 3β,17β-,[6] 7α-[7] 12α-hydroxysteroid dehydrogenase[7] and glucose dehydrogenase[6] are all B-stereospecific, so that high turnover numbers are not strictly necessary to obtain high isotopic purity, as they would be with enzymes of opposite stereospecificity.



Scheme Synthesis of regio- and stereo-specifically deuteriated bile acids with 3β,17β-, 7α- and 12α-hydroxysteroid dehydrogenase (β-, 7α- and 12α-HSDH).

As already found for the non-deuteriated bile acid analogues,[3] the reactions were completely regio- and stereo-specific furnishing (**2**), (**3**), and (**4**) with a purity ≥98%. The isotopic purity (≥94%) was determined by ^{1}H n.m.r. spectrometry, through comparison of integrated areas of residuals 3α-H, 7β-H, and 12β-H, respectively, with the area of the C-21 methyl group.

Besides commercial [1-^{2}H]glucose, which is rather expensive, we have also used deuteriated glucose, easily prepared by sodium borodeuteride-mediated reduction of δ-gluconolactone.[8] This compound, employed without any purification, gave deuteriated bile acids with the same purity as the ones obtained with commercial glucose.

In conclusion, the use of the [1-^{2}H]glucose/glucose dehydrogenase system with catalytic amounts of non-labelled coenzymes made it possible to prepare 0.5—1 mmol quantities of deuteriated bile acids in high yield and isotopic purity, suitable for metabolic studies. The [1-^{2}H]glucose/glucose dehydrogenase system is, in principle, applicable to any preparative-scale deuteriation involving NAD(P)-dependent enzymes.

Experimental

Glucose dehydrogenase (EC 1.1.1.47, 200 U mg^{-1} protein), 7α-hydroxysteroid dehydrogenase from *E. coli* (EC 1.1.1.159, 7 U mg^{-1} of protein), 3β,17β-hydroxysteroid dehydrogenase (EC 1.1.1.51, 26 U mg^{-1} of protein), and [1-^{2}H]glucose (98% ^{2}H) 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.*[9] All other reagents and compounds were of analytical grade. ^{1}H N.m.r. spectra were obtained on a Brucker 270 (270 MHz) instrument in deuteriated dimethyl sulphoxide with Me_4Si as internal standard. H.p.l.c. and t.l.c. analyses were carried out as already described.[3]

Synthesis of Deuteriated Bile Acids.—(a) [3α-^{2}H]-3β-*Hydroxy*-7,12-*dioxo*-5β-*cholan*-24-*oic acid* (**2**). A solution (100 ml) containing 0.05M potassium phosphate, 10% ethanol, 1mM DTT, 25 mM deuteriated glucose, 7.5 mM (320 mg) of the sodium salt of (**1**), 0.06mM NAD, 50 U of glucose dehydrogenase, and 100 U of 3β,17β-hydroxysteroid dehydrogenase was titrated to pH 6.7 and left to react at room temperature. The course of the reaction was monitored by t.l.c. and h.p.l.c. After *ca.* 8 h the reduction of (**1**) was complete. The solution was acidified to pH 4.5 with HCl and extracted with ethyl acetate (3 × 50 ml). The combined organic phases were dried (Na_2SO_4), evaporated under reduced pressure, and dessicated (86% yield). Chromatographic (h.p.l.c. and t.l.c.) and physical (m.p., $[\alpha]_D$) data were identical with those of the non-deuteriated analogue:[3] δ[270 MHz; $(CD_3)_2SO$]; 3.79 [1H, t, residual 3α-H (6%)], 1.26 (3 H, s, 19-CH_3), 0.98 (3 H, s, 18-CH_3), and 0.74 (3 H, d, 21-CH_3).

(b) [7β-^{2}H]-7α-*Hydroxy*-3,12-*dioxo*-5β-*cholan*-24-*oic acid* (**3**). The compound was prepared in the same way as (**2**) with 7α-hydroxysteroid dehydrogenase (100 U) instead of 3β,17β-hydroxysteroid dehydrogenase. The reaction was complete after 9 h and the yield was 84%. Chromatographic and physical data were identical to those of the non-deuteriated analogue:[3] δ[270 MHz; $(CD_3)_2SO$] 3.78 [1 H, q, residual 7β-H (5%)], 1.05 (3 H, s, 19-CH_3), 1.00 (3 H, s, 18-CH_3), 0.77 (3 H, d, 21-CH_3).

(c) [12β-^{2}H]-12α-*Hydroxy*-3,7-*dioxo*-5β-*cholan*-24-*oic acid* (**4**). The compound was prepared in a similar way to compound (**2**) with 12α-hydroxysteroid dehydrogenase (60 U) instead of 3β,17β-hydroxysteroid dehydrogenase and NADP (0.04 mM) instead of NAD. The reaction was complete after 6 h, with an 88% yield. Chromatographic and physical data were identical with those of the non-deuteriated analogue.[3] δ[270 MHz, $(CD_3)_2SO$] 3.83 (1 H, t, residual 12β-H (<3%)], 1.22 (3 H, s, 19-CH_3), 0.93 (3 H, d, 21-CH_3), and 0.63 (3 H, s, 18-CH_3).

Acknowledgements

We thank the Biotechnology Action Programme of the Commission of the European Communities for financial support of this work.

References

1 J. B. Jones, *Tetrahedron*, 1986, **42**, 3351.
2 C. H. Wong and G. M. Whitesides, *J. Am. Chem. Soc.*, 1983, **105**, 5012.
3 S. Riva, R. Bovara, P. Pasta, and G. Carrea, *J. Org. Chem.*, 1986, **51**, 2902.
4 G. Carrea and P. Cremonesi, *Methods Enzymol.*, 1987, **136**, 150.
5 S. Riva, R. Bovara, L. Zetta, P. Pasta, G. Ottolina, and G. Carrea, *J. Org. Chem.*, 1988, **53**, 88.
6 K.-S. You, *Methods Enzymol.*, 1982, **87**, 101.
7 G. Ottolina, S. Riva, G. Carrea, B. Danieli, and A. F. Buckmann, 1989, *Biochim. Biophys. Acta*, in the press.
8 M. L. Wolfrom and H. B. Wood, *J. Org. Chem.*, 1951, **73**, 2933.
9 I. A. Macdonald, J. F. Jellet, and D. E. Mahony, *J. Lipid Res.*, 1979, **20**, 234.

Received 20*th April* 1989; *Paper* 9/01682A