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Note

Synthesis of 7α-thiohydroxycholesterol involving the resolution of epimeric thiocholesterols by high-performance liquid chromatography

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Recently, work has been published describing the ability of certain sulphur-containing steroids to irreversibly inhibit specific isoenzymes of cytochrome P450. The compounds in question (spironolactone[1], 19-thioandrost-4-en-3,17-dione, 17β-hydroxy-10β-thioestr-4-en-3-one[2] and 7α-thiotestosterone[3] exert their effect by a process which has been called "suicide" inhibition. This involves the inhibitor occupying the active site of the enzyme and being oxidised by the enzyme's usual mechanism. However, a reactive species is generated which then destroys catalytic activity by binding irreversibly to the active site.

The specificity of "suicide" inhibitors for certain isoenzymes of cytochrome P450 make them valuable probes in the identification and isolation of these haemoproteins. Therefore, as part of our studies in this area, we wished to identify other sulphur-containing compounds falling within this category. Cholesterol 7α-hydroxylase is an important isoenzyme of cytochrome P450 which catalyses the hydroxylation of cholesterol to 7α-hydroxycholesterol, the rate-limiting step in bile acid biosynthesis in many species including man. The putative thiol "suicide" inhibitor of this enzyme would be 7α-thiohydroxycholesterol and therefore a synthetic route to this compound was required. 7-Thiohydroxycholesterol has been synthesised previously[4,5] but the stereochemistry of the product and its isomeric purity were not established. We here describe a synthesis of 7α-thiohydroxycholesterol involving the resolution of epimeric thiocholesterols by high-performance liquid chromatography (HPLC) and unambiguously define the stereochemistry of the reaction product by nuclear magnetic resonance (NMR) spectroscopy.

EXPERIMENTAL

Chemicals

Cholesteryl benzoate was supplied by Sigma (Poole, U.K.). N-Bromosuccinimide, benzoyl peroxide, thiolacetic acid, triethylamine and lithium aluminium hydride were obtained from Aldrich (Gillingham, U.K.). Hexane, benzene, diethyl ether, dichloromethane, ethyl acetate, tetrahydrofuran and acetone were all of AnalaR grade (BDH, Poole, U.K.). Tetrahydrofuran was dried prior to use by refluxing with and distilling from sodium metal under nitrogen.

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Analytical instrumentation

Mass spectra were recorded on a Finnigan-MAT 4500 combined gas chromatograph–quadrupole mass spectrometer system using a direct exposure probe. NMR spectra were recorded at 250 mHz on Varian and Brucker instruments with deuterochloroform as solvent and tetramethylsilane as reference.

Synthesis of 7α-thiohydroxycholesterol

7-Bromocholesteryl benzoate (II). Cholesteryl benzoate (4.90 g, 10 mmol) was dissolved in hexane (100 ml) and the system purged with nitrogen. N-Bromosuccinimide (2.1 g, 12 mmol) was added followed by benzoyl peroxide (50 mg) as a radical initiator. The solution was refluxed for 70 min, then cooled to room temperature and filtered to remove succinimide. After removal of the solvent by rotary evaporation, the crude reaction product was isolated as a yellow microcrystalline solid (5.18 g, 9.1 mmol, 91%).

7-Thioacetoxycholesteryl benzoate (III). To a stirred solution of 7-bromocholesteryl benzoate (2 g, 3.5 mmol) in benzene (30 ml) was added thiolacetic acid (500 μl, 7.0 mmol) followed by triethylamine (975 μl, 7.0 mmol). The solution rapidly became viscous, then gradually more fluid, and changed in colour from yellow to orange. After 4 h, the reaction mixture was diluted with ether (50 ml) and washed with water (2 × 50 ml), saturated sodium bicarbonate solution (2 × 50 ml), water (2 × 50 ml) and brine (50 ml). The organic phase was then dried over anhydrous magnesium sulphate, filtered and solvent removed by rotary evaporation. The solid residue was redissolved in a small volume of methylene chloride and passed through a short column of silica gel. Evaporation of the solvent left the reaction product as an off-white, crystalline solid (1.64 g, 2.9 mmol, 83%). Mass spectrometry (MS) (DCI, ammonia): 582 (100) $M.NH_4^+$, 367 (8)

Resolution of 7α-thioloacetoxycholesteryl benzoate (IIIa) and 7β-thioloacetoxycholesteryl benzoate (IIIb). An HPLC system consisting of an Altex Model 100A reciprocating pump, a Rheodyne Model 7125 six-port injection valve and an Altex Model 155 flow cell coupled to a Hitachi Model 100-10 variable-wavelength detector set at 250 nm was used. The system was equipped with a steel column, 25 cm × 10 mm I.D., packed with silica of 3 μm particle size (Shandon, Runcorn, U.K.) and the mobile phase was hexane–dichloromethane–ethyl acetate (75:25:0.3, v/v/v) at a flow-rate of 1 ml min^{-1}. Under these conditions, the retention times of the 7α and 7β isomers of 7-thioloacetoxycholesteryl benzoate were 11.4 and 13.6 min, respectively. The two compounds were isolated by the collection and combination of various fractions eluted from the HPLC column followed by evaporation of the solvent and the relative yield of the 7α to the 7β epimer was 1.4 to 1. *7α-thioloacetoxycholesteryl benzoate (IIIa)*: MS (DCI, ammonia): 582 (100) $M.NH_4^+$, 367 (8); NMR (250 mHz): δ 0.68 (3H, S, methyl), 2.35 (3H, S, methyl), 4.09 (1H, M, H at C7), 4.88 (1H, M, H at C3), 5.58 (1H, D, H at C6, J = 7.5Hz), 7.35-8.10 (5H, M, aromatic). *7β-thioloacetoxycholesteryl benzoate (IIIb)*: MS (DCI, ammonia): 582 (100) $M.NH_4^+$, 367 (8); NMR (250 mHz): δ 0.68 (3H, S, methyl), 2.29 (3H, S, methyl), 3.88 (1H, M, H at C7), 4.85 (1H, M, H at C3), 5.27 (1H, S, H at C6), 7.35–8.10 (5H, M, aromatic).

7α-Thiohydroxycholesterol (IV). 7α-thioloacetoxycholesteryl benzoate (339 mg, 601 μmol) was dissolved in anhydrous tetrahydrofuran (10 ml) under a nitrogen atmosphere. To the stirred solution at room temperature was added lithium alumini-

um hydride (2.4 mmol) in anhydrous tetrahydrofuran (2.4 ml) over a period of 10 min. The reaction was left for a further 3 h, after which 50% aqueous ethanol (10 ml) was added. The mixture was then acidified with N hydrochloric acid and extracted with ether (3 × 25 ml). The combined organic extract was washed with water (2 × 50 ml), saturated sodium bicarbonate solution (50 ml) and dried over anhydrous magnesium sulphate. After filtration and removal of the solvent, the reaction product was obtained as a semi crystalline solid (180 mg, 431μmol, 72%) which was recrystallised from acetone. MS (DEI): 418 (3) M^+, 385 (100), 367 (21); MS (DCI, ammonia): 453 (6) $M.N_2H_7^+$, 436 (6) $M.NH_4^+$, 419 (10) $M.H^+$, 402 (40), 385 (23), 367 (100); NMR (250 mHz): δ 0.69 (3H, S, methyl), 1.02 (3H, S, methyl), 1.58 (3H, S, methyl), 3.35 (1H, M, H at C7), 3.55 (1H, M, H at C3), 5.58 (1H, D, H at C6, J = 7.5Hz).

RESULTS AND DISCUSSION

The synthetic route used for the preparation of 7α-thiohydroxycholesterol is summarised in Fig. 1. Firstly, cholesteryl benzoate (I) was converted to 7-bromocholesteryl benzoate (II) by allylic bromination with N-bromosuccinimide in hexane and the radical catalyst benzoyl peroxide. This reaction has been used previously by Tachibana[6] for the 7-bromination of cholesteryl acetate. 7-Thioloacetoxycholesteryl benzoate (III) was then prepared from 7-bromocholesteryl benzoate (II) by reacting the latter with thiolacetic acid in the presence of triethylamine.

While the identity of the reaction product (III) was confirmed by MS, analysis of the material by HPLC revealed the presence of two isomeric compounds. These had very similar retention times on HPLC and hence efforts were directed toward finding the chromatographic conditions that gave the best separation. Using a 25 cm column packed with 3-μm silica, it was found that an isocratic solvent system contain-

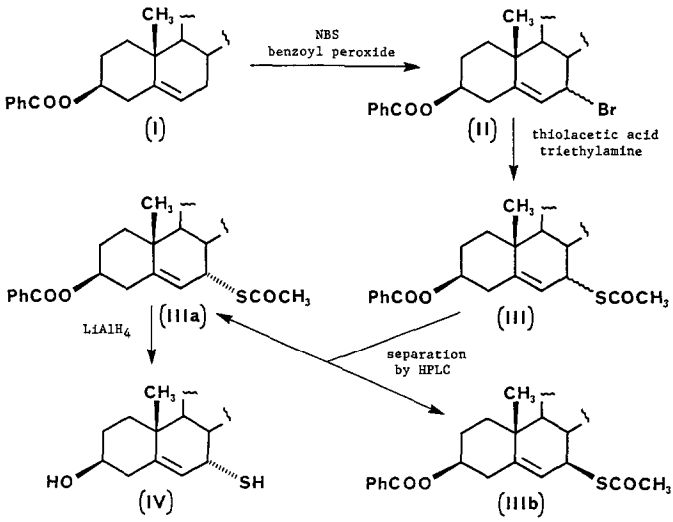

Fig. 1. Synthetic route for the preparation of 7α-thiohydroxycholesterol (IV). Ph = Phenyl; NBS = N-bromosuccinimide.

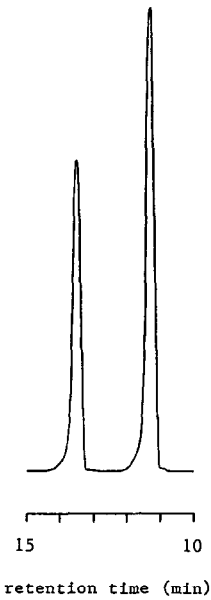

Fig. 2. HPLC separation of the 7α and 7β isomers of 7-thioloacetoxycholesteryl benzoate (III).

ing hexane–dichloromethane–ethyl acetate in the ratio 75:25:0.3 (v/v/v) gave baseline resolution of the two components (Fig. 2). The proportion of dichloromethane in the mobile phase could be increased to about 50% without any significant change in this separation but the amount of ethyl acetate present appeared to be crucial. Outside the range of 0.2 to 0.4%, resolution of the two compounds rapidly deteriorated.

Fractions of eluate from the HPLC column containing one of the two isomers of 7-thioloacetoxycholesteryl benzoate were collected. After removal of the solvent, NMR spectra were recorded in order to establish the stereochemistry of the two compounds. This can be done unambiguously by examining the vicinal coupling between the protons attached to C-6 and C-7 in the B ring of the steroid nucleus. The

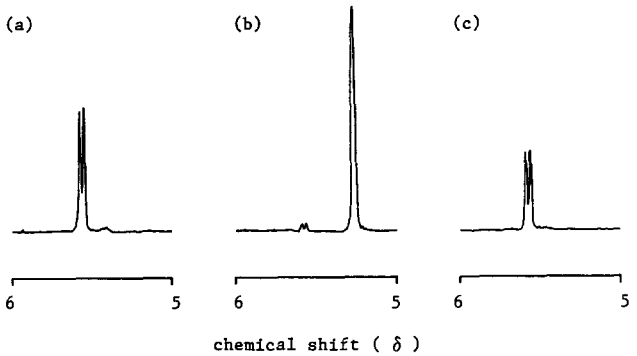

Fig. 3. Partial 250 mHz NMR spectra of (a) 7α-thioloacetoxycholesteryl benzoate (IIIa), (b) 7β-thioloacetoxycholesteryl benzoate (IIIb) and (c) 7α-thiohydroxycholesterol (IV).

NMR spectrum of the earlier-eluting component (retention time 11.4 min) contains a signal at δ 5.58 due to the proton at C-6. This absorbance is split into a doublet with a coupling constant of 7.5 Hz (Fig. 3a), indicating that a dihedral angle of less than 30° exists between the protons at C-6 and C-7. This is consistent with an equatorial orientation of the hydrogen atom at C-7, an axial orientation of the thioloacetoxy group at C-7 and a structure corresponding to 7α-thioloacetoxycholesteryl benzoate (IIIa). The NMR spectrum of the later-eluting compound (retention time 13.6 min) contains a singlet absorbance at δ 5.27 (Fig. 3b) due to the proton at C-6. The coupling constant of 0 Hz indicates a dihedral angle of 90° between the protons at C-6 and C-7 and hence axial orientation of the hydrogen atom at C-7. The thioloacetoxy group at C-7 is therefore in the equatorial plane and this occurs in the structure of 7β-thioloacetoxycholesteryl benzoate (IIIb). These NMR data are very similar to those reported by Johnson and Lack[7] for the isomers 7α-acetoxycholesteryl benzoate and 7β-acetoxycholesteryl benzoate.

How efficiently the two compounds had been separated by HPLC was assessed by examination of the NMR spectra and by HPLC analysis of the individual isomers. Heavy loading of the HPLC column for preparative purposes had resulted in some loss of resolution. However, it was estimated that, while the isomer eluted last (7β-thioloacetoxycholesteryl benzoate) was contaminated with approximately 5% of the 7α epimer, the required compound (7α-thioloacetoxycholesteryl benzoate) was $>99\%$ stereochemically pure.

7α-Thiohydroxycholesterol (IV) was then synthesised from 7α-thioloacetoxycholesteryl benzoate (IIIa) by reduction with lithium aluminium hydride. This reagent has been used by Johnson and Lack[7] for an analogous reaction, the convertion of 7α-acetoxycholesteryl benzoate to 7α-hydroxycholesterol, and these authors reported that there was no isomerisation at C-7 under these reaction conditions. Examination of the NMR spectrum of our reaction product (Fig. 3c) also indicated that the stereochemistry at C-7 had been maintained.

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