Journal of Chromatography, 289 (1984) 231–248
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 233

STUDIES OF THE SELECTIVE DERIVATIZATION OF METHYL HYOCHOLATE AND RELATED STEROIDAL RING B DIOLS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The 6α,7α-diol grouping in methyl hyocholate readily yields cyclic alkaneboronate esters, which are stable enough to permit some separate reactions of the 3α-hydroxy group, but which can be solvolysed to regenerate the diol. Mono-derivatives such as methyl hyocholate 3-acetate, so obtained, are of potential value in the preparation of a variety of "mixed" derivatives, and also of 6,7-*seco*-steroids of the 5β-H series. Gas chromatographic and mass spectrometric data are reported for eleven derivatives of methyl hyocholate, and the corresponding 6,7-*seco*-dialdehyde is characterized in the form of dioxime derivatives. Retention indices (OV-1) are reported for five derivatives of each of three isomers of methyl hyocholate: the 5β-3α,6β,7β-triol, 5α-3α,6α,7α-triol and 5α-3α,6β,7β-triol. Some distinctive features of the electron impact (70 eV) mass spectra are illustrated for the 3-trimethylsilyl ether 6,7-methaneboronates of these isomers.

INTRODUCTION

This model study of derivatives of methyl hyocholate was based on three elements of interest, *viz.*, the possible value of the 6,7-alkaneboronate esters (i) in providing selective derivatives for gas–liquid chromatography–mass spectrometry (GLC–MS), (ii) as protecting groups to allow selective reactions of the 3-hydroxy group and (iii) upon subsequent removal, in facilitating preparation of 6,7-*seco*-steroids of the 5β-series. In relation to the first two aspects, comparative studies of three isomeric *cis*-6,7-diols have also been made, but only on the microgram scale because the samples available each amounted to less than 1 mg.

The unique value of cyclic boronates[1,2] in the selective characterization of *vic*-diols (and related bifunctional substrates)[3] by GC–MS has been very substantially confirmed during the last 16 years. In the steroid field, the principal applications have been to side-chain diols[1–7] and ring D diols[8]; however, the recent discovery of brassinolide, possessing both a nuclear and a side-chain *vic*-diol grouping, has led to the convenient use of alkaneboronates in the "screening" of plant extracts for brassinosteroids[9]. Among other natural steroids containing *vic*-diol groupings, hyocholic (3α,6α,7α-trihydroxy-5β-cholan-24-oic) acid is the only major example of a 6,7-diol.

0021-9673/84/$03.00

Hyocholic acid is a characteristic bile acid of the pig[10], and as a minor component in man, was earlier employed as an internal standard for determining human faecal bile acids[11]; however, the occurrence of hyocholic acid in human meconium[12] and other neonatal body fluids[13], as well as in normal urine, and in urine of patients with liver cirrhosis[14], has stimulated a revival of interest in 6α-hydroxylation in the human. The recent developments in the treatment of gallstones by administration of 3α,7α- or 3α,7β-dihydroxy-5β-cholanoic acid[15,16] are also relevant in this respect.

Selectivity in paper chromatography, based on the interaction of *vic*-diol systems, including 6,7-dihydroxycholanoates, with borate (pH 5–8), was applied by Schneider and Lewbart[17] in an early study. The 6,7-acetonide of methyl hyocholate is a known cyclic derivative[18], but the use of alkaneboronates, both as derivatives and as removable protecting groups, for *cis*-6,7-diols in the bile acid series has hitherto been unexplored. Conventional derivatives applied to the gas-phase analysis of methyl hyocholate (and some of its stereoisomers) have included trifluoroacetates[19–21], heptafluorobutyrates[21], acetates[22–25] and trimethylsilyl ethers[19,20,26,27]. The latter two classes are now generally preferred. Drawbacks of hyocholate trifluoroacetates appear to include thermal instability[20] and some risk of incomplete derivatization[28]; the formation of double peaks in GLC has been reported[21]. The new derivatives studied in this work complement the established derivatives for purposes of GC–MS, and have particular value in facilitating selective reactions. There are few satisfactory chemical procedures for the selective transformation of individual hydroxy groups in trihydroxy bile acids, although the formation of 3-ethylcarbonates has been used for mass spectrometric studies[29], and partial trimethylsilylation has also been explored[30,31].

MATERIALS AND METHODS

Solvents and reagents

Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), N,O-bis(trimethylsilyl)acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and methaneboronic acid were obtained from Pierce and Warriner (Chester, U.K.). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *tert.*-butyldimethylchlorosilane (TBDMCS) was supplied by Regis Chemical Co. (Morton Grove, IL, U.S.A.). Methoxyl- and ethoxylammonium chlorides were purchased from Kodak (Liverpool, U.K.). 2,2-Dimethoxypropane (Fluka, Buchs, Switzerland) was obtained from Fluorochem (Glossop, U.K.). 1-Butaneboronic acid was supplied by Ventron (Karlsruhe, F.R.G.). Ethyl acetate (Nanograde) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Pyridine and N,N-dimethylformamide (AnalaR; BDH, Poole, U.K.) were redistilled over potassium hydroxide and barium oxide pellets, respectively, prior to use. Pyridinium dichromate and lead tetraacetate were obtained from Aldrich (Gillingham, Dorset, U.K.).

Bile acids

Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid), the only bile acid among those studied to be available in gram amounts, was obtained from C.P. Laboratories (Bishops Stortford, U.K.). A sample of β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) was provided by Professor D. N. Kirk (MRC Steroid

Reference Collection, Westfield College, London, U.K.), and samples of methyl esters of the two corresponding 5α-isomers (allohyocholic and allo-β-muricholic acid) were kindly donated by Professor W. H. Elliott (St. Louis University School of Medicine, St. Louis, MO, U.S.A.). Methyl hyocholate and β-muricholate [R_F 0.10 and 0.11, respectively (mobile phase, chloroform–ethyl acetate, 1:3, v/v); the corresponding acids remained at the origin] were obtained by heating at 60°C for 2 h in methanol–hydrochloric acid (prepared from dry methanol and acetyl chloride).

General procedures in derivative formation

Except where otherwise specified, reactions were carried out on samples of 20–200 μg; excess of volatile solvents, reagents and by-products were removed by evaporation under a stream of nitrogen, and the residues were taken up in ethyl acetate (50–100 μl) for analysis by thin-layer chromatography (TLC), GLC and/or GLC–MS. Most of the evidence of characterization of derivatives is given in the tables and figures, but other data (notably TLC R_F values) are cited in the Experimental section. NMR measurements (100 MHz) were carried out by Mr. J. Gall on solutions in [$^2H_5$]pyridine, with a Varian HR-100-12 instrument.

Gas–liquid chromatography

GLC was carried out with a Perkin-Elmer (Beaconsfield, U.K.) F-11 gas chromatograph equipped with a glass column (1.8 m × 4 mm I.D.) packed with 1% OV-1 on Gas-Chrom Q, 100–120 mesh (Phase Separations, Queensferry, U.K.) heated at 260°C; the nitrogen carrier gas flow-rate was 40 ml/min. Open-tubular GLC was performed with a Hewlett-Packard (Winnersh, U.K.) 5880A gas chromatograph, fitted with SE-54 and OV-1 wall-coated fused-silica capillary columns (25 m × 0.32 mm I.D. and 25 m × 0.25 mm I.D., respectively; GC^2, Northwich, Cheshire, U.K.), temperature programmed from 80°C (2 min) at 30°C/min to 200°C (2 min) and then at 3°C/min to 265°C. Helium was used as the carrier gas (3 ml/min, SE-54; 2 ml/min, OV-1) and make-up gas (25 ml/min). Injections were made with Grob-type injectors operated in the split (50:1) mode. Both instruments employed hydrogen flame-ionization detectors. *I* values quoted below refer to the packed column (1% OV-1) operated at 260°C, unless otherwise specified.

Gas–liquid chromatography–mass spectrometry

GLC–MS was carried out on an LKB 9000 instrument, equipped with a DB-1 bonded phase fused-silica capillary column (60 m × 0.32 mm I.D.) (J. and W. Scientific, Rancho Cordova, CA, U.S.A.) heated at 270°C, and a falling needle injector[32]. The helium carrier gas flow-rate was 7 ml/min (room temperature) and the make-up gas flow-rate was 25 ml/min. Mass spectra were recorded under electron-impact conditions (70 eV); accelerating voltage, 3.5 kV; trap current, 60 μA; source and separator temperatures, 270°C. A Kratos (Manchester, U.K.) MS12 mass spectrometer was used to record the mass spectra (70 eV) of the *seco*-dialdehyde (methyl 3α-hydroxy-6,7-*seco*-5β-cholane-6,7-dial-24-oate) by direct insertion (probe temperature 170°C; accelerating voltage, 8 kV; trap current, 500 μA).

Thin-layer chromatography

TLC was performed on silica gel 60 F_{254} pre-coated plates (5 × 20 cm; Merck,

Darmstadt, F.R.G.) obtained from McQuilkin (Glasgow, U.K.). Chloroform–ethyl acetate (3:1, v/v) was generally used as the mobile phase, and chloroform–ethyl acetate (1:3, v/v) for trihydroxy esters. The plates were sprayed with 1% cerium(IV) sulphate–1 *M* sulphuric acid solution and heated at 100°C for 20 min for detection.

Preparation of derivatives of methyl hyocholate and isomeric 3α,6,7-triols

Cyclic alkaneboronate esters. Methane- or butaneboronic acid (1.1 mol proportion) in dry pyridine was added to the triol (50–100 μg) and the mixture heated at 60°C for 30 min.

3-Trimethylsilyl ether 6,7-alkaneboronate esters. The bile acid boronates, prepared as above, were heated with BSTFA in dimethylformamide (1:3, v/v, total volume 20 μl) at 60°C for 5 min. Under the conditions outlined the 5β-bile acids were converted almost quantitatively to the required 3-trimethylsilyl ethers. In the 5α-series however, not only were traces of the original boronate still present, but small amounts of the required 3-TMS 6,7-boronate esters underwent deboronation, resulting in the production (approximately 5%) of di-TMS ethers (presumably at the 3,6 positions).

3-tert.-Butyldimethylsilyl ether 6,7-alkaneboronates. The bile acid boronates, prepared as above, were treated with MTBSTFA (containing 1% TBDMCS as catalyst) in dimethylformamide (1:10, v/v, total volume 10 μl) and heated at 60°C for 10 min. Partial deboronation followed by di-TBDMS ether production was observed to a similar extent to that described for the 3-TMS 6,7-alkaneboronates above.

3-Acetate 6,7-alkaneboronate esters. The bile acid boronates, prepared as above, were treated with acetic anhydride (8 μl) and dry pyridine (10 μl) and heated at 60°C for 30 min. The 6,7-boronate esters were completely stable to the acetylation conditions used in both the 5α and 5β series of bile acids.

Tri(trimethylsilyl) ethers. The triol was treated with BSA, HMDS and TMCS (2:1:1, v/v/v, total volume 20 μl) and heated in a Reactivial (Pierce and Warriner) overnight at 120°C.

Triacetates. The triol was treated with acetic anhydride (8μl) and dry pyridine (15 μl) and heated at 60°C for 48 h.

Solvolysis (exchange boronation) of acetate alkaneboronates of methyl hyocholate. The acetate alkaneboronates, prepared as above, were redissolved in propane-1,2-diol (2–4 μl), treated with distilled water (100 μl) and agitated on a Vortex mixer. The solutions were extracted with diethyl ether (200 μl), the extracts dried over anhydrous sodium sulphate, filtered and evaporated to dryness to yield methyl hyocholate 3-acetate (R_F 0.12). NMR data (δ, ppm) included 4.15 m (3β-H), 3.5 (br) (6β + 7β-H), 0.60s (18-H_3), 1.00s (19-H_3), 1.80s (Ac-H_3).

Verification of 3-monoacetate structure

3-Acetate 6,7-acetonide[18]. Dimethoxypropane (2 ml) containing *p*-toluenesulphonic acid (2.5 μg) as catalyst was added to methyl hyocholate 3-acetate (500 μg) and the solution refluxed for 1 h. The product had R_F 0.55.

3-Acetate 6,7-di(trimethylsilyl)ether. Methyl hyocholate 3-acetate (100 μg) was treated with BSA (10 μl), HMDS (5 μl) and TMCS (5 μl), and the solution heated in a Reactivial at 60°C for 30 min. The solution was diluted with ethyl acetate (50 μl) prior to analysis (R_F 0.66).

Reactions of methyl hyocholate 3-acetate. Methyl hyocholate 3-acetate (42 mg, 0.09 mmol) was placed in a round-bottomed flask (50 ml), thoroughly shaken with lead tetraacetate (0.02 *M* in glacial acetic acid, 10 ml) to ensure complete solution of the diol, and left at room temperature for 45 min. Following the sequential addition of potassium iodide/sodium acetate solution (0.1 g/1.2 g, 10 ml) and sodium thiosulphate solution (0.1 *M*, 20 ml), the mixture was extracted with diethyl ether (3 × 15 ml). The concentrated ether extract (10 ml) was washed with brine (1 ml) and water (0.5 ml), dried over anhydrous sodium sulphate and evaporated to dryness to yield the 3-acetate 6,7-*seco*-dialdehyde (36 mg; 85%) (R_F 0.37). The corresponding di-O-methyloxime (R_F 0.38 and 0.45) and di-O-ethyloxime (R_F 0.69) were prepared from 100-μg samples by heating at 60°C for 30 min with 20 mol proportion of the appropriate alkoxylammonium chloride in dry pyridine (25 μl).

Reactions of methyl hyocholate

Oxidative cleavage. Methyl hyocholate (42 mg; 0.1 mmol), oxidized with lead tetraacetate (as described for the 3-acetate, above) yielded the 6,7-*seco*-dialdehyde (34 mg; 81%), R_F 0.12; characterized without purification by solid probe mass spectrometry (see Table I). A portion (100 μg) was converted into the di-O-methyloxime, which was heated with BSTFA (20 μl) at 60°C for 10 min, affording the 3-TMS ether 6,7-*seco*-dialdehyde di-O-methyloxime, R_F 0.57 and 0.66.

3-Acetate 6,7-acetonide[18]. Acetonide formation followed by acetylation yielded the same product as that obtained from methyl hyocholate 3-acetate (see above): retention index on OV-1, I = 3470, R_F 0.55.

Methyl 3α,6α-diacetoxy-7-oxo-5β-cholan-24-oate. Methyl hyocholate (100 mg) was heated with acetic anhydride (1 ml) and pyridine (300 μl) at 60°C for 20 min. Analysis of the products by GLC and TLC indicated the presence of *ca.* 2% of triacetate[33] (I = 3575, R_F 0.53) and 98% of diacetate (I = 3465, R_F 0.42). NMR data (δ, ppm) included 4.56m (3β-H), 5.08dd (6β-H), 3.90m (7β-H), 0.66s (18-H_3), 1.00s (19-H_3). This almost pure 3,6-diacetate[33,34] (50 mg) was treated with pyridinium dichromate (188 mg) in dry dichloromethane (2 ml) and stirred at room temperature for 15 h. Dry diethyl ether (3 ml) was added and the mixture was filtered through a column of silica gel to remove chromium salts. Evaporation afforded the 7-ketone[34,35] of *ca.* 95% purity as judged by GLC (I = 3600) and TLC (R_F 0.55). NMR data (δ, ppm): 4.72m (3β-H), 5.48d (6β-H), 0.66s (18-H_3), 1.30s (19-H_3). Mass spectral data: m/z 504 (0.6%; M), 462 (2%; M − CH_2CO), 444 (10%; M − CH_3COOH), 402 (5%; M − CH_2CO − CH_3COOH), 384 (27%; M − 2CH_3COOH) and 43 (100%; CH_3CO^+).

Reactions of the 3,6-diacetate 7-ketone. A sample (1 mg) of methyl 3α,6α-diacetoxy-7-oxo-5β-cholanoate, heated with hydroxylammonium chloride (0.5 mg) in pyridine (0.25 ml) at 60°C for 3 h yielded a product having I = 3295, R_F 0.28. Trimethylsilylation with BSTFA–dimethylformamide (1:3, v/v; 20 μl) at 60°C for 30 min gave a product having I = 3375, R_F 0.70; m/z 621 (3%) (presumed to be M), 590 (4%; M − $CH_3O^•$), 561 (21%; M − CH_3COOH), 531 [35%; M − $(CH_3)_3SiOH$], 471 [36%; M − CH_3COOH − $(CH_3)_3SiOH$] and 147 {100%; probably $[(CH_3)_3SiOSi(CH_3)_2]^+$}. These data are consistent with the structure 3α-acetoxy-6α-trimethylsilyloxy-7-oxo-5β-cholanoate O-trimethylsilyloxime.

Methyl hyocholate 3,6-diacetate 7-trimethylsilyl ether. Methyl hyocholate 3,6-

diacetate (100 μg), heated with BSA (10 μl), HMDS (5 μl) and TMCS (5 μl) at 60°C for 30 min yielded the 7-TMS ether, I = 3535, R_F 0.68; m/z 518 (5%; M), 458 (100%; M − CH_3COOH), 428 [2%; M − $(CH_3)_3SiOH$], 386 (10%; M − $(CH_3)_3SiOH$ − CH_2CO) and 368 [17%; M − CH_3COOH − $(CH_3)_3SiOH$].

Borohydride reduction of methyl 3α,6α-diacetoxy-7-oxo-5β-cholan-24-oate. Sodium borohydride (3.8 mg in 1 ml of methanol) was added dropwise to the diacetoxyketone (10 mg) in methanol (0.5 ml). After 1 h at room temperature, the pH was adjusted to 7 with 10% hydrochloric acid. Ether extraction yielded a product containing 95% of methyl hyocholate 3,6-diacetate: I = 3470, R_F 0.45, with a minor impurity at R_F 0.29. The regeneration of the 7α-hydroxy group was further confirmed by formation of the 7-TMS ether, and also (via alkaline hydrolysis, etc.) of the 3-acetate 6,7-methaneboronate, both of which were identical, by TLC and GLC (including capillary GLC on SE-54 and OV-1 columns), with the authentic hyocholate derivatives.

RESULTS AND DISCUSSION

Sequential derivatization of methyl hyocholate

Methyl hyocholate readily underwent the reactions summarized in Fig. 1. The initial formation of a 6,7-cyclic alkaneboronate protected the *vic*-diol grouping, allowing monoacetylation at the 3α-hydroxy group. Solvolysis of the boronate ester by transesterification with propane-1,2-diol[1,36] afforded the 3-monoacetate, which could be converted into a variety of other mixed derivatives such as the 3-acetate 6,7-di-TMS ether. Both the latter derivative and the acetate alkaneboronates were satisfactory for GC, as exemplified in Fig. 2. It was also possible to effect selective silylation of the 3α-hydroxy group in the presence of 6,7-alkaneboronate esters, although partial displacement of the latter by silyl groups tended to occur as a minor (*ca.* 5%) side reaction. Thus two families of mixed derivatives could be readily obtained: those retaining a 6,7-boronate grouping, and those in which this had been replaced by other substituents after selective functionalization at the C-3 hydroxy group. The availability of a convenient preparative method for 3-monoesters and 3-monoethers also made possible the oxidative cleavage of ring B, with reduced risk of the formation of lactones or cyclic acetals between the 3α- and *seco*-6-substituents.

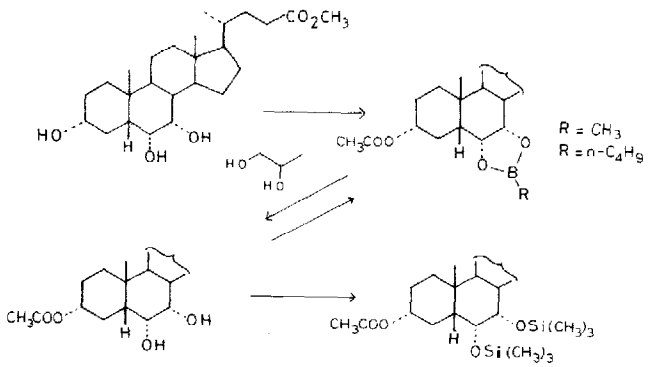


Fig. 1. Illustration of a route for the selective derivatization of methyl hyocholate.

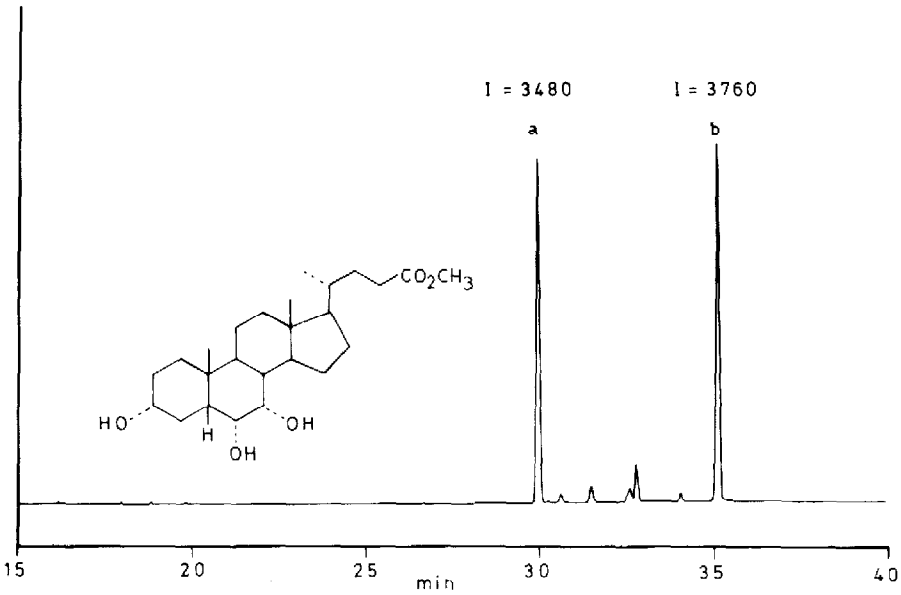


Fig. 2. Gas chromatographic separation of (a) the 3-acetate 6,7-di-TMS ether and (b) the 3-acetate 6,7-butaneboronate of methyl hyocholate. Column, SE-54 fused-silica capillary (25 m × 0.32 mm I.D.); column temperature, programmed from 80°C (2 min) at 30°C/min to 200°C (2 min), then at 3°C/min to 265°C; helium flow-rate, 3 ml/min.

GLC–MS data

Representative gas chromatographic and mass spectrometric data for products of the three kinds mentioned are given in Table I, together with data for methyl hyocholate triacetate and tri-TMS ether, as examples of types of derivatives that have been extensively studied by GLC[23,37] and GLC–MS[27,38]. Most GLC retention data for bile acid derivatives in the literature have been recorded as relative retentions, but methylene unit (MU) values have been reported recently[37] for a number of bile acid methyl ester TMS ethers on columns of OV-1 (at 245°C), OV-17 and SP-2340. There is an inexplicably wide disagreement between comparable results in ref. 37 and in our work: for example, we find $I^{OV\text{-}1}_{260^{\circ}C}$ = 3330 for methyl hyocholate tri-TMS ether, as against 36.95 MU; and for the parent compound, methyl 5β-cholanoate, $I^{OV\text{-}1}_{260^{\circ}C}$ = 2850 as against 32.6 MU, the column temperature difference being only 15°C.

Among the methyl hyocholate derivatives studied, only the TBDMS ether butaneboronate has an inconveniently long retention time, while the methaneboronate 3-TMS ether is eluted before the tri-TMS ether on OV-1. Some tailing of methaneboronate peaks occurs on this phase, and where this is objectionable it would be preferable to use a more polar phase such as OV-17 or SE-54 (*cf.*, Fig. 2).

A notable feature of the mass spectra is the low abundance of molecular ions, the butaneboronate yielding the highest relative abundance (5%). Data for the tri-TMS ether and TMS ether methaneboronate are discussed later in relation to the mass spectra of isomers. The fragmentation of the triacetate yields a number of ions typical of 3,6,7-trihydroxycholanoates, as detailed by (*inter alia*) Szcepanik *et al.*[22]; among these, the prominent ion of *m/z* 386 results from loss of ketene and acetic

TABLE I

MASS SPECTROMETRIC DATA (70 eV) AND KOVÁTS RETENTION INDICES (*I*) FOR DERIVATIVES OF METHYL HYOCHOLATE AND OF THE CORRESPONDING 6,7-*SECO*-DIALDEHYDE

Derivative	*I* (*OV-1*)	$M^{+\cdot}$ (*m/z*)	*Base peak*	*m/z for other principal ions (relative intensities in parentheses)*
Derivative of methyl hyocholate				
Methaneboronate	3330	466 (−)	55	444 (3) 428 (68) 413 (11) 386 (15) 379 (15) 368 (81) 353 (42) 314 (19) 313 (15) 286 (27) 271 (28) 253 (47) 211 (56) 199 (28) 197 (32) 159 (40) 157 (37) 147 (40)
Butaneboronate	3555	488 (5)	55	486 (2) 470 (30) 455 (6) 386 (15) 368 (60) 353 (27) 328 (19) 314 (22) 313 (24) 253 (36) 211 (50) 199 (21) 197 (22) 145 (33) 131 (30) 119 (41) 93 (60) 81 (83)
Acetate methaneboronate	3405	488 (−)	43	428 (78) 413 (13) 386 (21) 379 (17) 368 (89) 353 (47) 313 (25) 286 (36) 271 (34) 253 (54) 211 (68) 199 (29) 197 (29) 159 (38) 147 (40) 95 (78) 81 (89) 55 (86)
Acetate butaneboronate	3620	530 (1)	43	470 (40) 455 (6) 421 (7) 386 (15) 368 (64) 355 (20) 353 (30) 328 (24) 314 (19) 313 (24) 253 (44) 226 (23) 211 (49) 199 (20) 119 (35) 105 (60) 95 (65) 81 (88)
Acetate di-TMS ether*	3360	608 (−)	458	593 (1) 548 (2) 533 (4) 443 (10) 386 (4) 369 (58) 355 (4) 353 (6) 337 (5) 253 (8) 209 (9) 199 (10) 195 (15) 161 (13) 159 (20) 147 (36) 133 (14) 117 (19)
Acetate acetonide	3470	504 (−)	43	489 (27) 429 (6) 386 (19) 369 (73) 337 (7) 327 (38) 313 (4) 277 (10) 271 (7) 253 (10) 211 (10) 199 (8) 159 (24) 133 (18) 107 (27) 95 (38) 81 (36) 55 (46)
Triacetate	3575	548 (1)	43	488 (1) 446 (4) 428 (24) 397 (6) 386 (78) 368 (32) 353 (11) 313 (28) 253 (34) 227 (9) 226 (11) 211 (15) 199 (9) 197 (8) 159 (30) 105 (18) 95 (32) 81 (32)
Tri-TMS ether*	3330	638 (0.2)	458	548 (2) 533 (4) 459 (42) 443 (5) 369 (32) 368 (10) 355 (6) 353 (3) 253 (5) 199 (6) 195 (8) 161 (6) 159 (12) 147 (26) 145 (8) 133 (9) 131 (7) 129 (7)
TMS ether methaneboronate*	3295	518 (−)	386	476 (3) 461 (11) 458 (10) 443 (5) 369 (16) 368 (10) 353 (6) 271 (7) 253 (11) 211 (11) 197 (16) 195 (10) 159 (25) 147 (18) 133 (20) 129 (17) 107 (22) 105 (25)
TBDMS ether methaneboronate*	3520	560 (0.4)	369	545 (1) 503 (14) 461 (4) 428 (4) 386 (7) 337 (5) 327 (9) 253 (6) 213 (9) 209 (9) 199 (11) 173 (12) 161 (40) 159 (34) 145 (16) 133 (17) 117 (21) 105 (22)
TBDMS ether butaneboronate*	3740	602 (0.4)	369	545 (15) 470 (1) 461 (5) 421 (1) 387 (1) 353 (2) 337 (4) 327 (11) 295 (3) 253 (4) 213 (6) 209 (6) 199 (8) 173 (9) 161 (34) 159 (32) 147 (11) 133 (13)
Derivative of 6,7-seco-dialdehyde				
6,7-Dialdehyde***	—	420 (3)	95	402 (8) 390 (10) 356 (11) 262 (8) 249 (7) 199 (8) 175 (7) 147 (19) 141 (68) 133 (4) 123 (99) 105 (49) 93 (59) 91 (43) 81 (52) 79 (58) 67 (63) 41 (66)
Acetate dimethyloxime	3455** 3495	520 (8)	43	489 (56) 474 (12) 457 (14) 443 (10) 397 (18) 323 (20) 317 (16) 310 (12) 296 (12) 276 (12) 267 (12) 249 (14) 191 (24) 152 (32) 121 (48) 93 (56) 81 (82) 55 (94)
Acetate diethyloxime	3525** 3590	548 (6)	43	503 (53) 488 (21) 457 (11) 443 (17) 397 (19) 386 (8) 370 (23) 295 (8) 279 (15) 249 (12) 226 (15) 166 (20) 147 (30) 120 (28) 107 (32) 95 (51) 81 (40) 55 (73)
TMS ether dimethyloxime*	3355** 3405	550 (5)	519	535 (4) 504 (23) 487 (25) 471 (75) 460 (26) 397 (20) 370 (25) 338 (10) 317 (9) 305 (8) 278 (9) 242 (13) 188 (31) 152 (58) 147 (21) 133 (31) 129 (20) 107 (34)

* Mass spectra normalized above *m/z* 100.

** *I* value and mass spectra recorded for the more intense peak of an oxime doublet.

*** Recorded using a modified Kratos MS12 instrument (solid probe).

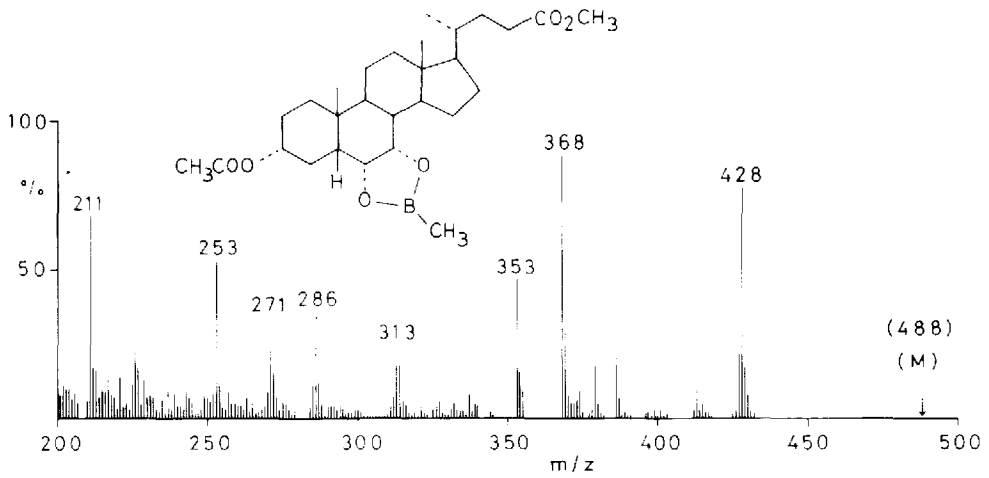


Fig. 3. Mass spectrum (70 eV electron impact) of methyl hyocholate 3-acetate 6,7-methaneboronate measured on an LKB 9000 gas chromatograph mass spectrometer. Column, DB-1 fused-silica capillary (60 m × 0.32 mm I.D.); column temperature, 270°C; helium flow-rate, 7 ml/min with 25 ml/min make-up gas; accelerating voltage, 3.5 kV; source temperature, 260°C; trap current, 60 μA.

acid, a well-known fragmentation of alicyclic *vic*-diol diacetates in general. The 3-acetate 6,7-acetonide yields the expected distinctive ion (M − CH_3•), of *m/z* 489.

Representative mass spectra (above *m/z* 200) of 3-acetate 6,7-alkaneboronates are shown in Figs. 3 and 4. The first major ions in the upper mass range [(M − CH_3COOH)] contain the boronate group: loss of $RB(OH)_2$ then affords prominent ions at *m/z* 368, as observed for many other types of derivative. Other characteristic ions retaining the boronate moieties occur at *m/z* 286 (methaneboronate: Fig. 3) and 328 (butaneboronate: Fig. 4); a possible ion structure is depicted in Fig. 5. In addition, boron-containing ions at *m/z* 313 and 355 in Figs. 3 and 4, respectively, can be ascribed largely to (M − CH_3COOH − side chain). It is of interest to note that a component species of the ion of *m/z* 271 prominent in the spectrum of the methaneboronate (Fig. 3), evidently retains the boronate grouping, even though this nominal

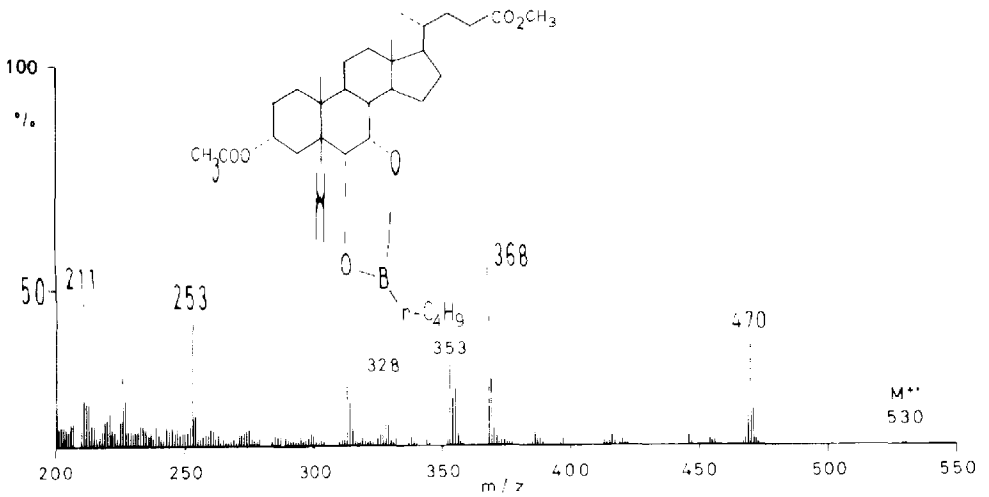
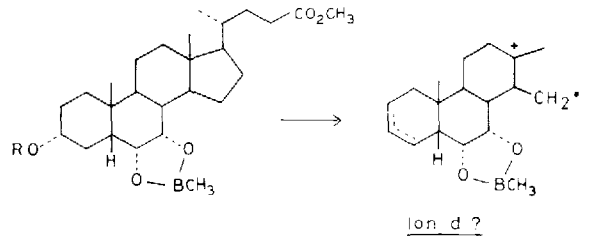


Fig. 4. Mass spectrum (70 eV) of methyl hyocholate 3-acetate 6,7-butaneboronate. GLC–MS conditions as in Fig. 3.



(R = H or CH_3CO) (a) m/z 428 M - ROH
(b) 368 a - $CH_3B(OH)_2$
(c) 253 b - side chain
(d) 286 $C_{18}H_{27}BO_2^{+\cdot}$
(e) 271 d - $CH_3^{\bullet}$

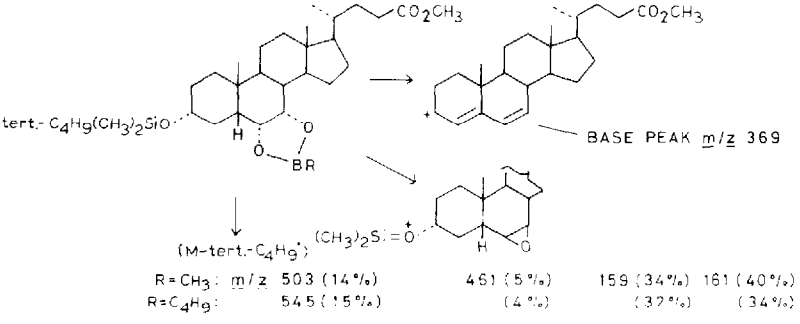
Fig. 5. Characteristic ions in the mass spectra of methyl hyocholate 6,7-methaneboronate and its corresponding 3-acetate. GLC–MS conditions as in Fig. 3.

m/z value also corresponds to a "6,7-epoxide" ion often formed from 3,6,7-triol derivatives[22,25]. On the other hand, elimination of RBO to yield ions of the "epoxide" type is observed in the mass spectra of 3-silyl ether 6,7-alkaneboronates, as exemplified in Table I and depicted in Fig. 6 for TBDMS ethers: the most useful ions from these derivatives are, of course, those of type (M − *tert.*-$C_4H_9^{\bullet}$) which are indicative of the molecular mass.

Derivatives of the 6,7-seco-dialdehyde

The cleavage of ring B in methyl hyocholate 3-acetate by the action of lead tetraacetate proceeded smoothly, but the product showed unsatisfactory behaviour in GLC, and was characterized by direct probe mass spectrometry and by conversion into di-O-alkyloximes, for which GLC–MS data are cited in Table I: peaks attributed to *syn* and *anti* isomers were observed, and the mass spectra were fully consistent with the expected structures, showing distinct molecular ions as well as the eliminations of RO$^{\bullet}$ followed by ROH, typical of di-O-alkyloximes.

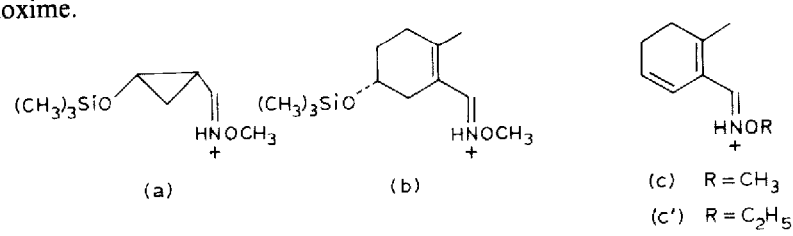
Oxidative cleavage of methyl hyocholate with lead tetraacetate afforded a dialdehyde, which was converted without purification into a di-O-methyloxime: trimethylsilylation at C-3 then yielded a derivative very suitable for GC–MS. In the mass spectrum of this product, notable features among the nitrogen-containing ions



R = CH_3: m/z 503 (14%) 461 (5%) 159 (34%) 161 (40%)
R = C_4H_9: 545 (15%) (4%) (32%) (34%)

Fig. 6. Characteristic ions in the mass spectra of 3-TBDMS 6,7-methane- and butaneboronates of methyl hyocholate. GLC–MS conditions as in Fig. 3.

were the base peak (*m/z* 519: $M - CH_3O^{\bullet}$); ions arising from separate and combined losses of trimethylsilanol, methanol and methoxyl; and ions of *m/z* 188 and 242 for which structures a and b may be postulated. In consonance with the latter proposal, ions of *m/z* 152 (c) were prominent in mass spectra of both the 3-acetate and 3-TMS ether di-O-methyloximes, and an ion of *m/z* 166 (c') arose from the 3-acetate di-O-ethyloxime.



Derivatives of cis-6,7-diols isomeric with methyl hyocholate

The three 5β-6,7-diols stereoisomeric with hyocholic acid (muricholic acids) were originally isolated from rat bile. Methods for the separation and characterization by packed column GLC and GLC–MS of methyl ester tri-TMS ethers of all four isomers[26,38] and of the four corresponding 5α-epimers[27,39–41] were developed more than 10 years ago. With the improvements in resolution that are potentially achievable on capillary columns[37,42–44] the utility of these types of derivative for both qualitative and quantitative analysis may be further enhanced. The mass spectra of the methyl ester tri-TMS ethers of the four 5β-triols are distinctive for each isomer[26], and the same holds true within the set of 5α-triols[27,40], but there is considerable similarity between pairs of 5α/5β epimers[40].

The present study has been limited to the two pairs of 5α/5β triols possessing 6,7-*cis*-diol systems suitable for formation of cyclic alkaneboronates (the possible formation of such derivatives from the diequatorial 6α,7β-diols has not yet been examined). We considered that "mixed" derivatives comprising a 6,7-alkaneboronate and a conventional mono-substituent at the 3-hydroxy group ought to promote significant differences in the mass spectrometric fragmentation of 5α/5β epimers, for the following reasons: (i) in the formation of the cyclic esters, there is a reduction in the original dihedral angle (nominally 60°) between the 6α,7α- or 6β,7β-C–O bonds involved: the consequent energy changes, arising from ring strain and from steric interactions, are unlikely to be equal for the 5α and 5β epimers; (ii) the cyclic boronate ring tends to suppress cleavage of the 6,7-C–C bond, which is a very facile process in 6,7-di-TMS ethers leading, particularly in the 6β,7β-derivatives, to prominent ions at *m/z* 285 and 195 in both 5α- and 5β-series[26,40]; consequently, one would expect reduced production of such ions, in which the distinctive 5-configurations are lost; (iii) the comparative stability of cyclic boronate rings under electron impact should allow more scope for observing fragmentations expressive of the original stereochemistry.

GLC data for four sets of derivatives based on 6,7-methaneboronates are shown in Table II, together with data for the corresponding tri-TMS ethers. Even though OV-1 is by no means the optimal phase, it is clear that the methaneboronate derivatives are of comparable value to tri-TMS ethers in separating isomers, as exemplified by the capillary GLC traces shown in Figs. 7 and 8 for the methyl hyocholate/methyl β-muricholate separations as tri-TMS ethers and methaneboronate

TABLE II

KOVÁTS RETENTION INDICES (*I*) (OV-1, 260°C) FOR DERIVATIVES OF METHYL 3α,6,7-TRIHYDROXYCHOLANOATE ISOMERS AND RETENTION INDEX INCREMENTS BASED ON TRI(TRIMETHYLSILYL) ETHERS

Derivative	*5β Series*				*5α Series*			
	3α,6α,7α-Triol		*3α,6β,7β-Triol*		*3α,6α,7α-Triol*		*3α,6β,7β-Triol*	
	I	*ΔI*	*I*	*ΔI*	*I*	*ΔI*	*I*	*ΔI*
Tri-TMS ether	3330	–	3340	–	3350	–	3340	–
Methaneboronate	3330	0	3310	−30	3350	0	3370	+30
Acetate methaneboronate	3405	+75	3405	+65	3380	+30	3415	+75
TMS ether methaneboronate	3295	−35	3280	−60	3300	−50	3290	−50
TBDMS ether methaneboronate	3520	+190	3535	+195	3530	+180	3505	+165

TMS ethers, respectively. As mentioned above, the preparation of the latter derivatives is not completely quantitative, because of minor displacements, slightly more marked in the 5α- than in the 5β-series, of boronate groups; no such drawback affects 3-acylation, and the 3-acetates, for example, are also satisfactory for GLC, as illustrated in Fig. 9, where three of the four *cis*-diol isomers are distinguished on an OV-1 capillary column.

Mass spectra of all the derivatives cited in Table II have been recorded and will be reported later. It is, however, pertinent to point out the marked dependence of the fragmentations of the methaneboronate 3-TMS ethers on the configurations of the parent methyl cholanoates. Some distinctive ions are noted in Table III, with brief indications of their likely origin. Striking features are (i) the abundance of molecular ions from the 5α-isomers and their insignificance in the spectra of 5β-

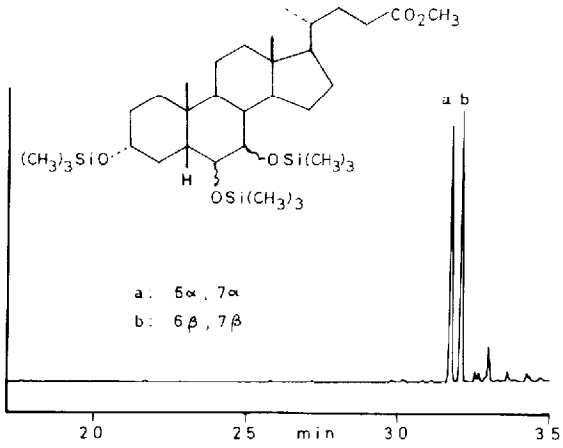


Fig. 7. Gas chromatographic separation of tri-TMS ethers of (a) methyl hyocholate and (b) its 6β,7β-epimer (methyl β-muricholate). Column, OV-1 fused-silica capillary (25 m × 0.25 mm I.D.); temperature programming conditions as in Fig. 2; helium flow-rate, 2 ml/min.

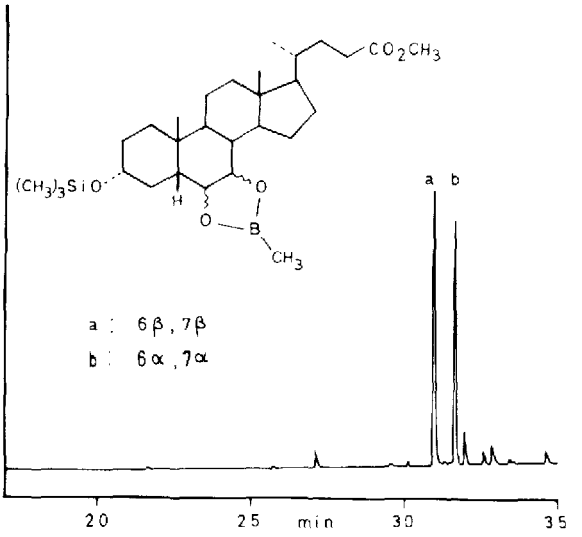


Fig. 8. Gas chromatographic separation of 3-TMS 6,7-methaneboronates of (a) methyl β-muricholate and (b) methyl hyocholate. GLC conditions as in Fig. 7.

isomers; (ii) similar prominence of three other high-mass ions (*m/z* 503, 476 and 458) in the 5α-isomers; (iii) preponderant ions (*m/z* 376) formally attributable to elimination of the (axial) 3α-substituent, followed by retro-Diels–Alder fragmentation in the 5α-isomers; and (iv) sharp distinctions between the 6α,7α- and 6β,7β-isomers of 5β-configuration, notably in the abundances of ions of *m/z* 428, 386 and 373, as illustrated in Fig. 10, reflecting more facile loss of the boronate moiety in the 6α,7α-isomer. The differences in the mass spectra of the 5α,6α,7α and 5α,6β,7β isomers are

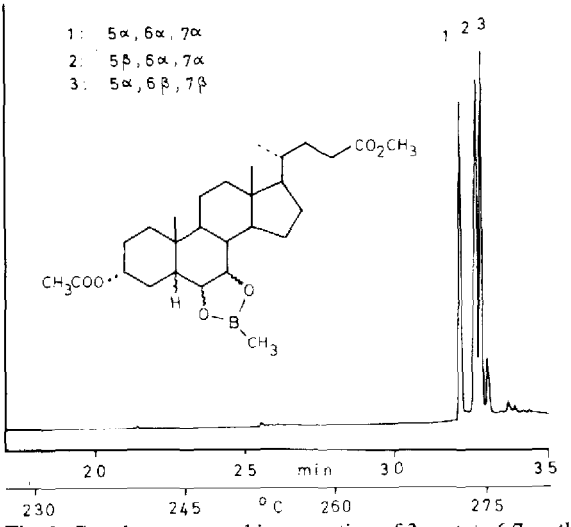


Fig. 9. Gas chromatographic separation of 3-acetate 6,7-methaneboronates of (1) methyl allohyocholate, (2) methyl hyocholate and (3) methyl allo-β-muricholate. GLC conditions as in Fig. 7.

TABLE III

DISTINCTIVE FRAGMENT IONS IN THE ELECTRON IMPACT (70 eV) MASS SPECTRA OF 3-TRIMETHYLSILYL ETHER 6,7-METHANEBORONATES OF METHYL 3α,6α,7α- AND 3α,6β,7β-TRIHYDROXYCHOLANOATES OF THE 5β AND 5α SERIES

Ion		*Ion abundances (relative to respective base peaks in range above m/z 100)*			
m/z	*Type*	*5β-Isomers*		*5α-Isomers*	
		3α,6α,7α	*3α,6β,7β*	*3α,6α,7α*	*3α,6β,7β*
518	M	–	–	48	40
503	$M-CH_3^{\cdot}$	–	8	6	37
476	$M-CH_3BO$	3	–	33	46
458	$M-CH_3B(OH)_2$	10	–	51	39
428	$M-CH_3SiOH$	–	100	53	87
386	$M-CH_3BO-(CH_3)_3SiOH$	100	5	35	65
376	"Retro-Diels–Alder" $M-C_4H_5OSi(CH_3)_3$	–	1	47	30
373	Ring A cleavage $M-C_4H_8OSi(CH_3)_3$	2	46	3	7
368	$M-CH_3B(OH)_2-(CH_3)_3SiOH$	10	7	100	75
353	$M-CH_3B(OH)_2-(CH_3)_3SiOH-CH_3^{\cdot}$	6	3	43	24
271	$M-CH_3BO-$[$(CH_3)_3SiOH$ + side-chain]	7	10	82	100
	$M-(CH_3)_3SiOH-$(ring D + side-chain + H)				

less pronounced, and discrimination between these would rest most reliably upon GLC, *e.g.*, of the tri-TMS ethers on OV-17[27], or the methaneboronate 3-acetates on OV-1 (Fig. 9). The mass spectra of the latter derivatives (Fig. 11), like those of the analogous 3-TMS ethers (Table III), fail to distinguish satisfactorily between the

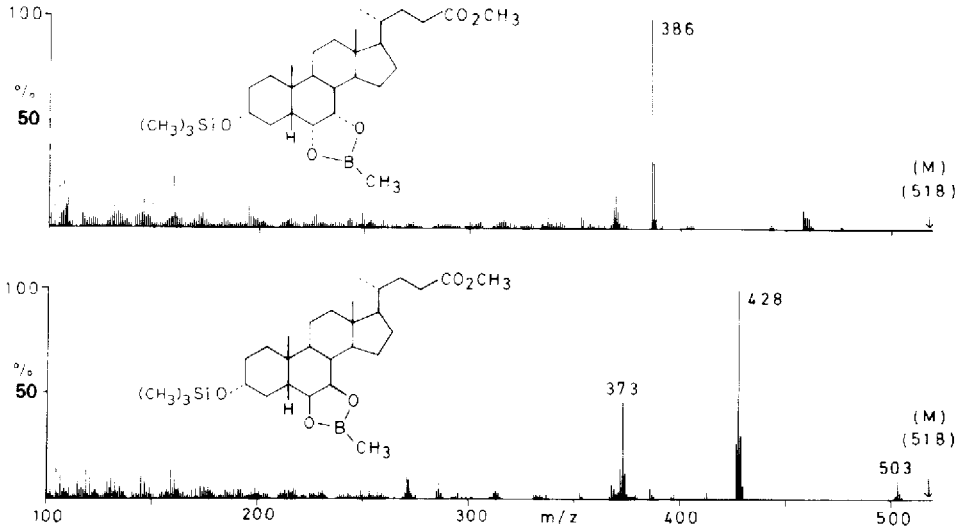


Fig. 10. Mass spectra (70 eV) of 3-TMS ether 6,7-methaneboronate of methyl hyocholate (top) and methyl β-muricholate (bottom). GLC–MS conditions as in Fig. 3.

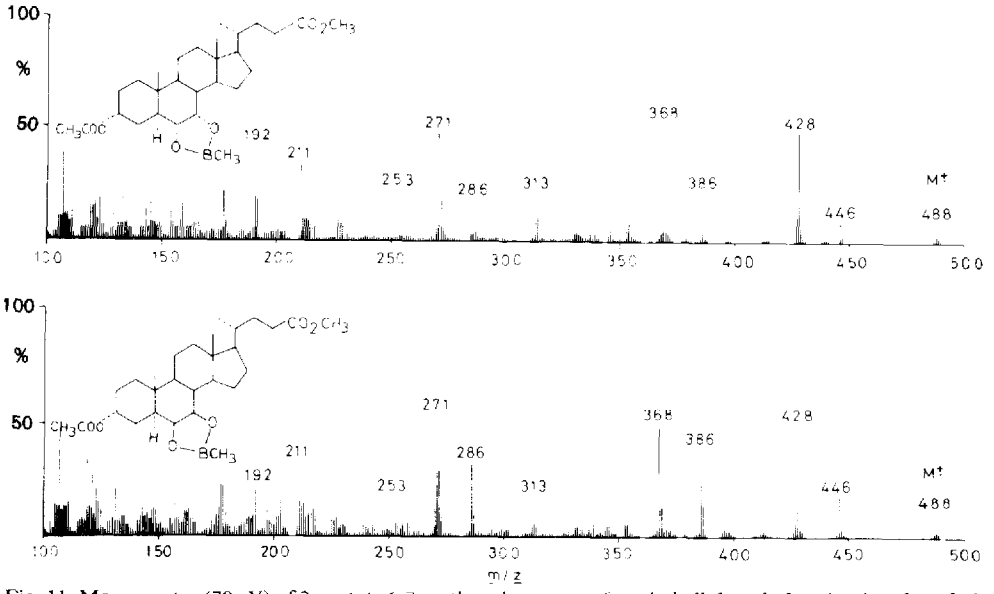


Fig. 11. Mass spectra (70 eV) of 3-acetate 6,7-methaneboronate of methyl allohyocholate (top) and methyl allo-β-muricholate (bottom). GLC MS conditions as in Fig. 3.

6α,7α- and 6β,7β-isomers, but do have the advantage of well defined molecular ions. A more general method of securing information on molecular weights is, of course, by means of chemical ionization mass spectrometry, for example using methyl ester acetate derivatives and isobutane[22], methane[45] or (most effectively) ammonia[46] as reagent gases.

Analytical applications of cyclic boronate derivatives

The results outlined above indicate that the four isomeric types of *cis*-6,7-diols in the methyl 3α,6,7-trihydroxycholanoate group all yield reasonably stable cyclic alkaneboronates, and that further reactions at the 3α-hydroxy group afford various mixed derivatives suitable for GLC–MS. The selectivity of cyclic boronate formation for vicinal (or other sufficiently proximal) diols[3,47] is of potential value in the analysis and characterization of new bile acids formed by metabolic hydroxylations. For example, the urine of healthy newborn infants[48], of neonates with intestinal obstruction[13] and of patients with cholestasis[14,49,50] contains tetrahydroxy acids, one of which has been characterized as 3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid[50,13]; this (as methyl ester) should readily yield a 6,7-methaneboronate 3,12-di-TMS ether of molecular weight 606 [conveniently lower than that (726) of the tetra-TMS ether]. Another neonatal bile acid, 2β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid[13], would also be expected (by analogy with 1,2,3,4-tetrahydronaphthalene-*trans*-2,3-diol[51]) to form cyclic 2,3-alkaneboronates useful for GLC–MS.

Preparative applications of cyclic boronate derivatives

The example of methyl hyocholate illustrates the utility of 6,7-cyclic alkaneboronates as temporary protecting groups, removable under mild conditions, yet

stable enough to permit selective derivatisation at the 3α-position. We are exploring the use of the 3-acetate and 3-TBDMS ether as intermediates for the preparation of 6,7-*seco*-dioic acids of the 5β-series, followed by regioselective hydride reductions of their anhydrides[52] to afford the 5β-analogue of the ring B lactone system in the plant hormone, brassinolide.

With the exception of the 3-monoacetate of cholic acid and related derivatives, few monoacylation products of trihydroxy bile acids have been reported, although in the 3,6,7-trihydroxy series methyl β-muricholate 3-acetate has been prepared by osmium tetroxide oxidation of methyl 3α-acetoxy-5β-6-cholen-24-oate[53,54].

Selective acetylation of methyl hyocholate

Although the 3,6-diacetate of ethyl hyocholate was obtained in crystalline form by Haslewood[33], characterization of the 3,6-diacetates of hyocholic acid[34] and methyl hyocholate[35] has not been entirely satisfactory. We have confirmed Ziegler's observation[35] that the latter derivative is unstable towards chromatography: it is partly converted into a product, possibly the 6,7-*ortho*-acetate, distinguishable by GLC, but not yet identified. In accordance with previous work[33–35], oxidation of the freshly prepared diacetate yielded the 7-ketone; this showed, in the ^{1}H NMR spectrum, the expected downfield shift[55] of *ca.* 0.3 ppm in the signal for the C-10 methyl protons. Products of mild hydrolysis, containing the 7,6α-ketol grouping[56], are of potential value for the preparation of the 6,7-*seco*-dioic acids mentioned above, while vigorous alkaline treatment affords 6,7β-ketols of the 5α-series[35,56] as established precursors of 5α-6,7-*seco*-dioic acids.

CONCLUSIONS

Cyclic alkaneboronates of methyl 3α,6,7-dihydroxycholanoates (*cis*-6,7-diols) are effective for the preparation of mixed derivatives (with retention or replacement of the boronate group) suitable for the differentiation, by GC–MS, between methyl hyocholate and its 5β,6β,7β-, 5α,6α,7α- and 5α,6β,7β-isomers. 3-Monoacyl and 3-monosilyl derivatives, obtainable selectively via boronate intermediates, are potentially useful for preparing 6,7-*seco*-dialdehydes and 6,7-*seco*-dioic acids, of interest in connection with the partial synthesis of brassinosteroid analogues.

ACKNOWLEDGEMENTS

We thank Professor W. H. Elliott (St. Louis) and Professor D. N. Kirk (MRC Steroid Reference Collection) for gifts of valuable samples, Mr. J. Gall, for NMR spectrometry, Mr. A. Ritchie, for mass spectra recorded by direct probe sampling, and Miss B. A. Brown, for the retention index value cited for methyl 5β-cholan-24-oate. The LKB 9000 instrument was provided by SRC grants Nos. B/SR/2398 and B/SR/8471.

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