Preparation and Hydrolysis of *N*-Glucuronides of Pyridine and Piperidine

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N-Glucuronides of pyridine and 3-methylpyridine were prepared and the chemical and enzymatic cleavage by base or β -glucuronidase studied respectively. A K_m value of 0.08 M was found using *E. coli* or *Helix pomatia* enzymes. Catalytic reduction of the *N*-glucuronide of pyridine caused cleavage of the glucuronide, except when the glucuronide was protected as methyl ester and peracetyl derivative. In this case the *N*-glucuronide of piperidine was identified by MS and NMR.

Glucuronides form a major class of compounds in the excretion of xenobiotics and endogenous metabolites. The presence of glucuronides is often proved by enzymatic methods utilizing β -glucuronidase (E.C.3.2.1.31) for the hydrolysis of the glucuronide conjugates.¹ As a result the more lipophilic aglycone can be determined more easily after extraction from a complex matrix. Structure elucidation and quantitation of intact conjugates have been performed, but it is a difficult task due to the administration of small doses and the numerous biotransformations a drug can undergo in a subject. Studies of the synthesis and stability of glucuronides may improve the possibilities for successful analysis of such compounds. The present work has been undertaken with the purpose of getting a more detailed picture of the *N*-glucuronides with regard to synthesis, hydrolysis and spectroscopic properties.

Administration of some xenobiotics results in the formation of *N*-glucuronides which are excreted in urine.²⁻⁶ However, the C-N bond of *N*-glucuronides and other *N*-glycosides is liable to

hydrolysis⁷ and rearrangement⁸⁻¹⁰ reactions depending on the functional group attached to nitrogen.

Ammonia and primary amines⁷ react spontaneously in aqueous solution with glucuronic acid salts, while secondary amines, like piperidine, require methanolic solution for the successful conversion to *N*-glycosides.^{11,12} Pyridine has been reacted with 1-bromo-1-deoxy-2,3,4,6-tetraacetyl glucose¹³ and galactose¹⁴ to give quaternary salts, glycosyl pyridinium bromides. A similar reaction between methyl 1-bromo-1-deoxy-2,3,4-triacetyl glucuronate and pyridine has been published.¹⁵ Deesterification gave a hygroscopic compound, β -D-glucopyranuronosyl pyridinium bromide, which was used as model compound for glucuronidase studies. However, no details were given for the glucuronide, nor have any results been published on the action of glucuronidase on this compound.

The present paper describes a different work-up procedure for the preparation of 1- β -D-glucopyranuronosyl pyridinium hydroxide, as inner salt, which was suitable for further studies on the enzymatic and chemical hydrolysis, as well as the catalytic hydrogenation of the compound.

RESULTS AND DISCUSSION

The *N*-glucuronides (*5a,b*) of pyridine and 3-methylpyridine were prepared in good yields as pure β -anomers (Scheme 1). 2-Methylpyridine failed to react with 3, probably due to steric hindrance caused by the methyl group. The structures were determined by ¹H NMR, IR, MS,

Scheme 1. Synthesis and hydrolysis of *N*-glucuronides of pyridines.

1. Glucurono-3,6-lactone.
2. Methyl 1-deoxy-1,2,3,4-tetracetyl- β -D-glucopyranuronate.
3. Methyl 1-bromo-1-deoxy-2,3,4-triacetyl- α -D-glucopyranuronate.
- 4a. 1-(Methyl 2,3,4-triacetyl- β -D-glucopyranuronosyl)-pyridinium bromide.
- 4b. 1-(Methyl 2,3,4-triacetyl- β -D-glucopyranuronosyl)-3-methylpyridinium bromide.
- 5a. 1-(β -D-Glucopyranuronosyl)-pyridinium hydroxide, inner salt.
- 5b. 1-(β -D-Glucopyranuronosyl)-3-methylpyridinium hydroxide, inner salt.

and elemental analysis. The presence of a broad doublet ($J=8$ Hz) at δ 5.70 ppm in D_2O of **5b** and a similar value for **5a** in $\text{DMSO}-d_6$ is evidence for the β -configuration at the anomeric carbon of the glucuronides. The enzymatic hydrolysis by β -glucuronidase is supporting the glycopyranosidic structure as well as the β -configuration, due to the high specificity encountered by this enzyme. IR showed the presence of a carboxylate group in the sample isolated in the ammonia eluate of a cation exchange column in agreement with the proposed inner salt structure. Acidification gave the free acid as shown by the IR resonance at

1740 cm^{-1} . Compounds **5a,b** were stable in solutions and as solids, except for basic solutions above pH 10, where slow hydrolysis occurred. A 0.1 M solution of **5a** was cleaved at a rate of 0.15 $\mu\text{M}/\text{min}$ at 38°C as determined by headspace analysis.

The Michaelis constant (K_m) and the rate of hydrolysis at saturation (V_{max}) were determined in a kinetic assay measuring the UV absorbance of the product formed by enzymatic hydrolysis using β -glucuronidase from *E. coli* and *Helix pomatia*. The product, pyridine, was measured at 254 nm and a graphic determination of K_m and

Fig. 1. $v/[S]$ versus v plot of kinetic data. Spectrophotometric determination of pyridine obtained by hydrolysis of *5a* in the presence of β -glucuronidase from *E. coli*. The rate of product formation, $\bar{v} = [P]/t$ ($M \min^{-1}$) is taken as ordinate and the rate divided by mean substrate concentration, $[\bar{S}] = ([S_0] + [S])/2$ (M) is taken as abscissa.

A_1 ($K_m = 0.076$ M, $V_{max} = 1.0 \cdot 10^{-4}$ M/min, $r = 0.992$).

A_2 ($K_m = 0.078$ M, $V_{max} = 1.9 \cdot 10^{-4}$ M/min, $r = 0.968$).

B_1 ($K_m = 0.075$ M, $V_{max} = 1.8 \cdot 10^{-4}$ M/min, $r = 0.891$).

B_2 ($K_m = 0.087$ M, $V_{max} = 6.8 \cdot 10^{-5}$ M/min, $r = 0.984$).

V_{max} (Fig. 1) was made using $v/[S]$ versus v plot of the Michaelis-Menten model.¹⁶ Reactions were allowed to proceed to about 5 % conversion to ensure sufficiently high absorbance values.

Usually less than 1–2 % conversion of the substrate is allowed in a kinetic assay where the initial rate ($v = -d[S]/dt$) is measured for a known substrate concentration $[S_0]$. However, it has been shown¹⁷ that conversion of larger fractions of the substrate can be allowed with negligible error if v is replaced by a mean rate ($\bar{v} = P/t$) and $[S_0]$ is replaced by $[\bar{S}] = ([S_0] + [S]) \times 1/2$.

Four experiments utilizing *E. coli* enzyme having pH optimum at pH 7 and two samples from different preparations (A and B in Fig. 1) gave a mean $K_m = 0.08(1)$ M. A single determination of *Helix pomatia* glucuronidase with *5a* as substrate gave $K_m = 0.08$ at the pH optimum of this enzyme (pH 5).

Further use of *5a, b* as substrates in the assay of β -glucuronidase in crude homogenates like faeces

and cancer tissue, measuring pyridine by head-space gas chromatography, is described elsewhere.^{18,19}

Preparation of the *N*-glucuronide of piperidine was attempted by hydrogenation of *5a* under mild conditions, but only piperidine formed by hydrolysis of the reduction product could be detected. However, hydrogenation of the protected *N*-glucuronide of pyridine, *4a*, successfully converted *4a* to the corresponding piperidine, *6*, determined by 1H NMR and MS (Scheme 2). The electron impact MS spectrum showed a weak molecular ion and a more intense $M-OAc$ at m/z 401 and 342, respectively. Cleavage at the anomeric center occurred, yielding m/z 317 due to loss of piperidine.

N-Glucosylpiperidine,¹¹ *7*, was prepared for comparison with regard to spectroscopic and hydrolytic behaviour (Scheme 2). This compound, like *6* showed no anomeric proton separate from other glycosidic protons in the 1H NMR spectrum. The electron impact MS spec-

Scheme 2. Syntheses of peracetylated glycosylpiperidines.

6. 1-(Methyl 2,3,4-triacetyl- β -D-glucopyranuronosyl)-piperidinium bromide.
7. 1-(β -D-Glucopyranosyl)-piperidine.
8. 1-(2,3,4,6-Tetraacetyl- β -D-glucopyranosyl)-piperidine.

trum of the peracetylated derivative, 8, showed a weak molecular ion at m/z 415 and more intense peaks at m/z 372 and 356 due to loss of acetyl and acetate, respectively. Compound 7 dissolved easily in water, yielding a solution of pH 7 which quickly rose to pH 11 indicative of a fast hydrolysis or rearrangement in agreement with the early findings of Hodge and Rist.¹⁰

EXPERIMENTAL

Materials. Solutions of β -glucuronidase (E.C.3.2.1.31) from *E. coli* (Boehringer) and *Helix pomatia* (Boehringer) were diluted with 1 M phosphate buffer prepared from NaH_2PO_4 in distilled water and 2 M NaOH to give a suitable pH.

Methods. Headspace analysis¹⁸ was carried out using 4 ml vials fitted with screw cap and laminated rubber-teflon septum. All samples were thermostated at 38.0 °C in a water bath at least 30 min before sampling was allowed. A gas-tight 1 ml syringe (SGE) heated to 60 °C was used for sampling and injection on the Hewlett Packard 5730 gas chromatograph using FID detection. The column was a 1.5 m $\times$ 2 mm i.d. glass coil packed with 10 % Apiezon L and 4 % KOH on Chromosorb WHP 100–120 mesh. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. Injector and detector were maintained at 200 °C and the column at 90 °C. Chromatograms were obtained with an Omniscrite recorder (Houston Instr.).

UV spectrophotometry for determination K_m was performed on a Kontron Uvicon LCD 725 measuring absorbance at 254 nm in 1 cm cells. NMR spectra were obtained on a Varian EM 360

L spectrometer. MS spectra were run on a VG Micromass 7070 F (electron impact) or a modified Varian CH 5 (field desorption) spectrometer. IR spectra were run on a Perkin Elmer 457 grating spectrometer. Optical rotations were measured on a Perkin Elmer 141 polarimeter.

Determination of K_m using 5a and β -glucuronidase from *E. coli*. Aqueous stock solutions of 5a (0.1535 M and 0.394 M) and dilutions thereof were thermostated at 38 °C. These solutions (200 μ l) were mixed with β -glucuronidase (200 μ l) in 1 M phosphate buffer at pH 7.0. After a suitable time, 0.25 M glucaro-1,4-lactone (200 μ l) was added followed by 1.0 ml chloroform. The vial was shaken and the content transferred to a bigger vial containing 4.0 ml chloroform. Absorbance was measured and pyridine concentration calculated from a standard curve. K_m was similarly determined using *Helix pomatia* juice at pH 5. In this case 1 ml 2 M NaOH was added before extraction with chloroform.

Preparation of 2 and 3 (Scheme 1). The commercially available glucuronic acid, γ -lactone (1) was used as starting material in some experiments. Alternatively 2 was purchased from Koch-Light. The detailed description of Bollenbach *et al.*²⁰ was followed in the syntheses of 2 and 3, the formation of products being followed by TLC. Yields of 2 and 3 were about 70 % and 85 %, respectively, giving an overall yield of about 60 % of the theoretical.

Preparation of 4a. 2 g (5 mmol) of 3 were dissolved in 25 ml pyridine and left at room temperature for 2 d or heated to 80 °C for a few hours with somewhat lower yield. The mixture was filtrated and the crystals washed with diethyl ether. Yield 1.4 g (58 %), no well defined m.p. ¹H NMR (60 MHz, DMSO- d_6): δ 1.90 (3H,s,CH₃CO), 2.03 (3H,s,CH₃CO), 2.05

(3H,s,CH₃CO) 3.68 (3H,s,CH₃-OCO), 4.8–6 (4H,m,CHOAc), 6.5–6.7 (1H,m,anomeric proton), 8.4–9.6 (5H,m,pyridinium protons). MS (field desorption) *m/z* 396 (M–Br).

Preparation of 4b. Preparation of 4b followed the procedure for preparation of 4a using 3-methylpyridine as reagent. Yield 1.9 g (73 %). ¹H NMR (60 MHz, D₂O): δ 2.0 (3H,s,CH₃CO), 2.13 (3H,s,CH₃CO), 2.19 (3H,s,CH₃CO), 2.60 (3H,s,CH₃Ar), 3.88 (3H,s,CH₃-OCO), 5–6 (4H,m,CHOH), 6.3 (1H,d, *J*=8 Hz, anomeric proton), 8–9 (4H,m,Ar).

Preparation of 5a. An amount of 1 g (2.1 mmol) of 4a was dissolved in 10 ml 1 M NaOH and left at room temperature overnight. The solution was applied to strongly acidic ion exchange resin (30 cm×1 cm i.d., Dowex 50×1, 50–100 mesh) and eluted with water to neutral reaction. Then elution was continued with 2 M ammonia and the first basic fractions (30 ml) containing U.V. absorbing material were collected. Evaporation of the solvent gave 450 mg (84 %) of 5a. Anal. C₁₁H₁₃NO₆: C, H, N. [*a*]_D²⁰+44.5 (c 1, water) ¹H NMR (60 MHz, DMSO-*d*₆): δ 3.5–4 (3H,m,CHOH), 4–5 (DOH), 5.8–6.1 (1H,m,anomeric proton), 8.2–9.4 (5H,m, pyridinium protons). MS (field desorption) of a sample treated with HCl *m/z* 256 (*M*+1). IR (KBr) cm⁻¹ 1610(s) and 1630(s) (carboxylate). HCl treated sample: IR (KBr) cm⁻¹ 1740 (carboxylic acid). Recrystallization of the product was done by dissolving the solid in water to give a saturated solution, approximately 0.3 M, and then adding ethanol to give a turbid mixture.

Preparation of 5b. 5b was prepared as 5a hydrolyzing 4b. Yield 0.8 g (83 %), [*a*]_D²⁰+45.96 (c 0.82, water). Anal. found: C 52.68, H 5.69, N 5.15; calc. for C₁₂H₁₅NO₆: C 53.54, H 5.62, N 5.20. ¹H NMR (60 MHz, D₂O): δ 2.60 (3H,s,CH₃-Ar), 3.5–4.3 (4H,m,CHOH), 5.70 (1H, broad d, anomeric proton), 7.8–9 (4H,m,Ar).

Catalytic hydrogenation of 4a. 440 mg (0.92 mmol) of 4a were dissolved in 40 ml distilled methanol. Hydrogenation was carried out in a glass vessel at 3 atm. using 5 % Rh on Alumina (Engelhardt), at room temperature overnight. The mixture was filtered through kieselguhr. The filtered solution was evaporated, giving 350 mg of compound 6, which was used directly for spectroscopic analysis. ¹H NMR (60 MHz, CDCl₃): δ 1.5–2.3 (15H,m), 2.9–3.5 (4H,m), 3.8 (3H,s), 4.5–5.6 (5H,m), MS (IP 70 eV, *m/z* (% rel. int.): 401(1,M), 342(1,M–OAc), 317(1,M–C₅H₁₀N), 228(7), 215(15), 186(38), 115(100).

Catalytic hydrogenation of 5a. 58 mg (0.23 mmol) of 5a was dissolved in 15 ml of water and

hydrogenated overnight at 3 atm. at room temperature using 5 % Rh on Alumina as catalyst. The mixture was filtered through kieselguhr and the solvent evaporated on a Rotavapor. TLC on silica using 2-propanol–water–acetic acid (8:3:1) as eluent. The plate was developed with ninhydrin in acetone. The visible spot had an *R_F* value (0.4) identical to piperidine.

Hydrolysis of 5a. A solution of 5a (11.5 mM) and 2-methylpyridine (5 mM) in 1 M phosphate buffer pH 7 was mixed with β-glucuronidase from *E. coli* (100 μl of each solution) and the evolution of pyridine was measured by headspace analysis at intervals. When the ratio of pyridine and the internal standard remained constant the concentration was measured from a standard curve.

The nonenzymatic hydrolysis of 5a in basic solution was determined by headspace analysis using a 0.134 M solution of 5a in 0.01 M borax buffer pH 10.

Preparation of 7 and 8. Preparation of 7 and 8 followed the procedure of Hodge and Rist.¹¹ Compound 7: ¹H NMR (60 MHz, CDCl₃): δ 1.4 (6H, broad s), 1.98 (3H,s,CH₃CO), 2.00 (3H,s,CH₃CO), 2.01 (3H,s,CH₃CO), 2.02 (3H,s,CH₃CO), 2.3–3.2 (4H,m,piperidine), 3.4–4.2 (4H,m,glycosidic), 4.8–5.2 (3H,m,glycosidic). MS (IP 70 eV, *m/z* (% rel. int.): 415 (5,M), 372 (1,M–Ac), 356 (19,M–OAc), 342 (14), 296 (1), 282 (3), 243 (9), 201 (1), 198 (3), 169 (19), 114 (100). *m* 342→282 obs. 232, calc. 232.5; 169→127 obs. 95.5, calc. 95.4, 156→114 obs. 83, calc. 83.3.

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