SYNTHESIS OF BROMOPHENYL β-D-GLUCURONIDES: HYDROPHILIC PRECURSORS OF LIPOPHILIC STANDARDS IN THE ANALYSIS OF ENVIRONMENTAL POLYCHLORINATED BIPHENYLS

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Dedicated to Professor Antonín Holý on the occasion of his 70th birthday in recognition of his outstanding contributions to the area of nucleic acid chemistry.

A rapid PCB-screening protocol, based on a combination of extraction techniques and instrumental nuclear activation analysis (INAA), required water soluble brominated internal standard that undergoes facile hydrolysis to a lipophilic brominated counterpart. Bromophenyl glucuronic acids **2**, **3**, **7** were synthesized for this application. Glucuronic acids **2** and **3** were prepared by bromination of phenyl β-D-glucopyranosiduronic acid in 81 and 54%, respectively, whereas compound **7** was prepared by coupling methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**) with 2,4,6-tribromophenol. Incubation with β-glucuronidase indicated that tribromophenyl derivative **7** is an excellent substrate, with near quantitative conversion to tribromophenol within seconds of incubation with the enzyme.
Keywords: Glycosides; Glucuronides; Bromophenyl glucuronic acids; Bromination; PCB analysis standards; Instrumental neutron activation analysis (INAA); Bromobenzenes.

Occupational studies show significant increases in cancer mortality in workers exposed to PCBs [1]. For example, cancer of the liver, gall bladder and biliary tract in capacitor manufacturing workers exposed to Aroclors 1254, 1242, and 1016, and malignant melanoma in workers exposed to

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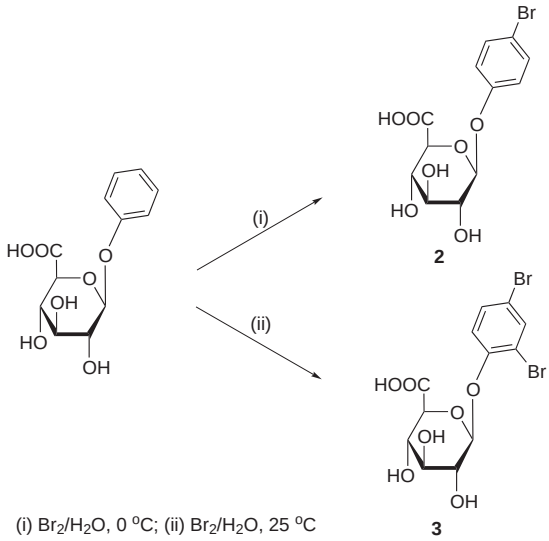
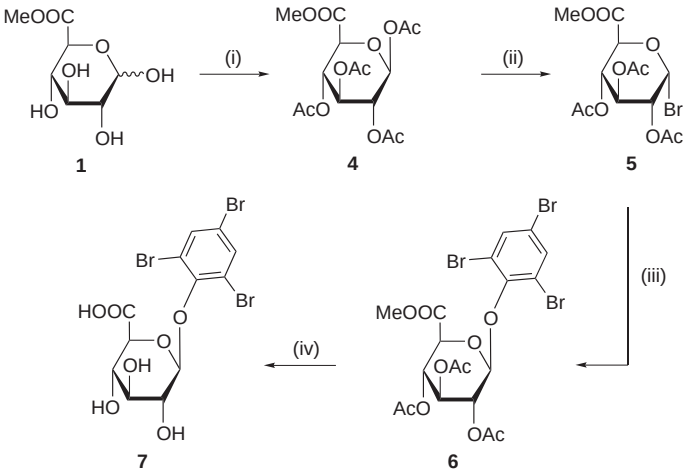
Aroclors 1242 and 1016, have been reported[2,3]. Concern for health risk associated with PCBs in environmental samples has necessitated the development of rapid, cost-effective screens for PCBs. However, routine screening of large numbers of environmental samples for polychlorinated biphenyls (PCBs) represents a major challenge for analytical laboratories doing this work. The SLOWPOKE laboratory[4] at the University of Alberta, in collaboration with Envirotest Ltd.[5], has developed a rapid screening system based on the estimation of 'organic chlorine' using a combination of extraction techniques and instrumental neutron activation analysis (INAA). Since most of the samples are water-based in origin, and because these samples invariably contain non-organic chlorine, it was necessary to develop an internal standard for the analysis that could easily be converted from hydrophilic to hydrophobic form and be assayed by INAA. Bromine-containing glucuronides were considered to be ideal candidates for use as internal standards for this purpose, since bromine has a high thermal neutron activation cross section, for high sensitivity, and since *O*-glucuronides undergo facile cleavage under the influence of commercially available enzymes such as β-glucuronidase.

This work describes the synthesis of mono-, di- and tribromophenyl glucuronic acids **2**, **3** and **7** (Schemes 1 and 2) and reports their enzymatic hydrolysis at the glycosidic bond to yield mono-, di-, and tribromophenols, respectively, by β-glucuronidase enzyme under conditions used within the assay procedure.



(i) Br_2/H_2O, 0 °C; (ii) Br_2/H_2O, 25 °C

SCHEME 1



(i) Pyridine/Ac_2O; (ii) HBr/AcOH; (iii) $Hg(CN)_2$/2,4,6-tribromophenol; (iv) NaOMe, MeOH

SCHEME 2

Direct bromination of the commercially available phenyl β-D-glucopyranosiduronic acid proceeded smoothly to afford 4-bromophenyl β-D-glucopyranosiduronic acid (**2**) and 2,4-dibromophenyl β-D-glucopyranosiduronic acid (**3**). It was observed that the degree of bromination depended on the temperature of the reaction[6]. Reaction of phenyl β-D-glucopyranosiduronic acid with liquid bromine at 0 °C yielded only **2** (81%) whereas, at 22 °C, only 2,4-dibromophenyl glucoside **3** (54%) was obtained. In both cases, it was necessary to add bromine dropwise until the color of bromine persisted.

Attempts to synthesize 2,4,6-tribromophenyl β-D-glucopyranosiduronic acid (**7**) using similar conditions were futile. Efforts to incorporate three bromine atoms on the benzene ring at elevated temperature (60 °C) resulted in severe decomposition, without any evidence of formation of the desired product. It was, therefore, deemed necessary to use a coupling reaction to synthesize the corresponding 2,4,6-tribromophenyl glucuronide **7**. Therefore, 2,4,6-tribromophenol was coupled with methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**) which, in turn, was obtained by bromination of methyl 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosiduronate (**4**) with 40% HBr in acetic acid at 22 °C. The synthesis of methyl (2,4,6-tribromophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**6**) involved reaction of an excess of 2,4,6-tribromophenol with **5** in the presence of mercuric cyanide[7] which was used here as a Lewis acid. This reaction af-

forded **6** in 52% yield. Deprotection of **6** yielded the desired compound **7** (69%) in good yield.

The effects of the degree of bromine substitution on lipophilicities and ^{13}C NMR chemical shifts of related carbons are presented in Tables I and II, respectively. Relative lipophilicity, estimated from the HPLC retention times of these products (**2**, **3** and **7**), indicated the anticipated increase as the extent of bromine substitution in the molecule increased (Table I). A comparison of ^{13}C NMR chemical shifts of these products revealed that an increase in the number of bromine substituents in benzene ring directly affected the electronic environment at C-1 of the aromatic ring and C-1′ of the glucuronide moiety. Since these positions participate in the formation of glycosidic bond, the susceptibility of glycosidic linkage to β-glucuronidase induced hydrolysis will depend on its increased electronic polarization (assisted by the extent of bromine substitution on benzene ring, Fig. 2). Thus, the shielding of C-1 of the aromatic ring increased as a function of increasing bromine substitution due to the strong *–I* effect of bromine, producing the largest upfield chemical shift for C-1 in the case of

TABLE I
Retention times of compounds **2**, **3** and **7**, their β-glucuronidase hydrolysis products (aglycone), and hydrolysis data

Compd	Retention time min	Retention time of Aglycone, min	Hydrolysis	Hydrolysis time min
2	2.3	26.5	100	5
3	5.8	33.4	88.6	0
7	7.6	32.0	100	0

TABLE II
Selected ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of compounds **2**, **3** and **7**

Compd	H-1′	H-5′	C-1′	C-4	C-1	COOH
2	5.0	3.80	100.92	115.71	156.55	176.04
3	5.0	3.40–3.66	101.35	115.96	153.14	175.93
7	5.2	3.50–3.60	103.80	119.14	149.80	171.60

tribromophenyl glucuronide **7**. The resulting high degree of polarization of the glycosidic bond in the case of tribromophenyl glucuronic acid **7** facilitates its virtually instantaneous enzymatic hydrolysis at "time zero" (Fig. 1), while monobrominated glucuronide **2** is hydrolyzed only partially (12%) at "zero time" and takes 5 min for complete hydrolysis (Table I). The C-1′ (sugar carbon) was more deshielded with the increase in the degree of bromination in the molecule (Table II). It also appears that the substitution on the aromatic ring orients the benzene ring in a plane that affected the electronic environment of carboxyl group at C-5′, since carbon chemical shifts for COOH was furthest downfield in **2** (monobromo-substituted) while it appeared at nearly 4.4 ppm upfield in case of **7** (tribromo derivative).

Compounds **2**, **3** and **7** were incubated with β-glucuronidase enzyme and their hydrolysis profiles were determined. Related data are provided in Table I. Figure 1 shows the respective HPLC chromatographic traces of the incubation mixture prior to and immediately after the addition of β-glucuronidase to tribromophenyl glucuronic acids. These analyses show that complete hydrolysis of tribromophenyl glucuronide occurred within seconds after the enzyme was introduced into the solution, thereby, confirming that tribromophenyl glucuronic acid is an excellent substrate for β-glucuronidase. Similar results were obtained for dibromophenyl glucuronide. The monobromophenyl glucuronide, however, was converted at a sub-

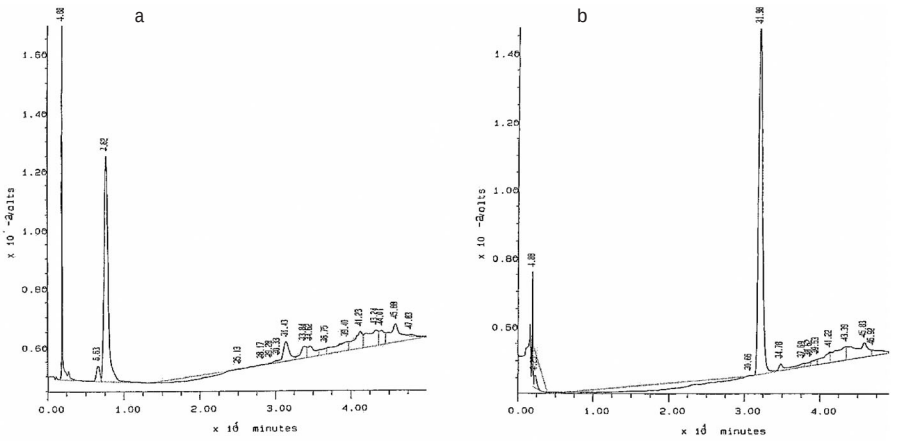


FIG. 1

HPLC chromatogram showing the hydrolysis of 2,4,6-tribromophenyl glucuronic acid (**7**). HPLC chromatogram of **7**: before hydrolysis (a), immediately (zero hour) after addition of 25 units of β-glucuronidase to 100 nmoles of tribromophenyl glucuronide (**7**) (b)

stantially slower rate, with 12% hydrolysis at "zero time", 64% after 1 min and 100% in 5 min (Table I). The slower rate of hydrolysis in case of **2** may be attributed to weaker *–I* effect of monobromophenyl moiety at C-1 in comparison to **7** and **3** where additional bromine substitutions afforded immediate hydrolysis at glucuronic linkage (Fig. 2).

In summary, mono and dibromophenyl glucuronic acids were prepared by direct bromination of commercially available phenyl glucuronide while the synthesis of tribromophenyl glucuronic acids was not possible using this approach, the latter being obtained only via a coupling procedure. All three glucuronides were excellent substrates for β-glucuronidase, under these incubation conditions, for use as internal standards for the analysis of organochlorine in environmental samples.

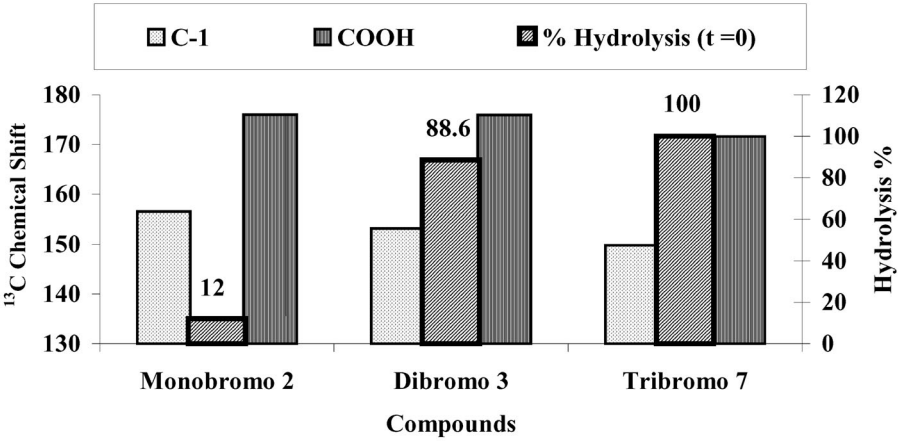


FIG. 2
Rate of enzymatic hydrolysis in relation to selected ^{13}C chemical shifts (δ, ppm)

EXPERIMENTAL

Melting points were determined on a Buchi capillary apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-300 spectrometer using $CDCl_3$ or D_2O as the solvents; the chemical shifts are given in ppm (δ-scale) downfield from tetramethylsilane as internal standard, coupling constants (*J*) in Hz. The ^{1}H NMR assignments were confirmed by selective decoupling experiments. The ^{13}C NMR resonances are assigned by using the J modulation spin echo technique to determine the number of hydrogen atoms attached to each carbon atom. Protons and carbon positions of the glucuronic acid moiety are represented by a single prime (′). Thin layer chromatography (TLC) was performed on Whatman MK6F silica gel micro TLC plates (25 μm thickness) using $CHCl_3$–MeOH–H_2O; 65:35:5, v/v/v (solvent system A) and hexanes–ethyl acetate; 60:40, v/v (solvent system B) as the developing solvents. Column chromatography was carried out using Merck 7734 silica gel

(100–200 μm particle size). The compounds were characterized by their elemental analyses for C, H and N or by Fast atom bombardment (FAB) mass spectra using a sodium probe on an AEI-MS-12 mass spectrometer. Phenyl β-D-glucopyranosiduronic acid, D-glucurone and β-glucuronidase (*E. coli*; 1000 units/ml) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). 2,4,6-Tribromophenol was synthesized by a reported procedure[8] (m.p. 92 °C (reported); 94 °C (observed)). Methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**) was prepared by the method of Bowering and Timell[9] (m.p. 104.5 °C (reported); 104 °C (observed)).

4-Bromophenyl β-D-Glucopyranosiduronic Acid (**2**)

Liquid bromine was added dropwise to a precooled (0 to 5 °C), stirred suspension of phenyl β-D-glucopyranosiduronic acid (0.1 g, 0.37 mmol) in water (1 ml), until the color of bromine persisted. The stirring was continued at this temperature for an additional 1 h. No starting material was detected (TLC) at this time. The solvent was removed by evaporation under vacuum and the mixture was purified on a silica gel column using solvent system A as eluent. The eluted product was recrystallized from methanol to afford 105 mg (81%) of pure **2**. R_F 0.60 (solvent system A); m.p. 210 °C (dec.). ^{1}H NMR (D_2O): 3.54–3.60 m, 3 H (H-2′, H-3′ and H-4′); 3.80 d, 1 H, *J*(4′,5′) = 8.7 (H-5′); 5.00 d, 1 H, *J*(2′,1′) = 7.5 (H-1′); 6.97 d, 2 H, *J*(2,3) = *J*(6,5) = 8.5 (H-3 and H-5 of phenyl ring); 7.42 d, 2 H, *J*(3,2) = *J*(5,6) = 8.5 (aromatic H-2 and H-6). ^{13}C NMR (D_2O): 72.37 (C-4′), 73.40 (C-2′), 75.99 (C-5′), 76.86 (C-3′), 100.92 (C-1′), 115.71 (aromatic C-4), 119.34 (aromatic C-2 and C-6), 133.24 (aromatic C-3 and C-5), 156.55 (aromatic C-1), 176.04 (COOH). FAB HRMS for $C_{12}H_{13}BrO_7$, M^+ relative abundance 5.06% (^{79}Br) and 5.27% (^{81}Br).

2,4-Dibromophenyl β-D-Glucopyranosiduronic Acid (**3**)

Phenyl β-D-glucopyranosiduronic acid (0.2 g, 0.74 mmol) was suspended in water (2 ml), and liquid bromine was cautiously added dropwise to this mixture while stirring, until bromine decolorization stopped. The reaction mixture was allowed to stir at 25 °C for 3 h. At this time TLC of the reaction mixture showed complete disappearance of the glucuronide and a new product appeared. Water was removed by evaporation under vacuum and the resulting impure syrup was chromatographed on a silica gel column using solvent system A as eluent to obtain 0.17 g (53.6%) of pure **3**. R_F 0.64 (solvent system A); m.p. 195 °C (dec.). ^{1}H NMR (D_2O): 3.40–3.66 m, 3 H (H-2′, H-4′ and H-5′); 3.76 d, broad, 1 H (H-3′); 5.00 d, 1 H, *J*(2′,1′) = 7.5 (H-1′); 7.04 d, 1 H, *J*(5,6) = 9.0 (aromatic H-6); 7.38 d, 1 H, *J*(6,5) = 9.0 of d, *J*(3,5) = 1.5 (aromatic H-5); 7.72 d, 1 H, *J*(5,3) = 1.5 (aromatic H-3). ^{13}C NMR (D_2O): 72.35 (C-4′), 73.31 (C-2′), 76.06 (C-5′), 76.99 (C-3′), 101.35 (C-1′), 113.68 (aromatic C-2), 115.96 (aromatic C-4), 119.05 (aromatic C-6), 132.49 (aromatic C-3), 136.07 (aromatic C-5), 153.14 (aromatic C-1), 175.93 (COOH). FAB HRMS for $C_{12}H_{12}Br_2O_7$, M^+ relative abundance 0.11% (^{79}Br), 0.13% ($^{79/81}$Br) and 0.11% (^{81}Br).

Methyl (2,4,6-Tribromophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate (**6**)

A mixture of 2,4,6-tribromophenol (4.17 g, 12.6 mmol) and mercuric cyanide (0.35 g, 1.39 mmol) was heated at 110 °C to form a homogenous melt. A solution of bromide **5** (0.5 g, 1.26 mmol) in anhydrous dichloromethane (2 ml) was added dropwise to this melt. After the addition of bromide **5** was complete, the reaction mixture was heated at 110 °C for

30 min and then at 50 °C for 2 h. A TLC examination of this mixture showed complete disappearance of bromide **5** and formation of a new product. Purification of this impure product on a silica gel column using sequential gradients of ethyl acetate (up to 14%) in hexanes afforded 0.43 g (52.8%) of pure **6**. R_F 0.44 (solvent system B); m.p. 180–182 °C. ^{1}H NMR ($CDCl_3$): 2.04, 2.07 and 2.13 3 × s, 3 H ($COCH_3$); 3.84 s, 3 H (OCH_3); 3.96 d, 1 H, J(4′,5′) = 9.5 (H-4′); 5.28–5.50 m, 4 H (H-1′, H-2′, H-3′ and H-5′); 7.68 s, 2 H (aromatic H-3 and H-5). ^{13}C NMR ($CDCl_3$): 20.42, 20.47, 20.77 (3 × $COCH_3$), 52.82 ($COOCH_3$), 69.26 (C-4′), 71.24 (C-2′), 71.90 (C-5′), 72.66 (C-3′), 100.35 (C-1′), 118.87 (aromatic C-4), 119.23 (aromatic C-2 and C-6), 135.39 (aromatic C-3 and C-5), 148.92 (aromatic C-1), 166.50, 169.19, 169.32 (3 × C=O), 170.09 (COOH). For $C_{19}H_{19}Br_3O_{10}$ (647.0) calculated: 35.27% C, 2.90% H; found: 35.31% C, 2.85% H.

2,4,6-Tribromophenyl β-D-Glucopyranosiduronic Acid (**7**)

A cold (–5 to 0 °C) solution of sodium hydroxide (0.03 g, 0.77 mmol) in methanol (2 ml) was added to a methanolic solution (2 ml) of **6** (0.1 g, 0.15 mmol) and the reaction mixture was stirred at 40 °C overnight. A chromatographic examination of the reaction mixture showed complete conversion of **6** to **7**. The solution was deionized with KA-2-(H^+) ion exchange resin, filtered and the solvent was removed in vacuo, to provide impure **7**. Purification on a silica gel column, using solvent system A as eluent, yielded 54.5 mg (69.6%) of pure **7**. R_F 0.65 (solvent system A); m.p. 185 °C (dec.). ^{1}H NMR (D_2O): 3.49 dd, 1 H, J(2′,3′) = 5.5, J(4′,3′) = 3.5 (H-3′); 3.52 dd, 1 H, J(3′,4′) = 3.5, J(5′,4′) = 9.5 (H-4′); 3.58 d, 1 H, J(4′,5′) = 9.5 (H-5′); 3.68 dd, 1 H, J(1′,2′) = 7.5, J(3′,2′) = 5.5 (H-2′); 5.20 d, 1 H, J(1′,2′) = 7.5 (H-1′); 7.76 s, 2 H (aromatic). ^{13}C NMR (D_2O): 72.38 (C-4′), 74.36 (C-2′), 76.13 (C-5′), 77.50 (C-3′), 103.80 (C-1′), 118.75 (aromatic C-2 and C-6), 119.14 (aromatic C-4), 136.06 (aromatic C-3 and C-5), 149.80 (aromatic C-1), 171.60 (COOH). FAB HRMS for $C_{12}H_{11}Br_3O_7$ (506.9), M^+ relative abundance 0.3%.

Enzymatic Hydrolysis

Enzymatic hydrolysis of bromophenyl glucuronic acids was studied using β-glucuronidase enzyme (500 units/ml in buffer) which was prepared by dissolving 1000 units of β-glucuronidase in 2 ml of 0.1 M acetate buffer (pH 5.0). The bromophenyl glucuronide (**2**, **3** or **7**, 1 mmol) solution was prepared with the same buffer. The hydrolysis was initiated by addition of β-glucuronidase to the bromophenyl glucuronic acids' solutions. The mixture was sampled at selected intervals. For analysis these samples were mixed with an equal volume of ice-cold methanol and then vortexed. After centrifugation at 12,800 rpm (Brinkman Microfuge) for 2 min, the supernatant was collected and frozen until analysis. The analysis was performed by high performance liquid chromatography (HPLC) using a C18 Radial-PAK cartridge (8 mm i.d., 10 μ particle size) with UV detection at 298 nm (di- and tribromophenyl glucuronic acids) or 280 nm (monobromophenyl glucuronic acid). A gradient system of water–methanol gradients (0–9 min, 20% methanol; 9–40 min, 20 to 80% methanol, linear increase; 40–60 min, 80% methanol) was used for elution.

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