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Glycosylation of bisphenol A by tobacco BY-2 cells

Nobuyoshi Nakajima, Yukiko Oshima, John S. Edmonds *, Masatoshi Morita

National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

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Abstract

Tobacco BY-2 cells in suspension culture absorbed and transformed bisphenol A dissolved in the culture medium. Major products were bisphenol A mono-*O*-β D-gentiobioside and the trisaccharide bisphenol A mono-*O*-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranoside. Also produced were the mono- and di- *O*-β-D-glucopyranosides. As glycosides of bisphenol A lack the estrogenic activity of the parent compound, these findings enhance the possibilities of phytoremediation of natural waters contaminated by bisphenol A.

Keywords: *Nicotiana tabacum* L.; Solanaceae BY-2 cells; Suspension culture; Bisphenol A; Glycosylation; Bisphenol A mono-*O*-β-D-gentiobioside; Bisphenol A mono-*O*-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranoside

1. Introduction

Bisphenol A (4,4′-isopropylidenediphenol, BPA; CAS No. 80-05-7) (Fig. 1, structure **1**) is used as a monomer in the manufacture of polycarbonate and epoxy resins, as a stabilizer or antioxidant for many types of plastics such as polyvinyl chloride (PVC), and as an inhibitor of end oxidation in PVC (Ash and Ash, 1995). Annual production capacity of this compound in the USA is about 865 000 tonnes (http://www.bisphenol-a.org). As early as 1936 BPA (**1**) was shown to have estrogenic activity in vivo when administered to rats by sub-cutaneous injection (Dodds and Lawson, 1936). Subsequent studies have confirmed its estrogenic properties (Matthews et al., 2001).

BPA (**1**) is released to the environment both accidentally and through permitted discharges (Staples et al., 1998) and its widespread distribution has been a major cause of concern to regulatory agencies and others (Safe, 2000). Its lifetime in the environment is sufficient for it to be virtually always detectable and it has been described as ubiquitous in surface waters (Kuch and Ballschmiter, 2001).

As part of a study into possible phytoremediation of contaminated water, we have recently shown that tobacco (*Nicotiana tabacum* L.) plants and cultured BY-2 cells rapidly absorb BPA (**1**) from the water to which their roots are exposed or from the culture medium, and metabolize it to a number of glycosidic compounds (Nakajima et al., 2002). One of these compounds was identified as BPA mono-*O*-β-D-glucopyranoside (**2**) We now report on the isolation and structure elucidation of three further glycosidic biotransformation products (**3**–**5**) of BPA (**1**) produced by tobacco BY-2 cells in suspension culture.

2. Results and discussion

HPLC examination of the methanol extract of cultured tobacco BY-2 cells exposed to BPA revealed 3 major peaks (A, B and C) when absorption at 217 nm was monitored. Although peaks C and B each contained a single compound (compounds **2** and **3** respectively), rechromatography of peak A under different conditions showed the presence of five compounds. Of these only two (compounds **4** and **5**) were in sufficient quantity for present examination.

* Corresponding author. Tel.: +81-298-50-2860; fax: +81-298-50-2870.
E-mail address: edmonds.john.s@nies.go.jp (J.S. Edmonds).

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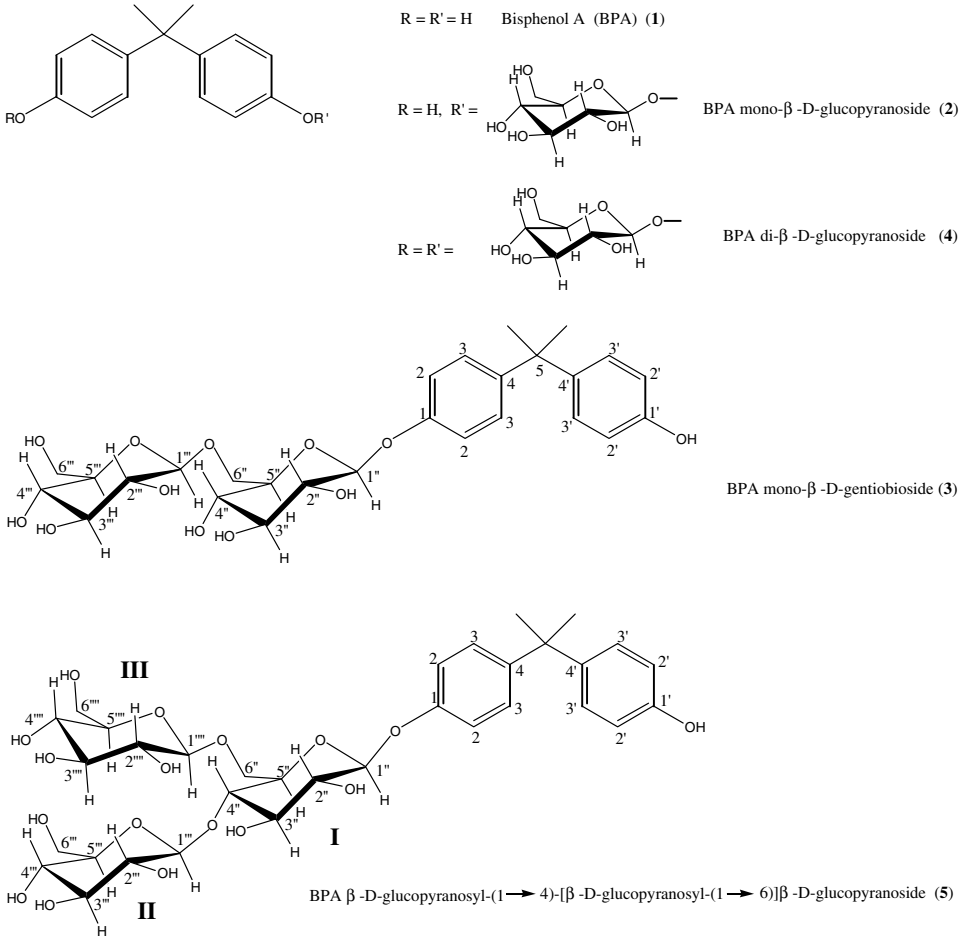

Fig. 1. 4,4′-Isotopropylidenediphenol (bisphenol A, BPA) (**1**) and products of its transformation by tobacco BY-2 cells (**2**–**5**).

The single compound (**2**) contained within peak C was identified as the previously reported BPA mono-β-D-glucopyranoside (Nakajima et al., 2002) by comparison of its ^{1}H NMR spectrum with that of an authentic synthetic specimen (Morohoshi et al., 2003).

The FAB MS spectrum of **3** revealed a molecular weight of 552 indicating that it was possibly either a di-mono-hexoside of BPA (**1**) (i.e., di-conjugated and symmetrical) or a mono-di-hexoside (mono-conjugated and asymmetrical). The ^{1}H NMR spectrum consisted of 28 non-exchangeable protons consisting of 4 signals, each of 2 protons, in the aromatic region (δ 6.82 to δ 7.27), and a 6-proton singlet at δ 1.63 which were readily assigned to the BPA residue; and signals for the remaining 14 protons at δ 3.29 to δ 5.08 which could be accommodated by the presence of a sugar moiety.

The presence of the 4 signals, each showing *ortho*-coupling, in the aromatic region of the spectrum, showed that the BPA was mono-conjugated, and thus most likely a mono-disaccharide, a conclusion consistent with the observation of 14 non-exchangeable protons in that part of the spectrum attributed to the sugar. Of the sugar resonances, of particular diagnostic value were one proton signals at δ 4.49 and δ 5.08, each showing spin-spin coupling of 8 Hz, which were readily assigned (Collins and Ferrier, 1998) as those of the anomeric protons of two sugar (hexose) residues. The spin coupling constants demonstrated that both linkages, that of the first hexose residue to a BPA phenolic hydroxyl group and that of the first hexose residue to the second, were β-orientated. A ^{1}H–^{1}H COSY NMR experiment facilitated the assignment of all proton signals in both hexose residues (see Section 3) and the identification of the resonances resulting from the methylene in the 6-position of the first residue suggested, because of their chemical shifts, that the linkage between the two hexoses was 1–6 (Bock and Thøgersen, 1982). Furthermore, the chemical shifts of the all individual protons in both hexoses indicated that both were glucose. Certainly, the match with glucose, in both cases, was closer than with either galactose or mannose. Available model compounds, 4-nitrophenyl-β-D-cellobioside and amygdalin – a gentiobioside, indicated that compound **3** was not a cellobioside but did not confirm that it was a gentiobioside. However the ^{13}C NMR spectrum of **3** confirmed the 1–6 linkage between the two hexose residues (shieldings of δ 61.01 ppm for the meth-

ylene carbon, C6, with unsubstituted hydroxyl group on the second hexose residue and δ 68.57 ppm for C6 carbon in the 1–6 link) (Orihara et al., 1992) and again indicated that both hexoses were glucose (Bock and Thøgersen, 1982). Acid hydrolysis of **3** (DCl/D_2O), carried out in an NMR tube and monitored by ^1H NMR, gave BPA and glucose as the only hydrolysis products. It was concluded on the basis of these data that compound **3** is the previously unreported bisphenol A mono-β-D-gentiobioside.

The FAB MS spectrum of compound **4** also revealed a molecular weight of 552. But in this case the ^1H NMR spectrum, although again showing 28 non-exchangeable protons, revealed in the aromatic region (2 resonances, each of 4 protons) that the BPA molecule was di-conjugated and symmetrical. The spectrum was identical to that of a sample of BPA di-β-D-glucopyranoside obtained by synthesis (Morohoshi et al., 2003).

The electrospray MS of compound **5** revealed a molecular weight of 714 which suggested that it was a trisaccharide of BPA (BPA plus three hexose residues). The ^1H NMR spectrum showed 35 non-exchangeable protons; 14 of these were readily attributed to a BPA moiety (8 aromatic protons and 2 methyls) and demonstrated that the BPA was asymmetrical and probably mono-conjugated. A ^1H–^1H COSY experiment enabled the assignment of all 21 non-exchangeable protons in the 3 sugar groups. The 3 most deshielded protons were easily assigned as the anomeric protons of the sugars and their spin coupling constants ($J_{H1,H2}$) showed that all linkages (sugar to aglycone and sugar to sugar) were β-orientated. The anomeric proton of the hexose residue bonded directly to the aromatic nucleus was identified as the most deshielded (δ 5.39). The chemical shifts of the 6_A and 6_B (methylene) protons of this residue revealed that a further hexose group was linked at the 6-position. The shifts of all protons in this further hexose (III) showed that it was not further substituted and that there was a close correspondence between the chemical shifts of all of its protons and those in the distal hexose of BPA mono-β-D-gentiobioside (compound **3**). Thus, that part of the trisaccharide composed of hexose residue I (bound to BPA) and residue III was directly analogous to the gentiobioside (**3**) with residue III not further conjugated. It followed that the trisaccharide (compound **5**) was branched with hexose residue I being further conjugated to residue II. Inspection of the chemical shifts of hexose I, and making the reasonable assumption that it was glucose (an assumption confirmed by subsequent acid hydrolysis), it was apparent that hexose residue III was linked to residue I at the 4-position. Thus a downfield shift, compared with glucose, of 0.54 ppm was seen for the 4-proton. This compared with a shift of 0.55 ppm for the equivalent proton in 4-nitrophenol-β-D-cellobioside and 0.44 ppm in β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranose (Percy et al., 1998). The free 6_A and 6_B (methylene) protons of residue II were more shielded (by about 0.4 and 0.7 ppm) than the free 6-protons in residue III and in β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranose (Percy et al., 1998). This observation suggested that the conformation of compound **5** in solution had the 6-protons of residue III close, and perpendicular, to one aromatic ring of the BPA moiety. Simple model making suggested the unconjugated BPA (**1**) ring might be involved. Acid hydrolysis of compound 3 (DCl/D_2O) yielded only BPA (**1**) and glucose. On the basis of these data the BPA trisaccharide (compound **5**) was concluded to be BPA mono-*O*-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranoside.

Eucalyptus perriniana cells in culture have been shown (Hamada et al., 2002) to hydroxylate BPA (**1**) in the 3-position and convert the later and BPA (**1**) to mono-and di β-D-glucopyranosides. BPA (**1**) was converted to compounds assumed to be carbohydrate conjugates (but not identified) by suspension cultures of cells of several plants including soybean (*Glycine max*), and wheat (*Triticum aestivum*) (Schmidt and Schuphan, 2002). However, the work reported here is the first to reveal mono di- and tri-saccharides of BPA (**1**). In addition, to our knowledge, this is the first report of a β-D-glucopyranosyl- (1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranoside conjugate (compound **5**) produced by plant tissue in response to exposure to a foreign aromatic compound. Glycosylation of xenobiotic phenols increases their water solubility and this may be integral to a detoxification process (Williams, 1967). The biosynthesis of complex glycosides of a phenol of low water solubility such as BPA (**1**) confers greater solubility than does transformation to a simple glucoside. Thus there may be advantages in the biosynthesis of complex rather than simple glycosides.

A previous study has shown that glycosides of BPA lack the estrogenicity of the parent compound (Morohoshi et al., 2003). Although this work further demonstrates the potential use of plants for remediation of contaminated environmental waters, previous work (Pridham, 1964) on the phenol glucosylation reaction in a wide range of plants suggests that the ability of aquatic plants to glycosylate xenobiotic phenols cannot be assumed. This is particularly so as algae and some aquatic angiosperms were reported (Pridham, 1964) to lack the phenol glucosylation reaction.

3. Experimental

3.1. General

NMR spectra were recorded in D_2O on a JEOLECA 800 spectrometer (JEOL, Tokyo, Japan) operating at

800 MHz (^{1}H) or 200 MHz (^{13}C). Shifts are reported relative to external TMS. ^{1}H assignments for compounds **3** and **5** were aided by ^{1}H–^{1}H COSY experiments. FAB+ MS were measured on A JEOL JMS-700 GC/MS (JEOL, Tokyo, Japan). ES MS was measured on a Quattro Ultima instrument (Micromass, UK). 4-Nitrophenyl-β-D-cellobioside and D-mandelonitrile β-gentiobioside (amygdalin) were obtained from Fluka Chemie AG, Buchs, Switzerland and Wako Pure Chemicals, Osaka, Japan, respectively. Solvents used were HPLC grade and were not further purified.

3.2. Plant cell culture, extraction and isolation of transformation products

Tobacco BY-2 cells were cultured in Murashige-Skoog medium 16 μM in 2,4-D (MSD medium) (11 of 50 ml bottles) for 2 weeks in the dark at 25 °C. BPA (**1**) was then added to each flask to a concentration of 10 mg/1 and the flasks were incubated with gentle . shaking for a further 24 h. The plant cells (120 g wet weight) were removed by filtrate and extracted with MeoH (360 ml). BPA (**1**) and its metabolites in the medium and in the methanolic extract of the cells were examined by HPLC (C-18 ODS reversed phase column, 150 × 3.9 mm, 40 °C, Symmetry, Nihon Waters, Tokyo, Japan) with aq. MeOH–H_2O (2:3) as mobile phase and a flow rate of 1 ml/min. Absorption at 217 nm was monitored. Three major peaks were observed and these were designated as A (retention time 4.70 min), B (8.77 min; compound **3**) and C (14.79 min; compound **2**), accounting for 4.5%, 70% and 11% of transformed BPA (**1**) respectively. Although subsequent operations showed that peaks B and C each consisted of a single compound, rechromatography of A on the ODS column with 25% MeOH–H_2O (1:3) as mobile phase revealed five compounds (retention times 13.00, 14.43, 20.13, 22.16 and 24.07 min). Of these only two were present in sufficient quantity (22.16 min, compound **4**, 49.5% of A; 24.07 min, compound **5**, 26% of A) to allow spectroscopic analysis. Thus BPA metabolites **2**–**5** were isolated from from the methanolic extract of the cells by repeated HPLC on the C-18 ODS column and the accumulated metabolites were examined by FAB MS, LC/ES MS,^{1}H NMR and ^{13}C NMR.

3.3. Acid hydrolysis

Portions of compounds **3** and **5** (approximately 100 μg) were each dissolved in D_2O (0.5 ml) and placed in an NMR tube. Concentrated DCl (100 μl) was added and tube maintained at 80 °C for 12 h. The ^{1}H NMR spectrum then showed the presence of BPA and glucose (α and β). No other compounds were present.

3.4. 4,4′-Isopropylidenediphenol mono-O-β-D-gentiobioside (compound 3)

FAB MS *m/z* 551 [M − 1], 553 [M + 1]. ^{1}H NMR(D_2O) δ 1.63 (6H, *s*, 2 × Me), 3.29 (1H, *dd*, *J* = 9.1), 9.1 Hz, H2‴, 3.37 (2H, *m*, H4‴,H5‴), 3.44 (1H, *m*, H3‴), 3.56 (2H, *m*, H2 ″H4″), 3.60 (1H, *dd*, *J* = 9.2, 9.1 Hz, H3″), 3.67 (1H, *dd*, *J* = 11.0, 3.7 Hz, H‴6_A), 3.78 (1H, *dd*, *J* = 8.2, 5.5 Hz, H5″), 3.87 (1H, *dd*, *J* = 11.9, 2 Hz, He″), 3.89 (1H, *dd*, *J* = 11.9, 11.9 Hz, H$6''_A$), 4.18 (1H, *dd*, *J* = 11.0, 2 Hz, H$6''_B$), 4.49 (1H, *d*, *J* = 8.2 Hz, H1‴), 5.08, (1H, *d*, *J* = 8.2 Hz, H1″) 6.82 (2H, *d*, *J* = 8.2 Hz, 2 × H2′), 7.06 (2H, *d*, *J* = 8.2 Hz, 2 × H2), 7.19 (2H, *d*, *J* = 8.2 Hz, 2 × H3′), 7.27 (2H, *d*, *J* = 8.2 Hz, 2 × H3). ^{13}C NMR (D^2O) δ 30.24 (2 × Me), 41.57 (C5), 61.01 (C6″), 68.57 (C6‴), 69.61, 69.94 (C4″, C4‴), 73.15 (C2‴), 73.40 (C2″), 75.49. 75.75, 75.95, 76.14 (C3″, C3‴, C5″, C5‴), 100.52 (C1″),100.52 (C1″), 102.86 (C1‴), 115.30 (2 × C2′), 116.48 (2 × C2), 128.29 (2 × C3′),128.33 (2 × C3), 143.14 (C4′), 146.41 (C4), 153.85 (C1), 154.60 (C1′).

3.5. Bisphenol A mono-O-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 6)] β-D-glucopyranoside (compound 5)

ES MS *m/z* 713 $[M-H]^-$, 732 $[M+NH_4]^+$, 737 $[M+Na]^+$. ^{1}H NMR (D_2O) δ 1.66 (6H, s, 2 × Me), 2.98 (1H, *dd*, *J* = 11.9, 2 Hz, H$6''_A$), 3.25 (1H, *dd*, *J* = 10.1, 2 Hz, H5‴), 3.28 (1H, m, H2⁗), 3.31 (1H, m, H2‴). 3.37 (2H, m, H4⁗, H5⁗), 3.43 (1H, m, H4‴), 3.44 (1H, m, H3⁗), 3:48 (1H, m, H$6'''_B$), 3.51 (1H, *dd*, *J* = 9.2, 9.1 Hz, H3‴), 3.61 (1H, *dd*, *J* = 9.1, 9.2 Hz, H3″), 3.68 (1H, m, H$6'''_A$), 3.81 (1H, m, H2″), 3.86 (2H, m, H4″, H5″), 3.88 (1H, m, H5⁗), 3.91 (1H, *dd*, *J* = 11.9, 11.9 Hz, H$6''_A$), 4.20 (1H, *dd*, *J* = 11.9, 2 Hz, H$6''_B$), 4.51 (1H, d, *J* = 8.2 Hz, H1⁗), 4.84 (1H, d, *J* = 8 Hz, H1‴), 5.39 (1H, *d*, *J* = 7.4 Hz, H1″), 6.80 (2H, *dd*, *J* = 8, 2 Hz, 2 × H2′), 7.07 (2H, *d*, *J* = 8.2 Hz, 2 × H2), 7.21 (2H, *dd*, *J* = 8, 2 Hz, 2 × H3′), 7.31 (2H, *d*, *J* = 8.2 Hz, 2 × H3).

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