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Purification of Polybrominated Biphenyl Congener 2

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Numerous toxicological studies of polybrominated biphenyls (PBB's) followed the 1973-74 accidental exposure of Michigan residents to Firemaster, a flame-retardant mixture of PBB's, in the food supply.

In this laboratory, the effective toxic activity of polyhalogenated aromatic compounds (PHA's) has been monitored with a rabbit ear test for keratogenic potential (Kimbrough et al 1977; Hill et al 1981). Rabbit ear hyperkeratosis is produced by the same compounds as those associated with chloracne in humans (Adams et al 1941). The principal congener (60%) of Firemaster, 2,2',4,4',5,5'-hexabromobiphenyl (PBB 4), administered in a 10-mg dose, did not produce hyperkeratosis of the rabbit ear. However, PBB 4 undergoes photodegradation, and its sunlight photolysis products did exhibit keratogenic activity (Patterson et al 1981). The individual compounds of the photolysis products must be isolated, identified, and purified before specific conclusions can be made from the bioassay. Here we describe a procedure for isolating the congener 2,3',4,4',5-pentabromobiphenyl (PBB 2) from the PBB 4 photolysis mixture.

Our primary objective was to develop a PHA congener separation method with selectivity related to molecular conformation, since coplanar conformation of halogenated biphenyls has been suggested as a requisite for toxicity (McKinney 1981). Halogen substitution in the position ortho to the biphenyl bridge decreases the likelihood that the two rings will freely rotate into coplanar arrangement. The lack of toxicity shown by PBB 4, which has two bromine atoms in ortho positions, follows the generalization in that regard. Chromatographic adsorbents which had been reported to selectively retard planar moieties were successfully used to separate PBB 2 from the other pentabromobiphenyls in the photolysis mixture.

Octadecylsilyl (ODS) high performance liquid chromatography (HPLC) columns (reversed phase) and charcoal adsorption chromatography are included in the methods reported. The result of the rabbit ear assay of PBB 2 is also reported, and its significance is discussed.

MATERIALS AND METHODS

Water used in mobile-phase solvents was purified by the Milli-Q Reagent Water System. All other solvents were purchased from Burdick and Jackson.

The PBB 4 congener was purified by repeated recrystallization from Firemaster, FF-1, to 96% purity by gas chromatography; the equal response of PBB congeners by flame ionization detection (FID) was assumed. The purified PBB 4 (300 mg) in 1.5 liters of hexane was allowed to photodegrade in a quartz vessel in the sunlight for 17 hours. The hexane solution was flash evaporated to dryness, leaving a crystalline residue. This material was dissolved in organic solvents for separation procedures.

Gas chromatography (GC) was performed on photolysis mixtures and fractions by using a 6-ft x 2-mm ID glass column packed with 3% SP-2250 DB on 100/120 mesh Supelcoport (Supelco). The Perkin-Elmer 3920 Gas Chromatograph equipped with FID was operated isothermally at 250°C. Helium was the carrier gas at 50 ml/min. Fractions were also analyzed on the Shimadzu GC - 6 AM with ^{63}Ni - ECD and N_2 as carrier, and a glass column, 5-ft. x 3-mm ID, was packed with the same material. The N_2 flow rate was 30 ml/min and the column temperature, 250°C.

Capillary GC was performed on the Hewlett-Packard 5840. The DB 1701 fused silica column (25-m x 0.32-mm ID, J & W) was used with the ^{63}Ni-ECD. Linear velocity of the He carrier gas was 40 cm/sec. The temperature program was from 250° to 280°C at 8°/min. Argon-methane served as makeup gas for the detector. The GC instrument was interfaced with the ZAB-2F double-beam focusing mass spectrometer with a SE 54 fused silica column (25-m x 0.32-mm ID, J & W). Mass spectra were examined to confirm the number of bromine substitutions per HPLC peak fraction.

The nuclear magnetic resonance (NMR) analysis was performed on purified PBB 2 in deuterated acetone, as previously described (Orti et al 1983).

For reversed phase HPLC (RPLC) we used a Waters 6000A pump at 0.6 ml/min mobile phase, propanol-2:water (7:3). Two columns, 25-cm x 4-mm ID, were placed in series: Whatman Partisil PXS 10/25 ODS-2 and Partisil PXS 5/25 ODS. Column eluant composition was monitored on two detectors: LDC Spectromonitor III UV at 261 nm and LDC UV-III (zinc lamp) at 214 nm. Sample volumes of 50 microliters were introduced via a Rheodyne M 7125 Injector into the HPLC system. Effluent fractions were collected and evaporated to dryness with heat (50°C) and a gentle stream of N_2. The residue was dissolved in hexane for GC. The collection and storage vessels were wrapped with aluminum foil to protect them from light.

Coconut charcoal (60/80 mesh, Analabs) was poured into dichloromethane in a glass column, 1.0-cm ID, to a height of 20 cm; it was

washed with 100 ml of dichloromethane:propanol-2 (7:3, Solvent A). Washing was continued with 50 ml toluene and 50 ml more of Solvent A. The PBB mixtures were applied in a small volume (20-50 ul) of acetone. Solvent A was applied in two 25-ml portions and collected as the first fraction (50 ml). Elution of the sample continued with the collection of a 50-ml portion of a mixture of Solvent A:toluene (8:1), 50 ml of a mixture 4:1, and a fourth 50-ml toluene fraction. All fractions were concentrated by evaporation and then dissolved in hexane for GC or in hexane:propanol-2 (1:5) for RPLC fractionation.

The procedure for assaying keratogenic activity of compounds has been published (Hill et al 1981). We used two experimental and two control rabbits. The purified and concentrated fractions of PBB 2 in hexane were pooled and dried to constant weight. The dry material was dissolved in toluene (1.95 mg in 2.0 ml) before dosing. The dose used was 1 mg per rabbit ear.

RESULTS AND DISCUSSION

Congener PBB 2 was successfully separated from the PBB 4 photolysis mixture with charcoal pretreatment and RPLC. The GC identities and relative proportions of major PBB constituents in the photolysis mixture are shown in Table 1.

Table 1. Percent Composition* of the PBB 4 Photolysis Mixture

Congener*	PBB Isomer	Relative Abundance (%)
1	2,2',4,5,5'	11.6
1[b]	2.2',4,4',5	3.9
1c	3,3',4,4'	3.2
2	2,3',4,4',5	8.8
4	2,2',4,4',5,5'	66.6
Other	tetras	5.6

*Based on GC-FID peak area only.

The RPLC fractionation of the photolysis mixture is shown in Figure 1a.

Figure 1b demonstrates the effect of the charcoal fractionation on the original photolysis mixture. Column effluent volumes corresponding to peaks were collected and analyzed by GC for identifying congeners (Figure 1). Tanaka et al (1982) had reported a selectivity of the ODS columns in RPLC that correlates with planarity of polynuclear aromatic hydrocarbons (PAH's); therefore, we used that stationary phase. PBB 2 appeared to be isolated from the other congeners (Figure 1); however, the use of two columns in series eliminated any detectable trace of PBB 4 in the PBB 2 fraction collected.

The purity of PBB 2 was checked by capillary GC-ECD and MS. The ECD chromatogram is shown in Figure 2. The mass spectrum was that of a pentabromobiphenyl.

Spectrophotometric characteristics of the RPLC fraction PBB 2 were measured in the UV detector. There were absorbance maxima at 192 and 218 nm and minima at 206 and 305 nm. Mobile-phase solvent was the reference.

The NMR spectrum of PBB 2 in hexadeuteroacetone is shown in Figure 3. There was a singlet at δ8.09 for a proton at 3 position. There was a singlet at δ7.79 for the proton at 6 position. The 2' proton gave a doublet (J 2',6' = 2.1) at δ7.81 and the 5', a doublet (J 5',6' =8.2) at δ7.85. A doublet of doublets (J 6',2' = 2.2, J 6',5' = 8.4) observed for the 6' proton was centered at δ7.40.

Although the RPLC system we used separated PBB 2 from the other congeners in the mixture, fractionation with charcoal minimized the loading of excessive PBB's on the HPLC column. Charcoal adsorbed all the biphenyls applied.

Desorption of doubly ortho-substituted hexabromobiphenyls and pentabromobiphenyls was readily accomplished in Solvent A and in the 8:1 and 4:1 mixtures of Solvent A:toluene. The tetrabromobiphenyls, lower molecular weight PBB's, also eluted in Solvent A (Table 2). The final toluene fraction was greatly enriched in the singly ortho-brominated PBB 2 (relative to the composition of the photolysis mixture, Fig. 1b).

Table 2
Percent[1] PBB Congener Recovery from Charcoal Adsorption of PBB 4 Photolysis Products

Fraction[2]	PBB Congener 1	1b & 1c	2	4
1 Solvent A[3]	35	50	nd	28
2 A:toluene (8:1)	16	32	nd	18
3 A:toluene (4:1)	24	nd[4]	nd	29
4 toluene	20	nd	67	27
5 toluene	6	nd	22	13

[1]GC-FID peak area ratios
[2]Fractions were 50 ml each for a 10-mg application of photolysis mixture on 5- to 10-g charcoal.
[3]Methylene chloride:propanol-2 (10:3)
[4]None detected

The purified PBB 2 was tested for toxicity in the rabbit ear assay and found to be negative at a 1-mg dose. This dose is equivalent to a greater amount of PBB 2 than was contained in the 10-mg dose of PBB 4 photolysis products that showed positive results on rabbit ears (Patterson et al 1981). Our results 1) indicate that PBB 2 does not contribute to the hyperkeratotic activity observed in Firemaster (Kimbrough et al 1977) and 2) again support our supposition that other minor PBB's in Firemaster are responsible for its toxic effects (Orti et al 1983).

Needham et al (1982) suggest that keratogenic activity of a toxicant in rabbit ears may parallel its effectiveness in 3-methylcholanthrene (MC)-type microsomal enzyme induction. PBB 2 has been reported to be a mixed MC- and phenobarbital-type microsomal enzyme inducer (Robertson 1980; Dannan 1982). Recently, Mills et al (1983) found that when PBB 2 was admininistered in vitro, it was not metabolized by liver microsomes from rats treated with MC. The PBB congeners that were metabolized had adjacent nonhalogenated ortho and meta carbons on at least one ring. The latter findings and our observations, including those of Needham et al (1982), suggest that the preparations used for earlier PBB 2 tests for enzyme induction might have been contaminated with PBB congeners that are potent inducers.

RPLC appears to hold great potential for separating PBB congeners and other PHA's. With RPLC of Firemaster, PBB 6 can also be isolated and tested.

Charcoal adsorption-desorption techniques can be used to advantage in fraction enrichment, and they show potential in separating PBB congeners. We find that charcoal binds the planar (toxic) congeners more strongly than other conformers.

In PBB congeners that previously lacked bromine in the ortho position and exhibited toxicity, ortho-bromination abolishes the toxic effect. This is the difference between PBB 2 and PBB 1c, a highly keratogenic congener (Orti et al 1983). On the basis of these observations, we believe that PBB 6, 2,3',4,4',5,5'-hexabromobiphenyl--an ortho-brominated PBB--would provide results similar to those provided with PBB 2.

RPLC and charcoal adsorption provide so much versatility with mobile phase or desorbant that many heretofore laborious methods of separating PBB's and similar congeners have been obviated. The advantages of RPLC far outweigh those of previous methods used for PBB congener separation.

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