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1,4-BIS[2-(3,5-DICHLOROPYRIDYLOXY)]BENZENE (TCPOBOP) AND RELATED COMPOUNDS AS INDUCERS OF HEPATIC MONOOXYGENASES

STRUCTURE–ACTIVITY EFFECTS

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Abstract—1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) resembles phenobarbital (PB) in its mode of induction of the hepatic drug-metabolizing enzymes in mice. The structural features of this molecule include: a linear tricyclic aromatic ether ring system, an internal 1,4-disubstituted benzene ring and two 3,5-dichloropyridyloxy substituents. Ten analogs of TCPOBOP have been synthesized and their activities as microsomal enzyme inducers evaluated. Dose–response induction of mice hepatic microsomal cytochrome P-450, aldrin epoxidase and dimethylaminoantipyrine *N*-demethylase gave ED_{50} values for TCPOBOP and five homologs. The results illustrate that changes in the structure of the pyridyloxy ring markedly affect enzyme induction activity. The order of activity for the substituents was 3,5-dibromopyridyloxy ~3,5-dichloropyridyloxy > 5-bromopyridyloxy ~5-chloropyridyloxy > 3-chloropyridyloxy > pyridyloxy. In addition, the effects of altered substitution pattern of the benzene ring and structural alterations of the internal ring moiety were evaluated by measuring hepatic microsomal coumarin hydroxylase activity. The results confirm the microsomal monooxygenase enzyme induction activity of TCPOBOP, and the observed structure-dependent potencies of several related homologs support a receptor-mediated mechanism of action for the process.

The hepatic cytochrome P-450 monooxygenase system is embedded in the endoplasmic reticulum and nuclear envelope of the cell and is responsible for the conversion of lipophilic endogenous and exogenous compounds to more readily excreted polar metabolites. This system consists of a flavoprotein, NADPH-cytochrome P-450 reductase, and a group of closely related heme-containing isozymes, the cytochromes P-450. Cytochrome P-450 represents the terminal oxygenase of this system in which reducing equivalents are transferred from reduced pyridine nucleotide to cytochrome P-450 via the reductase [1–6].

The induction of several isozymes of cytochrome P-450 by 3-methylcholanthrene (3-MC) and related polynuclear aromatic hydrocarbons and by certain polyhalogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been well documented [7–12]. The major isozyme induced by these compounds in the rat is cytochrome P-450c. This isozyme can be distinguished from cytochrome P-450b, the major phenobarbital (PB) inducible isozyme, by spectral, electrophoretic, immunologic and catalytic criteria [7–11, 13–17]. There is considerable evidence that the induction by 3-MC-type inducers is mediated by a high-affinity, low-capacity cytosolic receptor protein [18–25]. The use of an extremely potent agonist (TCDD) in a competitive binding assay has allowed the characterization of this receptor. Additional support for a receptor-mediated mechanism has been provided by genetic differences among inbred mouse strains and by structure–activity relationships within several classes of halogenated aromatic compounds including polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polybrominated biphenyls and polychlorinated biphenyls (PCBs) [18–22, 26–31].

In contrast to 3-MC-type induction, the mechanism of induction of microsomal monooxygenases by PB is not well understood. Pretreatment of rats with PB and a variety of structurally diverse xenobiotics, including polychlorinated biphenyls and organochlorine insecticides, produces a dramatic increase in cytochrome P-450b [7–11, 13–17]. Experimental evidence suggests that the PB-associated increase in microsomal cytochrome P-450b content can be attributed to an augmentation of cytochrome P-450b gene transcription [32–34]. It may be hypothesized that PB and inducers which produce a response similar to PB act via a receptor-mediated mechanism. In support of this hypothesis, recent *in vivo* studies in the rat with a series of 2,4-dichloro-substituted PCBs have demonstrated a marked effect of structure on the activity of the microsomal monooxygenases, dimethylaminoantipyrine (DMAP) *N*-demethylase and aldrin epoxidase [35]. However, the absence of a discernible structure–activity relationship among the various classes of inducers which evoke this response argues against the role of a receptor. The lack of a potent agonist had been taken as additional indirect evidence against a receptor hypothesis. Unlike TCDD, which has an ED_{50} of 1×10^{-9} moles/kg for AHH induction in the mouse, the ED_{50} for PB induction of DMAP *N*-demethylase is 3.3×10^{-4} moles/kg [36]. Recently, a highly potent

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Table 1. Summary of synthetic procedures

Reactants*	
2,3,5-Trichloropyridine† and hydroquinone	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
2,3,5-Tribromopyridine† and hydroquinone	1,4-bis[2-(3,5-dibromopyridyloxy)]benzene
2,5-Dichloropyridine and hydroquinone	1,4-bis[2-(5-chloropyridyloxy)]benzene
2,5-Dibromopyridine and hydroquinone	1,4-bis[2-(5-bromopyridyloxy)]benzene
2,3-Dichloropyridine and hydroquinone	1,4-bis[2-(3-chloropyridyloxy)]benzene
2-Chloropyridine and hydroquinone	1,4-bis[2-(pyridyloxy)]benzene
2,3,5-Trichloropyridine† and 2-chlorohydroquinone	1,4-bis[2-(3,5-dichloropyridyloxy)-2-chlorobenzene
2,3,5-Trichloropyridine† and 1,4-dihydroxynaphthalene	1,4-bis[2-(3,5-dichloropyridyloxy)]naphthalene
2,3,5-Trichloropyridine and 4,4′-biphenyldiol	4,4′-bis[2-(3,5-dichloropyridyloxy)]biphenyl
2,3,5-Trichloropyridine† and 1,3-dihydroxybenzene	1,3-bis[2-(3,5-dichloropyridyloxy)]benzene
2,3,5-Trichloropyridine† and catechol	1,2-bis[2-(3,5-dichloropyridyloxy)]benzene

* All the reactants were purchased from the Aldrich Chemical Co.
† Prepared via diazotization of their corresponding 2-amino-3,5-dichloro and 2-amino-3,5-dibromopyridines.

inducer of mouse hepatic DMAP *N*-demethylase activity has been identified: 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) [36, 37]. It has been estimated that TCPOBOP is 650 times more potent than PB as inducer of DMAP *N*-demethylase with an ED_{50} of 4.9×10^{-7} moles/kg.

The purpose of this report is to evaluate the structure–activity relationship within a group of TCPOBOP analogs by measuring select microsomal monooxygenase activities and total cytochrome P-450 content.

MATERIALS AND METHODS

Preparation of TCPOBOP and analogs. The halopyridine (2 μmoles), hydroquinone (1 μmole) (see Table 1) and potassium carbonate (180 mg) in dimethyl sulfoxide (3 ml) were heated with stirring at 180° for 1 hr. The mixture was filtered, and the solvent was allowed to evaporate in a fume hood. The residue was redissolved in chloroform, and the reaction product was isolated and purified by thin-layer chromatography. The 1,4-bis[2-(halopyridyloxy)]benzene coupling products (20–50% yields) were crystallized from methanol, and their structures were confirmed by their mass and nuclear magnetic resonance spectra. The purities of all the products (Table 1) were >97% as determined by gas chromatographic analysis.

Biochemicals. NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO). 4-Dimethylaminoantipyrine and semicarbizide hydrochloride were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Sodium phenobarbital was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ). Analytical grade aldrin and dieldrin were provided by the United States Environmental Protection Agency, Health Effects Research Laboratory, Environmental Toxicology Division, Research Triangle Park, NC. Ultra high purity carbon monoxide was obtained from Airco (Houston, TX). All other chemicals and reagents were of the highest quality commercially available.

Animal treatment and isolation of microsomes. Male B6D2F1/J mice 6 weeks of age were purchased from the Jackson Laboratory, Bar Harbor, ME. The mice were housed in plastic cages with hardwood bedding and allowed free access to Purina Rodent Chow, No. 5001, and water. They were maintained on a diurnal cycle of 12 hr of light and 12 hr of darkness.

All compounds were administered intraperitoneally. TCPOBOP and its analogs were dissolved in corn oil and administered on days 1 and 2. Sodium phenobarbital was administered in saline solution on days 2, 3 and 4. Administration of corn oil (10 ml/kg) on days 1 and 2 served as a control. All animals were starved for 24 hr prior to being killed on day 5. The livers were perfused via the hepatic portal vein with 15 ml of ice-cold isotonic saline containing 0.1 mM EDTA. Livers were weighed, homogenized with a Potter–Elvehjem homogenizer in 4 vol. of 0.25 M sucrose containing 0.1 mM EDTA, and centrifuged at 10,000 *g* for 20 min. The 10,000 *g* supernatant fraction was centrifuged for 1 hr at 100,000 *g*, and the microsomal pellet was resuspended in the homogenizing solution. Aliquots of the microsomal suspension were stored at −70° until catalytic or spectral properties were measured.

Assays. DMAP *N*-demethylase activity was determined as previously described [38]. The reduced

cytochrome P-450:CO binding difference spectrum was determined by the method of Omura and Sato [39] using a molecular extinction coefficient of 91 cm^{-1} mM^{-1}. Microsomal aldrin epoxidase activity was measured as the rate of formation of dieldrin as determined by gas chromatography [40]. Coumarin hydroxylase activity was measured by the method of Creaven *et al.* [41] as modified by Wood and Taylor [42]. Protein concentrations were determined by the method of Lowry *et al.* [43] using bovine serum albumin as a standard.

Calculations. Each dose–response experiment included a group of mice pretreated with corn oil and a group pretreated with phenobarbital. Dose–response data were normalized with respect to the control group pretreated with phenobarbital. The mean of enzyme activity or cytochrome P-450 content at each dose level was plotted against the logarithm of dosage level, and the ED_{50} was estimated graphically. The ED_{50} represents the total dose which produces one-half the maximum enzyme induction. The method of Dunnett [44] was utilized to test for statistical significance in the experiment measuring coumarin hydroxylase activity.

RESULTS

Pretreatment of mice with phenobarbital elicited a dose–response induction of hepatic microsomal cytochrome P-450 (Fig. 1). The dose of phenobarbital eliciting half the maximal induction (ED_{50}) of cytochrome P-450 was approximately 530 μmoles/kg. 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, 1,4-bis[2-(3,5-dibromopyridyloxy)]benzene, 1,4-bis[2-(5-chloropyridyloxy)]benzene and 1,4-bis[2-(5-bromopyridyloxy)]benzene also elicited dose-related increases in cytochrome P-450. 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene was the most potent inducer of cytochrome P-450 with an approximate ED_{50} of 0.18 μmole/kg. Although apparently less potent (ED_{50} = 5.0 μmoles/kg) than the chloro analog, 1,4-bis[2-(3,5-dibromopyridyloxy)]benzene produced the largest increase in cytochrome P-450 content with more than a 4-fold increase over control values. 1,4-Bis[2-(5-chloropyridyloxy)]benzene was less active than 1,4-bis[2-(3,5-dibromopyridyloxy)]-benzene with an ED_{50} of 35 μmoles/kg. An estimate of the ED_{50} for 1,4-bis[2-(5-bromopyridyloxy)]benzene was difficult to determine since the highest dose administered (500 μmoles/kg) did not produce a response equivalent to a maximum inducing dose of phenobarbital. Both 1,4-bis[2-(3-chloropyridyloxy)]-benzene and 1,4-bis[2-(pyridyloxy)]benzene demonstrated little activity as inducers of microsomal cytochrome P-450.

Measurement of hepatic microsomal DMAP *N*-demethylase activity demonstrated a dose-related increase after pretreatment with phenobarbital, and the 1,4-bis[2-(3,5-dibromo-, 3,5-dichloro-, 5-bromo- and 5-chloropyridyloxy)]benzene analogs (Fig. 2). The ED_{50} value for phenobarbital was 130 μmoles/kg. The 3,5-dibromopyridyloxy analog exhibited the greatest potency with an ED_{50} of 0.4 μmole/kg while the 3,5-dichloropyridyloxy compound was the next most potent compound with an ED_{50} of 1.8 μmoles/kg. The ED_{50} values for the 5-bromopyridyloxy and 5-chloropyridyloxy analogs were 3.5 μmoles/kg and 50 μmoles/kg respectively. 1,4-Bis[2-(3-chloropyridyloxy)]benzene and 1,4-bis[2-(pyridyloxy)]benzene were relatively weak inducers of DMAP *N*-demethylase.

A dose-related increase in hepatic microsomal aldrin epoxidase activity occurred after pretreatment of animals with phenobarbital, and all the 3,5-dihalo- and 5-halo-substituted analogs of TCPOBOP as illustrated in Fig. 3. The 3,5-dibromo- and 3,5-dichloro-substituted compounds produced similar dose–response curves with ED_{50} values of 0.89 μmole/kg

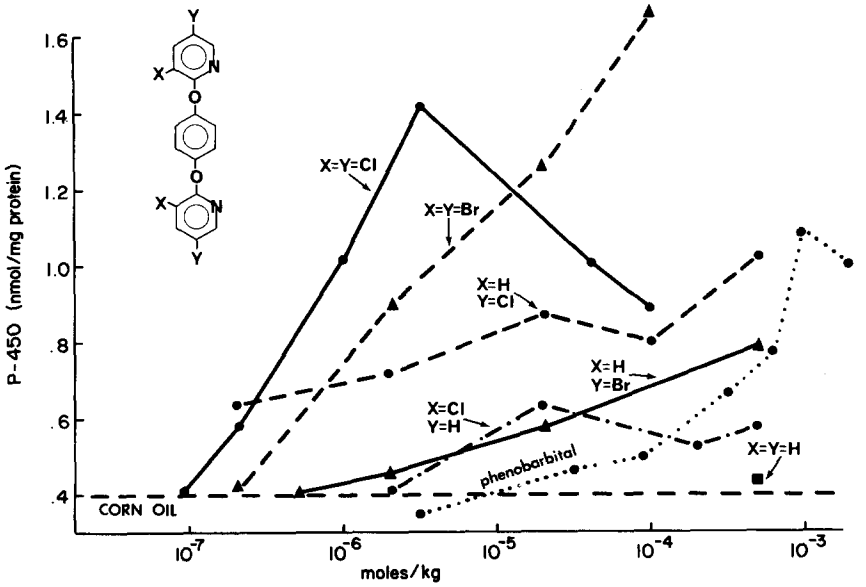

Fig. 1. Variation in hepatic microsomal cytochrome P-450 content with dose of compound. A phenobarbital control value of 1.000 nmoles/mg protein was utilized to normalize all data. Each point represents the mean of a group of four mice. Standard deviations ranged from 10 to 20% of the mean.

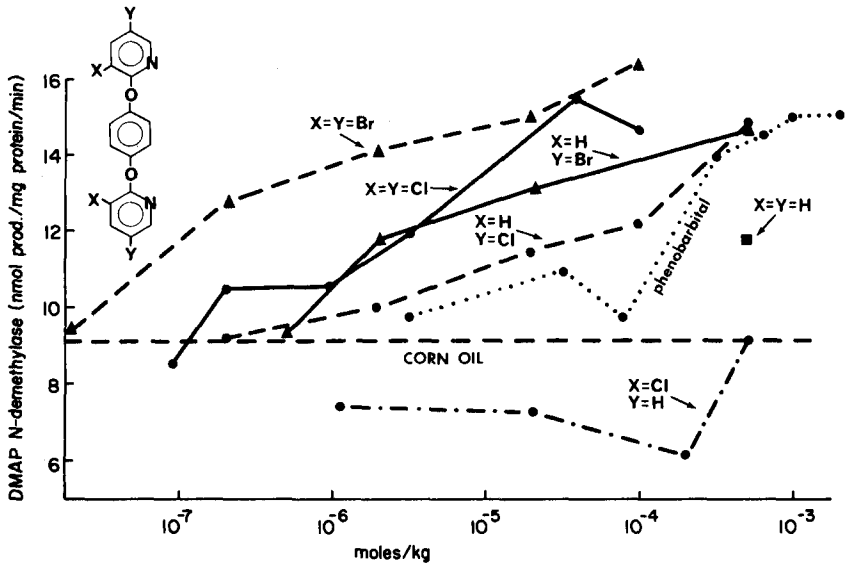

Fig. 2. Variation in hepatic microsomal DMAP *N*-demethylase activity with dose of compound. A phenobarbital control value of 15.00 nmoles·mg^{-1}·min^{-1} was utilized to normalize all data. Each point represents the mean of a group of four mice. Standard deviations ranged from 10 to 20% of the mean.

Table 2. TCPOBOP and related compounds as inducers of hepatic microsomal coumarin hydroxylase activity

Treatment	Coumarin hydroxylase activity (nmoles product/mg protein/min)
Corn oil (control)	0.497 ± 0.138
Phenobarbital (PB)*	1.932 ± 0.251†
1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)‡	1.544 ± 0.350†
1,4-Bis[2-(3,5-dichloropyridyloxy)]-2-chlorobenzene‡	1.371 ± 0.338†
4,4'-Bis[2-(3,5-dichloropyridyloxy)]biphenyl‡	0.423 ± 0.123
1,4-Bis[2-(3,5-dichloropyridyloxy)] naphthalene‡	0.599 ± 0.152
1,3-Bis[2-(3,5-dichloropyridyloxy)]benzene‡	0.410 ± 0.179
1,2-Bis[2-(3,5-dichloropyridyloxy)]benzene‡	0.526 ± 0.193

* Total dose administered was 1200 μmoles/kg.
† Different from control at the 1% (= 0.01) level of significance.
‡ Total dose administered was 20 μmoles/kg.

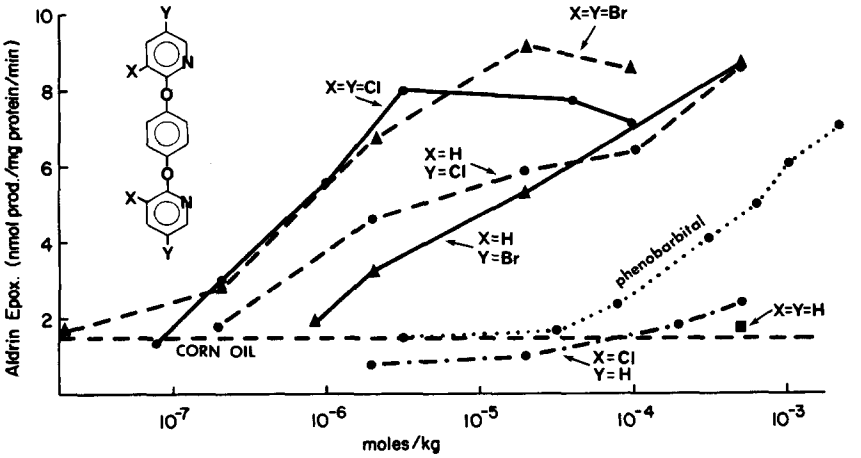

Fig. 3. Variation in hepatic microsomal aldrin epoxidase activity with dose of compound. A phenobarbital control value of 7.00 nmoles·mg^{-1}·min^{-1} was utilized to normalize all data. Each point represents the mean of a group of four mice. Standard deviations ranged from 10 to 20% of the mean.

and 0.56 μmole/kg respectively. The ED_{50} values of the 5-chloro- and 5-bromo analogs were 4.0 and 14 μmoles/kg. In comparison, the ED_{50} of phenobarbital for the induction of aldrin epoxidase was 320 μmoles/kg. 1,4-Bis[2-(3-chloropyridyloxy)]benzene and 1,4-bis[2-(pyridyloxy)]benzene were weak inducers of aldrin epoxidase, and these results correlated with their activities as inducers of microsomal DMAP *N*-demethylase and cytochrome P-450.

PB, TCPOBOP and the analogs 1,4-bis[2-(3,5-dichloropyridyloxy)]-2-chlorobenzene, 4,4′-bis[2-(3,5-dichloropyridyloxy)]biphenyl, 1,4-bis[2-(3,5-dichloropyridyloxy)]naphthalene, 1,3-bis[2-(3,5-dichloropyridyloxy)]benzene and 1,2-bis[2-(3,5-dichloropyridyloxy)]benzene were evaluated by measuring hepatic microsomal coumarin hydroxylase activity (see Table 2). Pretreatment with PB, TCPOBOP and the analog, 1,4-bis[2-(3,5-dichloropyridyloxy)]-2-chlorobenzene produced a significant increase in enzyme activity compared to corn oil controls.

DISCUSSION

In this report, the activities of TCPOBOP and ten analogs have been evaluated. TCPOBOP and the 3,5-dibromo analog possessed the greatest potency as determined from their ED_{50} values; compared to PB, both compounds shifted the monooxygenase dose–response curves to the left by approximately two to three orders of magnitude. It is difficult, however, to discriminate between the relative effects of the Cl vs Br substituents since their ED_{50} values varied with respect to the particular enzyme activity measured. Similarly, a difference in potency between the 5-bromo and 5-chloro analogs could also not be distinguished in these experiments. However, it is clear that these 5-haloderivatives are less active as microsomal enzyme inducers than the analogs substituted with chlorine or bromine at both the 3 and 5 positions of the pyridine ring. 1,4-Bis[2-(3-chloropyridyloxy)]benzene and 1,4-bis[2-(pyridyloxy)]-benzene were both weak microsomal enzyme inducers based on the three variables measured, and this result clearly indicates the importance of halogen substitution at the 5 position.

Three analogs have been examined in which the substitution pattern on the internal benzene ring has been altered. Both ortho (1,2) and meta (1,3) substitution of the benzene ring with dichloropyridyloxy substituents results in decreased enzyme-inducing activity. The addition of chlorine at the two position of the benzene ring results in an analog with significant induction activity. Two additional analogs were evaluated in which the benzene ring was replaced by either biphenyl or naphthalene. Neither analog was effective as a monooxygenase inducer at the dose tested. One caveat must be attached to an interpretation of these results. The role of bioconcentration and metabolism in producing a differential response has not been established. It would be helpful to determine the hepatic residues of individual analogs at the time of sacrifice. However, attempts to accurately establish residue levels by conventional extraction, cleanup and gas chromatographic analysis have not been successful due to low recovery yields of spiked samples and poor electron capture detection response factors.

Certain experimental observations employing TCPOBOP have been proposed to support a receptor mechanism for the induction of hepatic microsomal enzymes by PB and PB-like agonists [36, 37]. In summary, these observations include: (1) the identification of a highly potent agonist exhibiting a graded dose response, (2) tissue specificity for the induction response, and (3) species specificity for the induction response. Furthermore, the presence of a structure–activity relationship among a group of 2,4-dichloro-substituted PCBs supports such a mechanism [35]. This study, utilizing a group of TCPOBOP analogs, indicates that changes in the structure of the pyridyloxy or benzene ring markedly affect enzyme induction activity and therefore provides additional indirect evidence for a receptor-mediated response. Current studies in our laboratory involve the use of modified substituted biphenyls and pyridyloxybenzenes as probes for elucidating the mechanism of PB-associated increases in cytochrome P-450 monooxygenases.

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