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Modulation of cytochrome P450 by 5,5′-bis-trifluoromethyl-2,2′-dichlorobiphenyl, a unique environmental contaminant

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Abstract

5,5′-Bis-trifluoromethyl-2,2′-dichlorobiphenyl (5,5′CF_3-2,2′PCB) is representative of a unique class of trifluoromethyl polychlorinated biphenyls (CF_3-PCBs) found in sediments and fish of Lake Ontario, the Niagara river, and their tributaries. The potential hazard of 5,5′CF_3-2,2′PCB was assessed by exposing male Wistar rats to this agent in corn oil at a dose of 1 mg/kg/day or 75 mg/kg/day or corn oil alone (control) by oral intubation for 7 consecutive days. No lethality occurred during the course of exposure. A significant increase in liver weight and liver/body weight ratio and significant decrease in body weight gain were observed following exposure to the high dose of CF_3-PCB, relative to control. Exposure to the CF_3-PCB also resulted in a dose-related increase in the total hepatic cytochrome P450 content. This was associated with a dose-related increase in the O-deethylation of ethoxycoumarin, an activity which is mediated by several cytochrome P450s and thus provides a general representation of cytochrome P450 status. Specifically, a dose-dependent induction of the cytochrome P450s 1A1 and 1A2 (CYP1A1 and CYP1A2) proteins and their respective activities was observed, with significant induction occurring in both the low and high dose CF_3-PCB groups, compared to control. Additionally, CYP2B1 and CYP2B2 proteins and activities were induced following treatment with the high dose of 5,5′CF_3-2,2′PCB. Since, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds selectively induce CYP1A1 and CYP1A2, the results suggest that 5,5′CF_3-2,2′PCB has significant dioxin-like activity. Furthermore, 5,5′CF_3-2,2′PCB appears to be acting as a mixed-type inducer since phenobarbital-like induction of CYP2B1 and 2B2 was also associated with exposure. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cytochrome P450; CYP1A1; CYP1A2; CYP2B1; CYP2B2; 5,5′-bis-trifluoromethyl-2,2′-dichlorobiphenyl

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1. Introduction

The Great Lakes have become a focal point of many environmentalists due to their contamination with persistent toxic heavy metals and synthetic organic chemicals, such as polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). Humans can be exposed to these chemicals through the consumption of contaminated food and water (Kittner et al., 1987). The clinical manifestations of exposure to PCBs, PCDDs, and PCDFs includes effects on skin, liver, eyes, endocrine, and nervous systems as well as immunotoxic, carcinogenic, reproductive, and developmental effects (Murai and Kuroiwa, 1971; Kuratsune et al., 1972; Safe, 1984; Van den Berg et al., 1994; Safe, 1986). Specifically, PCBs have been shown to elicit detrimental effects on wildlife, including adverse effects on reproduction of fish (Ahlborg et al., 1992). In addition, PCB contamination was associated with declines in mink and otter populations around the Great Lakes (Ahlborg et al., 1992). Strong evidence suggests that many of the effects elicited following exposure to coplanar (non-*ortho* substituted) PCBs are mediated by the aryl hydrocarbon (Ah) receptor in the same manner as PCDDs and PCDFs (Poland and Knutson, 1982). The binding of ligand to the Ah receptor in the cytosol increases the affinity of the receptor for DNA and promotes translocation to the nucleus, resulting in alterations in gene expression (Vanden Heuvel and Lucier, 1993). A new class of environmental pollutants unique to this ecosystem, referred to as trifluoromethyl polychlorinated biphenyls (CF_3-PCBs), have been identified in lake sediments and fish samples collected from the Niagara River, Lake Ontario and their tributaries (Elder et al., 1981; Jaffe and Hites, 1985). The source of the CF_3-PCBs was traced back to the Hyde Park landfill in Niagara Falls, NY (Jaffe and Hites, 1986). Since no data are available on the toxicity of CF_3-PCBs, this study was designed to estimate the potential hazard of the CF_3-PCB shown in Fig. 1.

The potential hazard of 5,5′CF_3-2,2′PCB was characterized using the male Wistar rat and observing lethality, change in body and organ weight, and induction of hepatic cytochrome P450 content and activities. The male Wistar rat was utilized in this study because it was the in vivo model used in previous studies to determine the toxic potency and enzyme inducing potential for the structurally related PCDDs, PCDFs, and PCBs (Safe, 1986; Bandiera et al., 1984; Mason et al., 1985, 1986). Specifically, CYP1A1 and CYP1A2 proteins and activities were investigated because of their known association and induction by compounds that bind to the Ah receptor and as a consequence possess dioxin-like activity. The induction of hepatic CYP1A1 by dioxin-like compounds correlates with the ability of these compounds to elicit numerous toxic responses (Safe, 1990) and therefore can prove useful as a marker for the potential toxic potency of dioxin-like compounds. The O-deethylation of ethoxyresorufin (EROD) and the 4-hydroxylation of acetanilide (AcOH) are preferentially supported by CYP1A1 and CYP1A2 (Shimada et al., 1992; Tsyrlov and Duzchak, 1990; Liu et al., 1991), respectively, and used for assessment of enzymatic activity for the corresponding proteins. Additionally, since certain PCBs can also induce phenobarbital inducible cytochrome P450s, such as CYP2B1 and CYP2B2 (Ikegwuonu et al., 1996), the ability of CF_3-PCB to induce these proteins and their activities was investigated. The N-demethylation of benzphetamine was used to assess the enzymatic activity of CYP2B1 and CYP2B2, collectively (Lu et al., 1973). Since 5,5′CF_3-2,2′PCB is structurally related to PCBs, PCDDs, and PCDFs, the activity of this compound was compared with that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent and extensively studied PCDD.

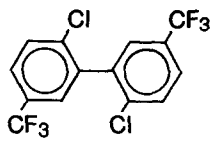

Fig. 1. 5,5′-bis-trifluoromethyl-2,2′-dichlorobiphenyl (5,5′CF_3-2,2′PCB).

2. Methods

2.1. 5,5'-bis-(trifluoromethyl)-2,2'-dichlorobiphenyl synthesis

The general procedure described by Jaffe and Hites (1985) was followed. A well-stirred mixture of 30 g of 4-chloro-3-iodobenzotrifluoride (Aldrich Chemical Co., Milwaukee, WI) and copper powder (20 g) in redistilled quinoline (300 ml) was heated under reflux for 5 h. The mixture was cooled to ambient temperature and poured onto 1 kg of ice containing 300 ml of concentrated hydrochloric acid. After stirring the mixture for 30 min, it was extracted with methylene chloride (2 × 500 ml). The combined methylene chloride extracts were washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to yield ~15.5 g of dark oil. The oil was chromatographed over dry column grade silica gel (EM Science) eluting the column with pentane. The individual fractions were monitored by TLC (silica gel, Analtech) developed in pentane. The fractions containing a single compound with an R_f ~0.60 were combined and concentrated to a small volume. Crystals separated on cooling were filtered, and recrystallized from pentane to yield 0.9 g (5%) of colorless flakes, m.p. 84–85°C; ^{1}H NMR ($CDCl_3$): δ 6.51 (s, 2 H), 7.64 (s, 4 H); mass spectrum: m/z 358 (M^+, 100), 339 (43.4), 323 (50), 303 (49), 288 (61), 254 (49), 219 (46).

The purity of the synthetic PCB was analyzed by GC/MS using a 30 m × 0.25 μ d_f SE-54 column (Alltech Assoc. Derfield, IL). While the synthetic approach made the presence of other congeners unlikely, the chromatograms were nonetheless evaluated for the presence of multiple components or for differences in peak shape or width, which would suggest unresolved components. The mass spectra of the eluting single peak was consistent with the proposed structure.

2.2. Treatment of animals

Male Wistar rats (Harlan Sprague Dawley Inc., Indianapolis, IN) weighing ~100 g were maintained on a 12 h light/12 h dark cycle and received water and food ad libitum throughout the study. The rats were randomly divided into four groups of 5 rats each. One group was designated control, while two of the groups received oral CF_3-PCB (1 mg/kg/day or 75 mg/kg/day for 7 consecutive days). The remaining group was treated with TCDD in corn oil (1.3 μg/kg on day 1 only). The CF_3-PCB was dissolved in acetone, mixed with corn oil and placed under a stream of N_2 to evaporate the acetone. The animals received the CF_3-PCB in corn oil or corn oil alone (control) for 7 consecutive days by oral intubation (3 ml/kg body weight). All animals were sacrificed on the 8th day and the liver, thymus, and spleen were removed, weighed, and frozen at −70°C.

2.3. Enzyme activity assays

Liver microsomes were prepared as described by Wroblewski et al. (1988). The liver was homogenized with 5 volumes (w/v) of homogenizing buffer (10 mM Tris base, 250 mM sucrose, 1 mM EDTA, pH 7.4 at 4°C) and centrifuged at 10 900 × g for 30 min. The supernatant was removed and spun in an ultracentrifuge at 100 000 × g for 60 min. The supernatant was discarded and reconstituting buffer (10 mM Tris base, 20% glycerol, 1 mM EDTA, pH 7.4 at 4°C) was added to the microsomal pellet at a concentration of 1 ml/g of original tissue and stored at −70°C. The protein concentration for the microsomal samples was determined by using the method of Bradford (1976).

The ethoxyresorufin-*O*-deethylase (EROD) and ethoxycoumarin-*O*-deethylase (ECOD) activities were determined by the fluorometric assay described by Prough et al. (1978). The wavelengths for excitation and emission were 530 nM and 585 nM, respectively for the EROD assay. For the ECOD, the wavelengths for excitation and emission were 360 nM and 460 nM, respectively. In both assays, the rate of fluorescence change was recorded prior to and after the addition of NADPH. The EROD and ECOD assays were calibrated with a known quantity of resorufin and 7-hydroxycoumarin, respectively.

The demethylation of benzphetamine (BND), a marker of CYP2B1/2 activity, was measured spec-

Table 1
Effect of 5,5′CF_3-2,2′PCB or TCDD exposure on body and organ weights of male Wistar rats[a]

Treatment group	Liver weight (g)	Liver/body weight ratio	Spleen weight (g)	Thymus weight (g)	Body weight gain (g)
Control $n=5$	7.99 ± 0.37	0.049 ± 0.003	0.497 ± 0.024	0.487 ± 0.051	51.0 ± 6.3
TCDD 1.3 μg/kg on day 1 $n=5$	9.18 ± 0.31[b]	0.055 ± 0.002[b]	0.506 ± 0.094	0.473 ± 0.080	38.8 ± 3.8[b]
CF_3-PCB 1 mg/kg/day × 7 days $n=5$	8.39 ± 0.84	0.049 ± 0.002	0.544 ± 0.089	0.549 ± 0.066	47.6 ± 5.6
CF_3-PCB 75 mg/kg/day × 7 days $n=6$	12.9 ± 0.97[b]	0.076 ± 0.004[b]	0.534 ± 0.062	0.473 ± 0.081	34.8 ± 8.6[b]

[a]Body and organ weight data (mean ± S.D.) were obtained on experimental day 8.
[b]Significantly different from the control group.

trophotometrically by quantitating the formation of formaldehyde using the NASH reagent (Nash, 1953; Lake et al., 1979).

The 4-hydroxylation of acetanilide, a marker for CYP1A2 activity, was determined essentially as described by Liu et al. (1991). Liver microsomal protein (0.2 mg) was incubated at 37°C for 40 min in a buffer comprised of 50 mM Tris (pH 7.5), 0.3 mM $MgCl_2$, 0.6 mM NADPH, bovine serum albumin (1 mg/ml), and 0.4 mM acetanilide. The 4-hydroxyacetanilide production was quantitated by reverse-phase HPLC analysis.

2.4. Cytochrome P450 protein analysis

The quantitation of the total cytochrome P450 content in the hepatic microsomes was determined by the method of Omura and Sato (1964), using a molar extinction coefficient of 91 mM^{-1} cm^{-1}.

The relative levels of specific cytochrome P450s (CYP1A1, CYP1A2, and CYP2B1/2) were determined by standard electrophoresis and immunoblotting. Briefly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to the method of Laemmli (1970). Proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) using a Mini-Genie electrophoresis blotter (Idea Scientific, Minneapolis, MN). Non-specific binding sites were saturated with 10% non-fat milk solution and the blots were subsequently incubated with primary antibodies directed against either rat CYP1A1, CYP1A2 (generously provided by Dr. J. Goldstein, NIEHS), or CYP2B1/2. Rat CYP2B1/2 antibody was a generous gift from Dr. James Halpert, University of Arizona. Chemiluminescent detection was employed utilizing either goat anti-rabbit or rabbit anti-goat IgG conjugated with alkaline phosphatase and developed using CDP-Star chemiluminescent substrate (Tropix, Bedford, MA) following the manufacturers' protocol.

2.5. Statistical analysis

Data are expressed as the mean ± S.D. Significant differences between groups were determined using the one-way analysis of variance with the Duncan's multiple range test and a P-value < 0.05.

3. Results

The potential hazard of a 5,5′CF_3-2,2′PCB was evaluated and compared to TCDD in male Wistar rats. Table 1 expresses the body and organ weight data collected from rats exposed to the CF_3-PCB (1 mg/kg/day or 75 mg/kg/day for 7 days), TCDD (1.3 μg/kg on day 1 only), or corn oil alone (control). The dose of TCDD was chosen to approximate the ED_{50} for CYP1A1 enzyme induction in male Wistar rats (Mason et al., 1986). All animals appeared normal and gained body weight throughout the exposure period with no

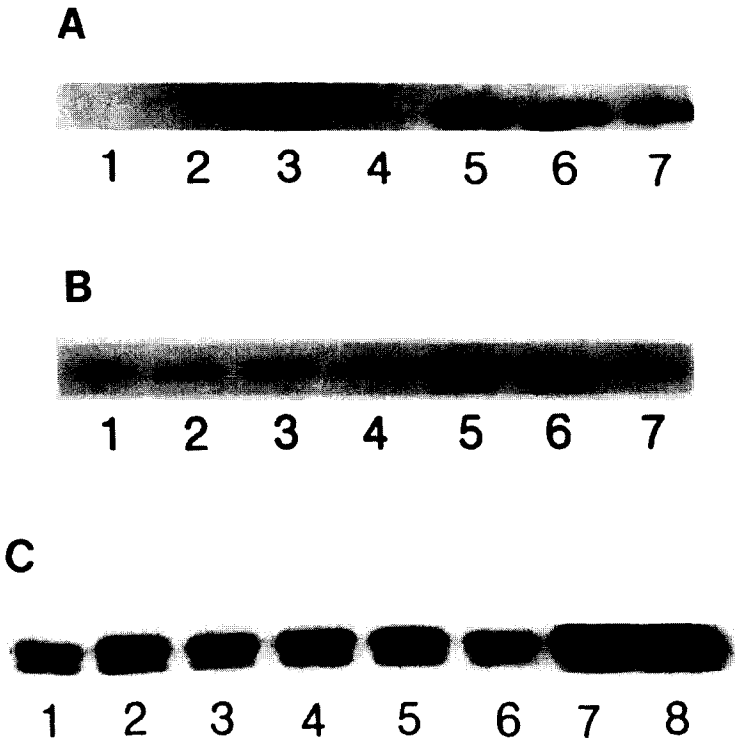

Fig. 2. Western immunoblot analysis of CYP1A1 (A), CYP1A2 (B), and CYP2B1/2 (C) in rat liver microsomes. Controls are represented by lanes 1 and 2 (A and B) and lanes 1–3 (C); low dose of 5,5′CF_3-2,2′PCB lanes 3 and 4 (A and B) and lanes 4–6 (C); high dose of 5,5′CF_3-2,2′PCB lanes 5 and 6 (A and B) and lanes 7 and 8 (C). Lanes 1–6 contain 5 μg (A) and 10 μg (B) microsomal protein, while lanes 1–8 contain 15 μg (C) microsomal protein. Lane 7 contains 1.2 pmol CYP1A1 (A) and 0.4 pmol CYP1A2 (B).

lethality occurring. Exposure to the high dose of the CF_3-PCB resulted in a significant increase in liver weight and liver/body weight ratio (Table 1) compared to control. The high dose also resulted in a decrease in body weight gain relative to control (Table 1). The low dose of the CF_3-PCB did not show any significant changes in organ or body weight compared to control. Treatment with TCDD produced responses similar to the high dose CF_3-PCB group, including an increase in liver weight and liver/body weight ratio (Table 1), as well as a decrease in body weight gain. No significant effect upon spleen or thymus weight was observed in any of the treatment groups relative to control.

The hepatic microsomal cytochrome P450 content and associated enzyme activities in the four groups are summarized in Table 2. Exposure to both the low and high dose of the CF_3-PCB resulted in a significant increase in the total cytochrome P450 content, ECOD, EROD, and acetanilide hydroxylase activities, compared to control, with the high dose having a much greater effect. The high dose group also showed a significant increase in the benzphetamine *N*-demethylase (BND) activity. While the low dose group showed an increase in the BND activity, it was not significantly different from control. Western blot analysis of randomly chosen samples from the control, low, and high dose CF_3-PCB treatment groups (Fig. 2) support the dose-related induction of CYP1A1, CYP1A2, and CYP2B1/2 proteins; correlating well with their representative enzymatic activities. As expected, TCDD treatment significantly increased the total hepatic cytochrome P450 content, ECOD, EROD and acetanilide hydroxylase activities, while producing no significant change in benzphetamine *N*-demethylase activity.

Table 2
Effect of 5,5′CF_3-2,2′PCB or TCDD exposure on hepatic microsomal cytochrome P450 and associated enzyme activities in male Wistar rats[a]

Treatment group	Total cytochrome P450 (nmol/mg protein)	ECOD (nmol/min/mg protein)	EROD (pmol/min/mg protein) (CYP1A1)	Benzphetamine *N*-demethylase (nmol/min/mg protein) (CYP2B1/2)	Acetanilide hydroxylation (pmol/min/mg protein) (CYP1A2)
Control $n=5$	0.443 ± 0.025	59 ± 15[b]	71 ± 17	5.74 ± 1.22	278 ± 37
TCDD 1.3 μg/kg on day 1 $n=5$	0.922 ± 0.113[c]	4730 ± 1800[c]	$13\,436 \pm 3117$[c]	6.04 ± 0.91	1778 ± 193[c]
CF_3-PCB 1 mg/kg/day × 7 days $n=5$	0.528 ± 0.024[c]	439 ± 265[c]	999 ± 593[c]	6.36 ± 1.10	535 ± 101[c]
CF_3-PCB 75 mg/kg/day × 7 days $n=6$	1.58 ± 0.142[c]	$5800 + 1040$[c]	$11\,052 \pm 1653$[c]	9.31 ± 1.28[c]	1978 ± 600[c]

[a]Data (mean ± S.D.) were obtained on experimental day 8.
[b]$n=4$.
[c]Significantly different from the control group.

4. Discussion

In the present study, the biological and toxicological activity of 5,5′CF_3-2,2′PCB was characterized in male Wistar rats by evaluating the effects of short-term exposure on organ and body weight and hepatic cytochrome P450 content and activities. Analysis and comparison of the data following exposure to either the CF_3-PCB or TCDD revealed similar responses, including increased liver weight and liver/body weight ratio. Additionally, the total hepatic cytochrome P450 content, ECOD, EROD, and acetanilide hydroxylase activities were also induced by both compounds. Since, EROD and acetanilide hydroxylase activities are preferentially supported by CYP1A1 and CYP1A2, respectively, and induction of these proteins is associated with exposure to dioxin-like compounds, it is concluded that the CF_3-PCB possesses significant dioxin-like activity. The CF_3-PCB, however, is significantly less potent than TCDD at inducing the aforementioned responses. For example, exposure to the high dose of 5,5′CF_3-2,2′PCB resulted in similar levels of induction of ECOD, EROD, and acetanilide hydroxylase activities compared to the TCDD treatment, but was $\sim$350 000 times less potent, on a molar basis. The low potency of the CF_3-PCB was expected, due to the lack of substitution of the 3,4-positions. It has previously been demonstrated that potency correlates with resistance to metabolism and PCBs lacking 3,4-substitution are the most easily metabolized (Goldstein et al., 1977). In addition, substitution in adjacent, lateral positions of the biphenyl results in greater affinity for the Ah receptor.

In addition to the induction of CYP1A1 and CYP1A2 proteins and activities, the CF_3-PCB also elevated CYP2B1/2 proteins and their corresponding activity, the demethylation of benzphetamine. The induction of CYP2B1/2 is a characteristic of phenobarbital exposure (Ikegwuonu et al., 1996). Thus, the CF_3-PCB appears to induce both dioxin responsive CYP1A1/2 and phenobarbital responsive CYP2B1/2. Typically, PCBs are classified in one of four categories based on their ability to induce specific hepatic cytochrome P450s. Coplanar PCBs, such as 3,3′,4,4′,5-pentachlorobiphenyl, are exclusively dioxin-like inducers (Albro and Mckinney, 1981; Parkinson et al., 1981). Other PCBs, such as 2,2′,4,4′,5,5′-hexachlorobiphenyl, are phenobarbital-like inducers (Goldstein et al., 1977). PCBs such as 2,2′,3′,4,4′,5 and 2,2′,3,3′,4,4′-hexachlorobiphenyl resemble both dioxins and phenobarbital in their induction profile and are classified as mixed inducers (Parkinson et al., 1981). The final group contains PCBs possessing little or no induction potential (i.e. 2,2′,3,3′,6,6′-hexachlorobiphenyl) (Goldstein et al., 1977). Using the data collected in this study, 5,5′CF_3-2,2′PCB can be classified as a mixed-inducer.

The CF_3-PCB's structure activity relationship appears to be unique compared to other PCBs. It generally does not follow established criteria required for enzyme induction. It has been established that chlorination of the *para* positions is a requirement for enzyme induction (Goldstein et al., 1977) and 3,4,3′,4′-chlorination is necessary for dioxin-like induction of CYP1A1/2 (Yoshimura et al., 1979). Moreover, mixed inducers require chlorination on both *para*, at least two *meta*, and two *ortho* positions, with one ring containing a 2,3,4-trichloro substitution pattern (Parkinson et al., 1981). The 5,5′CF_3-2,2′PCB meets none of these criteria. It does not possess the *para* chlorines required for enzyme induction, nor does it meet the structural criteria for being a mixed inducer. Nonetheless, the 5,5′CF_3-2,2′PCB exhibits an enzyme induction profile corresponding to the mixed type inducer.

While the dioxin-like PCBs are considered the most toxic PCB congeners (Yoshimura et al., 1979), the phenobarbital-like PCBs do possess the potential for toxicity. The phenobarbital-like PCBs have been shown to be capable of promoting the growth of preneoplastic foci in rodent liver (Buchmann et al., 1991). Additionally, the di-*ortho* PCBs such as 2,2′,5,5′, which is classified as a phenobarbital-like inducer (Yoshimura et al., 1979), are the most potent of all the PCB congeners at inhibiting gap junctional intracellular communication (GJIC) (De Haan et al., 1996). Inhibition of GJIC is an important part of the tumor promotion phase of carcinogenesis and is used as an indicator of the tumor promoting

potential of a chemical (De Haan et al., 1996). Moreover, the non-planar di-*ortho*-substituted PCBs (i.e. 2,2′,5,5′-tetrachlorobiphenyl) have been linked with the development of neurotoxicity (Seegal et al., 1990; Shain et al., 1991). Thus, while the non-planar di-*ortho*-substituted 5,5′CF_3-2,2′PCB possesses dioxin-like activity, it also has the potential to elicit numerous toxic effects associated with the phenobarbital-like PCBs. Additional studies are required to fully characterize the range of toxicological effects elicited by the CF_3-PCB.

In addition, it is necessary to identify the specific structure of the CF_3-PCBs which are persisting in Lake Ontario sediments and fish. Once specific congeners have been identified, it will be necessary to investigate the biological and toxicological activity of other CF_3-PCBs, since different structure activity relationships may apply to this new class of halogenated aromatic hydrocarbons.

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