Toxicokinetics of the Flame Retardant Hexabromocyclododecane Alpha: Effect of Dose, Timing, Route, Repeated Exposure, and Metabolism

David Taylor Szabo,*†§†† Janet Diliberto,† Heldur Hakk,‡ Janice K. Huwe,‡ and Linda S. Birnbaum§

*University of North Carolina in Chapel Hill Curriculum in Toxicology, †United States Environmental Protection Agency, Office of Research and Development, National Health Effects and Exposure Research Laboratory, Integrated Systems Toxicology Division, Research Triangle Park, North Carolina 27711; ‡Biosciences Research Laboratory, United States Department of Agriculture, Agriculture Research Service, 1605 Albrecht Blvd, Fargo, North Dakota, 58102-2765; and §National Cancer Institute and National Institutes of Health/National Institute of Environmental Health Sciences, PO Box 12233, Mail Drop B2-01, Research Triangle Park, North Carolina 27709

1To whom correspondence should be addressed at United States Environmental Protection Agency, Office of Research and Development, National Health Effects and Exposure Research Laboratory, Integrated Systems Toxicology Division, Mail Drop B143-01, 109 T.W. Alexander Drive, Research Triangle Park, NC 27711.

Fax: (703) 397-8693. E-mail: szabo.david@epa.gov.

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Alpha-hexabromocyclododecane (α-HBCD) is an emerging persistent organic pollutant present in the hexabromocyclododecane (HBCD) commercial mixture. HBCD is used as an additive flame retardant in a wide variety of household consumer products. Three main stereoisomers, alpha (α), beta (β), and gamma (γ), comprise roughly 10, 10, and 80% of the mixture, respectively. Despite its small contribution to HBCD global production and usage, α-HBCD is the major stereoisomer found in wildlife and human tissues including breast milk and blood in North America, European Union, and Asia. No mammalian or human data are currently available regarding the toxicokinetics of α-HBCD. This study was conducted in an effort to fully characterize the absorption, distribution, metabolism, and elimination of α-HBCD following a single and repeated exposure with respect to dose, time, and route of administration in female C57BL/6 mice. Results indicate that ~90% of the administered dose (3 mg/kg) was absorbed after oral exposure. Disposition was (1) dictated by lipophilicity, as adipose, liver, muscle, and skin were major depots and (2) was dose dependent with nonlinear accumulation at higher doses. Elimination, both whole-body and from individual tissues, was biphasic. α-HBCD-derived radioactivity was excreted in the feces as parent and metabolites, whereas urine only contained metabolites. Presence of polar metabolites in the blood and urine were a major factor in determining the rapid initial whole-body half-life after a single oral exposure. Initial half-lives were ~1–3 days and much longer terminal half-lives of 17 days were observed, suggesting the potential for α-HBCD bioaccumulation. A 10-day repeated study supports α-HBCD bioaccumulation potential. Stereosimzerization previously observed after exposure to γ-HBCD was not seen after exposure of α-HBCD. The toxicokinetic behavior reported here has important implications for the extrapolation of toxicological studies of the commercial HBCD mixture to the assessment of risk of α-HBCD which is the major stereoisomer found in wildlife and people.

Key Words: toxicokinetics; biotransformation; lipophilic; persistent organic pollutants; endocrine disruptors; risk assessment; brominated flame retardant; hexabromocyclododecane; mixture; metabolism.

1,2,5,6,9,10-hexabromocyclododecane (HBCD) is a brominated aliphatic cyclic hydrocarbon and a high production volume chemical used as a flame retardant primarily in polystyrene foams for insulation, with secondary uses in upholstered furniture, automobile textiles/cushions, packaging material, and electronic equipment. Concern for HBCDs has risen as it has been detected not only in wildlife (Covaci et al., 2006; Tomy et al., 2004) but also in human breast milk and serum (Meijer et al., 2008; Thomsen et al., 2007; Weiss et al., 2004). Time trend studies indicate that concentrations of HBCD in the environment, humans, and wildlife are increasing (Hermanson et al., 2010; Kakimoto et al., 2008; Sellström et al., 2003). The discovery of HBCD in Arctic marine ecosystems provides compelling evidence of long-range transport of this compound (de Wit et al., 2010). Field studies in marine mammals and aquatic wildlife have suggested HBCD is also highly bioaccumulative (Law et al., 2006).

Diet is likely a major source of HBCD exposure. Intake of HBCD was estimated at 16 ng/day, primarily from meat; however, fish and vegetables also had significant levels (Scheet et al., 2010). Dust is likely another important pathway of human exposure to HBCD due to levels present indoors (Roosens et al., 2009). Nondietary ingestion of dust may represent an important route of exposure especially for toddlers and young children (Lioy et al., 2000; Wilford et al., 2005; Wu and Takaro, 2007).

Commercially available HBCD is a mixture of different HBCD diastereomers (Fig. 1). Previous literature has focused on the three main diastereomers present in the commercial mixture, denoted as alpha (α), beta (β), and gamma (γ); with the γ-diastereomer predominating (>70%; Heeb et al., 2005).
It is well documented that there is a shift in the relative diastereomer contribution with increasing trophic levels in aquatic food webs (Covaci et al., 2006; Law et al., 2006). In general, lower level organisms contain mostly γ-HBCD closely mimicking the commercial mixture and certain environmental levels, whereas α-HBCD dominates the diastereomer pattern in apex predators. In fish and aquatic mammals, HBCD has been observed to accumulate in lipid rich organs including liver, gonads, muscle, and adipose tissue (Janák et al., 2005; Pock et al., 2008; Xian et al., 2008) and, where investigated, α-HBCD predominated. Presence of high concentrations of α-HBCD in marine mammals and birds of prey suggests persistence and biomagnifications up the food chain. Despite α-HBCD’s small contribution to HBCD global production and usage (~10% of commercial mixtures), α-HBCD is the major congener found in biota.

HBCD has been detected in the few human exposure studies conducted to date and, where investigated, α-HBCD predominates. Thomsen et al. (2007) reported that all serum samples tested in workers from an industrial plant occupationally exposed to the commercial mixture of HBCD had 60% α-HBCD and 39% gamma (ranged 6–856 ng/g lw). Ryan et al. (2006) reported that the mean concentrations of α-HBCD in human breast milk collected in Ontario, Canada (2002–2003), and Texas, USA (2002) were 3.8 and 0.5 ng/g lipid weight (lw), respectively. Kakimoto et al. (2008) analyzed breast milk from Japanese women (age 25–29) over the period 1973–2006; total mean HBCD concentrations over the period 2000–2006 ranged from 1 to 4 ng/kg lw, where α-HBCD predominated in nearly 90% of samples tested. Higher total HBCD (α, β, and γ) values were reported in breast milk of women living in Spain between 2006 and 2007 (Eljarrat et al., 2009) where the mean concentration was 27 ng/kg lw (range 3–188 ng/kg lw). α-HBCD dominated a smaller subset of the samples tested with γ-HBCD predominating in others. Thomsen et al. (2007) suggested that the elevated levels of γ-HBCD in some samples are an indication of recent exposure to the commercial mixture and with enough time; γ-HBCD will either be cleared from the system and/or converted to α-HBCD.

The field observations described in the current human and wildlife studies are unfortunately unable to fully characterize the exposure pathway, dose, and duration of exposure. The current hypothesis that may explain the elevated levels of α-HBCD found in biota is thought to be due to either diastereomer-selective uptake, diastereomer-specific metabolic rates (Law et al., 2006; Zegers et al., 2005), and/or in vivo stereoisomerization (Szabo et al., 2010). Stereoisomerization of purified γ-HBCD to α and/or β was reported in mice after a single oral exposure by Szabo et al. (2010) who demonstrated that in vivo stereoisomerization of γ-HBCD is rapid, 3–48 h postoral exposure, and is tissue specific in the liver, fat, and brain. However, more detailed information about the kinetics, toxicology, pathways of exposure, and bioavailability of the HBCD diastereomers are needed to help explain observations seen in the environment, wildlife, and humans.

In predicting human health risks posed by HBCD, it is necessary to accurately predict internal dose and the fate of these compounds. There are currently no toxicokinetic or toxicity data on α-HBCD in any mammalian species. This study is a companion paper to our previous work with γ-HBCD (Szabo et al., 2010) where the same methodology was used allowing a direct comparison of the kinetics between the two diastereomers. The dose concentrations selected for this toxicokinetic study were based on previous effect studies after exposure to the commercial mixture of HBCD. Developmental neurotoxicity was observed in mice after a single (acute) exposure with a lowest observed adverse effect level = 0.9 mg/kg bw/day (Eriksson et al., 2006). A 28-day repeated dose study in rats observed increased thyroid, liver, pituitary weight at 3.4, 29.9, and 50.6 mg/kg bw/day (benchmark dose lower confidence limit of 1.6, 22.9, and 29.9 mg/kg bw/day), respectively (van der ven et al., 2006). In a one-generation rat study, decreased concentration of apolar retinoids in the liver, increased immune response, and mineral bone density in F1 females were reported at 0.18, 1.45, and 5.1 mg/kg bw/day, respectively (Lilienthal et al., 2009). Increased liver weight was observed in a 90-day rat study with a LOAEL of 100 mg/kg bw/day (Chegels, 2001). The choices of dose in the current study were also driven by limited availability of the α-[14C]HBCD and the appropriate comparison with studies of γ-HBCD in mice.

The objective of this study was to characterize the fate of α-HBCD up to 14 days following an acute exposure, across a range of doses, and after repeated administration of a low dose to adult female mice.

MATERIALS AND METHODS

Chemicals

[14C]1,2,5,6,9,10-hexabromocyclododecane [14C]HBCD (2mCi/mmol) was purchased from American Radiochemicals Corporation (St Louis, MO) as a mixture of δ-[14C]HBCD and γ-[14C]HBCD diastereomers as determined by liquid chromatography-tandem mass spectrometer (LC/MS/MS) retention time comparisons with authentic standards (α-, β-, δ-, γ-, and ε-HBCD). Wellington Laboratories, Guelph, ON). The two diastereomers were separated by flash chromatography on a silica gel column eluted with hexane containing increasing amounts of methylene chloride (0–50%) (Szabo et al., 2010).
A fraction containing predominantly (>90%) γ-[14C]HBCD but some β-[14C]HBCD was thermally converted to α-[14C]HBCD by heating at 170°C for up to 3 h (Heeb et al., 2008). α-[14C]HBCD was purified by flash chromatography on a silica gel column as above, followed by preparative reverse-phase High Performance Liquid Chromatography on two Delta-Pak C18 cartridges (25 × 100 mm) (Waters Corp., Milford, MA) in series and isocratic elution with acetonitrile:water (80:20) at 20 ml/min. The radiochemical and diasteromic purities (98% each) were, respectively, determined by thin-layer chromatography (TLC) using silica gel plates (250 mm; Whatman Lab Div., Clinton, NJ) and developed with a 50:50 hexane:methylene chloride and by LC/MS/MS retention time comparison with authentic standards of α-, β-, δ-, γ-, and ε-HBCD (Wellington Laboratories, Guelph, ON). The impurities present in the α-[14C]HBCD were detected to be ≤1% each of the other two main stereoisomer β- and γ-HBCD. Radiochemical detection was performed on a System 2000 Imaging Scanner (Bioscan, Washington, DC). Retention times comparisons by LC/MS/MS were made on a Symmetry C18 column (2.1 × 100 mm) (Waters, Beverly, MA) using an isocratic program, which separated all five HBCD diasteromers (details below). Additionally, 1H-NMR of the α-[14C]HBCD performed on a Bruker AM 400 spectrometer (Bruker, Billerica, MA) matched the previously published spectrum of this diasteromer (Arsenault et al., 2007). Unlabeled α-HBCD was generously provided by Wellington Labs (98% purity). Other chemicals used were of the highest grade commercially available.

Dosing Solutions

Doses were selected based on published toxicity studies on the HBCD commercial mixture due to lack of any available α-HBCD whole animal studies. Furthermore, the low-sensitivity of the radiolabeled compound was a contributing factor in choosing 3 mg/kg as the low dose ensuring proper detection. A stock solution of α-[14C]HBCD was made by dissolving 19.23 mg of α-[14C]HBCD in toluene (400 μl). Aliquots were used directly from this solution for the dosing regimens. Dosing solutions were subjected to pre- and postdosage radioactivity examinations to ensure proper delivery of dose. All solutions were designed to deliver ~0.2 μCi to each mouse. Unlabeled α-HBCD was added to the α-[14C]HBCD to achieve desired mass (except the 3 mg/kg low dose) and was added directly to the dosing solution vial and dissolved in acetone. Com oil by weight was then added to the vials followed by the evaporation of toluene and acetone under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY). For intravenous (iv) treatment, the α-[14C]HBCD solvent from the stock solution, toluene, was allowed to evaporate in an amber vial under the flow of nitrogen, and the α-HBCD was resuspended in 95% ethanol followed by Emulphor. Deionized water was slowly added to a final volume to achieve a ratio of ethanol:Emulphor:water ratio was 1:1:8. Where applicable, glass was used in the handling, containment, transfer, and storage of the compound; no loss of radioactivity occurred with glass. Concentration of the dosing solution was verified by liquid scintillation chromatography (LSC).

Animals

Adult female mice were used in this study as limited supply of purified α-[14C]HBCD was available and to compare results with recent toxicokinetic mice studies on other related environmental chemicals (Diliberto et al., 2005; Szabo et al., 2010) including toxicity studies where mice were exposed to the commercial mixture of HBCD (Eriksson et al., 2006). Future mechanistic studies on disposition can be more easily tested in mice as transgenics are also readily available (Hakk et al., 2009a).

Female C57BL/6 mice (~20 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Females were chosen as they appear to elicit a more sensitive response after exposure to the commercial mixture of HBCD in several toxicity studies (Lilienthan et al., 2009; Van der ven et al. 2006). Animals were maintained on a 12 h light/dark cycle at ambient temperature (22°C) with relative humidity (56±5%), and were provided Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. Prior to the commencement of the study, mice were adapted (3 mice/cage) for 1 week to Nalgene metabolism cages (Nalgene, Rochester, NY). Mice were then assigned randomly to treatment groups (n = 4–8) and housed individually for the remainder of the study. All mice were 60 days old at time of treatment.

Route of exposure. “Oral treatment” (n = 4–8). A single dose (0, 3, 10, 30, and 100 mg/kg) was administered directly by oral gavage into the stomach of each mouse using a polytetrafluoroethylene (PTFE) animal feeding needle. Dose volume was 10 ml/kg. “Intravenous treatment” (n = 6–8). A single dose (3 mg/kg) was administered intravenously via the tail vein at a dosing volume of 2 ml/kg.

Treatment. Dose/response. A single dose was administered by gavage of either 3, 10, 30, or 100 mg/kg. The mice were held in metabolism cages for 4 days where urine and feces were collected daily.

Time course. Mice were treated by gavage at a single dose of 3 mg/kg and held for 14 days while urine and feces were collected daily.

Repeated. Mice were dosed for 9 days with 3 mg unlabeled α-HBCD/kg; and only on day 10 the mice were exposed to a single gavage of 3 mg α-[14C]HBCD/kg and then held for 4 more days (total of 14 days). This repeated dosing protocol allows comparison of “naïve” radioactive exposures to mice pretreated with unlabeled compound.

Animals were euthanized by CO2 asphyxiation followed by exsanguination via cardiac puncture at which point blood was collected. Liver, lung, kidneys, skin (ear, cheek, axillary, and cervical), small intestine (ileum), bladder, spleen, thymus, adipose (abdominal), muscle (abdominal), and brain were collected and weighed. Bile was removed directly from the gallbladder using a 0.2 mm bore needle and syringe.

Sample Analysis. Radioactivity in the tissues were determined by combustion with a tissue oxidizer (Packard 307 Biological Oxidizer, Downers Grove, IL) of triplicate samples when available (~100 mg/sample) followed by LSC (Beckman, Beckman 6000IC, Fullerton, CA) with limits of detections (LODs) of 50 (3x background) or 6.7 ng HBCD for the 3 mg/kg dose. Tissue data were reported based on wet weight and as percent of administered dose. Feces were air dried, weighed, and analyzed for radioactivity by combustion and LSC. Daily urine volume was recorded, and 100 μl aliquots (triplicate) were analyzed for [14C] by direct addition into scintillation cocktail.

Tissue Extraction and Analysis. Livers were weighed, pulverized and then homogenized in three volumes of 0.9% sodium chloride. Liver samples were extracted sequentially with three volumes of hexane, ethyl acetate, and methanol. The organic layers were pooled for each solvent and assayed by LSC. The liver sample extracts were applied to an acid silica gel column (0.5 × 7.0 mm, 40% concentrated sulfuric acid by weight) and eluted with hexane, methylene chloride. The collected fractions were analyzed by LC/MS/MS (see below).

Feces were dried and ground into a fine powder with a mortar and pestle and then extracted for 24 h stirring sequentially with 15 ml of hexane, ethyl acetate, and methanol. Fecal residues from the extractions were combusted and were the nonextractable fraction. The extractable fractions were reduced in volume to ~1 ml with nitrogen. Each extract was applied to a silica gel column (silica gel 60, EM Reagents, Cincinnati, OH; particle size <0.063 μm), preconditioned with hexane and eluted with a gradient from 100% hexane to 80:20 hexane/toluene. The gradient was increased in 2% increments, and fractions were assayed by LSC. Peaks of [14C] were collected, reduced in volume, and analyzed by LC/MS/MS (see below).

Serum was extracted with hexane followed by ethyl acetate. Each extract was applied to a silica gel TLC plate and developed in 50:50 hexane:methylene chloride. Bile was extracted with methanol and applied to silica gel
TLC plate, using a 50:50 hexane:methylene chloride as a developing solvent. TLC had LODs of 200 dpm (S/N = 3) or 0.03 ng HBCD for the 3 mg/kg dose. Gel permeation chromatography (GPC) was used to remove large macromolecules that can interfere with further analysis of low level of target molecules found in brain and adipose samples. Fat samples were pooled (n = 4) and homogenized in water, and 2 ml of hexane:acetone (2:7) was added and sonicated for 15 min. An additional 2 ml of hexane:acetone was added, sonicated, and combined with the previous organic layer. Four milliliters of hexane:ether (9:1) was added, vortexed, centrifuged, and decanted; this was repeated twice and organic layers were combined with the first extracts. The fat extract was assayed by LSC, evaporated with nitrogen, and eluted with methylene chloride. Fractions were collected, reduced in volume, applied to an acid silica column, and homogenized in water, and 2 ml of hexane:acetone (2:7) was added, sonicated, and combined with the previous organic layer. Four milliliters of hexane:ether (9:1) was added, vortexed, centrifuged, and decanted; this was repeated twice and organic layers were combined with the first extracts. The fat extract was assayed by LSC, evaporated with nitrogen, and eluted with methylene chloride. Fractions were collected, reduced in volume, applied to an acid silica column, eluted with hexane followed by 50:50 hexane:methylene chloride and analyzed by LCMS/MS (see below).

An LC/MS/MS was used to analyze selected samples and consisted of an Alliance 2695 Separation Model (Waters, Beverly, MA) equipped with a Symmetry C18 column (2.1 mm × 100 mm) and guard column (2.1 × 10 mm), and a quadrupole-time of flight mass spectrometer (Waters Q-TOF Ultima API-US; Waters, Beverly, MA). Isocratic elution conditions consisted of 15% aqueous 10 mM ammonium acetate and 85% methanol:acetone (80:20) prepared with 10 mM ammonium acetate. The flow rate of the mobile phase was 0.3 ml/min⁻¹. The mass spectrometer analysis was performed in negative ion mode (ES⁻) using a 634 m/z filter. LC/MS/MS had an estimated LOD of 0.3 ng (S/N = 3 calculated for a tissue matrix).

**Data Analysis.** To calculate the percent of dose in each compartment, mouse body composition estimates were used for blood, fat, skin, and muscle i.e., 8, 8, 12, and 35%, respectively (ILSI, 1994). Estimates were used for tissues for which it was not possible to obtain total weights from actual animals. The oral tissue disposition data refer to the mean of all data collected in which (a) animals were exposed orally with a 3 mg/kg dose and (b) 4-day time points where available. Intergroup comparisons were performed by a two-way ANOVA followed by Bonferroni post hoc tests were significant when p < 0.05. All data are presented as mean ± SD. GraphPad Prism 5.0, Hearn Scientific Software (Melbourne, Australia) was used to calculate half-life, the percent of dose, and statistical analysis.

**RESULTS**

**Tissue Disposition**

**Dose dependency** Tissue distributions, as a percent of α-[14C]HBCD administered dose in female C57BL/6 mice, are presented in Table 1. The top table (A) shows the disposition after administration of single and repeated oral doses of α-[14C]HBCD at concentrations of 3, 10, 30, or 100 mg/kg, or of 10-day repeated exposure to 3 mg/kg. Tissue distribution was analyzed 4 days after the administration of α-[14C]HBCD.

Data are represented in tabular form as it is useful for investigators who wish to model the data. All tissues examined had measurable levels 4 days after dosing. We found that the relative amount of α-[14C]HBCD-derived radioactivity was increased in tissues as a function of dose. The increases occurred at the 30 and/or 100 mg/kg exposures in tissues, which contained higher lipid content i.e., adipose, muscle, and skin, as well as the highly perfused liver tissue. Figure 2 represents this in a bar graph format.

**Repeated exposure** Tissue concentrations were altered after a 10-day repeated exposure. Levels in the adipose, liver, muscle, and blood were significantly increased between a single and 9 days of repeated oral exposure followed by α-[14C]HBCD at 3 mg/kg on the tenth day (Table 1A and Fig. 2). In fact, 4 days after, 10 d of repeated oral exposure, the percentage dose in the tissues are more similar to the 30 mg/kg as opposed to the 3 mg/kg single dose (Fig. 2).

**Time-course and half-life** From the dose/response study, the lowest dose was chosen (3 mg/kg) to conduct the time course (kinetic) study because it is within the range where effects have been seen (Erikkson et al., 2009), the specific activity of the starting radiolabeled material, and to compare the data with previous published kinetic data on γ-HBCD in mice at the same dose (Szabo et al., 2010). Only tissue distribution and elimination over time was performed after the 3 mg/kg administered dose. By observing tissue distribution over time, detectable concentrations were present at all time points investigated (Table 1B). This 14 days time course study showed a biphasic profile with an initial steep decline from 3 h to 2 days and a markedly slower decline between 2 and 14 days for liver, kidney, blood, and brain. Liver tissue had the highest observable level at 1 h. Although most tissues peaked in concentration at 1 day, α-[14C]HBCD-derived radioactivity was found to peak at 2 days in fat. Basically, the highly perfused tissues reached their highest observable peak at earlier time points (1 or 3 h), whereas the poorly perfused organs such as skin, fat, thymus, and muscle peaked later (at 2 days). As can be seen in Figure 2, α-[14C]HBCD-derived radioactivity is initially cleared rapidly from the blood, so that by 1 h postgavage, only 11.3% of the dose remained. By 1, 2, and 14 days, 2.0, 1.6, and 0.33% of the α-[14C]HBCD-derived radioactivity was detected in the blood, respectively. At all time points measured, liver tissue contained higher levels of α-[14C]HBCD-derived radioactivity than that found in blood. This was also observed in adipose tissue between 2 and 14 days, where higher levels were measured than those found circulating. A nonlinear regression two phase decay curve was used to fit the α-[14C]HBCD-derived radioactivity elimination from tissues. Half-lives and pool sizes were analyzed for each elimination phase (initial and terminal) separately where appropriate (Table 2). Although there is uncertainty for those tissues in which the calculated half-life exceeds the study’s data points (i.e., adipose tissue), we report its value for comparison to other tissues and the γ-HBCD stereoisomer (Szabo et al., 2010). Increased variance around the estimate is another factor contributing to this uncertainty.

**IV versus oral: absorption** By comparing disposition between iv and oral routes of exposure, it is possible to estimate the percentage of α-[14C]HBCD that was absorbed into the systemic circulation and delivered to the tissues. A comparison of the major tissue depots 4 days after treatment with the same oral and iv dose (3 mg/kg) is shown in Table 1C. The percent dose and concentration of the α-[14C]HBCD-derived radioactivity in the tissues were similar for both routes. Every tissue
### TABLE 1
Disposition of α-HBCD in Mice

<table>
<thead>
<tr>
<th></th>
<th>Skin</th>
<th>Liver</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Lung</th>
<th>Muscle</th>
<th>Kidney</th>
<th>Blood</th>
<th>Adipose</th>
<th>Brain</th>
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<tbody>
<tr>
<td>% dose</td>
<td>(mg/kg)</td>
<td>% dose</td>
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<td>% dose</td>
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<tr>
<td>3</td>
<td>2.5 ± 0.23</td>
<td>2.7 ± 1.20</td>
<td>0.02 ± 0.01</td>
<td>0.2 ± 0.08</td>
<td>0.4 ± 0.08</td>
<td>1.3 ± 0.21</td>
<td>0.04 ± 0.01</td>
<td>1.3 ± 0.29</td>
<td>3.70 ± 0.42</td>
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<td>10</td>
<td>2.5 ± 0.51</td>
<td>3.2 ± 0.31</td>
<td>0.05 ± 0.01</td>
<td>0.5 ± 0.03</td>
<td>0.2 ± 0.01</td>
<td>1.6 ± 0.16</td>
<td>0.06 ± 0.01</td>
<td>1.01 ± 0.46</td>
<td>3.93 ± 0.25</td>
<td>0.52 ± 0.45</td>
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<td>30</td>
<td>2.3 ± 1.41</td>
<td>4.16 ± 0.50</td>
<td>0.03 ± 0.01</td>
<td>0.3 ± 0.05</td>
<td>0.42 ± 0.02</td>
<td>1.7 ± 0.13</td>
<td>0.06 ± 0.01</td>
<td>1.32 ± 0.72</td>
<td>3.6 ± 0.36</td>
<td>0.51 ± 0.07</td>
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<td>100</td>
<td>4.9 ± 1.20</td>
<td>5.52 ± 1.11</td>
<td>0.05 ± 0.01</td>
<td>0.2 ± 0.03</td>
<td>0.43 ± 0.02</td>
<td>2.1 ± 0.31</td>
<td>0.06 ± 0.02</td>
<td>1.83 ± 0.39</td>
<td>8.91 ± 1.13</td>
<td>0.47 ± 0.03</td>
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<td>3 repeated</td>
<td>2.52 ± 0.51</td>
<td>3.75 ± 0.75</td>
<td>0.05 ± 0.01</td>
<td>0.2 ± 0.03</td>
<td>0.61 ± 0.03</td>
<td>1.9 ± 0.21</td>
<td>0.07 ± 0.03</td>
<td>2.4 ± 0.36</td>
<td>6.5 ± 0.93</td>
<td>0.48 ± 0.05</td>
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<td>(0027 ± 1836)</td>
<td>(13,500 ± 2700)</td>
<td>(180 ± 5.6)</td>
<td>(720 ± 108)</td>
<td>(2196 ± 108)</td>
<td>(17,100 ± 756)</td>
<td>(252 ± 108)</td>
<td>(8640 ± 1296)</td>
<td>(23,400 ± 3348)</td>
<td>(1728 ± 180)</td>
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<tr>
<td>1 h</td>
<td>0.0001 ± 0.000</td>
<td>13.25 ± 0.46</td>
<td>0.003 ± 0.01</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.02</td>
<td>0.80 ± 0.050</td>
<td>7.9 ± 0.001</td>
<td>11.31 ± 1.30</td>
<td>0.082 ± 0.001</td>
<td>3.5 ± 0.1</td>
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<td>3 h</td>
<td>0.0002 ± 0.010</td>
<td>12.24 ± 2.4</td>
<td>0.011 ± 0.02</td>
<td>0.39 ± 0.2</td>
<td>3.0 ± 0.51</td>
<td>1.0 ± 0.051</td>
<td>5.2 ± 0.31</td>
<td>7.03 ± 1.01</td>
<td>0.086 ± 0.001</td>
<td>1.9 ± 0.01</td>
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<td>8 h</td>
<td>0.013 ± 0.026</td>
<td>9.82 ± 0.55</td>
<td>0.016 ± 0.001</td>
<td>0.31 ± 0.01</td>
<td>2.1 ± 0.08</td>
<td>3.5 ± 0.02</td>
<td>3.6 ± 0.11</td>
<td>4.68 ± 1.21</td>
<td>0.072 ± 0.001</td>
<td>1.0 ± 0.01</td>
</tr>
<tr>
<td>1 day</td>
<td>1.53 ± 1.80</td>
<td>6.23 ± 1.3</td>
<td>0.17 ± 0.02</td>
<td>0.28 ± 0.01</td>
<td>0.62 ± 0.02</td>
<td>3.69 ± 0.22</td>
<td>0.8 ± 0.2</td>
<td>2.0 ± 1.24</td>
<td>2.30 ± 0.91</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>2 day</td>
<td>4.1 ± 1.21</td>
<td>4.21 ± 0.91</td>
<td>0.11 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.51 ± 0.01</td>
<td>1.8 ± 1.10</td>
<td>0.075 ± 0.01</td>
<td>1.60 ± 0.10</td>
<td>5.25 ± 0.5</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>4 day</td>
<td>2.5 ± 0.23</td>
<td>2.7 ± 1.2</td>
<td>0.02 ± 0.01</td>
<td>0.2 ± 0.08</td>
<td>0.4 ± 0.08</td>
<td>1.3 ± 1.21</td>
<td>0.04 ± 0.01</td>
<td>1.3 ± 0.29</td>
<td>3.70 ± 0.42</td>
<td>0.40 ± 0.23</td>
</tr>
<tr>
<td>7 day</td>
<td>2.1 ± 0.21</td>
<td>2.1 ± 0.9</td>
<td>0.01 ± 0.001</td>
<td>0.09 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.9 ± 0.10</td>
<td>0.02 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td>3.10 ± 0.01</td>
<td>0.21 ± 0.31</td>
</tr>
<tr>
<td>14 day</td>
<td>1.4 ± 0.11</td>
<td>1.2 ± 0.2</td>
<td>0.002 ± 0.001</td>
<td>0.03 ± 0.001</td>
<td>0.09 ± 0.004</td>
<td>0.29 ± 0.02</td>
<td>0.004 ± 0.001</td>
<td>0.33 ± 0.015</td>
<td>2.2 ± 0.001</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>(0540 ± 396)</td>
<td>(4680 ± 700)</td>
<td>(21 ± 1.1)</td>
<td>(11 ± 1.4)</td>
<td>(324 ± 0.6)</td>
<td>(720 ± 36)</td>
<td>(3240 ± 360)</td>
<td>(72 ± 72)</td>
<td>(2556 ± 72)</td>
<td>(11,160 ± 36)</td>
<td>(756 ± 116)</td>
</tr>
<tr>
<td><strong>C Route</strong></td>
<td><strong>Skin</strong></td>
<td><strong>Liver</strong></td>
<td><strong>Thymus</strong></td>
<td><strong>Spleen</strong></td>
<td><strong>Lung</strong></td>
<td><strong>Muscle</strong></td>
<td><strong>Kidney</strong></td>
<td><strong>Blood</strong></td>
<td><strong>Adipose</strong></td>
<td><strong>Brain</strong></td>
</tr>
<tr>
<td>iv</td>
<td>2.8 ± 0.24</td>
<td>3.7 ± 1.12</td>
<td>0.026 ± 0.01</td>
<td>0.31 ± 0.09</td>
<td>0.5 ± 0.09</td>
<td>1.7 ± 0.63</td>
<td>0.006 ± 0.003</td>
<td>1.51 ± 0.24</td>
<td>4.1 ± 0.9</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td>(010,900 ± 864)</td>
<td>(13,320 ± 4032)</td>
<td>(93.6 ± 32)</td>
<td>(1116 ± 324)</td>
<td>(1800 ± 324)</td>
<td>(920 ± 2268)</td>
<td>(92 ± 11)</td>
<td>(5436 ± 864)</td>
<td>(14,760 ± 3240)</td>
<td>(1620 ± 288)</td>
<td></td>
</tr>
<tr>
<td>po</td>
<td>2.5 ± 0.23</td>
<td>2.70 ± 1.20</td>
<td>0.02 ± 0.01</td>
<td>0.2 ± 0.08</td>
<td>0.4 ± 0.08</td>
<td>1.3 ± 0.21</td>
<td>0.04 ± 0.009</td>
<td>1.3 ± 0.29</td>
<td>3.70 ± 0.42</td>
<td>0.40 ± 0.23</td>
</tr>
</tbody>
</table>

**Notes.** Disposition of α-HBCD-derived radioactivity (A) 4 days following a single (3, 10, 30, and 100 mg/kg) and 10-day repeated (3 mg/kg) po dose, (B) at multiple time points following a single 3 mg/kg po dose, and (C) 4 days following a 3 mg/kg dose through iv or po. All data are mean ± SD; represented as percent dose (top value) or concentration of nanogram of administered dose per gram of tissue (ng/g; bottom value in parenthesis).

*Indicates significance as compared with lowest dose (p < 0.05).
measured had slightly higher levels after iv as compared with the oral route of exposure, but not all were statistically significant. Statistically significant differences are listed here as a percent of the oral to iv levels: liver (93%), muscle (89%), kidney (88%), and blood (92%). By comparing the calculated differences for each tissue between the two routes of administration (po vs. iv) for the four major tissues, the higher levels found in these tissues after iv administration indicate an approximate average oral absorption of 90 ± 3% for α-[14C]HBCD.

Elimination

Dose/response and repeated exposure Total cumulative elimination of the α-[14C]HBCD-derived radioactivity in the urine and feces after oral administration is shown in Figure 3 for the four increasing dose groups; 3, 10, 30, and 100 mg/kg/day. The data demonstrate a dose dependency on fecal but not urinary elimination. After exposure to increasing dose levels, total recovery of α-[14C]HBCD-derived radioactivity is similar between all dose groups. At the 3, 10, 30, and 100 mg/kg doses, ~84, 82, 80, and 81% total radioactivity was recovered, respectively. Average recovery of 14C, including that recovered from the tissues, urine and feces, was 82 ± 4%.

After 10 daily doses at 3 mg/kg, the fecal elimination profile more closely resembles that of a single exposure of 30 mg/kg, as compared with a single 3 mg/kg dose (Fig. 3). In the urinary elimination profile, a single exposure of 3 mg/kg is not different than the 10-day repeated exposure.

IV versus oral: absorption Figure 4 compares the cumulative percent of dose in the urine and feces over 4 days after

### TABLE 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α/β</th>
<th>β/β</th>
<th>α phase</th>
<th>β phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.4</td>
<td>3.0</td>
<td>13.9</td>
<td>4.18</td>
</tr>
<tr>
<td>Blood</td>
<td>0.1</td>
<td>0.5</td>
<td>16.6</td>
<td>3.56</td>
</tr>
<tr>
<td>Lung</td>
<td>0.3</td>
<td>15</td>
<td>3.97</td>
<td>0.93</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.2</td>
<td>2.1</td>
<td>7.06</td>
<td>0.15</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.3</td>
<td>8.0</td>
<td>22.4</td>
<td>1.59</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1</td>
<td>3.0</td>
<td>5.20</td>
<td>0.99</td>
</tr>
<tr>
<td>Fat</td>
<td>—</td>
<td>17</td>
<td>17.8</td>
<td>3.00</td>
</tr>
</tbody>
</table>

*Note.* Tissue-specific, biphasic half-lives were calculated from female mice given a single po dose (3 mg/kg) of α-[14C]HBCD. Calculations are derived from percent of administered dose; α and β phase time points are individually based on peak tissue concentrations. Where appropriate, the pool size was calculated by determining the y-intercept of each phase. All data presented in days.
either iv or oral exposure. Similar elimination profiles are observed between the iv and oral routes of administration. Comparison of the amount of [14C]-derived radioactivity eliminated in the urine and feces between the oral and iv routes of administration allows another approach to estimate absorption. Thirty-six percentage of the dose was eliminated in the feces of the iv administered mice on the first day, whereas 41% was eliminated in the oral treated animals. After 4 days, 45% of the dose was eliminated in the feces following iv exposure and 49% following oral. A measurable 4% difference is calculated between the two routes of exposure. This data support the conclusion that α-[14C]HBCD is well absorbed orally.

The iv route of exposure to α-[14C]-HBCD resulted in significantly higher levels of [14C]-derived radioactivity in the feces at days 1, 2, and 3. However, this difference suggested only slightly decreased oral absorption between the two routes. Urinary elimination was similar between iv and oral exposure with 15% of the dose eliminated in the urine following oral exposure versus 17% after the iv route in 4 days. Based on fecal and urinary elimination patterns, comparison between the iv route and oral route further indicates that α-[14C]HBCD is well absorbed orally (89 ± 2%). This agrees with the estimate derived from the difference found in tissue distribution when comparing the iv and oral routes of exposure (90 ± 3%).

Thin-layer chromatography The nature of the α-[14C]HBCD-derived radioactivity in the urine and feces was examined by TLC (Fig. 5). No parent compound was detected in the urine at 1 or 2 days after treatment. TLC consistently revealed one major retention (retardation factor [Rf] = 0.0) which contains polar metabolites. Similar metabolite(s) was seen in the blood and bile, with no parent compound detected.

This is in contrast to fecal elimination where the parent compound predominates in the extractable fraction. Preliminary studies using feces spiked with α-[14C]HBCD demonstrated complete extraction. Thirty-four percentage of the α-[14C]co-migrated-derived radioactivity that was extracted from the feces was parent. Given that ~90% of α-[14C]HBCD is orally absorbed (see above), at most 10% is unabsorbed α-[14C]HBCD. The remaining 24% was absorbed but eliminated unchanged in the feces. Sixty-six percentage of the extracted radioactivity was metabolite. The nonextractable radioactivity is assumed to be bound metabolites of α-[14C]HBCD. Our results indicated that by 1 day, greater than 66% of the radioactivity that was eliminated from the mice was no longer α-HBCD but several metabolites. To examine the identity of the extracted α-[14C]HBCD-derived radioactivity, the extracts, as well as standards of the three main diastereomers (α-, β-, and γ-[14C]HBCD), were assayed by TLC (Fig. 5). In this system, β-[14C]HBCD migrated with a shorter retention factor (Rf = 0.50) than α- and γ-[14C]HBCD which co-migrated at Rf = 0.61. One day postexposure, only one band was detected in the fecal extracts with a Rf = 0.54.
Interestingly, by 2 days postexposure, four bands were present in the fecal extract (Rf = 0.00, 0.12 and 0.14 and 0.54), demonstrating polar metabolites as well as parent compound.

Liver extracts were analyzed at 3 h shortly after the time of peak tissue concentration, 1 h. In the liver tissue, only one clear TLC band was detected with Rf = 0.60, similar to the fecal extracts (Rf = 0.54) and the α- and γ-HBCD standard peaks (Rf = 0.61). Slight Rf differences between standards and unknowns was attributed to matrix effects.

The GPC The nature of the α-[14C]HBCD-derived radioactivity in the brain and fat was initially examined by GPC. GPC separation is based on size but, unlike TLC, GPC is not useful for the identification of individual stereoisomers because all HBCD stereoisomers have the same molecular weight. GPC chromatograms revealed one major peak (data not shown) that was further analyzed using LC/MS/MS.

Liquid chromatography-mass spectrometer Due to the inability of TLC to differentiate α- from γ-HBCD, and the inability for GPC to resolve any HBCD stereoisomers, the [14C]-containing peaks from TLC and GPC were further characterized by LC/MS/MS, which can distinguish individual HBCD stereoisomers. The TLC peaks in the liver (Rf = 0.60) and fecal extracts (Rf = 0.54) were scraped from the plates, eluted with acetone, and analyzed by LC/MS/MS for comparison with authentic standards, α-, β-, and γ-HBCD, which had retention times of 3.40, 4.06, and 4.89 min, respectively. The results indicated that all peaks had an M-H of ~640.6 suggesting that they were HBCD stereoisomers. Both the liver and the fecal extract peaks had retention times of 3.40 min, which corresponds to α-HBCD. Furthermore, only α-HBCD was detected in the feces, fat, and brain at 1 day, and at the liver at 3 h postoral administration of α-[14C]HBCD (Fig. 6).

A summary of the parent, stereoisomer, and metabolite profiles in tissues and excreta after exposure to α-[14C]HBCD can be found in Table 3. Fat and brain contained only parent α-[14C]HBCD. Liver and feces both contained large amounts of parent compounds, 62 and 34%, respectively. No stereoisomerization products were detected in excreta and tissues. Polar metabolites were found at high levels in the liver and feces, 38 and 66%, respectively. The bile, serum, and urine contained exclusively polar metabolites. Metabolites in serum, tissues, and excreta are in the process of being characterized but beyond the scope of this report.

Mass Balance

Approximately 82% of the α-HBCD-derived radioactivity could be accounted for by measuring (1) the major mouse tissues and (2) excreta after oral and intravenous routes of exposure. This recovery is similar to recent publications on related lipophilic compounds in mice: γ-HBCD (Szabo et al., 2010), 2,3,7,8-TCDD (Hakk et al., 2009b), and BDE-47 (Staskal et al., 2005). Lack of total mass balance in this study could be attributed to a number of common factors. Approximate tissue masses (on a percent basis) for blood, skin, fat, and muscle from the literature (ILSI, 1994) were used for the calculation. Although, the [14C] in most major tissues were measured in this study, the carcasses were not and may have contained residual levels not included in the estimates of mass balance. In addition, any exhaled [14C] was not trapped.

TABLE 3

Summary of Metabolite Profiles in Female Mouse Tissue and Excreta after Oral Exposure to 3mg/kg α-[14C]HBCD

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Time (h)</th>
<th>Parent (%)</th>
<th>Isomerization (%)</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3</td>
<td>62</td>
<td>0</td>
<td>38% unextractable (38%)</td>
</tr>
<tr>
<td>Feces</td>
<td>24</td>
<td>34</td>
<td>0</td>
<td>66% Rf = 0.00; unknown (2%) Unextractable (64%)</td>
</tr>
<tr>
<td>Fat</td>
<td>24</td>
<td>100</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Brain</td>
<td>24</td>
<td>100</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Bile</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>100% Rf = 0.00; unknown</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The objectives of this study were to determine the absorption, distribution, metabolism, and excretion of α-HBCD in female mice to better evaluate its behavior. This study demonstrated that ~90% of an oral dose of α-HBCD was absorbed. Distribution of α-HBCD was initially to the highly perfused organs including liver, kidney, and lung, followed by redistribution to skin, muscle, and adipose tissues. Of the major tissues, adipose contained the highest levels detected across all doses measured. Tissue disposition of α-HBCD was dependent on dose at the higher dose levels (30 and 100 mg/kg) in the single (acute) dosing paradigm and also the 10-day repeated exposure paradigm at 3 mg/kg/day.

Fat was found to be a major tissue depot for α-HBCD-derived radioactivity after exposure to increasing α-HBCD concentrations. This is in contrast to γ-HBCD which failed to concentrate in fat due to its rapid metabolism and excretion (Szabo et al., 2010) but similar to another lipophilic BFR, PBDE-47 which accumulated in the fat (Staskal et al., 2005). In an industry report, the HBCD commercial mixture was found to partition to adipose tissue in rats (Yu and Atallah, 1980). HBCD was detected in fish oil supplements with a maximum concentration of 5.8 ng/g for total HBCD (UK Food Standards Agency, 2006).

Human adipose tissue obtained by liposuction in the Czech Republic had total HBCD concentrations ranging from <0.5 to 7.5 ng/kg lw (Pulkrobavá et al., 2009).

Elimination of α-HBCD either after oral or iv administration was primarily in the feces and to a lesser extent in the urine. Fecal elimination of α-HBCD-derived radioactivity decreased as dose increased, which was not observed for urinary elimination. An opposite trend in the liver, fat, muscle, and skin occurred, as the tissue concentration increased nonlinearly with increasing administered doses. Tissue concentrations for α-HBCD are in contrast to what was observed after oral exposure to γ-HBCD where the relative amount of γ-HBCD in excreta or tissues was independent of dose (Szabo et al., 2010). Van der Ven et al. (2009) reported similar findings in the liver of rats exposed daily to the HBCD commercial mixture (up to 175 days) where a dose-dependent hepatic increase in α-HBCD was observed. This rate of increase for α-HBCD was greater than for other HBCD stereoisomers measured. Similar to the distribution observed with α-HBCD, an industry report on the kinetics and distribution of the radiolabeled HBCD commercial mixture in rats detected higher levels of HBCD in the liver than the blood (Yu and Atallah, 1980). These finding suggests that there is a HBCD stereoisomer specific difference in hepatic function and clearance with increasing concentration. Although the mechanism for the nonlinear behavior of α-HBCD is not known, it is possible that inhibition, and/or saturation of metabolism, including binding to hepatic enzymes and transporters, may occur at higher concentrations.

Of the α-HBCD-derived radioactivity detected in the urine, bile, and blood, 100% consisted of polar metabolites after 24 h. Feces (24–48 h) and liver (3 h) contained both parent α-HBCD and polar metabolites. Differential metabolic capacities for γ-HBCD and α-HBCD have been observed in vitro with rat and harbor seal liver microsomes (Zegers et al., 2005) and are supported by the contrast between this present study of α-HBCD and our recently published report on γ-HBCD in mice (Szabo et al., 2010). α-HBCD was more slowly eliminated in female mice than γ-HBCD and therefore is believed to have a greater opportunity to bioaccumulate. Characterizing the toxicity of each stereoisomer remains to be determined.

The in vivo mouse data from this as well as the previous study with γ-HBCD (Szabo et al., 2010) suggest that two factors may be responsible for the shift observed from the predominance of γ-HBCD in the commercial mixture to α-HBCD in biota. First, γ-HBCD was more rapidly metabolized and eliminated with a terminal half-life of 17 days. Bioaccumulation may be even more pronounced after chronic exposure, as we observed over 20% of α-HBCD-administered dose remained in the mice in the 10-day repeated dose study, but <1% was found in the mice exposed to γ-HBCD at the same paradigm (Szabo et al., 2010). In addition, in vivo stereoisomerization (11–15%) of γ-HBCD to α- and β- was observed in female mice treated with γ-HBCD (Szabo et al., 2010); however, the stereoisomer shift was not seen for α-HBCD.

Thus, α-HBCD’s persistence and bioaccumulation in mice is relatively high and may explain the observed predominance of α in biota. These data lend support to a theory that the reason α-HBCD is the dominant HBCD stereoisomer in biota is its relatively slower metabolism, biological persistence, bioaccumulation potential, and absence of stereoisomerization.

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