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COMMUNICATIONS

Synthesis of 1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin and 1-Amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin as Haptenic Compounds

The chemical syntheses and characterization of 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin and 1-amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin are reported. These compounds can be used to couple with carrier proteins affording antigens for radioimmunoassay methodology.

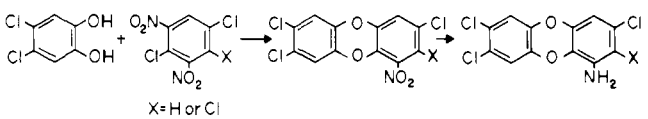
The high toxicity of certain polychlorinated dibenzo-*p*-dioxins has been demonstrated in a number of recent reports (Environmental Health Perspectives, 1973; McConnell and Moore, 1976). The most toxic member in this group is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which is a common contaminant of certain trichlorophenols and related compounds including the herbicide 2,4,5-T (2,4,5-trichlorophenoxyacetic acid). Because of its high chemical stability and lipophilicity, TCDD that is released into the environment could accumulate in the food chain. There is a need for a sensitive analytical method able to detect TCDD in biological tissue samples at levels well below its toxic dose. The common instrumental methods for analyzing TCDD in environmental samples are gas chromatography with an electron-capture detector or direct probe mass spectrometry. Electron-capture gas chromatography generally does not have the required high sensitivity and specificity for a single-method analysis. These methods, for most samples, require extensive sample cleanup and are relatively expensive to perform.

Radioimmunoassay (RIA) often permits specific measurement of compounds in the nanogram to picogram range. Since TCDD exhibits teratogenicity (Courtney et al., 1970; Courtney and Moore, 1971; Sparschu et al., 1971) and effects host resistance (Thigpen et al., 1975) even at sublethal doses, a RIA method would be useful for detecting minute quantities of the compound in biological systems as well as in various other environmental samples. In addition, RIA is readily adaptable to routine assay of large numbers of samples without extensive cleanup.

The TCDD and other chlorinated dibenzo-*p*-dioxins do not have functional groups in their molecule to bind with carrier proteins to form hapten–protein complexes (antigens). Therefore, it is necessary that they should be derivatized with such reactive groups as amino or carboxy groups. In this paper, the syntheses of 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin and 1-amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin are described. These derivatized dioxins can be covalently linked to carrier proteins affording antigens for RIA method development.

Certain nitro-substituted dioxins have been obtained by condensation of catechol dianions with various nitrohalobenzenes (Tomita, 1945; Loudon and McCapra, 1959).

Scheme I



Pohland and Yang (1972) also used this method to prepare halogenated dioxins. This reaction was successfully applied in the present study. The condensation of 4,5-dichlorocatechol dianion with di- or trichlorodinitrobenzene formed tri- or tetrachloronitrodibenzo-*p*-dioxin. The nitro derivatives were then reduced to the corresponding amino dioxins either by zinc dust or stannous chloride in hydrochloric acid (Scheme I). These products were purified by chromatographic techniques and characterized as dioxin derivatives by means of their spectral properties. The position of the amino groups was determined by deamination via the diazonium salts to the corresponding 2,3,7-tri- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

The relative toxicities of these nitro and amino dioxin derivatives have been demonstrated in guinea pigs and mice (McConnell and Moore, 1976). *Although these compounds are generally less toxic than their corresponding parent chlorodioxins, they are still sufficiently toxic to justify handling with extreme caution.*

EXPERIMENTAL SECTION

Elemental analyses were performed by Atlantic Microlab, Atlanta, Ga. Melting points were taken with a Hoover Uni-Melt melting point apparatus. The proton NMR spectra were obtained with Varian FT XL-100 instrument. Gas chromatography (GC) was done on a Varian Aerograph Series 2100 instrument using an electron-capture detector (Sc^3H). A 6 ft × 2 mm i.d. glass column containing 3% OV-210 on 80–100 mesh Gas Chrom Q was used. The column temperature was maintained at 200 °C, the injector and detector temperature at 240 °C. The carrier gas flow was nitrogen at a rate of 40 mL/min. The relative retention time is expressed based on the retention time of standard 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The gas chromatography–mass spectrometric (GC–MS) analysis was performed using a Finnigan Model 9500 GC interfaced by a glass jet separator to a Finnigan

Model 3300/F electron impact mass spectrometer. Ionization was done using 70 eV electrons with 500 μA collector current. The GC–MS analysis was done using a 3% OV-225 column (6 ft × 2 mm i.d., glass) at a programed temperature of 200 to 300 °C at 10 °C/min.

4,5-Dichlorocatechol. This compound was prepared according to the procedure of Willstätter and Müller (1911) in 23% yield, mp 115–117 °C (lit. mp 116–117 °C).

2,5-Dichloro-1,3-dinitrobenzene. To a mixture of 66.7 g (0.55 mol) of KNO_3 and 216 mL of concentrated sulfuric acid was added 95.0 g (0.5 mol) of 1,4-dichloro-2-nitrobenzene. The mixture was then heated with stirring at 70 °C for 3 h. After cooling to room temperature, the mixture was poured into 1.5 kg of crushed ice. The precipitated solid was filtered and recrystallized from ethanol. The product was dried in vacuo, wt 20.5 g (17.5%), mp 102 °C (lit. 104–105 °C; Holleman, 1920).

1,2,4-Trichloro-3,5-dinitrobenzene. To a suspension of 12 g (0.05 mol) of 1,2,4-trichloro-5-nitrobenzene in 50 mL of concentrated sulfuric acid was added 10 mL of fuming nitric acid dropwise with magnetic stirring over a 0.5-h period. The mixture was then heated at 80 °C in a water bath for 4 h. After cooling to room temperature, the mixture was poured into 150 mL of ice–water. The product was extracted with four 100-mL portions of ether. The combined etheral extracts were washed successively with 150 mL each of saturated sodium bicarbonate and water and finally dried over anhydrous sodium sulfate. After evaporation of the solvent, 13.5 g of the crude solid material was obtained. The crude product was recrystallized from ethanol, and 8 g (41%) of light-yellow crystals were obtained, mp 105 °C (lit. 103.5 °C; Hüffer, 1921): NMR ($CDCl_3$) δ 8.20 (s, 1 H). Anal. Calcd for $C_6HCl_3N_2O_4$: C, 26.54; H, 0.37; Cl, 39.18; N, 10.32. Found: C, 26.35; H, 1.12; Cl, 38.95; N, 10.24.

1-Nitro-3,7,8-trichlorodibenzo-*p*-dioxin. In a 250-mL round-bottom flask equipped with a condenser were placed 4.47 g (0.025 mol) of 4,5-dichlorocatechol, 6.9 g (0.05 mol) of K_2CO_3, and 150 mL of acetone. The mixture was refluxed for 0.5 h, and 5.9 g (0.025 mol) of 2,5-dichloro-1,3-dinitrobenzene was then added. The mixture was refluxed for 2 h, cooled to room temperature, and 100 mL of water was added. The precipitated solid, which was filtered and recrystallized from chloroform, weighed 5.3 g (64.0%): mp 175–176.5 °C; NMR ($CDCl_3$) δ 7.02 (s, 1 H), 7.14 (s, 1 H), 7.11 and 7.56 (d, J = 2.5 Hz); mass spectrum m/e M^+ 331 ($C_{12}H_4Cl_3NO_4$), M – NO_2 285, M – (CO+NO_2) 257, M – (Cl + NO_2) 250, M – (2CO + Cl + NO_2) 194. GC relative retention time was 3.10.

1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin. To a suspension of 5.01 g (15.07 mmol) of 1-nitro-3,7,8-trichlorodibenzo-*p*-dioxin in 150 mL of 95% ethanol and 15 mL of concentrated hydrochloric acid was added 11.9 g (52.74 mmol) of $SnCl_2 \cdot 2H_2O$ in 50 mL of 95% ethanol. The mixture was stirred at room temperature for 16 h. After partial removal of ethanol under reduced pressure, 100 mL of water was added and the mixture was adjusted to pH 8 with 1 N NaOH. The product was extracted with total 1-L of ether. The combined extracts were washed four times with 100-mL portions of water and dried over anhydrous sodium sulfate. After evaporation of the solvent, 4.45 g of crude product was obtained. The crude product which was a mixture of 1-nitro and 1-amino compounds was further purified in portions by column chromatography. The mixture (1 g) was dissolved in benzene and applied on silica gel column (2 × 43 cm, 60–200 mesh). The column was eluted with benzene and 50-mL fractions were collected. Fractions 4–6 contained pure 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin (0.57 g, 63%), mp 228.5–230 °C. The recovered 1-nitro derivative (fractions 1–3) was treated again with $SnCl_2 \cdot 2H_2O$. UV max ($CHCl_3$) 305 nm (ε 4840); NMR δ 3.85 (brd, 2 H), 6.95 (s, 1 H), 6.97 (s, 1 H), 6.28 and 6.37 (d, J = 2.4Hz); mass spectrum: m/e M^+ 301 ($C_{12}H_6Cl_3NO_2$), M – CO 273, M – Cl 266, M – (CO^+ Cl) 238. GC relative retention time was 2.39.

1-Nitro-2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The disodium salt of 4,5-dichlorocatechol was prepared by dissolving 1.5 g (8.4 mmol) of the catechol in a solution of 0.7 g (17.5 mmol) of sodium hydroxide in 20 mL of methanol. The solution was evaporated to dryness under reduced pressure and the residue was dissolved in 25 mL of Me_2SO. To this solution was added 1.9 g (6.4 mmol) of 1,2,4-trichloro-3,5-dinitrobenzene, and the mixture was refluxed for 16 h. After cooling, the mixture was poured into 150 mL of ice–water and the orange colored solid (2.5 g) was filtered.

The crude product was purified by silica gel column chromatography in portions. The solid (250 mg) was dissolved in 15 mL of warm hexane and applied on a column (2 × 24 cm) containing 25 g of silica gel. The column was eluted with (1) 100 mL of hexane, (2) 300 mL of hexane–benzene (95:5, v/v), and 50-mL fractions were collected. Fraction 4 contained pure 1-nitro-2,3,7,8-tetrachlorodibenzo-*p*-dioxin (80 mg, 34%): mp 202–203 °C; NMR ($CDCl_3$) δ 7.04 (s, 1 H), 7.06 (s, 1 H), 7.13 (s, 1 H); mass spectrum m/e M^+ 365, M – NO_2 319, M – (CO + NO_2) 291, M – (Cl + NO_2) 284, M – (2CO + Cl + NO_2) 228. GC relative retention time was 4.32. Anal. Calcd for $C_{12}H_3Cl_4NO_4$: C, 39.27; H, 0.82. Found: C, 39.34; H, 0.87.

1-Amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In a 250-mL three-necked round-bottom flask equipped with a reflux condenser were placed 600 mg (1.64 mmol) of 1-nitro-2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 1 g of purified zinc dust, and 80 mL of benzene. Concentrated hydrochloric acid (5 mL) was then added in portions. After 15 min of stirring at room temperature, another 1 g of zinc dust was added and the mixture was heated at 70 °C in a water bath for 15 min. After cooling to room temperature, 100 mL of water was added and the mixture was neutralized with 1 N NaOH. The mixture was transferred to a 500-mL separatory funnel and the benzene layer was removed. The aqueous layer was extracted in portions with total 1000 mL of benzene. The combined benzene extracts were washed once with 100 mL of warm water and dried over anhydrous sodium sulfate. After evaporation of the solvent, 425 mg (77%) of pure 1-amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin was obtained: mp 281 °C, UV max ($CHCl_3$) 305 nm (ε 4961); NMR ($CDCl_3$): δ 4.30 (brd, 2 H), 6.44 (s, 1 H), 6.97 (s, 1 H), 7.02 (s, 1 H); mass spectrum m/e M^+ 335, M – Cl 300, M – (CO + Cl) 272. GC relative retention time was 3.36. Anal. Calcd for $C_{12}H_5Cl_4NO_2$: C, 42.76; H, 1.49. Found: C, 42.75; H, 1.56.

Confirmation of Structure of 1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin and 1-Amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The amine (about 2 mg) was dissolved in 0.5 mL of concentrated sulfuric acid and 0.5 mL of water. The solution was cooled in an ice bath and 0.5 mL of sodium nitrite solution was added, followed by 2 mL of ethanol. The mixture was heated in a water bath at 80 °C for 15 min. After cooling to room temperature, the product was extracted with two 3-mL portions of benzene. The combined extracts were washed once with saturated sodium bicarbonate solution and water and dried over anhydrous sodium sulfate. The product was analyzed by gas chromatography, affording a GC retention time identical with standard 2,3,7-trichlorodibenzo-*p*-dioxin

(2.01 min) from 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (3.65 min) from 1-amino-2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The products had identical spectroscopic and chromatographic (NMR and GC–MS) properties with those of authentic standards.

In the synthesis of 1-nitro-2,3,7,8-tetrachlorodibenzo-*p*-dioxin, two other nitrotetrachlorodibenzo-*p*-dioxins were formed as by-products. These compounds, 1-nitro-3,4,7,8-tetrachlorodibenzo-*p*-dioxin and 2-nitro-1,4,7,8-tetrachlorodibenzo-*p*-dioxin, were isolated from the silica gel column as a mixture. The two compounds were separated on gas chromatography (relative retention times were 5.13 and 5.51) and identified by GC–MS (M^+ 365). However, no individual assignment was made to these two compounds. The mixture of nitro compounds was reduced to the corresponding amines and then deaminated via diazonium salts to form tetrachlorodibenzo-*p*-dioxin isomers. These two tetrachlorodibenzo-*p*-dioxins were identified by GC–MS (M^+ 320) and have GC retention times of 3.45 and 4.01 min which were not identical with that of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin isomer. No attempt was made to quantitate the deamination reactions but the expected products were the major GC peaks in all cases.

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Formation of the *N*-Trifluoroacetate of Carbofuran

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuranyl methylcarbamate) reacts rapidly and quantitatively with trifluoroacetic anhydride in deuteriochloroform in the presence of excess pyridine, which serves both as a catalyst and as a competitive base, preventing the formation of the unreactive trifluoroacetic acid conjugate of carbofuran.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad spectrum pesticide–nematocide with choline-esterase inhibiting properties. A number of analytical procedures, based on gas–liquid chromatography (GLC), have been developed for the detection of carbofuran and its metabolites. Seiber (1972) prepared the trifluoroacetate of carbofuran and a number of other *N*-methylcarbamates for GLC analysis and detection by both the alkaline-flame and electron-capture detectors. The derivatives were prepared in high yields by reaction of the *N*-methylcarbamates with a large excess of trifluoroacetic anhydride in benzene at elevated temperatures. Seiber concluded that two factors, solvent polarity and temperature, governed the rate of reaction and recommended either extended reaction times (16 h) for room temperature reactions or shorter periods (2 h) at elevated temperatures, e.g., 55 and 100 °C when ethyl acetate and benzene were used as solvents, respectively. Lau and Marxmiller (1970) previously *N*-trifluoroacetylated the commercial pesticide Landrin, a mixture of two isomeric *N*-methylcarbamates, for GLC electron-capture detection and employed ethyl acetate as solvent and overnight, room temperature reaction conditions. Wong and Fisher (1975) more recently reported on the determination of carbofuran and its toxic metabolites as their trifluoroacetates again prepared in ethyl acetate at elevated temperatures (45 °C) with a minimum reaction time of 16 h. In this work, it has been the practice (see Lau and Marxmiller, 1970, and Wong and Fisher, 1975) to employ several thousand to more than 100 000 mol excess of trifluoroacetic anhydride in the derivatization of the *N*-methylcarbamates for GLC detection. These practices have the disadvantage of requiring the decomposition of large excesses of trifluoroacetic anhydride with the addition of water following dilution of the reaction mixture with a suitable solvent such as hexane. The excess trifluoroacetic acid is subsequently removed by washing the organic phase with water, and finally the organic phase is dried and concentrated to a convenient volume for GLC analysis.

Table I. Yield of *N*-Trifluoroacetylcarbofuran in the Presence of Deuteriotrifluoroacetic Acid

Mol of F_3CCOOD/mol of carbofuran	Yield in 18 h, %
0	35
0.1	29
0.2	18
0.4	9
0.8	6

In the course of attempting a room temperature preparative scale trifluoroacetylation of carbofuran in $CDCl_3$ and employing more nearly stoichiometric proportions of trifluoroacetic anhydride than is normally the practice, the reaction was observed by NMR to approach a limiting yield of 50% after about 18 h. The reaction was repeated several times with varying proportions of reactants without effectively improving the yield. From this observation it was surmised that for each mole of *N*-tri-