Quantum Chemical and Metabolic Studies towards an Explanation of the Exceptional Status of the 2,3,7,8-Positions in Dibenzodioxin

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INTRODUCTION

Several authors previously have reported the highly selective tissue retention of 2,3,7,8-substituted PCDDs and PCDFs. Also, several reasons were held responsible for this behaviour[1], but at present a strong regioselective metabolic attack on either the dibenzodioxin or dibenzofuran molecule can be considered as the most plausible reason. Clearly, the regioselectivity and oxidation by cytochrome P450 must play an important role in this process[2]. On the other hand, very little information is available on this subject for PCDDs and PCDFs.
It is pointed out by Van den Berg[2] that for future purposes a more quantitative approach is required. From that standpoint one then should in principle be able to predict electron densities in these molecules to elucidate regioselectivity in biotransformation.
Regarding polychlorinated molecules of this size, quantum mechanical considerations and calculations are quite demanding and often unreliable regarding their outcome. The only theoretical study reported so far for the positions of oxygen insertion into an PFDF molecule[3], however, failed to account properly for the observed result[4].
Fluorine (like carbon and oxygen) belongs to the second row of the periodic table. Consequently, fluorinated dioxins are calculated much faster and more reliably regarding *ab initio* methods.
Whether these results can be translated or compared to their chlorinated analogues in any way, strongly depends on the effect of the substituent on the aromatic system. Regarding the aromatic π-system chlorine and fluorine substituents are of similar influence. Due to their strong electronegativity they lower the reactivity of the aromatic system for further electrophilic attack and (electrophilic) bisubstitution is directed to ortho and para positions. How any further substitution on polyhalogenated dibenzodioxins would proceed is difficult to estimate. The degree of comparability of the fluorinated and chlorinated congeners should become evident if their degradation rates and the resulting metabolites are compared in dependence of the substitution pattern.

MATERIALS AND METHODS

The chlorinated[5] and fluorinated[6] dibenzodioxins were synthesized as reported.
Liver homogenates were obtained from male NMRI mice weighing 20-23 g. The animals were pretreated with a total amount of 900 µg ß-naphthoflavone (i.p.; dissolved in corn oil) at three consecutive days prior to sacrifice. Livers were perfused via the potal vein with sterile saline, removed, and minced in 4 ml Tris/sucrose buffer (5 mM Tris, 250 mM sucrose, pH 7.4). The tissue was then homogenized in an ice-cooled Dounce homogenizer, centrifuged at 500 x g, and stored at -70°C. Incubations were performed at 37°C by mixing 200 µl 1M Tris-HCl (pH 7.4), 200 µl 1 mM $MgCl_2$, 200 µl 5 mM NADP, 200 µl 50 mM sodium isocitrate, and 40 µl isocitrate dehydrogenase (Boehringer, Mannheim) with liver homogenate containing 1.2 mg protein, and with a substrate mixture dissolved in 10 µl DMSO. At various timepoints, the incubations were

stopped by adding 1 ml toluene supplemented with analytical standards. Polyhalogenated dibenzodioxins were extracted twice for 15 min with 1 ml toluene under sonification. The organic phase was removed, the solvent was evaporated to a final volume of 40 µl, and the samples were analyzed by HRGC/LRMS.

For the fluorinated dioxins *ab initio* calculations of the *Restricted Hartree-Fock* type (RHF) were used. They were conducted with GAUSSIAN 94[7] on a Convex station at the „Zentrum für Datenverarbeitung" University of Tübingen. This RHF version is based on the LCAO molecular orbital theory, where the eigenfunctions are calculated using the self-consistent-field (SCF) approximation[8] and the formalism developed by Hartree and Fock and Roothan[9], and Hall[10]. Structures were optimized (without symmetry restrictions) on the RHF/3-21G level. The deduced molecular structures were confirmed by calculating the Hesse-matrices and vibrational frequencies and were shown to be stationary. Vibrational zero point energies (VZPE) were also determined and multiplied by 0.89 (empirical correction) to account for the usual overestimation of the VZPE at this theoretical level[8].

RESULTS AND DISCUSSION

Metabolic degradation of TCDD and TFDD in mouse liver homogenate

Enzymatic degradation took place in liver homogenates of mice that were pretreated with β-naphthoflavone. Due to this procedure the P450 concentration in the liver is risen to guarantee a better metabolization in the final homogenate.

Table 1: Rates of metabolization of PFDD in liver homogenates of mice after two hours of incubation (Σ PFDD = 2 nmol/mg protein).

PFDD-congener	non metabolized [%]	PFDD-congener	non metabolized [%]
O_8FD	99	1,3,6,8-T_4FDD	31
1,2,3,4,7,8-H_6FD	97	1,2,6,7-T_4FDD	28
1,2,3,4,7,8,9-H_7FD	95	1,2,3,4,6-P_5FDD	23
1,2,3,7,8-P_5FD	94	Σ1,2,6,8-/1,2,4,7-/1,2,4,8-T_4FDD	21
1,2,3,6,7,8-H_6FD	89	1,4,6-T_3FDD	20
1,2,3,7,8,9-H_6FD	84	1,2,7,9-T_4FDD	19
2,3,7,8-T_4FD	78	1,2,3,4,6,8-H_6FDD	19
Σ1,2,4,6,7,9-1,2,4,6,7,9-H_6FD	69	1,2,3,4,7-P_5FDD	18
		1,2,7-T_3FDD	17
1,2,4,6,9-P_5FD	67	Σ2,8-/2,7-D_2FDD	16
1,4,6,9-T_4FD	63	Σ1,2,4,6-/1,2,4,7-	16
1,2,3,4,6,7,9-H_7FD	61	1,2,4,7,9-T_4FDD	15
1,2,4,7,8-P_5FD	55	Σ1,3,7-/1,3,8-TFDD	14
1,4,7,8-T_4FD	32	1,2,3,4-T_4FDD	12
1,2,4,6,7-T_4FD	32	1,2,7,8-T_4FDD	11
1,2,4,8,9-P_5FD		1-MFDF	11

PFDD-congener	non metabolized [%]
1,2,6,9-/1,3,7,8-/1,3,6,9-/1,2,8,9-T_4FDD 2,3,7-/1,2,4-/1,3,6-T_3FDD 1,6-D_2FDD	between five and ten
1,3,9-/1,4,7-/1,2,6-/1,2,9- T_3FDD 1,7-/1,8-/1,9-/1,3-/2,3/1,2- D_2FDD 1-/2-M_1FDD	smaler than five

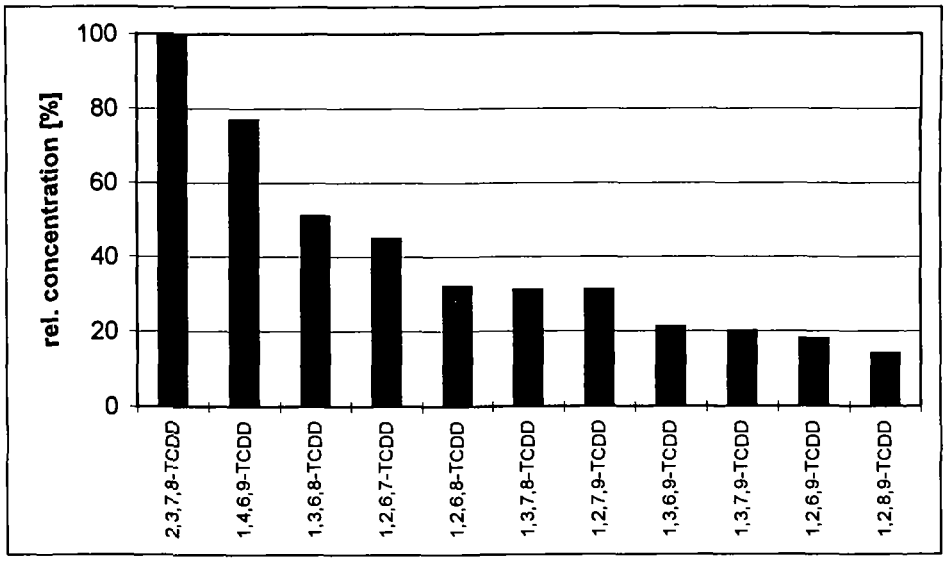

Figure 1: Relative concentrations of the non metabolized TCDD congeners after three hours

If the degradation rates of the tetrahalogenated dioxins are compared, the order of stability is nearly identical for the fluorine [Tab. 1] and chlorine [Fig. 1] congeners. In both cases, the 2,3,7,8-substituted congeners show the slowest degradation rates, but it is still much higher for fluorine than for chlorine. In all other cases, the stabilities of analogous congeners are quite comparable. Thus, fluorine and chlorine substitution in the case of dioxins results in a parallel behaviour of the aromatic system regarding metabolic attack. Interestingly, the 1,4,6,9-TXDD (X=Cl, F) are surprisingly stable. Although no lateral (2,3,7,8-) position substituted, their degradation rates are the 2nd lowest, following those of the 2,3,7,8-TXDD. This is in good agreement with in vivo studies carried out on rats that obtained injections of dioxin mixtures. Here, Abraham[11] and also Körner[12] still found 1,4,6,9-TCDD besides the 2,3,7,8-substituted congeners in the liver, 14 h and 24 h, respectively, after injection.

Identified metabolites

Also, investigating the metabolites of 2,3,7,8-TFDD and 1,7-dichloro-2,8-difluorodibenzofuran in comparison to their chlorinated species confirms the similarity of the fluorine and chlorine substituent in biodegradation. For 2,3,7,8-TFDD, only oxygen bridge cleavage was observed. Metabolites found were dihydroxytetrafluorobiphenylether and difluoro-ortho-benzoquinone. This is also an important degradation pathway for 2,3,7,8-TCDD[2,13] (only for 2,3,7,8-substituted chlorinated congeners this kind of degradation was found !). Another major metabolite in this case was dihydroxy-T_3CDD.

In the case of 1,7-dichloro-2,8-difluorodibenzofuran the aromatic ring was hydroxylated and the four monohydroxylated dichlorodifluoroDF as well as the monohydroxylated chlorodifluoroDF could be detected as metabolites. Such hydroxylated congeners could also be identified for the non-2,3,7,8-substituted PCDFs[2,14].

Quantum mechanical calculations

Preliminary remark: During the hydroxylation of aromatic systems by P450 oxygen has to be transferred from the enzyme towards the aromatic system (via epoxidation)[15,16]. In principle, an electrophilic aromatic substitution is held responsible for this step. Consequently, a high electron density in the aromatic system should facilitate this reaction. This is demonstrated by the fact that the metabolization rate is (on the average) decreased by an increase in the fluorination degree [Tab.1]. This also could be used to explain why the less polar TXDD (2,3,7,8; 1,4,6,9; 1,3,6,8; 1,2,6,7) show a slower degradation

than the more polar congeners (1,2,8,9; 1,3,7,9; 1,2,6,9) [Tab. 1 + Fig. 1] because their electron density is distributed more homogeneously over the whole molecular framework. Thus, they are less prone to a localized electrophilic attack. But a successful explanation for the exceptional position of the 2,3,7,8-substituted congeners is still missing. The reason for the specific hydroxylation of the unsubstituted dibenzodioxin in the lateral position also has to be found. It was concluded by the authors that by looking at the electron densities of the aromatic systems of interest one should get reasonable insight into this aspect.

Thus, the aromatic systems were investigated by means of quantum-mechanical calculations to give a first estimate of the electron density distributions in these systems. We used *ab initio* optimization of molecular geometry using the *Restricted Hartree-Fock* type (RHF) for the fluorinated dioxins leading to planar geometry of the aromatic systems. The total energies found (GAUSSIAN 94) were shown to refer to minima. These calculations yield by use of Mulliken's population analysis two kinds of electron distributions.

First, there are the total atomic charges [Tab.2]. They are an estimation for the total electron density at each atom. As expected a lower electron density is found at fluorine-substituted carbons. The charge density at the H-substituted carbons corresponds to that of dibenzodioxin (slightly higher because of the +M-effect of the fluorine). Dibenzodioxin itself shows a slightly higher electron density in the 1,4,6,9-positions as compared to the 2,3,7,8-positions. From this analysis the conclusion can be drawn that the Mulliken do not provide a good answer to the exceptional 2,3,7,8-position.

Secondly, for planar π-electron systems, the π-electron charges may be derived from Mulliken's gross orbital population [Tab.3]. These values clearly visualize the +M-effect of fluorine and oxygen in the aromatic system. It also turned out, that - regarding π-densities - the 1,4,6,9 positions possess higher relative charges (-0,0240) than the 2,3,7,8 positions (-0,0089) in the unsubstituted system. Anyhow, it does not provide any good reason for the exceptional 2,3,7,8-position, either.

It was postulated by Fleming[17] that the epoxidation of the aromatic systems proceed via an electrocyclic reaction, i.e., it is orbital-controlled. It is believed that polycyclic aromatic systems are attacked by P450 at the K-region. For this reaction it seems that the nucleophilic character of the K-region (the value of the HOMO-coefficients) plays an important role. Following these considerations the epoxidation by P450 or another electrocyclic reaction should take place at the position with the highest HOMO-coefficient (or more strictly the pair of positions with the highest HOMO-coefficients). These [Tab.4] and all other orbital coefficients can also be extracted from our quantum-mechanical calculations.

Regarding the HOMO of the unsubstituted dibenzodioxin, the positions with high orbital coefficients can be assigned to the 2,3,7,8-positions and the attack of P450 would be predicted to happen here. This situation is not changed when going to fluorine substituted congeners.

It has been shown for different polyaromatic systems that the introduction of fluorine at a potential site of metabolic attack suppresses the oxygenation[18].In this respect the possibility of blocking the formation of bay ring epoxides was of particular importance. This has been reported in the course of cancer studies (but without the aid of quantum-mechanical calculations).

Coming back to the problem of the observed slow biodegradation, it can be concluded that halogenation of dibenzodioxin at the 2,3,7,8-positions is expected to hinder the oxygenation.

But this hypothesis has to be verified from a more global and demanding point of view. For this purpose we suggest systematic investigations starting with different aromatic systems. If the HOMO-orbitals with the highest coefficients play that important role for the regioselectivity in hydroxylation (epoxidation) of aromatic systems it should also manifest itself in these systems and be observed. If blocking these positions the corresponding epoxidation should be remarkably slowed down. In contrast, halogenation at a different position should render this effect of substitution neglegible regarding its biodegradation characteristics.

Table 2: Total *Mulliken charges* of the optimized systems.

Comp.	DD	1-MFDD	2-MFDD	OFDD	1,2,6,7-TFDD	1,2,8,9-TFDD	1,3,6,8-TFDD	1,3,6,9-TFDD	1,3,7,9-TFDD	1,4,6,9-TFDD	2,3,7,8-TFDD
C1	-0.26195	0.37322	-0.30476	0.35721	0.33688	0.34046	0.39055	0.39177	0.39042	0.37788	-0.29531
C2	-0.23886	-0.27683	0.40033	0.36807	0.38491	0.38438	-0.31753	-0.31711	-0.31631	-0.26897	0.37560
C3	-0.23886	-0.23374	-0.28145	0.36807	-0.27307	-0.27229	0.41703	0.41618	0.41518	-0.26897	0.37560
C4	-0.26195	-0.26611	-0.25596	0.35721	-0.25668	-0.25857	-0.30458	-0.30348	-0.30344	0.37788	-0.29531
C6	-0.26195	-0.26039	-0.26145	0.35721	0.33696	-0.25858	0.39055	0.37675	-0.30344	0.37788	-0.29532
C7	-0.23886	-0.23817	-0.23722	0.36807	0.38489	-0.27230	-0.31753	-0.26942	0.41518	-0.26897	0.37562
C8	-0.23886	-0.23765	-0.23854	0.36807	-0.27307	0.38435	0.41703	-0.26829	-0.31631	-0.26897	0.37562
C9	-0.26195	-0.26046	-0.26011	0.36807	-0.25670	0.34056	-0.30458	0.37659	0.39042	0.37788	-0.29532
C1a	0.36853	0.33283	0.38560	0.37246	0.34606	0.35037	0.32090	0.32885	0.33536	0.35795	0.37668
C4a	0.36853	0.39600	0.36505	0.37246	0.39318	0.38950	0.41428	0.40777	0.40036	0.35795	0.37669
C6a	0.36853	0.36290	0.36903	0.37246	0.34608	0.38956	0.32090	0.34973	0.40036	0.35795	0.37669
C9a	0.36853	0.37089	0.36334	0.37246	0.39319	0.35034	0.41428	0.36473	0.33536	0.35795	0.37668

Table 3: π-electron densities of the optimized systems.

Comp.	DD	1-MFDD	2-MFDD	OFDD	1,2,6,7-TFDD	1,2,8,9-TFDD	1,3,6,8-TFDD	1,3,6,9-TFDD	1,3,7,9-TFDD	1,4,6,9-TFDD	2,3,7,8-TFDD
C1	-0.02400	-0.02223	-0.07551	-0.06125	-0.06907	-0.06407	0.00387	0.00445	0.00270	-0.03596	-0.04748
C2	-0.00893	-0.06068	-0.00460	-0.07673	-0.05022	-0.05140	-0.10868	-0.10648	-0.10430	-0.03350	-0.05087
C3	-0.00893	0.01393	-0.06193	-0.07673	-0.03389	-0.03263	0.02130	0.01957	0.01752	-0.03350	-0.05087
C4	-0.02400	-0.04544	-0.00070	-0.06125	-0.01408	-0.01999	-0.09129	-0.08931	-0.09017	-0.03596	-0.04748
C6	-0.02400	-0.02131	-0.02336	-0.06125	-0.06903	-0.02004	0.00387	-0.03645	-0.09017	-0.03596	-0.04748
C7	-0.00893	-0.00709	-0.00462	-0.07673	-0.05023	-0.03264	-0.10868	-0.03577	0.01752	-0.03350	-0.05087
C8	-0.00893	-0.00525	-0.00778	-0.07673	-0.03390	-0.05142	0.02130	-0.03152	-0.10430	-0.03350	-0.05087
C9	-0.02400	-0.01895	-0.01958	-0.06125	-0.01411	-0.06398	-0.09129	-0.03773	0.00270	-0.03596	-0.04748
C1a	-0.04213	-0.09216	-0.01727	-0.08122	-0.07368	-0.07705	-0.12651	-0.12164	-0.11482	-0.07715	-0.04626
C4a	-0.04213	-0.02002	-0.06429	-0.08122	-0.04962	-0.04635	0.00216	-0.00426	-0.00912	-0.07715	-0.04626
C6a	-0.04213	-0.04827	-0.04135	-0.08122	-0.07370	-0.04630	-0.12651	-0.08215	-0.00912	-0.07715	-0.04626
C9a	-0.04213	-0.04408	-0.04941	-0.08122	-0.04961	-0.07712	0.00216	-0.07033	-0.11482	-0.07715	-0.04626

Table 4: HOMO coefficients of the optimized systems.

Comp.	DD	1-MFDD	2-MFDD	OFDD	1,2,6,7-TFDD	1,2,8,9-TFDD	1,3,6,8-TFDD	1,3,6,9-TFDD	1,3,7,9-TFDD	1,4,6,9-TFDD	2,3,7,8-TFDD
C1	-0.09898	-0.10704	-0.05204	-0.06685	-0.05483	-0.03206	-0.11025	-0.14422	-0.16120	-0.08702	-0.07296
C2	0.30516	0.27838	0.29196	0.30152	0.29480	0.30647	0.32367	0.31573	0.28181	0.29063	0.30985
C3	0.30516	0.27660	0.31629	0.30148	0.31144	0.29957	0.27222	0.30496	0.29906	0.29063	0.30985
C4	-0.09898	-0.08498	-0.13353	-0.06691	-0.11674	-0.13913	-0.05105	-0.00709	0.01345	-0.08702	-0.07298
C6	-0.09898	-0.08358	-0.11033	-0.06691	-0.05492	-0.13925	-0.39310	-0.04571	0.01342	-0.08700	-0.07294
C7	0.30516	0.33038	0.29826	0.30158	0.29478	0.29956	0.32367	0.29860	0.29902	0.29059	0.30987
C8	0.30516	0.31777	0.31684	0.30165	0.31145	0.30648	0.27222	0.24532	0.28181	0.29059	0.30987
C9	-0.09898	-0.10432	-0.07764	-0.06677	-0.11680	-0.03208	-0.05105	-0.12616	-0.16113	-0.08700	-0.07295
C1a	-0.34531	-0.34399	-0.31824	-0.34784	-0.31646	-0.33193	-0.39310	-0.41239	-0.38655	-0.35383	-0.34470
C4a	-0.34531	-0.31150	-0.36208	-0.34784	-0.35705	-0.03206	-0.30362	-0.31964	-0.30578	-0.35383	-0.34470
C6a	-0.34531	-0.36716	-0.34647	-0.34795	-0.33361	-0.35466	-0.39310	-0.32886	-0.30577	-0.35378	-0.34473
C9a	-0.34531	-0.36582	-0.35028	-0.34788	-0.35696	-0.33187	-0.30362	-0.33619	-0.38653	-0.35378	-0.34473

META (po)

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