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From Dioxin to Drug Lead—The Development of 2,3,7,8-Tetrachlorophenothiazine

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Polychlorinated dibenzo-p-dioxins are persistent environmental pollutants. The most potent congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), causes a wasting syndrome and is a potent carcinogen and immunosuppressant in the rat at high doses. However, low doses cause opposite effects to some of those observed at higher doses, resulting in chemoprevention, stimulation of the immune system, and longevity in experimental animals. The new TCDD analogue, 2,3,7,8-tetrachlorophenothiazine (TCPT), was developed to take advantage of the low-dose effects of dioxins that have potential application as therapeutics. Its development marked a deviation from the traditional scope of phenothiazine drug design by deriving biological effects from aryl substituents. TCPT was synthesized in three steps. The key ring-closing step was performed utilizing a Buchwald-Hartwig amination to provide TCPT in 37% yield. Its potency to induce CYP1A1 activity over 24 h was 370 times lower than that of TCDD in vitro. The elimination half-life of the parent compound in serum was 5.4 h in the rat and 2.7 h in the guinea pig, compared to 11 and 30 days, respectively, for TCDD. These initial findings clearly differentiate TCPT from TCDD and provide the basis for further studies of its potential as a drug lead.

Introduction

As one of the best-studied chemicals in toxicology, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been reported to exert a broad range of effects in vitro and in vivo. The most biologically effective dioxins are halogenated in the 2, 3, 7, and 8 positions, with TCDD being the most potent congener. Different dioxin congeners vary in potency.[1,2] Their effects are additive and diverse but well-characterized. They include a wasting syndrome[3,4] and carcinogenicity (lungs, liver)[5] at high doses, liver injury, immunosuppression,[6] reproductive effects,[7] and reduced serum IGF-1 (insulin-like growth factor-1) levels[8] at medium doses, and effects on thyroid hormones,[9,10] thymic atrophy,[11] and a variety of enzyme induction[12] at low doses.

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TCDD is a teratogen in the rat, with a NOEL (no observable effect level) at a cumulative dose of 0.3 $\mu g\,kg^{-1}$ TCDD administered orally during gestation.[13]

Much research has been conducted to elucidate the mechanism of TCDD toxicity,[14–16] most of which has been related to the aryl hydrocarbon receptor (AhR). However, it has been argued that a single mechanism is unlikely to explain a complex profile of effects such as that displayed by dioxins[17,18] extending over several orders of magnitude along the dose-scale.

Reports on differences between low-dose and high-dose effects of chemicals date back to the 19th century.[19] In principle,[20] low doses trigger a system to be removed from equilibrium (homeostasis), which can be counteracted by responses on the same or a different pathway, leading to overcompensation which causes the observed low-dose effects. As this overcompensation pushes homeostasis in the opposite direction of the counteracted cause, it by definition causes effects opposite to those of high doses. Effects can be reversed based on the kinetics of a compound or the dynamics of its effects. Kinetic reversibility includes distribution, biotransformation, and/or excretion. An organism adapts by increased elimination, for example, by enzyme induction. Dynamic reversibility can be due to repair or reversibility of receptor interactions, with adaptation occurring by, for example, protein up- or down-regulation. High doses, however, force the system beyond kinetic or dynamic recovery.

Chronic low-dose effects of TCDD were first documented in the rat by Kociba et al. in 1978.[5] At life-long dose rates of 0.1 $\mu g\,kg^{-}day^{-1}$ TCDD, they observed a reduction in the occurrence of spontaneously occurring age-related tumors in the pituitary, uterus, pancreas, adrenal, and especially mammary tissue. Only 49% of the female rats treated with TCDD showed benign mammary tumors, and none showed malignant tumors, compared to 85% and 9%, respectively, in controls.

Recently, chronic toxicity including carcinogenicity of 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD) has been investigated in female Sprague-Dawley rats.[21] Lung cancer was the major effect and overall chronic toxicity obeyed Haber's Rule of Inhalation Toxicology:[22–26]

$$c\,t = k \qquad (1)$$

where c = concentration, t = time, and k = constant. Increasing doses have been found to reduce the time to death in an entirely predictive manner (the constant for longevity and chemoprevention by HpCDD is $k = 598 \pm 28.7\ mg\,kg^{-1}\,day^{-1}$, whereas the constant for reduced life expectancy by HpCDD is $k = 1212 \pm 53.4\ mg\,kg^{-1}\,day^{-1}$). A single subthreshold dose (1,000 $\mu g\,kg^{-1}$) of HpCDD prolonged the life of rats by about two months over controls (8% increase in life-span). No lung or liver cancer occurred in these rats, as compared to 2.8% and 5.6% respective cancer prevalence in controls.[21]

The mechanism of action for the abovementioned effects has not been fully determined. However, TCDD has been reported to reduce serum IGF-1 levels,[8] which is assumed to be a key mediator for several medium- to low-dose effects such as longevity, inhibition of ovulation,[27,28] and permanently reduced body weight.[29] Another presumed mechanism of action for low-dose effects is mediated through the AhR. Agonists of this receptor-mediated pathway induce cytochrome P450 CYP1 A1 enzyme activity. This induction of phase I metabolism of ubiquitous toxic exogenous and endogenous compounds could account for reducing concentrations at target sites from reaching threshold levels for effects, such as cancer, to occur.

Given these beneficial effects of dioxins, it was reasonable to contemplate possible medicinal uses of this class of compounds.[30] Effects such as reduced cancer rates, lowered body weight, increased insulin sensitivity,[31] and inhibition of ovulation can all be desirable effects and suitable analogues could find application in the prevention of cancer, treatment of obesity, and for diabetes type II, or as a contraceptive. However, the high toxic potency and unfavorable kinetics of dioxins themselves strictly prohibit their use as therapeutic agents. With an average elimination half-life ($t_{1/2}$) of 7.8 years[32] and a calculated LD_{50} value of 6 $mg\,kg^{-1}$[33] in humans, TCDD poses risks that far outweigh potential benefits. The use of congeners with lower potency (that is, higher degree of chlorination) is also prohibitive, as the elimination half-life increases with increasing chlorination to the point of exceeding human life-expectancy.[32] These two prohibitive properties (kinetics and potency) had to be eliminated for the development of a dioxin analogue with potential therapeutic applications.

During the search for such a new compound, the striking structural similarity between dioxins and the medicinally important class of phenothiazines became apparent (Figure 1).

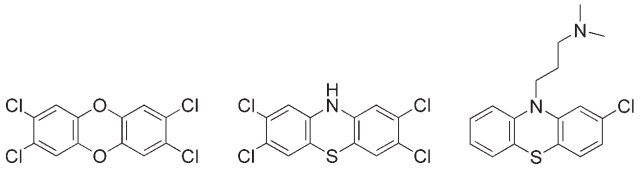


Figure 1. Structural comparison of TCDD (left), TCPT (middle), and a representative classical phenothiazine (chlorpromazine, right).

Both classes consist of a heterocyclic central ring in a tricyclic twelve-carbon structure. Phenothiazines have long been known for their antipsychotic as well as their sedative, antihistaminic, antiemetic, antidiabetic, and anthelmintic effects.[34] These drugs show a wide therapeutic index and a flat dose-response curve, rendering them ideal for therapeutic use.[34] Adverse reactions include cardiovascular effects; however, these have been mostly observed after suicidal self-administration.[35] Most phenothiazine drugs showed no fetotoxic, embryotoxic, or teratogenic effects in rodent models at doses that did not affect the pregnant female; only doses that were toxic to the dam were also toxic to the fetus and embryo.[36] Epidemiological data in humans could not associate phenothiazine drugs administered during pregnancy to increased mortality or morbidity.[37–39]

Although the chloro-substituent in the first big-market neuroleptic phenothiazine drug, chlorpromazine (CPZ), was documented to be a key component for its efficacy,[40] no attempt had yet been made to investigate the effects of higher chlorinated, N-unsubstituted phenothiazine derivatives. Historically, the pharmacological activity and other favorable properties such as a wide therapeutic index and flat dose-response curves of phenothiazine therapeutics were mostly attributed to their dialkyl-amino side chain. In contrast, a lateral chlorination pattern of polychlorinated dibenzo-*p*-dioxins (PCDDs) is known to be the determinant for their biological activity. Therefore, to develop a new dioxin-analogue, the N-unsubstituted laterally chlorinated phenothiazine was desired: 2,3,7,8-tetrachlorophenothiazine (TCPT). The effects of this structural deviation from classical phenothiazine drugs remains an important aspect of future research. Pharmacological properties and possible adverse effects need to be investigated. A key mechanism for the therapeutic efficacy of psychoactive phenothiazines such as CPZ is their interaction with phospholipid membranes.[41] Although CPZ was reported not to cause drug-induced phospholipidosis,[42,43] this endpoint is characteristic for other phenothiazine drugs and such investigations are required in the further development of TCPT as a drug lead.

The pharmacokinetic profile of phenothiazines differs dramatically from that of PCDDs, which addresses the first prohibitive property of dioxins, unfavorable kinetics. The most prominent member of the class, CPZ, shows an elimination half-life of 9.1 h in the rat[44] and 30 h in humans.[34] CPZ, like all conventional phenothiazine drugs, forms sulfoxo-, ring hydroxyl-, and side-chain metabolites. However, TCPT does not contain side chains and its aryl positions are sterically hindered by chloro-substituents, preventing ring hydroxylation from occurring.

Rapid metabolism is expected to occur at the thio-ether moiety, which can be readily oxidized to the sulfoxide or sulfone, and the secondary amine, which can be oxidized to the hydroxylamine, conjugated by phase II enzymes, and readily excreted. In contrast, biotransformation is the slowest and hence rate-limiting step in PCDD-elimination,[45] making nonbiliary intestinal elimination by desquamating enterocytes and redistribution of PCDDs into fecal fat to become the main route of very slow excretion.

Concerns regarding the second prohibitive factor, potency, were addressed by considerations of the sterics of phenothiazines. Whereas dioxins are essentially planar molecules,[46,47] phenothiazines are folded along the N-S axis. Semiempirical calculations of the molecular structure of TCPT[48] predicted a deviation from planarity by $19.8^\circ \pm 0.7^\circ$ with a preferred H-intra configuration. As AhR-mediated CYP1A1 induction has typically been described for mostly planar ligands,[49,50] this slight sterical difference between phenothiazines and dioxins was expected to affect interactions with endogenous targets and to cause a reduced potency of TCPT as compared to TCDD.

Results and Discussion

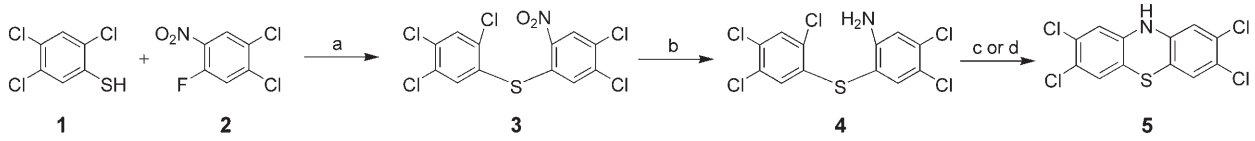
Synthesis

Previously, the synthesis of 2,3,7,8-TCPT had been reported[51,52] in which the unsubstituted phenothiazine was exposed to chlorine gas in acidic solution in the presence of a Friedel–Crafts catalyst. However, attempts to reproduce this synthesis led only to the isolation of 1,3,7,9-TCPT and its sulfoxide as analyzed by GC/MS and finally confirmed by crystal structure analysis.[48] Variation of the conditions for catalyzed chlorination also did not produce any 2,3,7,8-TCPT.[53]

Phenothiazine derivatives have been used for their therapeutic effects as of the 1950s. Numerous synthetic approaches to the phenothiazine backbone have been developed[54,55] and were applied in the attempted synthesis of TCPT. However, only one of these routes successfully produced the target compound in isolable quantities. As the compound was to be used in biological testing, a synthetic approach was needed which could be easily reproduced and readily scalable. The final synthetic route (Scheme 1) was chosen based upon the commercial availability of the starting materials, the ease of preparation of the cyclization substrate, and the possibility for new methods to be developed in the ultimate ring-closing step.

The three-step reaction sequence commenced with the coupling of the aromatic thiol **1** and the aryl fluoride **2** in the presence of potassium carbonate under anhydrous conditions, producing diarylsulfide **3** (Scheme 1). The yield of the coupling reaction was found to be solvent dependent, with polar solvents such as DMF and acetone giving low yields (32% and 35%, respectively). However, a less polar solvent (dichloromethane) improved the coupling step, resulting in a 97% yield. In the second step, the nitro group was reduced with Fe in acetic acid to the corresponding amine **4** with the Bechamp method[56,57] in 86% yield after purification. These reduction conditions proved to be superior to other methods in which $SnCl_2$ (10% yield) or Pd/H_2 (18% yield) were used.

Compound **4** was then set up for an intramolecular cyclization to yield the target compound **5**. Ullmann-type coupling



Scheme 1. Synthesis of TCPT in three steps, based on commercially available starting material: formation of the diarylsulfide, reduction to the amine, then alternatively intramolecular Ullmann-type condensation, or Maes-modified Buchwald-Hartwig coupling. Reagents and conditions: a) K_2CO_3, $CaCO_3$, 3Å molecular sieves, CH_2Cl_2, reflux, 21 h, 97%; b) Fe filings, Glacial HOAc, Acetone, DI H_2O, reflux, 2.5 h, 86%; c) Cu, CuI, Na_2CO_3, DMF, reflux, 24 h, 5%; d) $Pd(OAc)_2$, 2-(dicyclohexylphosphano)-biphenyl, NatBuO, DMF, 200 °C microwave, 2 min, 37%.

conditions modified from historical phenothiazine syntheses[58] resulted in a 5% yield of the desired tricyclic compound. Critical for inducing cyclization was the use of purified cuprous iodide and fresh copper. Further investigations into the Ullmann-type coupling reaction kinetics identified an increase in production of TCPT up to 24 h, followed by decay of the cyclized compound presumably due to the harsh conditions, despite the continuous presence of starting material. Additional supplementation of the reaction with catalysts after 24 h did not increase the yield nor influence the kinetics of formation and degradation.

In an attempt to shorten the reaction time and improve the yield, a microwave-mediated Ullmann-type coupling was attempted. Ullmann-type couplings under microwave conditions have been reported to reduce reaction time and to improve yields.[59] However, these reaction conditions allowed no conversion to product, and only starting material was recovered.

Key to our synthesis of multigram quantities of the compound was the discovery that a Buchwald-Hartwig amination could be used to induce the cyclization. After minimal success with the Ullmann-type coupling, a catalytic system employing palladium was used to perform the cyclization and increase the yield of the ultimate step. The Buchwald-Hartwig amination is known to proceed better for substrates having electron-deficient ring systems, as the carbon-halogen bond becomes more activated for oxidative insertion.[60] However, Buchwald has demonstrated that functional groups located *ortho* to the coupling position of the substrate reduces reactivity because of steric hindrance.[61] The reactivity of the catalytic system has also been shown to be sensitive to the electronics and size of the palladium ligand used.[62]

Bearing these facts in mind, a catalytic system was chosen because of its ability to couple many types of activated and unactivated substrates. This catalytic system, based upon the work of Maes,[63] was shown to successfully couple aryl chlorides with amines under microwave heating. The ligand used in the reaction, 2-(dicyclohexylphosphino)-biphenyl (DCPB), has been demonstrated by Buchwald to be effective in Suzuki couplings of sterically hindered substrates[64] and in coupling of both activated and unactivated reactants.[63] Using $Pd(OAc)_2$ and DCPB as the ligand, the reaction was completed after two minutes of microwave irradiation at 200 °C using 10 mol% catalyst, resulting in a 37% yield after recrystallization from chloroform (Table 1, entry 3). Attempts to reduce the amount of catalyst resulted in decreased yields and incomplete conversion, even after prolonged reaction times. Yields decreased when the reaction time was extended for longer than two minutes using 10% catalyst (entries 4 and 5), presumably because of product decomposition as was observed in the Ullmann-type coupling. The only solvent that produced the desired compound was DMF; toluene and dioxane both proved unsuitable for cyclization.

Table 1. Variation of the reaction conditions in the palladium-catalyzed ring closing to form TCPT.[a]

#[b]	*t* [min]	*T* [°C]	% Catalyst	Yield[b]
1	5	150	1	No Reaction
2	10	150	1	No Reaction
3	2	200	10	37%
4	4	200	10	27%
5	5	200	10	26%
6	60	150	10	35%[c]

[a] See Scheme 1 d. [b] after recrystallization. [c] The reaction was run under normal reflux conditions.

A control experiment was run using conventional heating methods to determine if microwave heating displayed any distinct advantages over conventional heating. The Buchwald-Hartwig amination was heated in an oil bath at 150 °C for 1 h—conditions corresponding to 200 °C and 2 min in the microwave reactor.[65] Although some starting material remained after 1 h, the reaction produced the desired product which was subsequently purified and recrystallized to yield 35% of TCPT (entry 6). This control experiment showed that a reduction in reaction time was the only inherent advantage in utilizing microwave heating, thus providing more suitable access for large-scale synthesis. Maes-modified Buchwald-Hartwig animation improved the total yield from 4.2% (Ullmann-type condensation) to 30.9%.

Determining the crystal structure of TCPT revealed the expected butterfly structure of the compound (Figure 2). The two central heteroatoms form an axis along which the molecule is folded, causing a deviation from planarity by 18.5°. This supports the validity of semiempirical calculations, which predicted $19.8 \pm 0.7°$.[48]

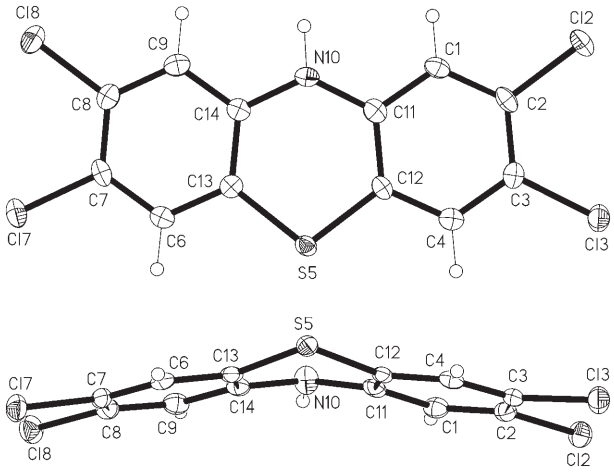


Figure 2. Crystal structure of TCPT, displaying its deviation from planarity by 18.5°.

Solid TCPT is not completely stable under ambient conditions, resulting in oxidation of trace amounts. When dissolved in acetone or THF and exposed to light, TCPT is rapidly photolyzed. TCPT is insoluble in most other common solvents. However, TCPT is highly soluble (280 mg mL^{-1}) and stable when dissolved in DMSO at room temperature even when exposed to light.

In vitro Enzyme Induction

EROD-specific activity increased over time after exposure to TCDD. The induction by TCPT, however, declined after 24 h and was virtually completely reversed for the concentrations tested after 72 h (Figure 3). An ED_{50} of 85.1 pg/well was calculated for the induction of EROD by TCPT after 24 h, as compared to 0.23 pg/well by TCDD, demonstrating a 370-fold difference in terms of induction potency. This clearly illustrated the influence of the underlying tricyclic backbone on potency: The high potency as displayed by dioxins was diminished by the introduction of different heteroatoms without substantially compromising efficacy of TCPT regarding the induction of CYP 1A1-specific enzyme activity. The average ED_{50} across all time points tested was $ED_{50}=90.5$ pg/well (± 6.78) for TCPT, and $ED_{50}=0.20$ pg/well (± 0.03) for TCDD. The decline of induction by TCPT at the concentrations tested after 24 h is indicative of its metabolism by hepatocytes and the formation of inactive metabolites. This conclusion is further supported by constant ED_{50}s throughout the time points and concentrations studied. Potency is a substance-specific property and, thus, independent of dose or concentration. Therefore, 24 h data correlated well with the 48 h and 72 h measurements for both compounds across the studied dose range. The maximal induction of EROD activity after 24 h incubation with TCDD was 636.7 pmol min^{-1} mg $protein^{-1}$, whereas TCPT elicited a calculated induction of 494.1 pmol min^{-1} mg $protein^{-1}$, representing 77.6% efficacy of the maximal induction by TCDD. The concentrations used in this assay were noncytotoxic.

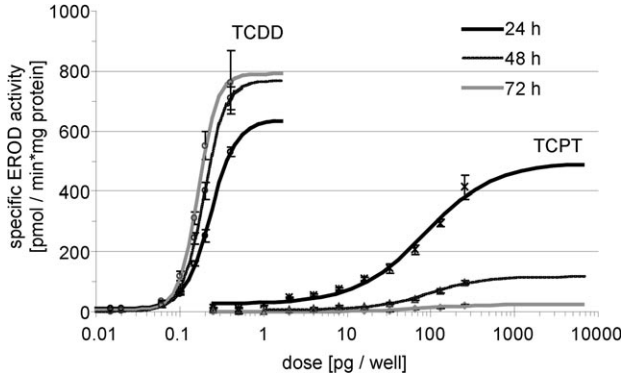


Figure 3. Time-dependent dose-responses of TCDD (left, increasing with time) and TCPT (right, decreasing with time) in an in vitro EROD-assay.

The binding of potential drug candidates to the AhR with subsequent induction of CYP 1A1 activity is considered detrimental because of the alleged association of CYP 1A1 with the metabolic activation of potential carcinogens.[66,67] However, it has been stated that "intact animal data contradict pharmaceutical company policies" that eliminate drug leads that induce CYP1 activity "for fear of possible toxic or carcinogenic effects".[68] Furthermore, the induction of CYP 1A1 activity is not only a hallmark effect of many polychlorinated organic compounds, also many naturally occurring compounds such as indoles from cruciferous vegetables, chrysin derivatives, and carotinoids are AhR ligands.[49,50] Furthermore, caffeine[69] and drugs such as omeprazole[70] and chlorpromazine[71] are known CYP1A1 inducers. Although the elimination half-lives of naturally occurring compounds and drugs are comparatively short, daily intake still causes chronic exposure. As evidenced by these compounds, induction of CYP1A1 can occur without a documented increase in cancer prevalence. In fact, induction of CYP1A1 by HpCDD during the entire life span of rats resulted in decreased cancer prevalence,[5,21] and the induction of EROD activity should therefore not compromise TCPT as a drug lead.

Serum Kinetics in Rats and Guinea Pigs after Intravenous Injection

The kinetics of TCPT were identified by classical curve feathering as well as computational regression to follow a two-compartment model in both species investigated:

$$\text{Rat}: \; C_p = 3.700e^{-0.843t} + 1.862e^{-0.128t} \;\; R^2 = 0.99 \;\; (n = 8) \qquad (2)$$

$$\text{Guinea Pig}: \; C_p = 6.132e^{-1.658t} + 4.518e^{-0.256t} \;\; R^2 = 0.99 \;\; (n = 9) \qquad (3)$$

The calculated half-lives in rats were $t_{1/2}=0.8$ h for distribution and $t_{1/2}=5.4$ h for elimination (Figure 4). In guinea pigs, half-lives were $t_{1/2}=0.4$ h and $t_{1/2}=2.7$ h, respectively (Figure 5). Based on comparison with CPZ, an extrapolation of the elimination half-life from rats (CPZ = 9.1 h)[44] to humans (CPZ = 30 h)[34] suggests an approximate elimination half-life of 18 h for TCPT in humans.

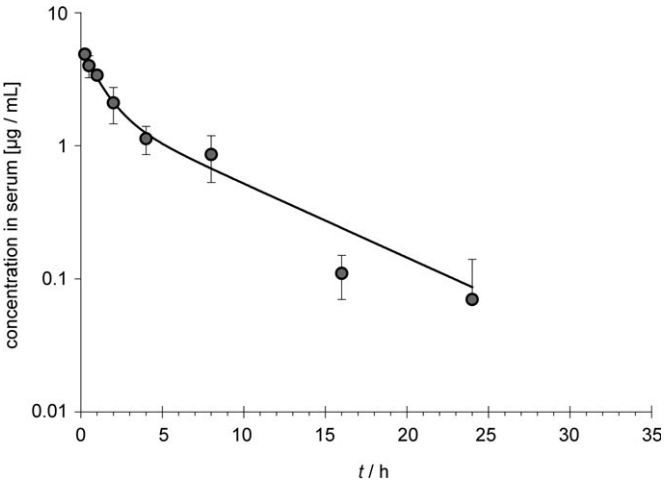


Figure 4. Serum profile of TCPT in rats after administration of i.v. 5 mg kg^{-1} TCPT with two-compartment curve-fit ($C_p = C_1e^{-k_1t} + C_2e^{-k_2t}$). $C_1=3.700$ (SD 0.744), $k_1=0.843$ (SD 0.284), $C_2=1.862$ (SD 0.832), $k_2=0.128$ (SD 0.057), $R^2=0.99$ ($n=8$), $t_{1/2(\text{distribution})}=0.82$ h, $t_{1/2(\text{elimination})}=5.42$ h.

The volumes of distribution were $V_{d(central)}=0.9$ L kg^{-1}, $V_{d(apparent)}=1.8$ L kg^{-1}, $V_{d(peripheral)}=2.3$ L kg^{-1} in rats and $V_{d(central)}=0.9$ L kg^{-1}, $V_{d(apparent)}=2.6$ L kg^{-1}, $V_{d(peripheral)}=4.1$ L kg^{-1} in guinea pigs. The apparent volumes of distribution indicate a distribu-

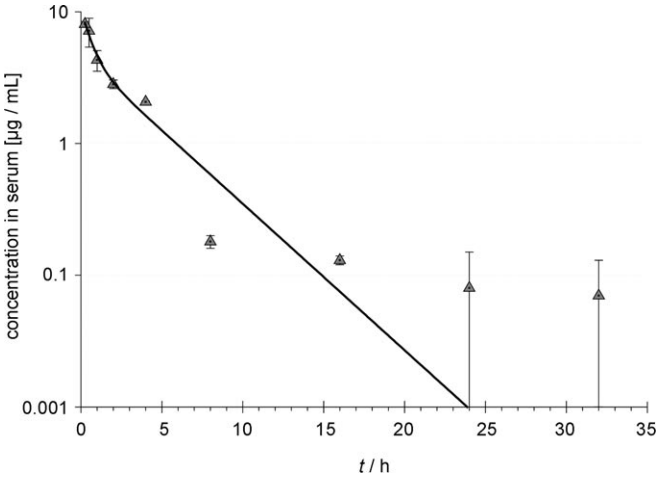


Figure 5. Serum profile of TCPT in guinea pigs after administration of i.v. 10 mg kg^{-1} TCPT with two-compartment curve-fit ($C_p = C_1e^{-k_1t} + C_2e^{-k_2t}$). $C_1 = 6.132$ (SD 1.840), $k_1 = 1.658$ (SD 1.022), $C_2 = 4.518$ (SD 2.254), $k_2 = 0.256$ (SD 0.125), $R^2 = 0.99$ ($n = 9$), $t_{1/2(\text{distribution})} = 0.42$ h, $t_{1/2(\text{elimination})} = 2.71$ h.

tion slightly larger than total body water. One possible explanation is that TCPT, like TCDD, exerts binding affinity to proteins such as the AhR or CYP1A2 resulting in tissue sequestration.

It is noteworthy that TCPT exhibits a faster elimination rate than CPZ and that at a lower apparent volume of distribution (CPZ in the rat: 29.1 L kg^{-1} [72]). These kinetic properties indicate a lack of prolonged storage of the compound in a peripheral compartment and, thus, further support the suitability of TCPT as a drug lead.

Conclusions

The new TCDD-analogue TCPT was synthesized for the first time by classical Ullmann-type coupling. Application of Pd-catalyzed conditions provided a sevenfold increase in yield, rendering ready access to this compound and demonstrating the advantages of this new technology. TCPT was developed for potential therapeutic applications[73] and as a model compound for the study of dioxin-like compounds and their mechanisms of action under different pharmacokinetic conditions. The two major prohibitive properties for the medicinal exploitation of low-dose effects of dioxins were eliminated: Compared to TCDD, TCPT has favorable kinetics and a much reduced enzyme-inducing potency, yet maintaining high efficacy regarding the induction of CYP1 A1-specific enzyme activity as presented in this study.

Experimental Section

NMR: Nuclear Magnetic Resonance Laboratory, University of Kansas, 400 MHz DRX-400 NMR, Bruker, Billerica, Massachusetts. 2-D spectra: 500 MHz Bruker Avance System. Peaks were calibrated using an internal standard and determined to be >95% pure by NMR integration. **GC/MS:** GC Model 5890 Series II Hewlett Packard, Rockville, Maryland. Column: J&W Scientific, Folsom, California, USA, DB-5 ms, 60 m, ID 0.25 mm, ft 0.10 µm, w/helium 1720 hPa. Injector temp 280 °C. Temperature program: 90 °C (1 min), w/20 °C/min to 170 °C (4 min), 170 °C (7.5 min), w/3 °C/min to 280 °C (37 min), 280 °C (10 min), Transfer line to MS: 280 °C, EI detection (70 eV, 150 °C). **UV-VIS:** UV-Visible Spectrometer Cary 1, Varian, Victoria, Australia. **Kofler Melting point apparatus:** Thomas Hoover capillary melting point apparatus Unimelt, Arthur H. Thomas Company, Philadelphia, Pennsylvania, USA. All microwave reactions were performed on an Emrys Creator by Personal Chemistry (Personal Chemistry, Inc., Foxboro, MA 02035 USA).

6′-Nitro-2,3′,4,4′,5-pentachlorodiphenylsulfide (3). 2,4,5-Trichlorothiophenol (**1**, 20.7 g, 97.0 mmol, purchased from Lancaster Inc., Pelham, NH, in 97% purity), 1,2-dichloro-4-fluoro-5-nitrobenzene (**2**, 22.4 g, 107 mmol, purchased from Aldrich Chemical Company Inc., Milwaukee, WI, in 95% purity), K_2CO_3 (67.0 g, 485 mmol), $CaCO_3$ (7.31 g, 73.0 mmol) and two weight equivalents of activated 3 Å molecular sieves were stirred and heated in refluxing CH_2Cl_2 (500 mL) for 21 h. The reaction mixture was then filtered and dried to yield 37.1 g (94.1 mmol, 97%) of a yellow solid: mp 162–164 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.38$ (s, 1H), 7.80 (s, 1H), 7.72 (s, 1H), 6.76 ppm (s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 139.75$, 138.80, 138.61, 136.82, 135.94, 132.89, 132.55, 131.30, 130.53, 129.09, 128.63, 127.77 ppm. GC/MS: $t_R = 43{:}08$ min, 401 (10% ($C_{12}H_4Cl_5NO_2S^{+\cdot}$)), 354 (2% ($C_{12}H_3Cl_5S^+$)), 337 (3% ($C_{12}H_4Cl_5NO_2S^{+\cdot} - 64$)) 320 (8% ($C_{12}H_4Cl_4S^+$)), 302 (75% ($C_{12}H_4Cl_4NO_2S^{+\cdot} - 64$)), 276 (3% ($C_{12}H_3Cl_3NO_2S^{+\cdot} - 64$)), 250 (13% ($C_{12}H_4Cl_2S^+$)), 176 (12% ($C_{12}S^+$)).

6′-Amino-2,3′,4,4′,5-pentachlorodiphenylsulfide (4). Four portions of acid-activated Fe filings (4×20 g) were added in 30 min intervals to a refluxing solution of **3** (44.4 g, 110 mmol), glacial acetic acid (375 mL), acetone (1.5 L), and distilled water (375 mL). The reaction was refluxed and stirred for an additional 30 min. After cooling, the solution pH was increased to eight with 1 M NaOH solution and the resulting solution was filtered through Celite. The Celite plug was washed with NaOH solution, followed by water, acetone, and Et_2O until the solvent became colorless. The eluent was extracted with Et_2O, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was washed with hexanes, yielding 35.3 g (94.6 mmol, 86%) of a bright yellow solid: mp 191 °C. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.50$ (s, 1H). 7.45 (s, 1H), 6.93 (s, 1H), 6.65 (s, 1H), 4.33 ppm (s, 2H, NH_2); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 148.43$, 138.35, 136.53, 135.27, 132.24, 131.01, 130.36, 130.04, 127.34, 121.56, 116.83, 111.42 ppm. GC/MS: $t_R = 44{:}13$ min, 371 (57% ($C_{12}H_6Cl_5NS^{+\cdot}$)), 335 (44% ($C_{12}H_5Cl_4NS^+$)), 301 (60% ($C_{12}H_6Cl_3NS^+$)), 266 (10% ($C_{12}H_6Cl_2NS^+$)), 230 (4% ($C_{12}H_5Cl_1NS^+$)), 192 (17% ($C_{12}H_2NS^+$)).

TCPT (5) by Ullmann-type-Coupling. Aniline **4** (8.40 g, 22.5 mmol) was dissolved in DMF (1 L) and Na_2CO_3 (2.49 g, 23.5 mmol), freshly generated copper (0.29 g, 4.50 mmol) and freshly purified CuI (0.86 g, 4.5 mmol) were subsequently added and heated at reflux for 24 h. After cooling, the crude product was isolated by precipitation from cold brine solution and filtration. The resultant residue was dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography with *tert*-butyl methyl ether/hexanes (1:9) afforded 379 mg (1.13 mmol, 5%) of an off-white solid.

TCPT (5) by Microwave Buchwald-Hartwig amination. A solution containing $Pd(OAc)_2$ (4.65 mg, 0.0207 mmol) and 2-(dicyclohexylphosphano)-biphenyl (14.5 mg, 0.0414 mmol) in anhydrous DMF (4.65 mL) was prepared and added to **4** (75.0 mg, 0.207 mmol) and sodium *tert*-butoxide (27.0 mg, 0.282 mmol) in a microwave reaction vessel. The contents were flushed with argon for 5 min, capped, and exposed to microwave irradiation at 200 °C for 2 min. After the reaction was complete, the DMF was removed by pouring the reaction solution into a separatory funnel containing aqueous sodium hydrosulfite (40 mL) and extracting with 50:50 hexane-

s:EtOAc (4×40 mL). The solution was dried over Na_2SO_4, concentrated under reduced pressure, and purified by the flash chromatography conditions specified above. The solid was recrystallized from $CHCl_3$ to yield 26 mg (0.077 mmol, 37%) of the off-white solid.

TCPT (5) by standard conditions Buchwald-Hartwig amination. A solution containing $Pd(OAc)_2$ (4.65 mg, 0.0207 mmol) and 2-(dicyclohexylphosphano)-biphenyl (14.5 mg, 0.0414 mmol) in anhydrous DMF (4.65 mL) was prepared and added to **4** (75.0 mg, 0.207 mmol) and sodium *tert*-butoxide (27.0 mg, 0.282 mmol) under an argon atmosphere. The reaction was stirred and heated at 150 °C for 1 hour. After the reaction was complete, the DMF was removed by pouring the reaction solution into a separatory funnel containing aqueous sodium hydrosulfite (40 mL) and extracting with 50:50 hexanes:EtOAc (4×40 mL). The solution was dried over Na_2SO_4, concentrated under reduced pressure, and purified by the flash chromatography conditions specified above. The solid was recrystallized from $CHCl_3$ to yield 25 mg (0.073 mmol, 35%) of the off-white solid: mp 225 °C; UV-VIS absorption maxima: 203.8 nm 43%, 221.4 nm 53%, 264.4 nm 100%, 336.8 nm 11%; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.01$ (s, 2H), 6.63 (s, 2H), 5.83 ppm (s, 1H, NH); ^{1}H NMR (500 MHz, [D_6]acetone): $\delta = 8.33$ (s, 1H, NH), 7.16 (d, $^5J = 1.5$ Hz, 2H), 6.86 ppm (d, $^5J = 1.2$ Hz, 2H); ^{1}H NMR (500 MHz, [D_8]THF): $\delta = 8.10$ (s, 1H, NH), 7.09 (s, 2H), 6.65 ppm (s, 2H); ^{13}C NMR (125.8 MHz, $CDCl_3$): $\delta = 139.72$, 131.11, 127.39, 125.93, 117.63, 115.67 ppm; GC/MS (TCPT): $t_R = 49{:}34$ min, 335 (73% ($C_{12}H_5Cl_4NS^+$)), 300 (62% ($C_{12}H_5Cl_3NS^+$)), 264 (11% ($C_{12}H_4Cl_2NS^+$)), 230 (9% ($C_{12}H_5ClNS^+$)), 194 (4% ($C_{12}H_4NS^+$)), 168 (12% ([$C_{12}H_5Cl_4NS^{\cdot}]^{2+}$)), 132 (14% ($C_{12}H_4Cl_2NS^{2+}$)).

In vitro Enzyme Induction

An in vitro ethoxyresorufin-O-deethylase (EROD) assay was conducted with H4IIEC/T3 rat hepatoma cells[74,75] in 96-well plates according to standard procedures.[76] Cells were plated at a density of about 10000 cells/well and cultured for 72 h prior to exposure. TCPT was then added in concentrations of 0.25–256 pg/well (2.5–2560 ng L^{-1}). TCDD served as positive control in concentrations of 0.015–0.4 pg/well (0.15–4 ng L^{-1}). Plates were incubated at 37 °C and 7% CO_2 for 24, 48, and 72 h. After incubation, media was discarded and hepatocytes were exposed to 7-ethoxyresorufin for 30 min. Subsequently, the generated resorufin was quantified by detecting fluorescence at 590 nm after excitation at 535 nm. Protein content was measured with the BCA (bicinchoninic acid) protein assay and absorption was measured at 540 nm.[77,78] Cytotoxicity was determined by the resazurine assay. Data was processed using the Microsoft Excel Solver option, which was applied to yield a sigmoidal regression to a four-parameter equation. Each concentration was measured in quadruplicate, error bars depict the standard deviation.

Serum Kinetics in Rats and Guinea Pigs after Intravenous Injection

All animal studies were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (228–264 g) and female Dunkin-Hartley guinea pigs (445–461 g) were purchased from Harlan, Indianapolis, Indiana, and Charles River Laboratories, Wilmington, Maine, respectively. Animal numbers were two per species, based on inherently low interindividual variability regarding kinetics. In both species, the *Vena jugularis* was cannulated by the supplier. Animals were housed in a climate-controlled facility with 12 h dark/light cycle and ad libidum access to feed and water. Rats were administered 5 mg kg^{-1} TCPT i.v. in freshly prepared acetone solution (1 mL acetone kg^{-1}), guinea pigs received 10 mg kg^{-1} TCPT i.v. in acetone (0.5 mL kg^{-1} acetone). Blood samples were drawn in 500 μL volumes from both species at $^1/_4$, $^1/_2$, 1, 2, 4, 8, 16, and 24 h, and 36 h (guinea pigs only) after dosing and stored on ice. The sample volume withdrawn was replaced by saline injections. Coagulated blood samples were centrifuged at 9,000 g at 4 °C for 16 min, and serum stored at −80 °C until analysis.

Analytics were performed by HPLC, using a 655A-11 liquid chromatograph, L-5000 LC controller with D-6000 interface, L-3000 photo diode array detector, and the software D-6000 HPLC Manager version 2 (Merck-Hitachi, Darmstadt, Germany). The analytical column Nukleosil C18 5μ 250×4.0 mm (VDS Optilab Chromatographie Technik GmbH, Berlin, Germany) was temperature-controlled at 25 °C and protected by an upstream Security Guard C18 ODS 4×3 mm (Phenomenex, Aschaffenburg, Germany). All samples were prepared online using the alkyl-diol-silica (ADS) technology developed by Boos et al.[79–83]. The cartridge employed was LiChroCART 25-4 LiChrospher RP-4 ADS, generously provided by Prof. Dr. Boos, Klinikum Großhadern, Ludwig-Maximilians-Universität München, Germany and protected by an upstream inline filter ADS (Merck KGaA, Darmstadt, Germany). Solvents for analytics (water and methanol) were of HPLC grade (Fisher Scientific, Loughborough, Leicestershire, UK). Calibration was conducted with nine concentrations from 0.01–20 mg/mL TCPT in rat serum ($R^2 = 0.99$) and five standards of 0.1–20 μg mL^{-1} in guinea pig serum ($R^2 = 0.99$). Limit of quantification was set at 0.05 μg mL^{-1}.

Curve regression was performed by manual curve feathering and computational regression according to one–compartment ($C_p = C_1 e^{-k_1 t}$), two–compartment ($C_p = C_1 e^{-k_1 t} + C_2 e^{-k_2 t}$), and three-compartment models ($C_p = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} + C_3 e^{-k_3 t}$) using Sigma Plot 4.0 (SPSS Inc., Chicago, Illinois), where C_p = concentration in plasma, $C_{1,2,3}$ = concentrations in compartments, $k_{1,2,3}$ = compartmental rate constants, and t = time.

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