Structure of a Laccase-Mediated Product of Coupling of 2,4-Diamino-6-Nitrotoluene to Guaiacol, a Model for Coupling of 2,4,6-Trinitrotoluene Metabolites to a Humic Organic Soil Matrix

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This work presents laccase-mediated model reactions for coupling of reduced 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix. The structure of an isolated coupling product of 2,4-diamino-6-nitrotoluene (2,4-DANT) to guaiacol as humic constituent was determined. Among several structures, the compound was identified conclusively to be the trinuclear coupling product 5-(2-amino-3-methyl-4-nitroanilino)-3,3'-dimethoxy-4,4'-diphenylquinone. The compound has a weight of 409 g mol⁻¹ and may serve as a model reaction for the biogenic formation of bound residues in soil from TNT by coupling aminotoluenes (reduced TNT metabolites) to humic constituents. A linear correlation of the substrate consumption to the enzyme activity was detected. Based on this observation, the described reaction of 2,4-DANT coupling to guaiacol may be used for determination of laccase activity since the reaction was not inhibited by other compounds of culture supernatants. We propose a two-step mechanism for the coupling reaction because 2,4-DANT was not transformed by laccases in the absence of guaiacol and guaiacol oxidation was independent of the presence of 2,4-DANT. The first reaction step is a laccase-mediated dimerization of two guaiacol monomers with subsequent oxidation to a diphenylquinone. The second step is the nucleophilic addition of 2,4-DANT to the *ortho* position of the carbonyl group of the diphenylquinone structure.

2,4,6-Trinitrotoluene (TNT) was the most widely used military explosive during World Wars I and II. Soil and ground-water TNT contaminations at large ammunition plants are the result of extensive manufacturing and the loading, assembling, and packing of ammunition. The hazardous nature of TNT contaminations stems from its toxic and mutagenic potential (13, 17, 41). Due to the low solubility in water, TNT is still present as a contaminant at the sites and may continuously leak into the groundwater. Since manufacturing of TNT needs extensive consumption of water, the plants were built in water-rich regions threatening today's drinking-water resources.

Recent research has demonstrated the metabolism of TNT by bacteria and fungi (37). However, mineralization to carbon dioxide was found only in a few cases with low rates even under optimized laboratory conditions (3, 4, 28). The most extensive mineralization of TNT was exhibited by the cometabolic oxidation process of white-rot fungi, e.g., *Phanerochaete chrysosporium* (9, 24). Microbial transformation of TNT often occurs by consecutive reduction of the nitro groups to corresponding amino derivatives (3, 42), which were detected at the most contaminated sites (30). The monoaminodinitro- or diaminonitro-derivates could be formed by microorganisms under aerobic conditions, whereas further reduction to 2,4,6-triaminotoluene (TAT) requires strictly anaerobic conditions (18, 29). Due to the basic amino groups, TAT is a highly reactive compound and cannot be detected in contaminated soils (30).

The balancing of the TNT degradation in soil often yielded a balance gap of metabolites. It has been suggested that the gap is due to an irreversible binding of TNT metabolites to the organic soil matrix (18, 19). Composting experiments using ¹⁴C-labeled TNT showed that 22.1% of the TNT initially used is bound to the humic fraction (14). Other studies demonstrated that 35% of the ¹⁴C-TNT was not extractable from soil 65 days after amendment (27). Long-term sorption experiments with soil slurries revealed that 34.6% of the ¹⁴C-TNT was not extractable from solid humic particles (5). However, the recovery of the radiolabeled residues of TNT and metabolites did not allow any statement on the structure of such bound TNT molecules. The measured radioactivity accounts only for single labeled carbon atoms.

Binding of pollutants to the organic soil matrix may reduce the amount of material available to interact with the biota (23, 25) and may represent a way of decreasing toxic effects. The most persistent residues resulted from the covalent binding to humic substances of soil (12, 31). Such covalent bonds may arise from oxidative coupling, which is one of the most important reactions within the humification processes in soils (2, 44). Several extracellular oxidoreductases including laccases (benzenediol:oxygen oxidoreductases) catalyze oxidative coupling reactions (36). Laccases are predestined enzymes for investigations of such coupling reactions. In contrast to H₂O₂-dependent peroxidases, these enzymes simply use oxygen and are widely distributed in fungi. Therefore, laccases have been used several times to characterize binding mechanisms involved in the humification of pesticides derived from aniline compounds (21, 32, 38).

Despite conclusive demonstration of TNT residues in soils,

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the reaction mechanisms of TNT incorporation have not been clarified yet. Moreover, no proof of the covalent coupling of reduced TNT metabolites to humic compounds exists. So far, there is only one report of a binding reaction of TNT metabolites to humic substances of soil (15).

The aim of the present work was to examine the impact of fungal extracellular laccases on the incorporation, and binding, of TNT and of reduced TNT metabolites into the organic soil matrix. The metabolites aminodinitro- and diaminonitrotoluene were investigated since these compounds were usually found in contaminated soils. The experiments were conducted in model systems containing laccase and the humic constituent guaiacol as structural element of humic matter. Results of the laccase-catalyzed coupling of guaiacol to 2,4-diamino-6-nitrotoluene (2,4-DANT) will be discussed, and the structure of the coupling product will be presented.

MATERIALS AND METHODS

Chemicals. 2,4-DANT and 4-amino-2,6-dinitrotoluene (4-ADNT) were obtained from U. Steinbach (Institute of Organic Chemistry, Philipps-University, Marburg, Germany). Guaiacol (2-methoxyphenol) was purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Enzyme assays. The laccase (EC 1.10.3.2) of *Pyricularia oryzae* (lot 54H3398) was purchased from Sigma-Aldrich Chemie GmbH. The laccase of *Trametes versicolor* G3 (isolated by G. Grams, Department of Technical Microbiology, Friedrich-Schiller-University, Jena, Germany) was obtained from concentrated culture supernatants.

Laccase activity was determined spectrophotometrically by using the substrate syringaldazine (4-hydroxy-3,5-dimethoxybenzaldazine) (20). One unit was defined as the amount that causes a change in optical density of 0.001/min at 530 nm (34). Enzyme assays were conducted in potassium-phosphate buffer (pH 6.5) with the laccase of *P. oryzae* and in citrate-phosphate buffer (pH 5.0) with the culture supernatant of *T. versicolor*. The assay mixtures contained 2.2 ml of buffer (0.1 M), 0.2 ml of syringaldazine solution in methanol (0.216 mM), and 0.5 ml of enzyme solution, and the assays were carried out at 28°C. Heat-inactivated enzymes were used as controls.

Culture conditions. *T. versicolor* was maintained on malt extract agar slants. Agitated submerged liquid cultures were grown at 24°C in nitrogen-limited medium modified from that of Kirk et al. (16) containing (per liter) KH_2PO_4 , 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; CaCl_2 , 0.1 g; glucose, 20 g; 2,2-dimethylsuccinate, 1.5 g; NH_4 -tartrate, 0.22 g; mineral solution, 10 ml; and vitamin solution, 1.5 ml. The medium was adjusted to pH 4.5. Flasks (500 ml) containing 100 ml of the medium were inoculated with eight agar plugs (1 cm² each) from well-grown cultures on malt extract agar. During growth of the culture, laccase production was induced by adding 2,5-dimethylaniline to a final concentration of 0.5 mM (8). The culture supernatant was concentrated by ultrafiltration (PM-10; Amicon, Witten, Germany) and showed mainly laccase activity (EC 1.10.3.2). Catechol oxidase activity (EC 1.10.3.1) was observed to a negligible extent. Neither lignin peroxidase activity (EC 1.11.1.14) nor Mn(II)-dependent peroxidase activity (EC 1.11.1.13) was detected as already reported (33).

Reaction conditions. The coupling reaction was carried out in the dark in triplicate sets at 28°C. Two milliliters of citrate-phosphate buffer (pH 5.0) was used for the culture supernatant of *T. versicolor*, and 2 ml of phosphate buffer (pH 6.5) was used for the laccase of *P. oryzae*. If not stated otherwise, the buffer contained 300 U of laccase ml⁻¹, which was incubated with 1.75 mM guaiacol and 0.25 mM 2,4-DANT. Enzyme activity was stopped by shifting the pH of the assay to 2.5 with trichloroacetic acid. The precipitated coupling products were dissolved by adding acetonitrile to aqueous samples, and precipitated proteins were removed finally by filtration with a 0.45- μm -pore-size cellulose membrane (Sartorius, Göttingen, Germany). One hundred fifty milliliters of the reaction mixture was used for isolation and characterization of the coupling product.

Analytical methods. (i) **HPLC.** Substrates and products were separated and analyzed by high-performance liquid chromatography (HPLC) using a reversed-phase column (RP-18 [5 μm , 125 by 4 mm], LiChroCART; Merck), an L-6200 pump, an AS-2000 autosampler, and an L-4500 diode array detector with system manager D-6500 (all from Merck). The mobile phase consisted of a mixture of acetonitrile-water-methanol (35:50:15, vol/vol/vol) at a flow rate of 1.0 ml min⁻¹. UV detection was performed at 210 nm. The error bars in the figures show standard deviations for triplicate samples.

(ii) **Thin-layer chromatography.** Thin-layer chromatography was used to isolate the coupling product. Samples were applied to a 20- by 20-cm by 0.5-mm silica gel plate (SIL G-50; Machery-Nagel, Düren, Germany). The plate was developed with a mobile phase of methylene chloride-acetonitrile-acetic acid (4:1:3, vol/vol/vol). The separated product was then extracted from the silica gel by using methanol containing 1% acetic acid.

(iii) **Mass spectrometry.** Molecular weights and acidic proton number were determined by electron impact ionization and chemical ionization mass analyses using an SSQ 710 mass spectrometer (Finnigan MAT). In both cases, sample introduction took place by a direct-exposure probe. H_2O and D_2O were used as reactant gases for chemical ionization. High-resolution mass spectroscopy analysis was performed by electron impact analysis on an AMD 402/2 mass spectrometer (AMD Intectra GmbH).

(iv) **NMR.** Proton and ¹³carbon nuclear magnetic resonance (¹³C NMR) spectra were recorded on a Bruker DRX 400 instrument using 400 MHz for ¹H spectra and 100 MHz for ¹³C spectra. Dimethyl sulfoxide-d₆ was used as solvent for ¹H NMR experiments. The two-dimensional (H,H)-COSY (correlated spectroscopy) experiment was performed with dimethyl sulfoxide-d₆; CD₂Cl₂ was used for both the two-dimensional (H,H)-TOCSY (total correlated spectroscopy) and the two-dimensional (C,H)-correlation (HMQC [heteronuclear multiple quantum correlation]) experiments. Data acquisition and processing were carried out by using Bruker software package XWIN-NMR.

Infrared spectrometry. The infrared spectra were obtained on a Perkin-Elmer 2000 infrared spectrophotometer in Nujol mull.

RESULTS

To examine the oxidative coupling of TNT and metabolites to humic constituents, TNT, 4-ADNT, and 2,4-DANT were each incubated with guaiacol and the laccase of *P. oryzae*. The disappearance of substrates and the formation of products were monitored by HPLC analyses. In comparison to the control with guaiacol and laccase only, no additional peaks of products were observed after incubation with TNT and only traces were observed with 4-ADNT. No significant decrease of the nitrotoluene substrates was detected. The peaks of the control were later identified by mass spectrometry as dimeric, trimeric, and traces of tetrameric products of guaiacol coupling to itself as already described (35).

The occurrence of greater amounts of red precipitates in the reaction solution with 2,4-DANT clearly showed the formation of water-insoluble coupling products by this TNT metabolite. Therefore, the coupling products of 2,4-DANT were further investigated. Beside decreased peaks known from the controls, seven small peaks and one major peak occurred additionally with 2,4-DANT. The small peaks accounted for yields much lower than 10%, whereas the major peak accounted for a total yield of 37%. We will use the term "coupling product" for this main product, which is characterized below.

Similar patterns of reaction between 2,4-DANT and guaiacol were observed during incubation with culture supernatants of *T. versicolor*. In addition, both incubation with the laccase of *P. oryzae* and incubation with the laccase of the culture supernatant of *T. versicolor* yielded the same substrate consumption of 2,4-DANT and guaiacol (Fig. 1). From a practical point of view, this reaction was not sensitive to interference by proteins or other compounds of the culture supernatants. Therefore, we performed the following experiments with the culture supernatants of *T. versicolor*. Figure 2 shows the consumption of 2,4-DANT and guaiacol as well as the formation of the coupling product. Without guaiacol, 2,4-DANT was not affected by the laccase, whereas the enzyme caused an extensive transformation of guaiacol with or without 2,4-DANT present, indicating a complex type of reaction. During simultaneous incubation with guaiacol, this TNT metabolite disappeared and the red coupling product was formed. The formation of the coupling product decreased after consumption of about 70% of guaiacol. However, no additional reaction products were observed after this time. Thus, the formation of the coupling product depends on the concentration of guaiacol. Enzyme catalysis of the reaction was proven by use of heat-inactivated enzymes, and no transformation of guaiacol or 2,4-DANT was observed.

Considering a complex coupling reaction with parallel consumption of guaiacol by oligomerization without coupling of

FIG. 1. Substrate consumption of 2,4-DANT (0.25 mM) (circles) and of guaiacol (0.25 mM) (squares) by laccases (13 U ml⁻¹) of *P. oryzae* (filled symbols) and of *T. versicolor* (open symbols).

2,4-DANT, we determined the impact of the substrate ratio on the yield of the coupling product of 2,4-DANT and guaiacol. Maximum 2,4-DANT substrate consumption and maximum yield of the coupling product were obtained with an initial ratio of 2,4-DANT to guaiacol of 1:7, mol:mol (total reaction ratio). A pronounced linear correlation between the amount of substrate transformation and the enzyme activity was observed with this ratio (2,4-DANT, $r = 0.989$; guaiacol, $r = 0.999$) (Fig. 3).

After isolation and purification, the coupling product was characterized by different analytical methods. High-resolution mass spectral analysis revealed an elemental composition of C₂₁H₁₉N₃O₆ (calculated, 409.1257; observed, 409.1274). This corresponds to a trinuclear compound that consists of one 2,4-DANT and two guaiacol molecules, indicating a partial reaction by coupling of 2,4-DANT to a guaiacol dimer. No evidence of higher-molecular-weight compounds was obtained, and no residues were observed during the preparations. Figure 4A presents the mass spectrum of the coupling product based on chemical ionization with H₂O as reactant gas. In order to detect acidic protons, we treated the product with partially deuterized methanol (CH₃OD). This led to the exchange of acidic protons and deuterium. The mass spectrum of the mod-

FIG. 2. Substrate consumption (2,4-DANT [○], 0.25 mM; guaiacol [■], 1.75 mM) and formation of the coupling product (x) (as determined by HPLC [E₂₁₀]) by the laccase (300 U ml⁻¹) of *T. versicolor*. x coupling product.

FIG. 3. Consumption of 2,4-DANT (0.25 mM) and of guaiacol (1.75 mM) correlated to the laccase activity of *T. versicolor*. ○, 2,4-DANT ($r = 0.989$); ■, guaiacol ($r = 0.999$).

ified product obtained by chemical ionization with D₂O as reactant gas is shown in Fig. 4B. The molecular ion M⁺ of m/e 412 (referring to molecular ion [M⁺+D]⁺ of m/e 414) exhibits a difference of $m/e = 3$ with respect to the unaltered compound. Hence, the coupling product possesses three acidic protons.

Another structural analysis was performed by using infrared spectroscopy. The infrared spectrum of the coupling product

FIG. 4. Mass spectra of the product resulting from the laccase-mediated coupling reaction between 2,4-DANT and guaiacol, obtained by chemical ionization with reactant gas H₂O (A) or with reactant gas D₂O after the exchange of acidic protons of the product (B).

TABLE 1. ¹H NMR data (400 MHz) of the coupling product

Chemical shift(s) (ppm)	Proton assignment
2.03	CH ₃ group from the parent 2,4-DANT molecule
3.69 and 3.78	Two OCH ₃ groups from the parent guaiacol molecules
3.73 and 3.83	Two OCH ₃ groups caused by an E,Z isomerism ^a
5.71	NH ₂ group from the parent 2,4-DANT
6.2 to 7.29	Protons of aromatic and quinonoid rings
9.38	NH group
9.42	NH group caused by an E,Z isomerism ^a

^a See Discussion.

exhibits a broad absorption at 3375 cm⁻¹, which can be assigned to a stretching vibration of a (possibly associated) NH₂ group (data not shown). Stretching-vibration bands of a secondary amino group ($\nu_{\text{N-H}}$, 3550 to 3100 cm⁻¹) may be obscured by this broad absorption. The deformation (scissoring) vibration of the NH₂ group causes an absorption at 1629 cm⁻¹. The stretching vibration band of a C=O group appearing at 1656 cm⁻¹ suggested the existence of a quinonoid structure. The asymmetric stretching-vibration band of a nitro group occurs at 1524 cm⁻¹. The symmetric stretching-vibration band of this nitro group is obscured by the absorption of the infrared solvent Nujol at 1377 cm⁻¹.

Further structural analysis of the coupling product was carried out by NMR spectroscopy. The resonance signal assignment of the ¹H NMR spectrum is summarized in Table 1. The chemical shifts of the NH₂ group and the NH group were confirmed by a two-dimensional (H,C)-HMQC experiment (data not shown). ¹H NMR chemical shifts showed protons located at the aromatic and quinonoid rings of the coupling product. These protons were assigned by two-dimensional (H,H)-COSY and (H,H)-TOCSY. Table 2 presents the results of these correlation experiments including our calculated coupling constants. The proton assignments are discussed below (Fig. 5). We also found additional NMR signals for two OCH₃ groups, for one NH group, and for the protons located at the quinoid rings which indicate the occurrence of an isomerism (see Discussion). The chemical shifts of the CH₃ and NH₂ groups and of the two protons located at the aromatic ring from the parent 2,4-DANT molecule were not affected.

DISCUSSION

In this work we demonstrate reaction conditions under which a covalent bond between the TNT metabolite 2,4-DANT and the humic constituent guaiacol was coupled enzymatically. The structure of the coupling product was proven by different analytical methods. The binding was catalyzed by the purified

laccase of *P. oryzae* as well as by the laccase from the culture supernatant of *T. versicolor*. To our knowledge, we present the first conclusive data on the structure of the isolated product of the laccase-mediated reaction of 2,4-DANT coupling to guaiacol. The coupling shows that TNT metabolites already having two reduced nitro groups may couple enzymatically to quinoid moieties of humic matter, resulting in biogenic formation of TNT residues in soil, which were found by several authors (5, 14, 28). Considering a linear correlation of substrate consumption to the enzyme activity, this reaction might be used for determination of laccase activities. The reaction is of little sensitivity to interference with other compounds in unpurified culture supernatants.

The structure of the coupling product was determined based on the following considerations and experimental results. Three types of covalent bonds between aromatic amines and humic substances are known from literature. (i) Formation of imines or anils is rapid but generally easily reversible (6, 11, 26). This type of bond has only little stability in aqueous environments because of its hydrolyzability. (ii) Aniline binding to quinones occurs in several steps involving 1,4 addition followed by tautomerization and oxidation to anilinoquinones which stabilize the compound (11, 26, 39, 40). Covalent bonds are achieved after nucleophilic attack to aromatic carbonyl moieties. (iii) The incorporation of aromatic amino nitrogen into heterocyclic ring structures may occur during the oxidative condensation of anilines with catechols (12). Similar heterocyclic structures may arise also through further oxidation of anilinoquinones (26). Thorn et al. (39) showed direct spectroscopic evidence of the formation of such structures in natural organic matter.

Based on the known bonding types, we present five possible structures for the coupling product of the reaction between 2,4-DANT and guaiacol (Fig. 5). Some of the structures may be excluded according to our analytical data. At first, we consider structures A, B, and C. These structures do not match the data since their molecular weights are not in accordance with the value of the molecular ion of the coupling product determined by mass spectroscopy. Moreover, structures A, B, and C do not possess the determined number of acidic protons. On the other hand, structures D and E are in correspondence with all experimental data. A distinction between D and E could not be drawn from infrared spectroscopy due to the obscuration of characteristic vibrational bands by other absorptions.

For further determinations, two-dimensional NMR spectroscopy was used to elucidate the coupling pattern of the five protons located at the diphenoquinone structure. From the values of the coupling constants we concluded that the number of bonds between the mutually corresponding coupling protons is three or four. This analysis allowed the assignment of structure D to 5-(2-amino-3-methyl-4-nitroanilino)-3,3'-dime-

TABLE 2. Data and assignment for protons of aromatic and quinonoid rings arising from the (H,H)-COSY and (H,H)-TOCSY spectra (400 MHz) of the coupling product

Chemical shift(s) (ppm)	Splitting (integral)	Coupling constant(s) (Hz)	Proton assignment
6.2	Doublet (1 H)	2.41 (⁴ J)	H _y
6.49 and 6.46	Doublet (1 H and 0.7 H)	2.0 (⁴ J)	H _b and H _b caused by an E,Z isomerism ^a
6.56 and 6.57	Doublet (1 H and 0.7 H)	2.0 (⁴ J)	H _a and H _a caused by an E,Z isomerism
6.83 and 6.77	Doublet (1 H and 0.7 H)	8.2 (³ J)	H _e and H _e caused by an E,Z isomerism
7.02 and 6.84	Doublet of doublets (1 H and 0.7 H)	8.2/2.0 (³ J/ ⁴ J)	H _d and H _d caused by an E,Z isomerism
7.13 and 6.97	Doublet (1 H and 0.7 H)	2.0 (⁴ J)	H _c and H _c caused by an E,Z isomerism
7.28	Doublet (1 H)	2.44 (⁴ J)	H _z

^a See Discussion.

FIG. 5. Structures of potential products resulting from a laccase-mediated coupling of 2,4-DANT to guaiacol (structure D was concluded from the data presented in Fig. 4 and in Tables 1 and 2).

thoxy-4,4'-diphenylquinone as the only matching structure of the coupling product that fits the experimental data (Fig. 6). In this structure formula, 2,4-DANT is linked to the *ortho* position of the C=O group of the diphenylquinone arising from the guaiacol monomers. This position is also supported for reactivity reasons since the C=C double bond of the quinone is activated by the adjacent C=O group. Several authors suggested and found that quinoid moieties of humic polymers are the principal sites for covalent attachment of aniline residues (26, 39, 43). The detected doublet NMR signals for the two OCH₃ groups, for the NH group, and for the protons located at the quinoid rings can be explained by the occurrence of an E,Z isomerism caused by a rotation of the rings of the diphenylquinone around the C=C double bond. Hence, such double signals were not detected for the more distant CH₃ and NH₂ groups and the two protons located on the aromatic ring from the parent 2,4-DANT molecule.

The data suggest that coupling of 2,4-DANT was produced by a two-step reaction mechanism, since 2,4-DANT was not transformed by laccases in the absence of guaiacol and the guaiacol oxidation was independent of 2,4-DANT. The first reaction step resulted in a laccase-mediated dimerization of two guaiacol monomers with subsequent oxidation to a diphenylquinone. The second step might be performed as a nucleophilic addition of 2,4-DANT to the *ortho* position of the carbonyl group of the diphenylquinone structure. The addition is followed by tautomerization and oxidation yielding the 5-(2-amino-3-methyl-4-nitroanilino)-3,3'-dimethoxy-4,4'-diphenylquinone. The selective attack on the diphenylquinone structure may explain the fact that other guaiacol dimers without cou-

pling of 2,4-DANT were observed in the experiments and may account for higher guaiacol consumption in the total enzyme reaction in comparison to the formation of the coupling product. The formation of the guaiacol dimers was already proven by Simmons et al. (35). The proposed scheme of the second reaction step is presented in Fig. 6. The coupling reaction requires high reactivity of the TNT metabolites. Indeed, TNT and the TNT metabolite 4-ADNT, a precursor of 2,4-DANT during microbial reduction of TNT (3, 30, 42), did not react with the humic constituent guaiacol to a significant extent. This clearly underlines the importance of reduced functional groups of the TNT metabolites for such coupling reactions. 4-ADNT exhibits a much lower basicity owing to the second electron-withdrawing nitro group instead of the electron-donating amino group of 2,4-DANT. Kaplan and Kaplan (15) pointed out that in peroxidase-catalyzed reactions with humic acids only the diamino metabolites, but not TNT itself or other TNT metabolites, act as reactive binding species. Therefore, it is very likely that binding of TNT derivatives to humic substances depends on the reduction of at least two nitro groups to amino groups. The increased basicity of the highly reactive metabolite TAT may explain why this compound is not detectable in contaminated soils (30).

In this work we demonstrated the coupling of 2,4-DANT to a guaiacol dimer. The detected enzyme-mediated coupling reaction between 2,4-DANT and a humic constituent may serve as model reaction and explains the incorporation of reactive TNT metabolites into the organic soil matrix by C=N single bonds. Mayaudon and Sarkar (22), for instance, isolated a laccase from soil which is similar to that produced by *T. versicolor*. Consequently, it is very likely that processes analogous to those we have observed may occur in natural environments and may cause a biogenic formation of bound residues. This covalent coupling to soil humic constituents should be considered for detoxification of pollutants as proposed in principle by Berry and Boyd (1). Due to water-insoluble coupling products, the migration in soil and the danger of leaching of toxic compounds into the groundwater can be diminished. Moreover, once a pollutant is covalently coupled to humic substances, the long-term release of small quantities is often accompanied by a simultaneous mineralization (7). Haider and Martin (10) pointed out that radiolabeled residues from anilines bound to

FIG. 6. Proposed reaction scheme for the nucleophilic addition of 2,4-DANT to the diphenylquinone derived from laccase-mediated dimerization of two guaiacol monomers.

humic substances exhibit even higher mineralization mediated by white-rot fungi in comparison to the parent compounds.

The described coupling reaction of 2,4-DANT took place in the *ortho* position to the C=O group of quinonoid structures of humic constituents. Other authors found this type of reaction with anilines (11, 26, 39, 43). Therefore, this reaction seems to be specific for this type of binding site, and thus, it should be possible to analyze reactive sites of potentially coupling active quinonoid or amino structures in soil organic matter by this reaction. This might, for instance, allow the quantification of the coupling capacities of different soils.

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