Chemoenzymatic Synthesis of Trinitrobenzyl Halides as an Alternative Approach to Hexanitrostilbene

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This paper is dedicated to Prof. Roger Sheldon on the occasion of his 60th birthday.

Abstract: The selective oxidation and halogenation of nitroaromatics is a difficult task both chemically and enzymatically. We have discovered that vanadium chloro- and bromoperoxidases from *Curvularia inaequalis* and *Corralina officinalis*, respectively, are capable of catalyzing the hydroxylation, halogenation, and demethylation of 2,4,6-trinitrotoluene (TNT) under alkaline conditions. At pH 8, the conversions for hydroxylation and demethylation reached 38 and 45%, respectively, while direct halogenation was minimal. Vanadium chloroperoxidase generated trinitrobenzyl alcohol with initial rates of 0.27 μM/h-unit enzyme as compared with 0.11 μM/h-unit enzyme for the vanadium bromoperoxidase. The products of the enzymatic reaction were easily separated and purified and the unreacted substrate recovered. In the presence of PCl_5, the trinitrobenzyl alcohol produced by vanadium chloroperoxidase was readily converted to trinitrobenzyl chloride (TNBCl). This chemoenzymatic synthesis may be useful in the environmentally benign synthesis of hexanitrostilbene, a key component of heat-resistant explosive materials.

Keywords: benzylic hydroxylation; chemoenzymatic synthesis; halogenation; vanadium chloroperoxidase

Introduction

The oxidation of aromatic compounds bearing electron-withdrawing substituents is a difficult task in biocatalysis. Peroxidases are able to catalyze the oxidation, hydroxylation, and halogenation of toluene derivatives;[1] however, these reactions have not been shown to be feasible on toluene compounds with strongly electron-withdrawing substituents, such as nitro groups. Nonetheless, such substrates are of practical importance in a number of synthetic transformations. For example, the selective oxidation of 2,4,6-trinitrotoluene (TNT), *via* a benzyl chloride intermediate, is used in the formation of hexanitrostilbene (HNS), a common explosive capable of resisting temperatures of 260 °C for a period of 2 h.[2] HNS has found numerous applications in the aerospace and mining industries, and as military ordinance.[3]

HNS has been synthesized chemically in two ways. Kompolthy and coworkers[4] used an oxidative linkage of two TNT molecules to generate HNS. A more commonly used method, however, was developed by Shipp in the early 1960's,[2] and involves the coupling of two 2,4,6 trinitrotoluene (TNT) molecules *via* a halogen containing intermediate.[5] The mechanism of HNS synthesis from TNT using the Shipp synthesis has been elucidated. Under alkaline conditions, TNT is dehydrogenated to form the trinitrobenzyl anion, which is rapidly halogenated in the presence of NaOCl to yield trinitrobenzyl chloride (TNBCl). Subsequent reaction with base produces the benzyl chloride anion, which undergoes C-C bond formation and dechlorination to give HNS.[6] Despite the ease of synthesis, there are several problems associated with the Shipp synthesis. For example, the reaction is hard to control and is inefficient, with yields of HNS below 35%. Furthermore, because of the harsh alkaline conditions employed, the chemical process generates a large number of undesirable byproducts and large amounts of tetrahydrofuran and methanol wastes – up to 65 pounds of waste per pound of HNS produced.

The key step in the Shipp synthesis is the formation of the TNBCl, and this is the step that is most inefficient chemically. For this reason, we set out to establish whether a biocatalytic alternative was feasible. Peroxidases are known to catalyze hydroxylation and halogenation reactions on a wide range of substrates;[7] however, they exhibit very low catalytic activity under alkaline conditions.[8] Nevertheless, such conditions are critical to support a sufficient concentration of the benzyl anion of TNT (pKa *ca.* 14.5).[2] Therefore, we set out to identify a peroxidase capable of halogenating activity under basic conditions. We identified several peroxidases with sufficient alkaline activity to support

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both halogenation and hydroxylation activity, and in the process we achieved a chemoenzymatic strategy for the synthesis of TNBCl.

Results and Discussion

Peroxidase-Catalyzed Transformation of TNT

A variety of peroxidases were screened for their ability to oxidize and/or halogenate nitrotoluene derivatives under conditions ranging from pH 2 to 10. These enzymes included vanadium chloroperoxidase (VCPO) from *Curvularia inaequalis*, vanadium bromoperoxidase (VBPO) from *Corallina officinalis*, chloroperoxidase (CPO) from *Caldariomyces fumago*, soybean peroxidase (SBP), and a *Coprinus cinereus* peroxidase mutant (NS 50014) from Novo Nordisk, the latter engineered to have high stability in alkaline media. Reactions were performed in aqueous buffer (containing 5%, v/v, CH_3CN added to aid in TNT solubility) with 10 mM KBr, 10 μM TNT, and a slow feed of H_2O_2 up to a concentration of 2.5 mM. Neither SBP nor CPO was active on TNT under these conditions. In the presence of 100 μM Na_3VO_4, VCPO and VBPO were found to transform TNT at pH 8.0, as noted by the decrease in the substrate concentration and the formation of three distinct products, as shown by HPLC (Figure 1). Above and below pH 8, the reactivity of both enzymes decreased substantially with no measurable activity below pH 7 or above pH 9.

The two major reaction products, eluting at 4.45 and 6.43 min, have parent ions of *m/z* 242 and 210, likely the benzyl alcohol derivative of TNT (TNT-OH) and a demethylated compound likely that of trinitrobenzene, respectively. A third and very minor product eluting at 11.65 min had two parent ions of equal intensity of *m/z* 304 and 306, a characteristic MS signature for bromination. Unfortunately, this product was highly unstable under the reaction conditions employed and was unable to be isolated. NMR analyses on the two major products confirmed the chemical structures of the early compounds to be the TNT-OH and trinitrobenzene, respectively.

A series of control reactions was performed. No reactions were observed in the absence of enzyme (VCPO or VBPO) or H_2O_2. Reactions conducted in the absence of bromide yielded a single product corresponding to the hydroxylated derivative with minimal conversion (less than 1% after 48 h) and did not yield trinitrobenzene. Reactions with KCl instead of KBr also led to negligible yields of TNT-OH as the single reaction product, suggesting that neither VBPO nor VCPO was capable of oxidizing Cl^- under these alkaline conditions or that HOCl, if generated, was unable to react with TNT under the conditions employed. In no case were 4-nitrotoluene, 2,4-dinitrotoluene or 2,6-dinitrotoluene modified by any of the enzymatic systems employed, suggesting some relationship between the formation of a benzylic anion and the enzymatic reaction, as the pKa of the TNT is significantly lower than that of 4-nitrotoluene and 2,4-dinitrotoluene. These results suggest that the generation of HOBr was truly enzymatic and that this chemical agent was responsible for the observed transformations.

The formation of trinitrobenzene is intriguing, and led us to investigate the reaction of *chemically* generated HOBr with TNT. Reactions conducted with molecular bromine (Br_2) in the presence of sodium hydroxide in MeOH/THF solutions led to the complete conversion of TNT to trinitrobenzene. This is similar to the results obtained by Emmrich[9] who demonstrated that the alkaline hydrolysis of TNT by calcium hydroxide solutions (pH 10–11) led to a similar formation of trinitrobenzene along with various trace amino derivatives. Thus, TNT is highly susceptible to demethylation and reduction in the presence of various oxidants. This

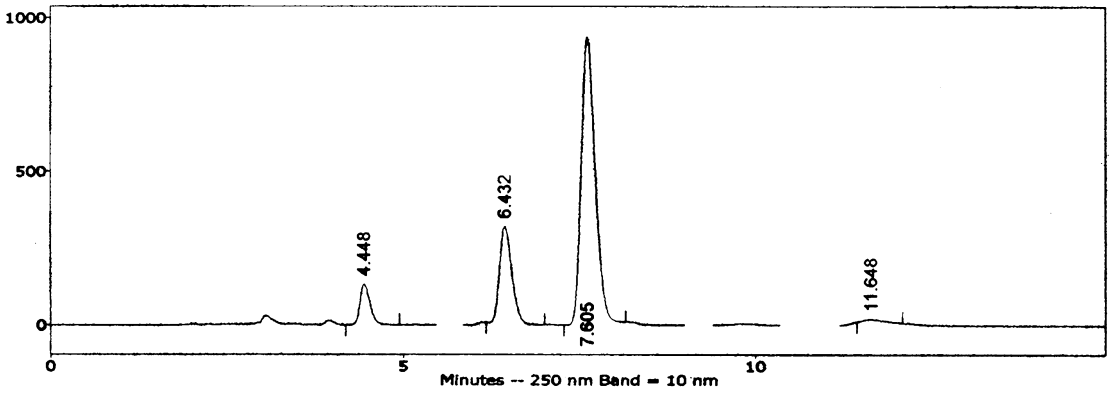


Figure 1. HPLC trace for the vanadium chloroperoxidase (60 μM) reaction with TNT (10 μM) containing 10 mM KBr, 2 mM H_2O_2, and 1 mM Na_3VO_4. Chromatogram represents product distribution after 24 h. Vanadium bromoperoxidase catalysis gave a similar reaction profile, albeit at lower yields.

observation also supports the likelihood that enzymatically-generated HOBr is responsible for the carbon-carbon bond cleavage rather than a direct peroxidative action by VCPO.

Reactivity of Vanadium Peroxidases on TNT

The catalytic activities and product distributions of both VBPO and VCPO were also evaluated. To properly compare the relative activities of these different enzymes, standard assays for the chlorination of monochlorodimedone (MCD) were conducted to establish unit activities for both enzymes using standard methods at pH 5. Acidic conditions were employed, as neither enzyme was able to oxidize chloride at pH 8. The VCPO catalyst was subsequently diluted several fold to maintain an equivalent unit activity. Figures 2A and B show the reaction time courses for TNT and the VCPO and VBPO catalyzed products. Both enzymes catalyze the formation of both TNT-OH and trinitrobenzene in equimolar amounts; however, VCPO exhibits higher activity with an initial rate of TNT oxidation of *ca.* 0.27 μM/h-unit E, compared with 0.11 μM/h-unit E for VBPO. No visible TNT consumption or product formation was found in VBPO systems for up to 2 h, indicating that insufficient amounts of HOBr were likely present to act as an effective hydroxylating/demethylating agent. However, once enough HOBr was generated, hydroxylation and demethylation ensued. It is critical to note that the lower rate of VBPO *vs.* VCPO is intrinsic, and not due to lower enzyme concentration in the reaction, as we normalized VBPO and VCPO activities using the MCD assay prior to the reaction.

Product conversions for the VCPO catalyzed reactions after 24 h were 38 and 45% for TNT-OH and trinitrobenzene, respectively (data not shown). This compares to only the 16 and 15% conversion for the TNT-OH and trinitrobenzene, respectively, in the same time period with VBPO as biocatalyst. Further addition of H_2O_2 to either reaction after this time failed to alter the final chemical composition of the reaction mixtures, indicating the likely inactivation of the enzymes. Various attempts were made to improve both product conversion and increase yields of the hydroxylated derivative over the demethylated compound. These included varying the TNT, vanadate, and bromide concentrations, employing anaerobic environments, and using alternative oxidants such as *tert*-butyl hydroperoxide (instead of H_2O_2). In no case was overall product conversion or distribution altered. Higher enzyme concentrations led to higher initial rate of TNT oxidation. VCPO catalysis was also performed in non-aqueous systems (including CH_3CN, MeOH, THF, and ethyl acetate), saturated with KBr and VO_4^{3-} with VCPO added as a lyophilized suspension (up to 1 mg/mL). However, no reactions were observed in the non-aqueous media, using either H_2O_2 or *tert*-butyl hydroperoxide as oxidants.

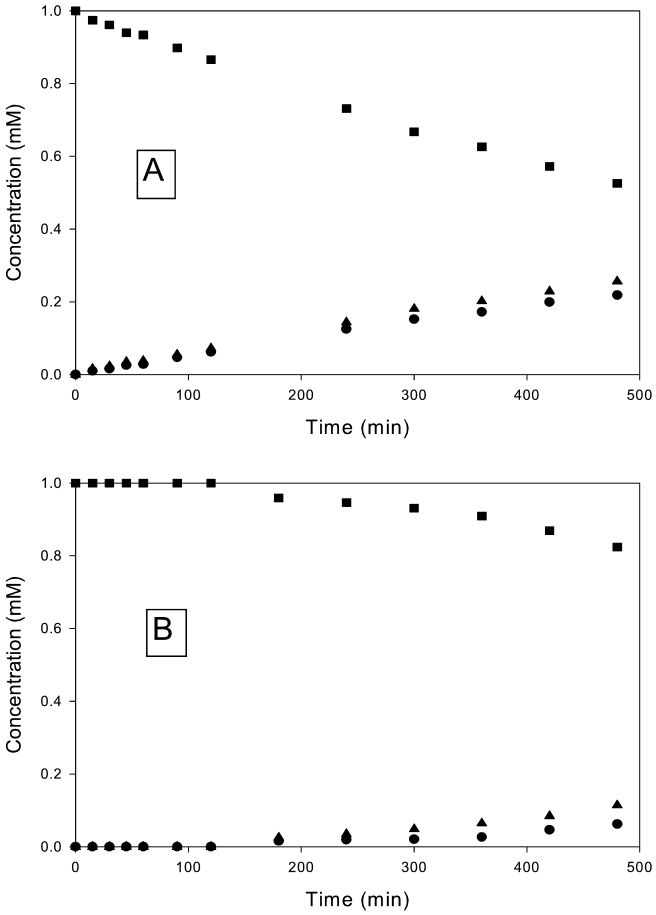
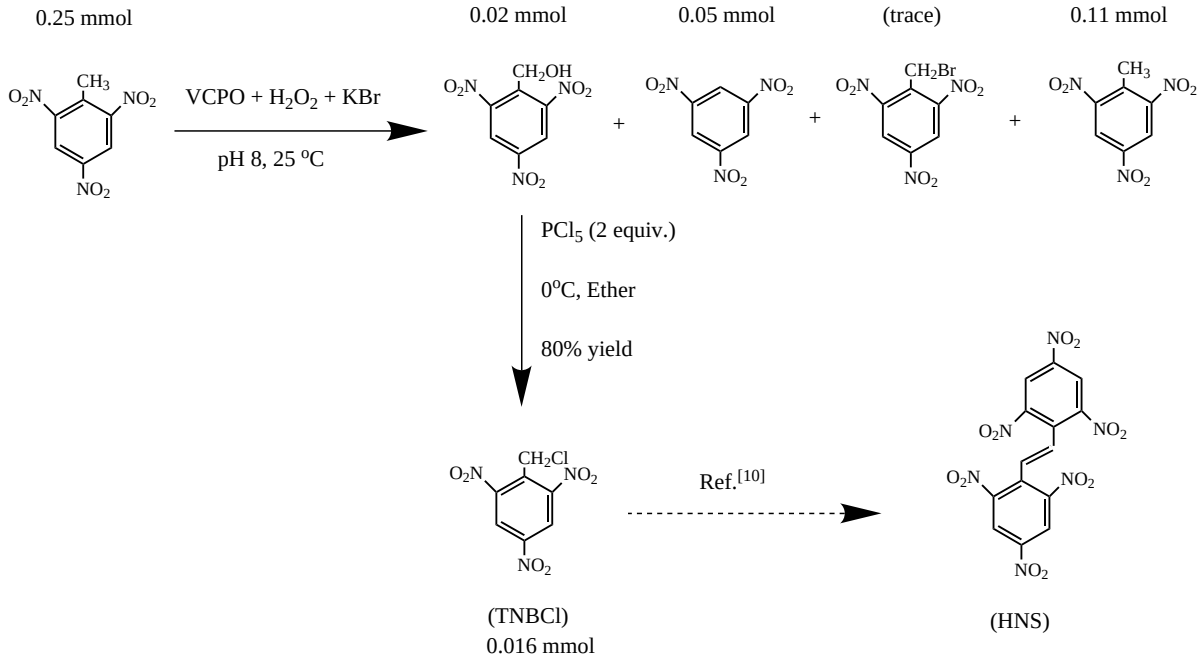


Figure 2. Reactivity of (**A**) VCPO and (**B**) VBPO toward TNT (■) in aqueous systems (pH 8.0), producing trinitrobenzyl alcohol (●) and trinitrobenzene (▲). Reaction conditions are described in the text.

Chemoenzymatic Synthesis of Trinitrobenzyl Chloride

Larger scale reactions were then performed to obtain sufficient product for subsequent chemical transformations. TNT (0.25 mmol) was used in aqueous buffer containing 30% (v/v) CH_3CN. The product was worked up as described in the Experimental Section, and from the 45 mg residue obtained, 4.8 mg of TNT-OH (0.02 mmol) and 10.0 mg of trinitrobenzene (0.05 mmol) were isolated along with 25 mg of unreacted TNT (0.11 mmol).

The enzymatically derived TNT-OH was then converted to the corresponding chlorinated derivative using PCl_5 with complete conversion in 30 min to give the pure trinitrobenzyl chloride (TNBCl) (Scheme 1). Similar results were obtained for the synthesis of the trinitrobenzyl bromide using PBr_3. The chemical chlorination was facile, with *ca.* 80% recovered yield (0.016 mmol,



Scheme 1. Chemoenzymatic synthesis of trinitrobenzyl halides (synthesis completed to TNBCl).

4.16 mg). In this chemoenzymatic strategy, the only products were the TNBCl and the trinitrobenzene. This is far cleaner than the 16 products and byproducts from the Shipp synthesis.

Conclusions

We have demonstrated that vanadium chloroperoxidase and vanadium bromoperoxidase are capable of catalyzing the oxidation of bromide in alkaline media. The resulting chemical species (HOBr) remains highly stable under these conditions and is able to catalyze the demethylation, hydroxylation, and halogenation of TNT, the latter producing a highly unstable compound visible only in trace amounts. VCPO exhibited nearly three-times higher activity at pH 8.0 than VBPO. The enzymatically derived trinitrobenzyl alcohol was then transformed to the corresponding benzyl chloride, a key intermediate in the synthesis of HNS, using PCl_5. There are many references and patents describing the formation HNS from the chlorinated TNT, the most effective being one developed by Angres[10] with overall product yields of HNS typically exceeding 50% from TNBCl. Based on such a subsequent chemical step, we would expect to achieve *ca*. 19% yield of HNS from the TNT starting material. Nonetheless, there are several potential benefits to this approach. For example, simpler purification of the TNBCl or HNS from the reaction mixture is ensured by the fewer number of products inherent in the chemoenzymatic route. Additionally, the enzymatic reaction is controllable, and this may be important for the selective oxidation of TNT derivatives or related compounds. Finally, from an enzymatic standpoint, the reactivity of peroxidases has now been shown to occur under alkaline conditions for the oxidation of a highly recalcitrant nitroaromatic compound. This is an important finding that may impact other synthetically relevant reactions where peroxidase may be considered as a viable biocatalytic alternative to conventional chemical synthesis.

Experimental Section

Materials

A recombinant form of the vanadium chloroperoxidase (VCPO) from *Curvularia inaequalis* was obtained from the Biotechnology Application Centre – a division of Unilever (Naarden, The Netherlands) as a crude suspension (5.8 mg/mL). The isolation and purification of the recombinant enzyme in the *Saccharomyces cerevisiae* expression system is described in the work of Hemrika et al.[11] Before use the enzyme was dialyzed against 0.01 M sodium phosphate buffer (pH 8.0) using a 3500 MWCO membrane (Pierce Tubing, Rockford IL) to remove unwanted salts. Vanadium bromoperoxidase (VBPO) from *Corallina officinalis* was purchased from Sigma as a partially purified lyophilized powder (~130 units/mg). Chloroperoxidase (CPO) from *Caldariomyces fumago* and soybean peroxidase were purchased from Sigma. A *Coprinus cinereus* peroxidase mutant (NS 50014) was obtained as a gift from Novo Nordisk The TNT used in the kinetic studies was purchased from Cerrilliant (Austin, TX, USA) as a dilute solution (1 mg/mL in CH_3CN). TNT (5 mg/mL in CH_3CN) used in the large-scale syntheses was donated by Los Alamos National Laboratory (Los Alamos, NM). 2,6-Dinitrotoluene,

2,4-dinitrotoluene and 4-nitrotoluene, phosphorus pentachloride and phosphorus tribromide were purchased from Aldrich Chemical (Milwaukee, WI, USA). Hydrogen peroxide (30% solution in water) was obtained from EM Science (Gibbstown, NJ, USA) and silica gel (flash chromatography grade) was purchased from J.T. Baker (Phillipsburg, NJ). Reversed-phase C-18 silica-gel was purchased from Aldrich. All other chemicals employed in this research were of the highest purity commercially available.

Determination of VCPO and VBPO Reactivity

The activity of VCPO and VBPO toward TNT was assessed by monitoring both the disappearance of the substrate, and the formation of reaction products by HPLC. A typical system consisted of 1 mM TNT dissolved in 2 mL of 50 mM sodium phosphate buffer (pH 8.0) containing 5% (v/v) CH_3CN, 10 mM NaBr, 100 μM Na_3VO_4 and either 30 μM VCPO or 10 units/mL VBPO. Reactions were initiated upon the addition of H_2O_2 to a final concentration of 2.5 mM and the reactions shaken on an orbital shaker at 100 rpm at 25 °C. Periodically samples were removed and analyzed using HPLC (Shimadzu 10A VP) equipped with a Phenomenex Kingsorb-Phenyl (150 × 4.6 mm) column running an isocratic mobile phase of 57% CH_3CN and 43% water. Detection of reaction components was performed at 255 nm using a photodiode array detector (Shimadzu 10AS). Under these conditions, TNT eluted at 7.61 min and the reaction products of trinitrobenzyl alcohol, trinitrobenzene and trinitrobenzyl bromide eluted at 4.45, 6.43 and 11.65 min, respectively.

Enzymatic Product Identification

Initial product characterization was conducted using LC-MS with a PE-SciEx 7000 mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) detector, operating in both positive and negative ionization modes. The reaction conditions and HPLC parameters were identical to those discussed in the previous section. As a result of the relatively high activity of the VCPO, this enzyme was utilized in large-scale synthetic reactions to isolate and purify products for structural confirmation. In this case, 0.5 mM TNT was dissolved in 500 mL of 30% (v/v) CH_3CN in aqueous buffer system containing 60 μM VCPO. The reaction was terminated after 48 h by extracting the substrate and products with an equal volume of ethyl acetate. The organic layer was subsequently removed and concentrated under reduced pressure. The reaction components were separated using flash chromatography (C-18 reversed phase) initially eluting 95% CH_3CN: 5% H_2O with a linear gradient to 50:50 mixture and dried under vacuum. The residues (~45 mg) were separated by reversed-phase C-18 chromatography using $CH_3CN:H_2O$ (20:1, 10:1 and 5:1) to afford pure hydroxy-TNT (4.8 mg), trinitrobenzene (TNB, 10.0 mg) and unreacted TNT (25 mg).

Various spectroscopic methods were used to determine the chemical structure of the enzymatically-synthesized products, including: ^{1}H NMR, ^{13}C NMR, and FT-IR with the following chemical shifts and peaks. ^{1}H NMR and ^{13}C NMR were recorded on a Unity 500 spectrometer in $CDCl_3$. Chemical shifts are reported in parts per million (ppm). Atomic pressure chemical ionization (APCI) MS was measured on an Agilent mass spectrometer. FT-IR spectra were recorded on a Perkin-Elmer spectrometer.

Hydroxy-TNT: ^{1}H NMR (500 MHz, CD_3Cl): $\delta = 8.87$, 5.13; IR (KBr): $\nu = 3435.62$, 3098.36, 2963.79, 1713.43, 1616.91, 1546.67, 1350.34, 1263.10, 1096.71, 1036.40 cm^{-1}; APCI MS: [M$^-$] 243.0.

2,4,6-Trinitrobenzene: ^{1}H NMR ($CDCl_3$): $\delta = 9.39$ (s, 3H); ^{13}C NMR ($CDCl_3$): $\delta = 149.2$, 124.0; IR (KBr): $\nu = 3436.8$, 3107.4, 2923.4, 1624.7, 1546.1, 1346.9, 1078.9, 921.9 cm^{-1}.

Trinitrobenzyl alcohol: ^{1}H NMR ($CDCl_3$): $\delta = 8.82$ (s, 2H), 5.13(s, 2H); FT-IR (KBr): $\nu = 3435.6$, 3098.4, 2963.8, 1713.4, 1619.9, 1350.3, 1263.1, 1096.7, 1036.4 cm^{-1}; APCI MS: [M$^-$] 213.0.

Generation of Trinitrobenzyl Chloride and Trinitrobenzyl Bromide

To hydroxy-TNT (3 mg) in dry ether (1 mL) was added PCl_5 (2.0 equiv., 5.5 mg) at 0 °C and the reaction mixture was stirred at 0 °C for 4 h. The reaction mixture was then washed with saturated NaCl and dried over $MgSO_4$. After evaporation of ether, pure desired product (2.8 mg) was obtained (80%). ^{1}H NMR (500 MHz, CD_3Cl): $\delta = 8.92$ (s, 2H), 5.11 (s, 2H); APCI MS: [M$^-$] 243.0.

The bromide derivative was prepared in a similar manner: to hydroxy-TNT (3 mg) in dry ether (1 mL) was added PBr_3 (3.0 μL) at 0 °C, and the reaction mixture was stirred at 0 °C for 2 h. After evaporation of ether, pure desired product (3.3 mg) was obtained (82%). ^{1}H NMR (500 MHz, CD_3Cl): $\delta = 8.85$, 5.59.

Demethylation of TNT by HOBr

To TNT (30 mg) in THF/MeOH (1:1, 1 mL) was added HOBr solution, which was generated by mixing bromine (30 mL) and NaOH solution (1.0 N, 1 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. After evaporation of the solvents, the crude product was washed with saturated NaCl, and purified by silica gel chromatography using hexane : EtOAc (5:1 and 3:1). The product had an NMR spectrum identical to that identified as 2,4,6-trinitrobenzene, as described above for the enzymatic reaction.

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