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Detection of ketyl radicals using ^{31}P NMR spin trapping

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Our recent work has allowed the development of ^{31}P NMR spin trapping techniques for the detection and, at times, absolute quantification of many oxygen- and carbon-centered free radical species. These methods are based on the ability of the nitrone phosphorus compound, 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO), to react with free radical species and form stable radical adducts, which are suitably detected and accurately quantified using ^{31}P NMR. Our continuing efforts have now been focused on the application of this powerful system for the trapping of ketyl radicals, which are very difficult intermediates to be detected and quantified with traditional techniques (i.e., EPR). Ketyl radicals were initially produced using photochemical reactions of acetophenone, whose excited triplet state is able to abstract hydrogen from an H donor. As such, the ^{31}P NMR signals for the radical adducts of the DIPPMPO spin trap with the ketyl radicals were assigned. Furthermore, in an effort to confirm the structure of these adducts, their mass spectra and fragmentation patterns were carefully examined under Gas Chromatography–Mass Spectrometry (GC–MS) conditions. Subsequently, the DIPPMPO spin trapping system was applied to the oxidation of 1-(3,4-dimethoxyphenyl)ethanol in the presence of horseradish peroxidase (HRP), hydrogen peroxide, and 1-hydroxybenzotriazole (HBT) as the electron carrier (mediator). Our work confirmed that the mechanism consists of a hydrogen abstraction reaction from the α position, involving the ketyl radical: during the oxidation, the hydroxyl, hydroperoxyl, and ketyl radical intermediates were all detected. These efforts demonstrate the efficacy of our methodology that provides for the first time a facile means for the detection of the otherwise elusive ketyl radical species, with important implications in biology, chemistry, and biochemistry. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: DIPPMPO; GC–MS; ketyl radical; ^{31}P NMR; photochemistry; peroxidase; spin trap

INTRODUCTION

Ever since the introduction of spin trapping techniques, studies in this field have proliferated with numerous applications focusing on the detection of free radicals formed in solution chemistry as well as in biology and medicine.[1–3] Recently, a novel spin trap, the 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO), was proposed.[4] This compound was demonstrated to be a powerful tool for detecting and precisely differentiating oxygen-centered radicals, namely ·OH and $O_2{}^{\cdot -}$,[5] and carbon-centered radicals, such as $\cdot CH_3$, $\cdot CH_2OH$, $\cdot CH(OH)CH_3$, $\cdot C(O)CH_3$[5] as well as phenoxy radicals,[6] when coupled with ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR). Figure 1 shows the general trapping chemistry of the DIPPMPO spin trap system.

The radical species (R·) reacts with the nitrone spin trap DIPPMPO (**a**) to form the nitroxide paramagnetic adduct (**b**), which is Electron Paramagnetic Resonance (EPR) detectable. The paramagnetic species (**b**) decays with time and undergoes unimolecular and/or bimolecular decomposition to give the corresponding diamagnetic species, the hydroxylamine (**c**) and the nitrone (**d**) via a disproportionation reaction.[7] The stable diamagnetic products (**c** + **d**), derived from the radical adducts, coupled with the uniqueness of the quantitative ^{31}P NMR technique can be exploited further to perform qualitative analyses, since the ^{31}P NMR signal is specific to each trapped free radical.[5,6] In many cases the chemical species of the radical adducts (**c** + **d**) could be characterized by Gas Chromatography–Mass Spectrometry (GC–MS), thus elucidating and confirming their structures.[8]

In an effort to explore and extend the potential of this methodology for trapping the ketyl radicals, the well-known radical pathway involved in the photochemistry of acetophenone was studied. The derived knowledge was then applied in elucidating the mechanism of enzymatic oxidation of non-phenolic compounds. Both these systems are known to have the ketyl radical as the common free radical intermediate.

More specifically, the hydrogen abstraction reaction is amongst the most intensely studied photochemical reactions of acetophenones.[9–11] The excited n,π^* triplet state of acetophenone is the demonstrable reactive state in both aliphatic and benzylic hydrogen abstraction systems.[11,12] Furthermore, in the presence of a hydrogen donor, such as alcohols, the reaction leads to the formation of a radical intermediate.[13,14] This simple reaction was used to explore and understand the ability of DIPPMPO to trap ketyl radicals.

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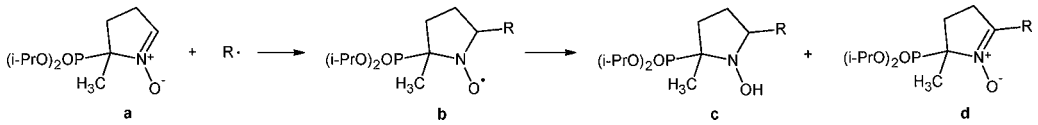


Figure 1. The general trapping chemistry of the DIPPMPO spin trap system

As the second most abundant biopolymer on the planet, lignin is a common substrate for enzymatic transformations involving laccases and/or peroxidases. This is because the presence of the phenolic functionality on lignin, which has a redox potential that matches those of the enumerated enzymes.[15]

For phenolic substrates, electron abstraction and subsequent deprotonation gives rise to phenoxy radicals.[16] The DIPPMPO spin trapping system has been demonstrated to be rather useful for the elucidation of reactions involving phenoxy radicals.[6] Alternatively, the oxidation of non-phenolic substrates is prevented by their high redox potential.[17–19] However, in the presence of a radical mediator such as 1-hydroxybenzotriazole (HBT), an increase in enzymatic reactivity has been demonstrated.[20,21] In general, such mediator molecules could be considered as electron shuttles that, after being oxidized, diffuse away from the active site to oxidize substrates that are not directly susceptible of oxidation by the enzyme itself.[22] Despite the large body of information that exists on the reaction of enzymes such as laccases, with several substrates and mediators, a comprehensive understanding of the reaction mechanism is lacking. Recently it was shown that HBT reacts with non-phenolic compounds via a radical mechanism involving hydrogen atom transfer (HAT), forming the ketyl radical species as an intermediate.[18] This radical species seems to play a pivotal role in lignin chemistry.[23] Support for this important conclusion has so far been provided by indirect evidence. This evidence involves the study of the intermolecular selectivity of oxidation using appropriate substrates, followed by Hammett correlations for the oxidation of a series of 4-X-substitued benzyl alcohols, measuring the kinetic isotopic effect, and correlating the product pattern with suitable probe precursors.[17]

This important enzymatic system, the horseradish peroxidase (HRP) in presence of 1-hydrobeznotriazole (HBT) as mediator, was applied in the oxidation of 1-(3,4-dimethoxyphenyl)ethanol and was examined in order to further understand the application potential of the proposed techniques.

RESULTS AND DISCUSSION

Photochemical generation of ketyl radicals

In an effort to examine the efficacy of the DIPPMPO spin trapping system to react with ketyl radicals, the 1-phenyl-ethanol-1-yl radical ($\cdot C(OH)CH_3Ph$) was generated photochemically, since hydrogen abstract is one of the best known and most extensively studied photochemical reactions of aryl ketones.[9–11] A mixture of acetophenone and 1-phenylethanol, under UV irradiation, is known to produce a single radical product, the 1-phenyl-ethanol-1-yl radical.[14] In the absence of spin trap the reaction leads to the formation of the corresponding pinacols (*d*, *l*, and *meso* forms), which were detected and characterized by detailed GC–MS work.

$$PhCOCH_3 + h\nu \rightarrow [PhCOCH3]^* \quad (1)$$

$$[PhCOCH_3]^* + PhCH(OH)CH_3 \rightarrow 2PhC\cdot(OH)CH_3 \quad (2)$$

$$2PhC\cdot(OH)CH_3 \rightarrow meso + d,l - [PhC(OH)CH_3]_2 \quad (3)$$

During UV irradiation, ground-state ketones are converted to excited singlets, which via intersystem crossing form the corresponding triplets (1). The excited triplets then abstract a hydrogen atom from the corresponding alcohol (2) to give ketyl radicals. The ketyl radicals react via pinacol coupling to form 2,3-diphenyl-2,3-butanol in a racemic mixture (3).

In the presence of the spin trap DIPPMPO, the 1-phenyl-ethanol-1-yl radical was trapped and detected by ^{31}P NMR. As in our previous work, the chemical shift of the adducts was observed at 28.2 ppm.[6,8] The structure of these adducts were confirmed by detailed GC–MS analyses. Previous work has shown that the bimolecular decomposition of nitroxide radical adducts of cyclic nitrones can occur to give the corresponding nitrone and hydroxylamine via disproportionation reaction[7] (Fig. 2). In case of ketyl radical adducts the hydroxylamine further reacts, undergoing water elimination to form the corresponding alkene.

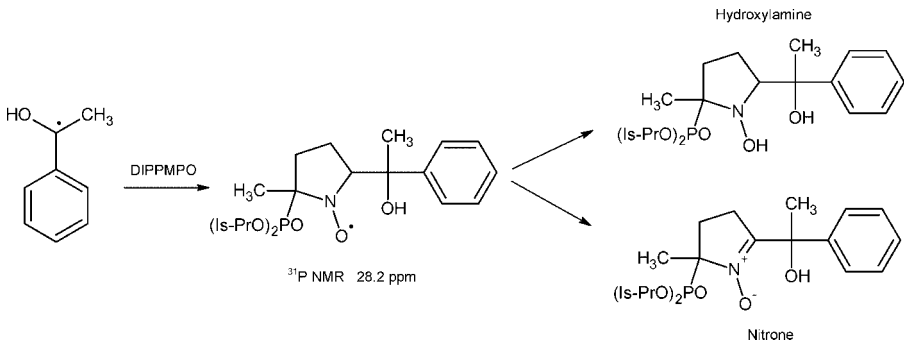
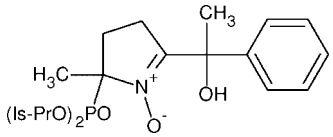
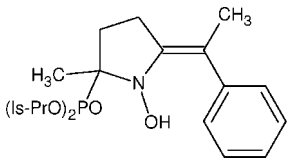


Figure 2. Spin trapping reactions of the ketyl radical with DIPPMPO and the disproportionation reaction of the radical adducts to form the corresponding nitrone and hydroxylamine

Table 1. Mass spectra and ^{31}P NMR data for DIPPMPO and the depicted ketyl radical adducts

Species	δ ^{31}P NMR (ppm)	Mass spectrum (*m/z*)
DIPPMPO	22.2	263 (5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21).
	28.2	383 (2), 365 (5), 340 (4), 281 (7), 264 (5), 256 (8), 238 (4), 218 (6), 200 (100), 158 (9), 141 (4), 123 (11), 105 (20), 77 (12).
	28.2	367 (7), 352 (1), 325 (3), 310 (2), 266 (3), 247 (4), 202 (100), 184 (44), 158 (15), 144 (7), 123 (31), 105 (15), 82 (30).

This reaction could explain why the nitrone and the hydroxylamine have the same chemical shift in ^{31}P NMR, while for methyl radical trapping with DEPMPO as spin trap, the chemical shift in ^{31}P NMR of hydroxylamine and nitrone is quite different.[24] The GC–MS analyses confirmed this reaction since both forms of the radical adducts were detected (Table 1).

The interpretation of the fragmentation pathway of DIPPMPO under Electron Impact ionization (EI) conditions is shown as the first entry of Table 1 with details of this fragmentation pattern having been discussed elsewhere.[8] The fragmentation pattern for the nitrone form of the ketyl radical adduct is shown as the second entry of Table 1: the molecular peak (M·+) at 383 *m/z* undergoing loss of water (18 u) gives rise to the peak at 365 *m/z*. The loss of the diisopropyl(oxido)phosphoranyl radical ·P(O)(O-C_3H_7)$_2$ (165 u) leads to the formation of the main peak at 200 *m/z*. In an identical manner the mass spectrum of the hydroxylamine adduct, shown as the third entry in Table 1, could be interpreted as follows: after formation the hydroxylamine undergoes water elimination; in fact the molecular peak (M·+) was detected at 367 *m/z* (385-18 u). The loss of the diisopropyl(oxido)phosphoranyl radical ·P(O)(O—C_3H_7)$_2$ (165 u) leads to the formation of the main peak at 202 *m/z*.

In order to generate the 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical, a mixture of 1-(3,4-dimethoxyphenyl)ethanol and acetophenone was irradiated by UV light. The possibility of forming the 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical as a unique radical product by UV irradiation of a mixture of 3,4-dimethoxy acetophenone and 1-(3,4-dimethoxyphenyl)ethanol is precluded by the lowest π,π^* triplet state nature of this ketone.[13] This is because the n,π^* triplet, such as acetophenone, is the reactive state in aliphatic and benzylic hydrogen abstraction reactions and ketones with lowest π,π^* triplet states known to react predominantly via the higher energy n,π^* states, populated thermally from a lower energy state.[12] The mechanism of this reaction is shown in Fig. 3a. Upon irradiation, ground-state acetophenone is converted to an excited singlet, which via intersystem crossing yields the corresponding triplet. The excited triplet then abstracts a hydrogen atom from the 1-(3,4-dimethoxyphenyl)ethanol to give a mixture of ketyl radicals: the 1-phenyl-ethanol-1-yl and the 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical.

The data from the GC–MS analyses of the reaction products have confirmed the reaction pathways depicted in Fig. 3b. The reaction products detected were, 3,4-dimethoxy acetophenone **1** and pinacol **2**. The 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical is thought to react via disproportionation, leading to the formation of the corresponding ketone (**1**); furthermore, the 1-phenyl-ethanol-1-yl radical undergoes a coupling reaction to form the pinacol (**2**).

In the presence of DIPPMPO, it was possible to trap the UV-generated ketyl radicals (Fig. 3c) and characterize the radical adducts by ^{31}P NMR and GC–MS. The ^{31}P NMR analyses showed the presence of two different radical adducts with chemical shifts of 28.2 and 27.3 ppm, respectively. These adducts, whose ^{31}P NMR signal appeared at 28.2 ppm, were related to the trapping of the 1-phenyl-ethanol-1-yl, (the ketyl radicals from acetophenone), on the basis of the previously described experimental data. The new radical adduct at 27.3 ppm is thought to be due to the ketyl radical from 1-(3,4-dimethoxyphenyl)ethanol. The GC–MS data confirmed these hypotheses and the mass spectrum of these adducts confirmed the proposed structures. The chromatogram showed the presence of the radical adducts of 1-phenyl-ethanol-1-yl radical, in addition to another radical adduct whose mass spectrum is shown in Fig. 4.

As reported in Fig. 4a, the 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical was generated via the acetophenone triplet state H-abstraction at the α (benzylic) position. This was then trapped by DIPPMPO, leading to the formation of a paramagnetic adduct that, after loss of water from the tertiary alcohol, decayed to a diamagnetic hydroxylamine. In our work the decay reaction to the nitrone form was not observed. As shown in Fig. 4b, it is possible to observe the molecular peak (M·+) at 427 *m/z*, that undergoes loss of the diisopropyl(oxido)phosphoranyl radical ·P(O)(O—C_3H_7)$_2$ with a loss of 165 u, leading to the formation of the main peak at 262 m/z. As reported previously, the main peak of ketyl radical adducts in EI corresponds to the main mass peak of DIPPMPO (98 u) in addition to the mass of the radical (182 u)

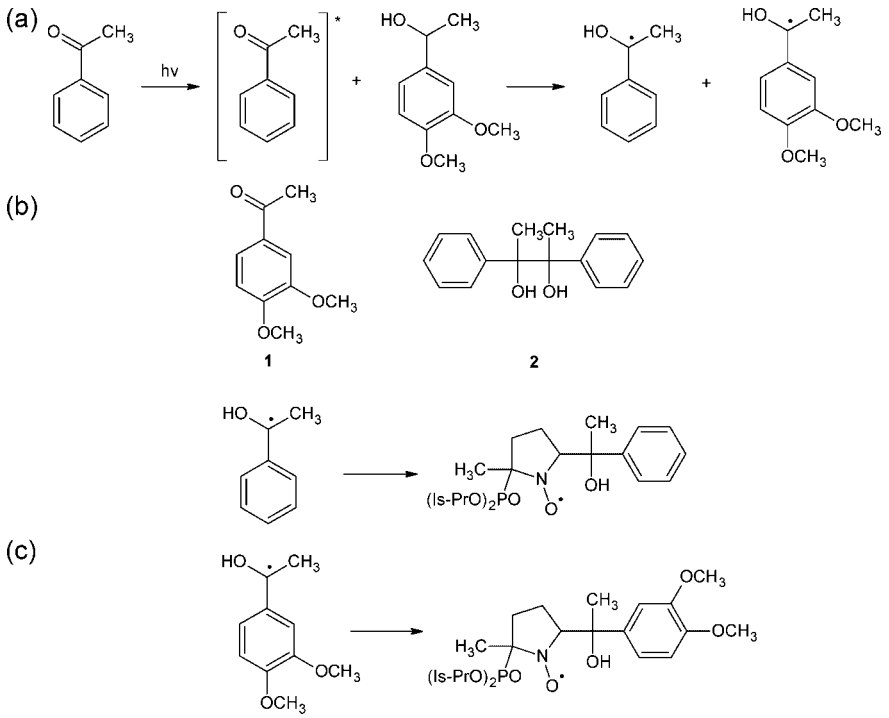


Figure 3. (a) Photochemical pathways thought to occur upon irradiation of a mixture of acetophenone with 1-(3,4-dimethoxyphenyl)ethanol; (b) Reaction products detected by GC–MS; (c) Trapping reaction of ketyl radicals in the presence of DIPPMPO

with loss of water (18 u).[8] These data confirm the fact that DIPPMPO allows the trapping of the ketyl radical from 1-(3,4-dimethoxyphenyl)ethanol permitting its ^{31}P NMR detection with a signal at 27.3 ppm.

Enzymatic oxidation of 1-(3,4-dimethoxyphenyl)ethanol in the presence of DIPPMPO

Spin trap systems could be a powerful tool to understand the radical intermediates involved in lignin chemistry, which represents the second most abundant biopolymer on the planet. In this respect the DIPPMPO system has been shown to be successful for the study of the trapping reaction of phenoxy radicals in HRP oxidation of phenolic lignin precursors.[6] The oxidation of non-phenolic benzyl alcohol and 1-phenylethanols with laccases and peroxidases, mediated by appropriate radical mediators such as HBT, yields the analogous aldehydes and ketones, whereas enzymes cannot oxidize these non-phenolic substrates directly.[19–21] The oxidation step is thought to be carried out by the oxidized form of the mediator (Med_{ox}), generated via its interaction with the enzyme (Fig. 5). The Med_{ox} species can follow a radical HAT route for the oxidation of the substrate.

This effort aims to provide additional support to the above reactions mechanism by utilizing the proposed spin trapping

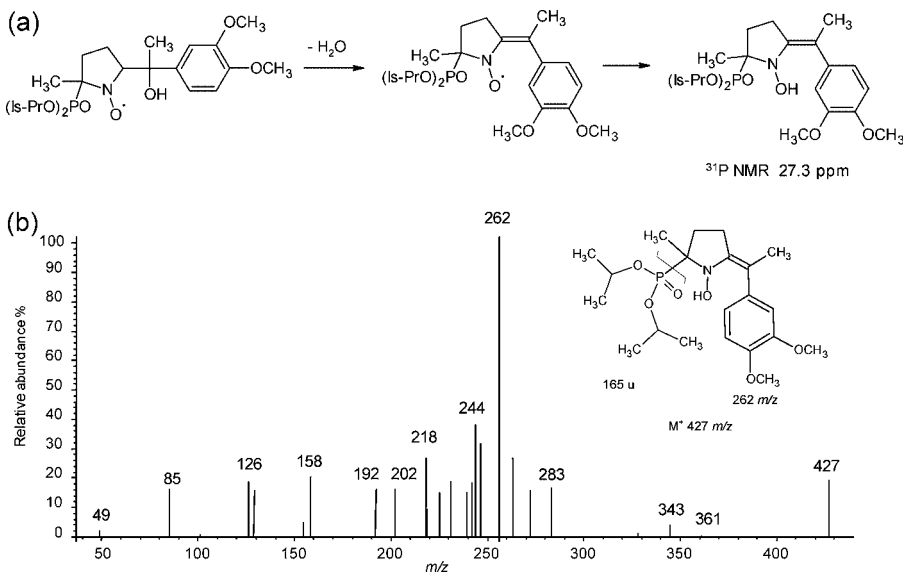


Figure 4. Spin trapping reaction of 1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical with DIPPMPO (a) associated mass spectrum and its interpretation (b)

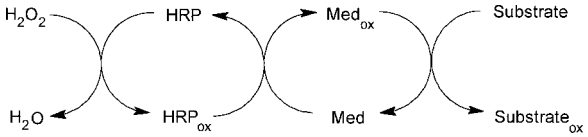


Figure 5. The role of a mediator in HRP catalyzed oxidation for a non-phenolic substrate

technique. This is done by clarifying the role of the ketyl radical in the oxidation of non-phenolic substrate such as 1-phenylethanols.

The oxidation reaction of a non-phenolic substrate, 1-(3,4-dimethoxyphenyl)ethanol with HRP in the absence or in the presence of the mediator HBT was first examined. During this effort the recovered starting substrate and the oxidized product was monitored by GC–MS. As anticipated, in the absence of HBT the enzyme was not able to oxidize the substrate, which was quantitatively recovered (99%). This is because of the difference between the redox potentials of the enzyme (E° 0.8–1.0 V *versus* normal hydrogen electrode NHE) and that of the substrate (E° 1.5 V *versus* NHE).[17–18] In the presence of the mediator the activity of the enzyme increased and it was possible to quantify the reaction product. After 4 h, the GC–MS showed the formation of the corresponding ketone, 3,4-dimethoxyacetophenone at around 66% yield (Fig. 6).

The >N-O· species generated from HBT by the HRP, in view of the matching value of the bond dissociation energy (85 kcal/mol for HBT *versus* 84–87 kcal/mol for the benzylic proton),[20] is thought to perform the hydrogen abstraction reaction from the α position affording the corresponding ketyl radical. Furthermore, a disproportionation reaction causes the formation of the corresponding carbonyl compound (Fig. 7).

During early work, it was confirmed that no reaction occurred between DIPPMPO and the HRP enzyme in the presence of H_2O_2. This confirmed that the HRP enzyme was not able to oxidize the spin trap since the ^{31}P NMR showed a single signal at 22.2 ppm due to the starting unreacted DIPPMPO (Fig. 8a). However in the presence of the mediator HBT, the ^{31}P NMR showed that the DIPPMPO was able to trap and create two different adducts (Fig. 8b). More specifically, the two adducts were observed giving rise to ^{31}P NMR signals at 23.5/23.8 (doublet) and at 17.9 ppm, together with signals due to trace amounts of OH· and HOO· radical adducts at 25.3 and 16.9–17.0 ppm, respectively.[5] This data was interpreted as being the result of adduct formation between the >N—O· species generated from the interaction of

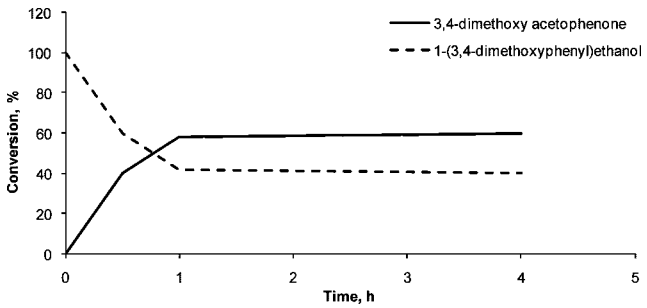


Figure 6. Percent conversion of 1-(3,4-dimethoxyphenyl)ethanol in 3,4-dimethoxy acetophenone as a function of time during the oxidation with horseradish peroxidase in the presence of 1-hydroxybenzotriazole (HBT) as mediator

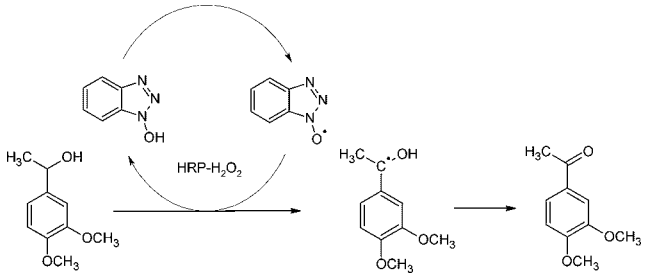


Figure 7. The HRP oxidation reaction of 1-(3,4-dimethoxyphenyl)ethanol mediated by HBT

HBT by HRP with the DIPPMPO.[6] The specific structures of these radical adducts is currently under investigation.

Finally, the oxidation of 1-(3,4-dimethoxyphenyl)ethanol with HRP/HBT in the presence of DIPPMPO was examined. After a reaction period of 4 h the ^{31}P NMR spectra showed the presence of different adducts, related to the HBT radical, as well as a low field adduct at 27.2 ppm (Fig. 8c). The latter signal was interpreted on the basis of our earlier work on photochemical generation of ketyl radical (27.3 ppm) as being such a structure generated from 1-phenylethanol via H-abstraction (Table 2). Moreover, GC–MS analyses of the reaction mixtures showed the presence of the corresponding ketone as being the major oxidation product (Fig. 9). These analyses also showed that after 4 h of treatment, approximately 33% of the ketone was formed. This value was lower with respect to the oxidative conversion in the absence of DIPPMPO (Fig. 6). This can be rationalized on the basis of the possibility that the HBT and ketyl radicals, generated during the reaction, were trapped by the spin trap, leading to lower amounts of the ketone (Fig. 9).

As reported previously, during the oxidation of 2,4,6-tri-*tert*-butyl-phenol, the DIPPMPO showed the ability to trap only the phenoxy radical generated by the HRP/HBT system. The formation of a single adduct was apparent (related to the phenoxy radical at 25.2 ppm), without any signals related to the trapping of the radical due to HBT.[6] In the trapping reaction under investigation (oxidation of 1-(3,4-dimethoxyphenyl)ethanol

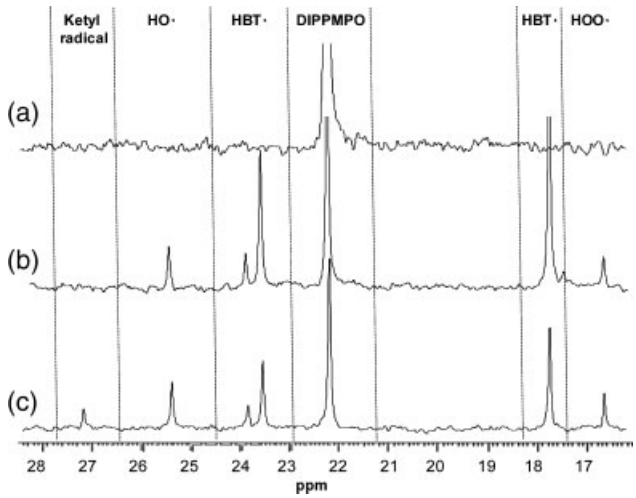


Figure 8. ^{31}P NMR spectra of: (a) DIPPMPO; (b) DIPPMPO and HBT; and (c) DIPPMPO and 1-(3,4-dimethoxyphenyl)ethanol. The oxidation system was horseradish peroxidase (HRP)/H_2O_2. The signal assignments are for the indicated adducr species with DIPPMPO

Table 2. ^{31}P NMR data for spin trapping experiments with DIPPMPO

Substrates	Oxidation system	Chemical shift (ppm)
—	—	22.2
—	HRP-H_2O_2	22.2
—	HRP-HBT-H_2O_2	23.5/17.9
1-(3,4-dimethoxyphenyl)ethanol	Acetophenone + UV Light	27.3
1-(3,4-dimethoxyphenyl)ethanol	HRP-HBT-H_2O_2	27.2/23.5/17.9

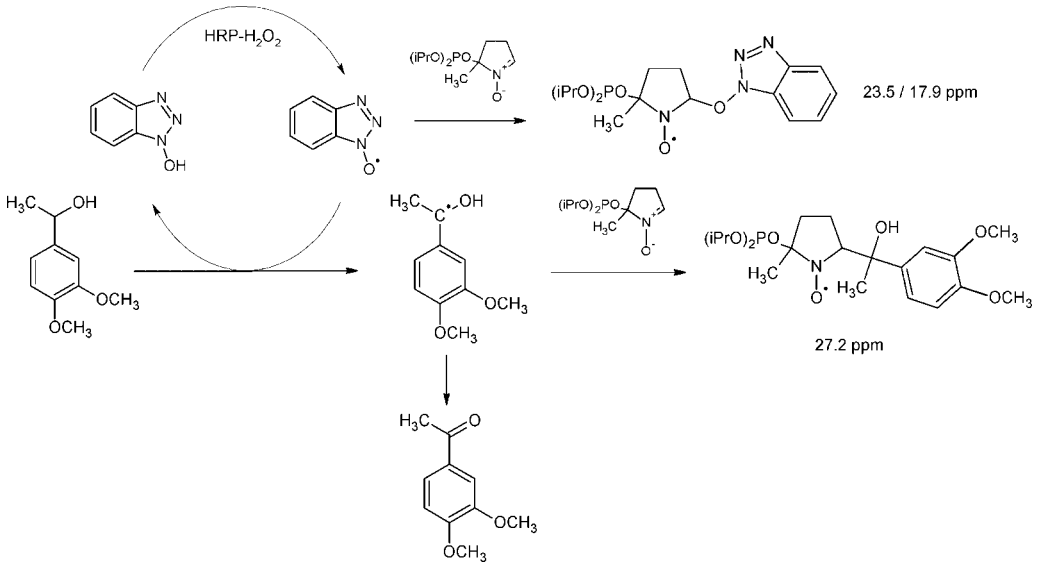


Figure 9. Proposed mechanism of 1-(3,4-dimethoxyphenyl)ethanol oxidation by the HRP/HBT system in the presence of DIPPMPO

performed by HRP/HBT system), the radical adducts related to the HBT were detected at 17.9 and 23.5 ppm together with the signal related to the ketyl radical (27.2 ppm). These results are in agreement with the higher H-abstraction rate reaction performed by HBT on phenolic substrates, than from a given benzylic position. In fact for phenols the normalized second-order rate constant of H-abstraction by the HBT radical has been measured to be 66 M^{-1} s^{-1} (at 25 °C in acetonitrile), while for a non phenolic substrate such as 3,4-dimethoxybenzyl alcohol, the same constant has been measured to be 11 M^{-1} s^{-1}.[25]

Finally, on the basis of the information supplied in this effort, coupled with literature accounts[21] the mechanism of interaction of non-phenolic structures with HRP in the presence of HBT as the mediator is shown in Fig. 9. The ketyl radical adducts formed in subsequent reactions with DIPPMPO are also shown.

More specifically, the HRP enzyme in the presence of hydrogen peroxide catalyzes the oxidation of the mediator HBT to the radical active form, which could be trapped by DIPPMPO, leading at the formation of a mixture of radical adducts (under investigation with ^{31}P NMR signals at 23.5 and 17.9 ppm). The oxidized form of HBT could also abstract a hydrogen atom from the benzylic position of the 1-(3,4-dimethoxyphenyl)ethanol, to form the corresponding ketyl radical. The ketyl radical could be trapped by DIPPMPO and the radical adduct is detected by ^{31}P NMR with a signal at 27.2 ppm. This can be further decayed via a disproportionation reaction to yield 3,4-dimethoxy acetophenone. Based on the accumulated data and the proposed mechanism the HAT route could be the prevalent mechanism in such systems.

CONCLUSIONS

The present set of experiments have shown the power of the DIPPMPO spin trapping system toward understanding the chemistry and the intermediate ketyl radicals involved in the interaction of the non-phenolic compound 1-(3,4-dimethoxyphenyl)ethanol under HRP/HBT oxidative conditions, confirming a hydrogen abstraction reaction mechanism. Overall, the proposed spin trapping system allows ketyl radicals involved in such enzymatic reactions as well as photochemical reactions to be readily and reliably detected. These preliminary results create the foundations for a targeted understanding of the nature, identity, and mechanisms of ketyl radical activity in a variety of chemical processes.

MATERIALS AND METHODS

Materials

DIPPMPO was synthesized according to a modified two step procedure, in which the catalytic amount of the Lewis acid, boron trifluoride diethyl etherate was added to shorten the reaction time of diisopropyl-(2-methyl-1-pyrrolidin-2-yl) phosphonate

from 12 days to 3 days in high yield (96%).[5] It was then oxidized with H_2O_2 using catalytic amounts of Na_2WO_4. The ^{31}P NMR spectra showed a single resonance at 22.2 ppm, in agreement with the literature.[4] The chemical shifts as well as the multiplicities for the proton resonances were: δ_H (400.13 MHz; $CDCl_3$; Me_4Si) 6.822 (1H, q, $J_{H,P}$ 2.8, $J_{H,H}$ 2.8, HC = N), 4.81 (1H, d sept., $J_{H,P}$ 0.6, $J_{H,H}$ 6.3, $OCHMe_2$), 4.72 (1H, d sept., $J_{H,P}$ 1.1, $J_{H,H}$ 6.3, $OCHMe_2$), 2.65–2.81 (2H, m, CH_2), 2.44–2.55 (1H, m, CH_2), 1.92–2.08 (1H, m, CH_2), 1.60 (3H, d, $J_{H,H}$ 14.79, CH_3), 1.306 (3H, dd, $J_{H,P}$ 0.24, $J_{H,H}$ 6.3,CH_3), 1.286 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.282 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.277 (3H, d, $J_{H,H}$ 6.3, CH_3). EI Mass Spectrum *m/z* (%): 263 (M+, 5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21). The spin trap was stored under argon at −78 °C.

The 1-(3,4-dimethoxyphenyl)ethanol was prepared by reacting 3,4-dimethoxy acetophenone with 2 equivalents of $NaBH_4$. The reaction was carried out in MeOH-H_2O (3:1) and heated at reflux for 3 h. The reaction mixture was neutralized with carbon dioxide, and extracted with 1,2-dichloroethane. A quantitative conversion of the 3,4-dimethoxy acetophenone was obtained. EI Mass Spectrum *m/z* (%): 182 (M+, 59), 167 (87), 153 (47), 139 (100), 124 (32), 108 (21), 93 (50), 77 (21), 65 (25).

All chemicals were purchased from Sigma-Aldrich and used as received.

Photochemical generation of free radicals

1-phenyl-ethanol-1-yl radical

A solution of acetophenone (50 mmol L^{-1}) and 1-phenylethanol (50 mmol L^{-1}) in benzene was added to argon purged Pyrex tubes and degassed by a series of freeze-thaw cycles. The sealed tubes were irradiated with a 75 W xenon lamp for different times at room temperature. The reaction was monitored by GC–MS after suitable dilution in hexane. The products of the reaction were the corresponding pinacols in *d*, *l*, and *meso* forms (2,3-diphenyl-2,3-butanol) and were characterized by GC–MS: EI Mass Spectrum *m/z* (%) 242 (M+, 2), 181 (5), 121 (100), 107 (12), 91 (7), 77 (17).

A solution of acetophenone (50 mmol L^{-1}), 1-phenylethanol (50 mmol L^{-1}), and DIPPMPO (10 mmol L^{-1}) in benzene was added to argon purged Pyrex tubes and degassed by a series of freeze-thaw cycles. The sealed tubes were irradiated with a 75 W xenon lamp at variable times at room temperature. The products of reaction were monitored by GC–MS after suitable dilution in hexane and the radical adducts were detected by ^{31}P NMR after dilution in $CDCl_3$, in the presence of trimethylphosphate as an internal standard and chromium acetylacetonate as a relaxation agent. The mass spectrum of the radical adducts and their ^{31}P NMR chemical shifts are reported in the text.

1-(3,4-dimethoxyphenyl)-ethanol-1-yl radical

A solution of acetophenone (50 mmol L^{-1}) and 1-(3,4-dimethoxyphenyl) ethanol (50 mmol L^{-1}) in benzene was added to argon purged Pyrex tubes and degassed by a series of freeze-thaw cycles. The sealed tubes were irradiated with a 75 W Xenon lamp at different times at room temperature. The reaction was monitored by GC–MS after suitable dilution in hexane. The products of reaction were the pinacol of acetophenone and the 3,4-dimethoxy acetophenone. EI Mass Spectrum *m/z* (%): 180 (M+, 100), 165 (41), 137 (10), 122 (9), 107 (7), 94 (5), 79 (11), 77 (12).

A solution of acetophenone (50 mmol L^{-1}), 1-(3,4-dimethoxyphenyl)ethanol (50 mmol L^{-1}), and DIPPMPO (10 mmol L^{-1}) in benzene was added to argon purged Pyrex tubes and degassed by freeze-thaw cycles. The sealed tubes were irradiated with a 75 W xenon lamp for different times a room temperature. The products of reaction were monitored by GC–MS after suitable dilution in hexane and the radical adducts were detected by ^{31}P NMR after dilution in $CDCl_3$, in presence of trimethylphosphate as internal standard and chromium acetylacetonate as relaxation agent. The mass spectrum of the radical adducts and their ^{31}P NMR chemical shifts are reported in the text.

Oxidation of 1-(3,4-dimethoxyphenyl)ethanol

The oxidation was performed in a dimethylformamide/water (1:5) solvent mixture. The 1-(3,4-dimethoxyphenyl)ethanol (0.3 mmol) was dissolved in dimethylformamide/acetate buffer pH 4.5 (0.05 mol L^{-1}, 5 ml, 1:5) in the presence or absence 1-hydroxybenzotriazole (HBT, 0.07 mmol), and treated with horseradish peroxidase (HRP, activity 1000 units/mg, 20 U ml^{-1}). Then water peroxide (H_2O_2, 30%) was added to the reaction mixture (0.3 mmol). During the spin trapping experiment, the DIPPMPO was added (0.1 mmol). The reaction was conducted under argon, at 25 °C with magnetic stirring. For ^{31}P NMR analysis, the samples were diluted in D_2O with trimethylphosphate as internal standard and chromium trichloride as relaxation agent. The aqueous solution was extracted 3 times with ethyl acetate and the organic phase was dried with Na_2SO_4, filtered and the solvent was evaporated with rotavapor. The residual was dissolved in chloroform for GC–MS analyses.

^{31}P NMR spectra

^{31}P NMR spectra were acquired on a Bruker-300 MHz NMR spectrometer (operating at 121.49 MHz). The chemical shifts reported are relative to external orthophosphoric acid (85%). All spectra were acquired with proton decoupling. The total number of scans for all experiments was 256–1024 with an acquisition time of 1.60 s. Trimethylphosphate was used as the internal standard for quantification (20 mmol L^{-1}), and chromium trichloride or chromium acetylacetonate were used as relaxation agent (30–35 mmol L^{-1}), added to the mixture prior to NMR measurement.

Gas chromatography/mass spectrometry (GC–MS)

The analyses were performed by injecting 2 μl of the samples in a Hewlett Packard 5972 mass spectrometer (Electron Impact, EI 70 eV) interfaced to a Hewlett Packard 5890-A gas chromatograph. Chromatographic separation was performed on a DB-5 30 m·0.25 mm fused silica capillary column (J. and W. Scientific Agilent Technologies). Chromatographic conditions: initial temperature 60 °C, 2 min isothermal, 10 °C min^{-1} up to 200 °C, 6 °C min^{-1} up to 280 °C, 20 min isothermal. Carrier gas: He (purity 99.995%), constant flow 1.0 ml min^{-1}.

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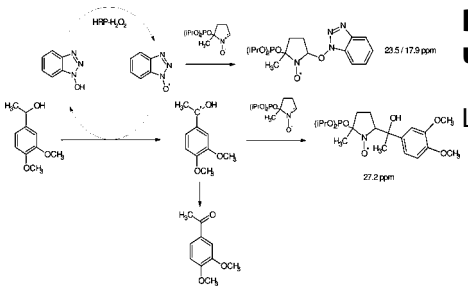
Our efforts have been focused at applying the DIPPMPO system for the trapping of the radicals involved in the photochemistry of acetophenone. The derived knowledge was then applied in elucidating the mechanism of enzymatic oxidation of non-phenolic compounds. Both of these systems are known to have the ketyl radical as the common free radical intermediate.



Detection of ketyl radicals using ^{31}P NMR spin trapping

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