Unusual Double Beckmann Fragmentation Reaction under Physiological Conditions

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S Supporting Information

ABSTRACT: Pyridinium aldoximes, which are best-known as therapeutic antidotes for organophosphorus chemical warfare nerve-agents and pesticides, have been found to markedly detoxify polyhalogenated quinones, which are a class of carcinogenic intermediates and recently identified disinfection byproducts in drinking water. However, the exact chemical mechanism underlying this detoxication remains unclear. Here we demonstrate that pralidoxime can remarkably facilitate the dechlorination/hydroxylation of the highly toxic tetrachloro-1,4-benzoquinone in two-consecutive steps to generate the much less toxic 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone, with rate enhancements of up to 180 000-times. On the contrary, no accelerating effect was noticed with *O*-methylated pralidoxime. The major reaction product from pralidoxime was identified as its corresponding nitrile (2-cyano-1-methylpyridinium chloride). Along with oxygen-18 isotope-labeling studies, a reaction mechanism was proposed in which nucleophilic substitution coupled with an unprecedented double Beckmann fragmentation reaction was responsible for the dramatic enhancement in the detoxification process. This represents the first report of an unusually mild and facile Beckmann-type fragmentation that can occur under normal physiological conditions in two-consecutive steps. The study may have broad biomedical and environmental significance for future investigations of aldoxime therapeutic agents and carcinogenic polyhalogenated quinones.

INTRODUCTION

Polyhalogenated quinones are a group of reactive intermediates which can cause various harmful consequences in vivo, such as acute nephrotoxicity, hepatotoxicity, and carcinogenesis.^{1,2} Pentachlorophenol (PCP) has been extensively used for wood preservation. In addition, PCP was used to prevent snail fever as a snail-killing compound in China and other developing countries. The U.S. EPA has identified that at least 20% of National Priorities List sites are contaminated with PCP, and the International Association for Research on Cancer (IARC) has recently considered PCP as a Group-I environmental carcinogen.^{3,4} During the oxidation and destruction processes for PCP and other polychlorinated aromatic compounds via different chemical and enzymatic systems, tetrachloro-1,4-benzoquinone (TCBQ) has been generated as a reactive and transient intermediate or final product.^{3,5–8} Recently, TCBQ was detected as one of many disinfection quinoid byproducts in chlorination of drinking and swimming pool waters.^{9,10} TCBQ was often used as a powerful oxidizing or dehydrating agent in organic synthesis.

Pyridinium aldoximes have been used as acetylcholinesterase activators since the 1950s for the medical treatment of poisoning with organophosphorus nerve agents and pesticides. More than 300 000 fatalities every year are due to organophosphorus pesticide poisoning worldwide, resulting in a serious public health problem. In addition, organophosphorus nerve agents remain a persistent threat to the public due to terrorist attacks and armed conflicts. Aldoximes possess high nucleophilicity that can displace the phosphoryl group from the catalytic serine to restore catalytic activity of the enzyme.^{11–16}

In recent years, researchers have devoted enormous efforts to the synthesis and development of new compounds as potential antidotes. Only four pyridinium aldoximes (i.e., pralidoxime or 2-PAM, trimedoxime, obidoxime, and HI-6), are in clinical use.^{13,17,18} Pralidoxime and obidoxime are the two currently clinically used drugs, and trimedoxime (TMB-4) and HI-6 have been tested in humans and are available for human use in some countries.¹²

Received: August 19, 2017

Published: November 2, 2017

Figure 1. Dechlorination and dihydroxylation of TCNQ to DDBQ was dramatically accelerated by 2-PAM, other pyridinium aldoximes and oximes (a, b). 2-PAM: pralidoxime; TMB-4: trimesoxime bromide; obdioxime: obdioxime chloride; HI-6: asoxime chloride; 2-*syn*-PAM: *syn*-2-pyridinealdoxime; APM: acetophenone oxime; 2-MPM: methyl 2-pyridyl ketoxime; BAM: benzaldehydeoxime; 2-Me-BAM: 2-methyl-benzaldehydeoxime. TCNQ, 0.05 mM; oximes, 5.0 mM. Chemical structures of the aldoximes and oximes used in this study (c). 2-PAM, but not *O*-methyl-2-PAM, enhanced the dechlorination and hydroxylation of TCNQ and TrCBQ-OH to DDBQ (d–g). 2-PAM enhanced hydrolysis of TCNQ (d) and TrCBQ-OH (e) in a concentration-dependent manner. (f) 2-PAM accelerated hydrolysis of TCNQ in a pH-dependent manner; TCNQ, 0.05 mM; 2-PAM, 0.50 mM. (g) *O*-Methyl-2-PAM did not accelerate hydrolysis of TCNQ even when the pH value reached 10. (d–g) The concentration of 2-PAM is indicated, TCNQ, 0.05 mM; TrCBQ-OH, 0.05 mM. The experiments were conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4) at 25 °C. DDBQ formation was quantified for 36 min by HPLC analysis.

During our recent studies on the molecular mechanism of the protection of pyridinium aldoximes against the toxicity of organophosphorus and haloquinoid pesticides, we unexpectedly found that pyridinium aldoximes can efficiently detoxify TCNQ

and other polyhalogenated quinoid compounds, the carcinogenic metabolites of PCP, and other persistent organic pollutants. Nevertheless, the detoxification mechanism remains not clear. We extensively examined the molecular mechanism

Figure 2. 2-Cyano-1-methylpyridinium chloride (2-CMP) was identified as the major product of 2-PAM after reacting with TCBQ by HPLC/MS: (a) Chromatogram of 2-PAM, TCBQ/2-PAM (TCBQ: 2-PAM = 1:2, TCBQ: 0.05 mM, 36 min), and 2-CMP (authentic standard). The MS spectra of 2-PAM (b) and TCBQ/2-PAM (c) (2-PAM: TCBQ = 1:10, 2-PAM: 0.05 mM, 40 s), MS/MS spectrum of 2-CMP (d). The synthetic 2-CMP has the same retention time (a), molecular ion peak and fragment ion peak (d) as the final major reaction product from 2-PAM. The reactions of (a), (b), (c), and (d) were conducted in Chelex-treated $\text{CH}_3\text{COONH}_4$ buffer (pH 7.4, 100 mM) at 25 °C. The time and dose-dependent formation of 2-CMP from the reaction between 2-PAM and TCBQ (e, f): (e) TCBQ, 0.05 mM; 2-PAM, 0.5 mM. (f) 2-PAM, 0.05 mM, 36 min (2-PAM + TCBQ); TCBQ, 0.05 mM, 36 min (TCBQ + 2-PAM). The reactions of (e) and (f) were conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4) at 25 °C.

of TCBQ and pyridinium aldoximes recently, using HPLC, HPLC/MS, NMR, and oxygen-18 isotope-labeling methods. Unexpectedly, this effective detoxication was found to be an unusually mild and facile Beckmann-type double fragmentation reaction.

RESULTS AND DISCUSSION

Pyridinium Aldoximes Accelerate the Hydrolysis of TCBQ Dramatically, and 2-Pyridinium Aldoxime Was the Most Effective One. TCBQ was first hydrolyzed to trichlorohydroxy-1,4-benzoquinone (TrCBQ-OH), and then very slowly hydrolyzed to the final 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone (chloranilic acid, or DDBQ) at neutral pH.¹⁹ In this study, the four pyridinium aldoximes can markedly accelerate this hydrolysis process to DDBQ. Interestingly, we found that the two 2-pyridinium aldoximes (2-PAM and HI-6)

are more effective than the two 4-pyridinium aldoximes (obidoxime and TMB-4) (Figure 1a), and among the four studied pyridinium aldoximes antidotes, 2-PAM was determined to be the most effective one. Next, we compared 2-PAM with five other oximes that have very similar structures, including *syn*-2-pyridinealdoxime (2-*syn*-PAM), benzaldehydeoxime (BAM), 2-methyl-benzaldehydeoxime (2-Me-BAM), acetophenone oxime (APM), and methyl 2-pyridyl ketoxime (2-MPM), and 2-PAM was also determined to be the most effective at accelerating this hydrolysis process. Interestingly, 2-PAM was much more efficient than 2-*syn*-PAM, indicating that the methylation of the nitrogen in pyridine may increase the nucleophilicity of aldoximes (i.e., the $\text{p}K_{\text{a}}$ values of 2-PAM and 2-*syn*-PAM are 7.68 and 8.41, respectively)^{14,20} (Figure 1b).

On the basis of these results and that 2-PAM, which is the first and most clinically used aldoxime since 1955, possesses the

simplest chemical structure among the four pyridinium aldoxime antidotes, 2-PAM was chosen as a model pyridinium aldoxime for further mechanistic investigations.

In our previous research, we observed that TCBQ can be hydrolyzed to the initial transient intermediate TrCBQ–OH and final product DDBQ.^{21–24} The TCBQ/2-PAM system was determined by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS), and the same results were observed. The generation of TrCBQ–OH and DDBQ was dependent on the molar ratios of 2-PAM/TCBQ. When the molar ratios are ≤ 1 , TCBQ was primarily converted to TrCBQ–OH. In the presence of excess 2-PAM (the molar ratios > 1), TCBQ was initially converted to TrCBQ–OH, and then to the final product DDBQ (for details, see Figure S1a,c–f of the Supporting Information, SI). To determine whether TrCBQ–OH can be directly converted into DDBQ by 2-PAM, TrCBQ–OH was synthesized as previously described, and this transformation was confirmed (see Figure S1b,g).

The hydrolysis rate also depends on the molar ratios of 2-PAM/TCBQ and 2-PAM/TrCBQ–OH (Figure 1d,e). The rate increased as the molar ratio increased. For instance, the half time of TCBQ (0.05 mM) to DDBQ was observed to be 10, 2.39, 0.5, 0.25, and less than 0.17 min, respectively (pH 7.4), in the presence of 0.2, 0.5, 1.0, 2.5, and 5.0 mM 2-PAM. As the half-time is 21-days for TCBQ hydrolysis to DDBQ,¹⁹ our data suggest that 2-PAM can enhance TCBQ hydrolysis to DDBQ up to 180 000-fold (TCBQ, 0.05 mM; 2-PAM, 5.0 mM).

It is important to note that the ratios of DDBQ were not 100%, even at a high 2-PAM concentration and pH, which may due to a side reaction between TCBQ (or TrCBQ–OH) and 2-PAM. This process will require additional study in the future.

When TCBQ was replaced by other tetrahalogenated quinones, including tetrabromo- and tetrafluoro-1,4-benzoquinones (TBrBQ, TFBQ) and tetrachloro-1,2-hydroquinone (TCHQ), as well as tetrachloro-1,2-benzoquinone (O-TCBQ), similar accelerated hydrolysis effects were observed (see Figures S2–S5). As previously mentioned (Figure 1a,b), when 2-PAM was replaced with other aldoximes, such as trimedoxime, obidoxime, HI-6, 2-syn-PAM, BAM, and 2-Me-BAM, analogous results were noticed. Our results demonstrate that this reaction is common among all aldoximes and tetrahalogenated quinoid compounds.

The Free Pralidoxime Anion Is Crucial for the Enhancement of TCBQ Conversion to DDBQ. The pH of the buffer is crucial for the rate of 2-PAM-mediated TCBQ conversion to DDBQ. Hydrolysis was not observed at a pH of ≤ 4 . However, as the pH increased, the rate of hydrolysis gradually increased (Figure 1f). On the basis of these results, we assume that the free pralidoxime anion may be the reactive form of 2-PAM ($pK_a = 7.68$). In this case, we expect that the TCBQ hydrolysis will not be accelerated when the free anionic oxygen is blocked. To further confirm this hypothesis, O-methyl-2-PAM ($Py^+(CH_3)-CH=N-O-CH_3$) was synthesized.²⁵ As expected, O-methyl-2-PAM did not accelerate TCBQ hydrolysis, even when the pH value reached 10 (Figure 1g). These findings demonstrated that the free pralidoxime anion is required for substantial enhancement of TCBQ conversion to DDBQ.

The Major Reaction Product of 2-PAM Was Identified as Its Corresponding Nitrile (i.e., 2-Cyano-1-methylpyridinium Chloride). Since the rate of TCBQ hydrolysis with 2-PAM is greatly accelerated compared with its spontaneous

hydrolysis, this process may be catalyzed by 2-PAM. However, during the interaction with TCBQ, 2-PAM was consumed, and a new product with m/z at 119 was simultaneously generated (Figure 2b,c). This result demonstrated that 2-PAM participate in the reaction rather than act as a catalyst.

To further explore the underlying molecular mechanism between 2-PAM and TCBQ, we isolated and purified the major product.^{26,27} Interestingly, 2-cyano-1-methylpyridinium chloride (2-CMP, eq 1) was found to be the main reaction product of 2-PAM, with a yield of up to 97.4% (Figure 2a,d–f). Similar results were also observed for TrCBQ–OH/2-PAM (see Figure S6).

This raised a new question: what pathway would result in 2-CMP being generated from 2-PAM? After consulting the literature, we determined that the classical Beckmann fragmentation was responsible for this reaction. Beckmann fragmentation is the reaction that involves the transformation of an oxime ($R_1-(R_2)C=N-OH$) into the corresponding nitrile ($R_1-C\equiv N$) via a nitrilium cation and/or carbocation intermediate.^{28–32} The conversion of the *N*-hydroxy group of the oxime into a better leaving group (via protonation, etherification, or esterification), is necessary to initiate this reaction.^{30,32} Therefore, the activation of the oxygen atom of oxime by an activating agent (usually by strong electrophiles such as diethyl chlorophosphate and chlorosulfonic acid) is very important for the Beckmann fragmentation to occur (A is the activator by losing a halogen or other active group, and OA is an acceptable leaving group, eq 2):

It is important to note that if TCBQ was replaced by other tetrahaloquinones, like TBrBQ, TFBQ, TCHQ, and O-TCBQ, 2-CMP was also identified as the major reaction product of 2-PAM, and the yield was determined to be 97.1%, 96.8%, 74.2%, and 100%, respectively (see Figure S7); when 2-PAM was replaced by other pyridinium aldoximes, the corresponding nitrile products were also identified by HPLC-ESI-MS when they reacted with TCBQ (see Figure S8). On the basis of these experimental results, an analogous, but previously unknown Beckmann-type fragmentation pathway may occur between pyridinium aldoximes and tetrahalogenated quinoid compounds.

Molecular Mechanism of 2-PAM-Mediated Enhancement of TCBQ Hydrolysis: Nucleophilic Substitution Coupled with an Unprecedented Double Beckmann Fragmentation. On the basis of our results, and pralidoxime anion being an effective α -nucleophile, we proposed a novel mechanism for 2-PAM-enhanced hydrolysis of TCBQ (Scheme 1): A nucleophilic attack of the pralidoxime anion ($Py^+(CH_3)-$

Scheme 1. Proposed Molecular Mechanism of 2-PAM-Mediated Enhancement of TCBQ Hydrolysis: Nucleophilic Attack Coupled with an Unusually Facile Double Beckmann Fragmentation under Physiological Conditions

CH=N-O⁻) on TCBQ forms an unstable transient intermediate $\text{Py}^+(\text{CH}_3)\text{-CH=N-O-trichloro-1,4-benzoquinone}$, which undergoes a Beckmann-type fragmentation. This fragmentation leads to the formation of the monohydroxylation intermediate TrCBQ-O^- and the cationic nitrilium ion $\text{Py}^+(\text{CH}_3)\text{-C}\equiv\text{NH}^+$, which is in equilibrium with carbocation $\text{Py}^+(\text{CH}_3)\text{-C}^+=\text{NH}$. Then, a proton loss results in the formation of the nitrile 2-CMP. In the presence of excess 2-PAM, TrCBQ-O^- may further react with 2-PAM to form analogous reaction intermediates, which can further convert to dihydroxylation final product DDBQ and another molecule of 2-CMP via a second-step Beckmann-type fragmentation reaction.

If this proposed double Beckmann-type fragmentation mechanism is correct, the source and origin of the oxygen atom in TrCBQ-OH and DDBQ can be traced from the hydroxyl group of 2-PAM not from the reaction buffer.²⁴ To further confirm the proposed mechanism, TCBQ (or TrCBQ-OH) was reacted with 2-PAM in $\text{CH}_3\text{COONH}_4$ buffer using oxygen-18-labeled H_2O ($^{18}\text{O}\text{-H}_2\text{O}$) as the solvent. If H_2O is the source and origin of the oxygen atom in either TrCBQ-OH and DDBQ, then the mass spectra of TrCBQ-OH and DDBQ should demonstrate the shift of 2 and 4 mass units compared to the unlabeled compounds. However, the mass

Figure 3. Mass spectral analysis of the dechlorination and hydroxylation products for TCBQ in buffer solution prepared with oxygen-18-labeled H_2O . The MS spectra of 2-PAM (a), DDBQ (b), TCBQ (c), TCBQ:2-PAM = 1:50 (d), TrCBQ-OH (e), and TrCBQ-OH :2-PAM = 1:20 (f) in Chelex-treated $\text{CH}_3\text{COONH}_4$ (pH 7.4, 100 mM) using oxygen-18-labeled H_2O ($^{18}\text{O}\text{-H}_2\text{O}$, 97%) as solution. TCBQ, 0.1 mM; TrCBQ-OH , 0.1 mM; 2-PAM, 2 mM, or 5 mM, 25 °C.

Figure 4. (a) Inhibition by 2-PAM of hydroxyl radicals produced by TCNQ/H₂O₂ as measured by fluorescent method using TPA as a probe. TCNQ: 0.1 mM, H₂O₂: 1 mM; TPA: 20 mM. (b) Protection by 2-PAM against TCNQ/H₂O₂-induced DNA double-strand breaks. The plasmid pBR322 DNA (5 μg/mL) was incubated at 37 °C for 1 h in Chelex-treated phosphate buffer (0.1 M, pH 7.4), TCNQ: 0.1 mM, H₂O₂: 1 mM.

spectra of TrCBQ–OH or DDBQ did not shift (Figure 3). The isotope-labeling results provided more convincing experimental evidence and further confirmed the proposed Beckmann-type fragmentation mechanism (Scheme 1).

Why this Beckmann Fragmentation Reaction Is Unique? The activation of the oxygen atom of aldoximes is crucial for Beckmann fragmentation. In previous studies, the Beckmann fragmentation is typically achieved by using O-activated aldoxime derivatives (i.e., aldoxime ethers or esters). Other activation methods of free aldoximes often employ various activating agents, such as concentrated H₂SO₄, diethyl chlorophosphate, chlorosulfonic acid, either via protonation, etherification, or esterification (see Table S1).^{32,33} To the best of our knowledge, this study is the first report of the use of TCNQ and other tetrahalogenated quinones as the special activation agents for free aldoximes, affording unstable O-trihaloquinonated aldoximes that spontaneously undergo two consecutive Beckmann-type fragmentations.

Numerous studies have demonstrated that Beckmann-type fragmentation reactions occur in only one step under strong acidic conditions by heating to a required temperature in organic solvents.^{30,32} In the current study, the reaction between 2-PAM and TCNQ occurred via a two-step or double Beckmann fragmentation reaction, which took place at room temperature and under neutral or even alkaline pH in aqueous buffer solutions (i.e., under normal physiological conditions). This reaction may be due to the unusually rapid and facile fragmentation of the proposed reaction intermediate (i.e., O-trichloroquinonated 2-PAM or O-trichloroquinonated aldoxime ether). A recent study reported that the stability of haloquinone-activated benzohydroxamate intermediates was well correlated with the pK_a value of the corresponding conjugate acids.³⁴ We speculated that this correlation may also apply to O-trichloroquinonated aldoxime ethers. Both

TrCBQ–OH (pK_a: 1.10) and DDBQ (pK_{a1}: 0.58; pK_{a2}: 2.72) possess strong acidity, and are the conjugate acids of the anionic leaving groups in the proposed mechanism (Scheme 1). Therefore, the fragmentation rate of the proposed reaction intermediates should be very fast. Once formed, these intermediates should fragment extremely rapidly under physiological conditions, which may result in 2-PAM and other aldoximes substantially enhancing the hydrolysis of TCNQ (or TrCBQ–OH) to DDBQ at room temperature and under neutral pH. This explanation may also be the reason why the proposed reaction intermediates cannot be isolated and identified in the current study, unlike other O-substituted oximes.

Pyridinium Aldoximes Provide Marked Protection against TCNQ-Mediated DNA Damage. In our previous studies, TCNQ produced highly reactive hydroxyl radicals (•OH) in the presence of H₂O₂ through a metal-independent mechanism, which can lead to DNA strand breakage^{3,21} and base oxidations.^{35,36} The transformation of covalently closed circular double-stranded supercoiled DNA (form I) to relaxed open circle (form II) and linear forms (form III) has been widely employed to investigate DNA strand breakage using a gel electrophoresis method.³⁷ We determined that 2-PAM can provide strong protection against TCNQ/H₂O₂-induced DNA double-strand breaks in isolated DNA (Figure 4b). The protection by 2-PAM was due to its efficient inhibition of •OH formation by TCNQ/H₂O₂, and was measured using fluorescent methods and terephthalic acid as a trapping agents (Figure 4a).

Pyridinium Aldoximes Provide Marked Protection against TCNQ-Induced Cytotoxicity in Two Model Cell-Culture Systems. Then the study was extended from isolated DNA to bacterial and mammalian cells using *P. phosphoreum* and human hepatoma (HepG2) as two model cell culture

Figure 5. Protection by 2-PAM against TCBQ-induced toxicity in *P. phosphoreum* and HepG2 cells. (a) The *P. phosphoreum* experiment was performed at 20 °C for 15 min in Chelex-treated sodium phosphate buffer (100 mM, pH 7.4, 3% NaCl); TCBQ: 0.01 mM. (b) The HepG2 experiment was conducted via incubation of cells in RPMI 1640 medium at 37 °C for 2 h; TCBQ: 0.008 mM. (c) The IC₅₀ values of TCBQ, TrCBQ-OH, DDBQ, 2-CMP, and 2-PAM in *P. phosphoreum* and HepG2 cells. Each point was repeated twice, and the SD was less than 5%.

systems. The *P. phosphoreum* toxicity assay, which is commonly known as the Microtox assay,³⁸ is a conventional method for evaluating chemicals' potential acute aquatic toxicity, especially environmental samples and products generated from waste treatment or remediation processes. The luminescence of *P. phosphoreum* is directly related to respiratory activity, and the intensity of the luminescence is a good indicator for the metabolic activity. Therefore, the compound's general cytotoxicity can be determined.¹⁹

Using this model system, the protection of 2-PAM for TCBQ-induced toxicity was remarkable and dose-independent. A 2-PAM concentration of 1.0 mM protected against TCBQ-induced toxicity by 67.2% (Figure 5a). The IC₅₀ values of TCBQ, TrCBQ-OH, DDBQ, 2-PAM, and 2-CMP were measured by *P. phosphoreum* (Figure 5c). On the basis of these results, DDBQ is much less toxic than TCBQ and TrCBQ-OH (The IC₅₀ values for DDBQ, TCBQ, and TrCBQ-OH were 676.9 μM, 2.6 μM, and 19.5 μM, respectively).

Since halogenated quinones and their parent phenols have been shown to cause hepatotoxicity,^{1,2,35} HepG2 cells were chosen as a model human liver cell culture model for further study. The experiment was conducted by pretreating the cells with 2-PAM followed by treatment with TCBQ, and the cell survival was measured using a MTT assay.³⁹ Analogous protective effects were observed with the HepG2 cells (Figure 5b). The IC₅₀ values for TCBQ, TrCBQ-OH, and DDBQ were determined to be 2.0 μM, 94.0 μM, and 613.9 μM, respectively (Figure 5c). Interestingly, the IC₅₀ values for DDBQ were much higher than those of TCBQ in both *P. phosphoreum* and HepG2 cells, which suggests that the conversion of TCBQ to DDBQ by 2-PAM is an efficient detoxification process in the two model cell culture systems. Similar protective effects were also observed for other pyridinium aldoximes (i.e., HI-6, TMB-4, and obidoxime) (see Figure S9).

Pyridinium aldoximes have been widely employed as antidotes to treat organophosphorus-based agents intoxications for more than 60 years. On the basis of these experimental results, we propose that pyridinium aldoximes may also be

employed as potential antidotes for detoxifying carcinogenic polyhalogenated quinones in the future.

CONCLUSIONS

On the basis of our results, we can see that this unusual Beckmann-type fragmentation reaction mechanism is a universal mechanism for all haloquinoid and pyridinium aldoxime compounds, not limited to TCBQ and 2-PAM. Therefore, this research represents broad environmental and biomedical significance, because a great deal of widely used polyhalogenated aromatic compounds can be dehalogenated to form the corresponding quinones.^{1-3,5-7,40-43} Polychloroquinones have also been detected in discharges from paper and pulp industries,²² and more importantly, 12 haloquinones have been newly identified as a class of carcinogenic disinfection byproducts, which are potential bladder carcinogens.⁸ These halogenated quinones could induce stronger cytotoxicity and/or stronger developmental toxicity than most of the other regulated disinfection byproducts.¹⁰ Cellular mechanistic studies have demonstrated that haloquinones can produce reactive oxygen species (ROS), deplete cellular glutathione levels, and influence cellular antioxidant enzymes, which can induce oxidative stress and oxidatively damage cellular DNA, proteins, and lipids.^{1,3,10,21} ROS generation along with activation of MAPK signaling could represent essential mechanisms that explain how PCP triggers tumor promotion.⁴⁴ Our recent studies have also demonstrated that polyhaloquinones along with H₂O₂ and/or organic hydroperoxides can effectively cause various DNA damages, oxidize 5mC to form methyl oxidation products,^{35,45} and induce lipid peroxidation.⁴⁶ Therefore, these polyhaloquinones are potential carcinogens, and it is critically important to detoxify them under mild conditions.

In fact, four pyridinium aldoximes (pralidoxime, trimefoxime, obidoxime, and HI-6) have been employed into clinical treating the poisonings by organophosphorus pesticides or nerve-agents.^{12,13} The good activity of pyridinium aldoximes is because of the strong binding of the positively charged pyridinium to the enzyme active site as well as the proper

orientation of oxime group for replacement of the phosphyl moiety. As demonstrated in the current study, these compounds are also effective for detoxification of polyhaloquinones, which are a class of carcinogenic intermediates and recently identified disinfection byproducts. Interestingly, pyridinium aldoximes react directly with organophosphorus chemical warfare agents, or activate the inhibited enzyme by forming phosphyloximes, which decompose to produce phosphilic acid and the corresponding nitriles in vitro,^{12,16} via a potentially analogous Beckmann-type fragmentation reactions. In addition, pyridinium aldoximes are beneficial in carbamate pesticides poisoning and can inhibit the cholinergic activity. The detoxification courses are similar to poisoning by organophosphorus agents.^{47,48} Therefore, this unusual Beckmann-type fragmentation may be a universal, but previously unrecognized new detoxification molecular mechanism for the currently clinically used pyridinium aldoxime therapeutic agents. Our results represent broad environmental and biomedical significance for future investigation of polyhalogenated aromatic pollutants and pyridinium aldoximes, which are two important classes of compounds that have attracted the attention of both the general public and academic researchers. Additional study is required to determine whether pyridinium aldoximes can be effectively and safely used to prevent or treat various human diseases, for example, bladder or liver cancer related to haloquinones.

EXPERIMENTAL SECTION

Chemicals. Tetrachloro-1,4-benzoquinone (TCBQ) was purchased from Fluka. Pyridine-2-aldoxime methochloride (2-PAM), chloranilic acid, and trimedoxime bromide (TMB-4) were purchased from Sigma-Aldrich. Asoxime chloride (HI-6) and obidoxime chloride were purchased from Toronto Research Chemicals. The oxygen-18-enriched H₂O (97%, [¹⁸O]-H₂O) was purchased from Aladdin. Trichlorohydroxy-1,4-benzoquinone (TrCBQ-OH), 2-cyano-1-methylpyridinium iodine (2-CMP), and *O*-methyl-2-PAM were synthesized according to the previously published methods.^{21,25,26}

Quantification of DDBQ and 2-CMP. DDBQ. DDBQ was measured according to the previously published method.¹⁹

2-CMP. The quantification of 2-CMP employed the same equipment and reaction buffer as DDBQ. However, the reaction was monitored at 269 nm using a 3.9 × 300 mm 10-μm μPorasil column (Waters Associates).²⁷ The mobile phase consisted of water:acetonitrile (10:90), in which the aqueous component contained 8.36 mM tetraethylammonium chloride dissolved in 3/1000 aqueous acetic acid solution, and the pH was 2.9. The flow rate was 1.0 mL/min.

Fluorescent Method for Hydroxyl Radical Detection. Terephthalic acid (TPA, nonfluorescent) provides a single product, 2-hydroxyl-TPA (fluorescent) in reactions with hydroxyl radicals. First, 200 mM TPA was dissolved in a 500 mM NaOH solution. Next, TPA was added to phosphate buffer (100 mM, pH 7.4). Then, 2-PAM, TCBQ, and H₂O₂ were added to the buffer in the following order: TCBQ, 0.1 mM, H₂O₂, 20 mM, TPA, 20 mM. The fluorescence intensity of the reaction mixture (1 mL) was measured by excitation at 310 nm and emission at 425 nm.

The *Photobacterium phosphoreum* Toxicity Assay. *P. phosphoreum* were grown in the dark at 18 °C in liquid medium, and the cultures were harvested when an absorbance of 0.6 at 600 nm was reached. The liquid medium was composed of 1 g/L KH₂PO₄, 5 g/L Na₂HPO₄, 30 g/L NaCl, 3 mL/L glycerol, 5 g/L yeast extract, and 5 g/L tryptone and adjusted to a pH of 6.5. When the *P. phosphoreum* reached logarithmic phase, it was diluted in Chelex-treated sodium phosphate buffer and 3% NaCl (0.1 M, pH 7.4) in a 2:50 ratio. The experiment was conducted at 18 °C in a 200 μL *P. phosphoreum* solution by treating 2-PAM with TCBQ for 15 min (TCBQ: 0.01

mM). Then, the luminescence intensity was measured at 475 nm using a Thermo Scientific Varioskan Flash.

2-Cyano-*N*-methylpyridinium Iodine (2-CMP). The ¹H NMR and ¹³C NMR spectra were recorded on a Bruker ARX 400 spectrometer using D₂O as solvent. ¹H NMR δ = 4.72 (s, 3H), 8.49 (t, 1H), 8.75 (d, 1H), 8.87 (t, 1H), 9.28 (d, 1H); ¹³C NMR δ = 48.9 (CH₃), 127.5 (CN), 110.6, 132.0, 134.3, 146.8, 149.5 (PyC). The synthetic 2-CMP has the same retention time, molecular ion peak, and MS/MS fragment as the product of TCBQ/2-PAM (TCBQ, 0.2 mM; 2-PAM, 0.1 mM; pH 7.4, 25 °C) (Figure S10).

***O*-Methyl Pralidoxime (*O*-Methyl-2-PAM).** The ¹H NMR and ¹³C NMR spectra were recorded on a Bruker ARX 400 spectrometer using D₂O as the solvent. ¹H NMR δ = 4.24 (s, 3H), 4.48 (s, 3H), 8.09 (t, 1H), 8.50 (d, 1H), 8.60 (t, 1H), 8.77 (s, 1H), 8.86 (d, 1H); ¹³C NMR δ = 46.8, 63.9, 126.5, 128.0, 141.5, 145.6, 146.6, 146.7 (Figure S11).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b02106.

Some MS and HPLC data, the protection by other pyridinium aldoximes against TCBQ-induced toxicity in HepG2, the reaction conditions to convert aldoximes to corresponding nitriles, and the relevant ¹H and ¹³C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by CAS grant (XDB01020300); NSFC Grants (21577149, 21477139, 21237005, 21621064, 21321004, and 21407163); NIH Grants ES11497, RR01008, and ES00210.

REFERENCES

- (1) Song, Y.; Wagner, B. A.; Witmer, J. R.; Lehmler, H.-J.; Buettner, G. R. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 9725.
- (2) Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J. *Chem. Res. Toxicol.* **2000**, *13*, 135.
- (3) Zhu, B.-Z.; Shan, G.-Q. *Chem. Res. Toxicol.* **2009**, *22*, 969.
- (4) Guyton, K. Z.; Loomis, D.; Grosse, Y.; El Ghissassi, F.; Bouvard, V.; Benbrahim-Tallaa, L.; Guha, N.; Mattock, H.; Straif, K. *Lancet Oncol.* **2016**, *17*, 1637.
- (5) Gupta, S. S.; Stadler, M.; Noser, C. A.; Ghosh, A.; Steinhoff, B.; Lenoir, D.; Horwitz, C. P.; Schramm, K.-W.; Collins, T. J. *Science* **2002**, *296*, 326.
- (6) Meunier, B. *Science* **2002**, *296*, 270.
- (7) Sorokin, A.; Meunier, B.; Séris, J.-L. *Science* **1995**, *268*, 1163.
- (8) Zhao, Y.; Qin, F.; Boyd, J. M.; Anichina, J.; Li, X.-F. *Anal. Chem.* **2010**, *82*, 4599.
- (9) Huang, R.; Wang, W.; Qian, Y.; Boyd, J. M.; Zhao, Y.; Li, X.-F. *Anal. Chem.* **2013**, *85*, 4520.
- (10) Li, J.; Wang, W.; Moe, B.; Wang, H.; Li, X.-F. *Chem. Res. Toxicol.* **2015**, *28*, 306.
- (11) Kiderlen, D.; Eyer, P.; Worek, F. *Biochem. Pharmacol.* **2005**, *69*, 1853.

- (12) Eyer, P. A.; Worek, F. In *Chemical Warfare Agents: Toxicology and Treatment*, 2nd ed.; Marrs, T. C., Maynard, R. L., Sidell, F. R., Eds.; Wiley: New York, 2007; p 305.
- (13) Jokanovic, M.; Prostran, M. *Curr. Med. Chem.* **2009**, *16*, 2177.
- (14) Kalisiak, J.; Ralph, E. C.; Zhang, J.; Cashman, J. R. *J. Med. Chem.* **2011**, *54*, 3319.
- (15) Mercey, G.; Verdet, T.; Renou, J.; Kliachyna, M.; Baati, R.; Nachon, F.; Jean, L.; Renard, P.-Y. *Acc. Chem. Res.* **2012**, *45*, 756.
- (16) Lee, J. Y.; Lee, Y. H.; Byun, Y. G. *Phosphorus, Sulfur Silicon Relat. Elem.* **2012**, *187*, 641.
- (17) Sharma, R.; Gupta, B.; Sahu, A. K.; Acharya, J.; Satnami, M. L.; Ghosh, K. K. *Chem.-Biol. Interact.* **2016**, *259*, 85.
- (18) Wei, Z.; Liu, Y.-q.; Wang, S.-z.; Yao, L.; Nie, H.-f.; Wang, Y.-a.; Liu, X.-Y.; Zheng, Z.-b.; Li, S. *Bioorg. Med. Chem.* **2017**, *25*, 4497.
- (19) Sarr, D. H.; Kazunga, C.; Charles, M. J.; Pavlovich, J. G.; Aitken, M. D. *Environ. Sci. Technol.* **1995**, *29*, 2735.
- (20) Dewangan, H. K.; Nagwanshi, R.; Ghosh, K. K.; Satnami, M. L. *Catal. Lett.* **2017**, *147*, 602.
- (21) Zhu, B. Z.; Kalyanaraman, B.; Jiang, G. B. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 17575.
- (22) Zhu, B.-Z.; Mao, L.; Huang, C.-H.; Qin, H.; Fan, R.-M.; Kalyanaraman, B.; Zhu, J.-G. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 16046.
- (23) Li, F.; Huang, C.-H.; Xie, L.-N.; Qu, N.; Shao, J.; Shao, B.; Zhu, B.-Z. *Sci. Rep.* **2016**, *6*, 39207.
- (24) Zhu, B.-Z.; Zhu, J.-G.; Mao, L.; Kalyanaraman, B.; Shan, G.-Q. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20686.
- (25) Lees, G.; Holmes, F.; Underhill, A.; Powell, D. J. *Chem. Soc. A* **1971**, 337.
- (26) Kammer, M. N.; Koplitz, L. V.; Mague, J. T. *Acta Crystallogr., Sect. E: Struct. Rep. Online* **2013**, *69*, o1281.
- (27) Schroeder, A. C.; Digiovanni, J. H.; Von Bredow, J.; Heiffer, M. H. *J. Pharm. Sci.* **1989**, *78*, 132.
- (28) Grob, C. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 535.
- (29) Gawley, R. E. In *Organic Reactions*; Kende, A. S., Paquette, L. A., Overman, L. E., Denmark, S. E., Eds.; Wiley: New York, 1988; Vol. 35, p 3.
- (30) Pereira, M. M. A.; Santos, P. P. In *The Chemistry of Hydroxylamines, Oximes and Hydroxamic Acids*; Rappoport, Z., Liebman, J. F., Eds.; Wiley: New York, 2009; p 386.
- (31) Drahl, M. A.; Manpadi, M.; Williams, L. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 11222.
- (32) Chandrasekhar, S. In *Comprehensive Organic Synthesis II*; Knochel, P., Molander, G. A., Eds.; Elsevier: Amsterdam, 2014; Vol. 7, p 770.
- (33) De Luca, L.; Giacomelli, G.; Porcheddu, A. *J. Org. Chem.* **2002**, *67*, 6272.
- (34) Shan, G.-Q.; Yu, A.; Zhao, C.-F.; Huang, C.-H.; Zhu, L.-Y.; Zhu, B.-Z. *J. Org. Chem.* **2015**, *80*, 180.
- (35) Shao, J.; Huang, C.-H.; Kalyanaraman, B.; Zhu, B.-Z. *Free Radical Biol. Med.* **2013**, *60*, 177.
- (36) Yin, R.; Zhang, D.; Song, Y.; Zhu, B. Z.; Wang, H. *Sci. Rep.* **2013**, *3*, 1269.
- (37) Zhu, B.-Z.; Har-El, R.; Kitrossky, N.; Chevion, M. *Free Radical Biol. Med.* **1998**, *24*, 360.
- (38) Ribo, J. M.; Kaiser, K. L. *Toxic. Assess.* **1987**, *2*, 305.
- (39) Witte, I.; Zhu, B.-Z.; Lueken, A.; Magnani, D.; Stossberg, H.; Chevion, M. *Free Radical Biol. Med.* **2000**, *28*, 693.
- (40) Chignell, C. F.; Han, S. K.; Mouithys-Mickalad, A.; Sik, R. H.; Stadler, K.; Kadiiska, M. B. *Toxicol. Appl. Pharmacol.* **2008**, *230*, 17.
- (41) Teuten, E. L.; Xu, L.; Reddy, C. M. *Science* **2005**, *307*, 917.
- (42) Kelly, B. C.; Ikonomou, M. G.; Blair, J. D.; Morin, A. E.; Gobas, F. A. P. C. *Science* **2007**, *317*, 236.
- (43) Gao, H.-Y.; Mao, L.; Li, F.; Xie, L.-N.; Huang, C.-H.; Shao, J.; Shao, B.; Kalyanaraman, B.; Zhu, B.-Z. *Environ. Sci. Technol.* **2017**, *51*, 2934.
- (44) Chen, H.-M.; Lee, Y.-H.; Wang, Y.-J. *Chem. Res. Toxicol.* **2015**, *28*, 339.
- (45) Zhao, B.; Yang, Y.; Wang, X.; Chong, Z.; Yin, R.; Song, S.-H.; Zhao, C.; Li, C.; Huang, H.; Sun, B.-F. *Nucleic Acids Res.* **2014**, *42*, 1593.
- (46) Qin, H.; Huang, C. H.; Mao, L.; Xia, H. Y.; Kalyanaraman, B.; Shao, J.; Shan, G. Q.; Zhu, B. Z. *Free Radical Biol. Med.* **2013**, *63*, 459.
- (47) Natoff, I. L.; Reiff, B. *Toxicol. Appl. Pharmacol.* **1973**, *25*, 569.
- (48) Fukuto, T. R. *Environ. Health Perspect.* **1990**, *87*, 245.