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Unexpected transformation of dissolved phenols to toxic dicarbonyls by hydroxyl radicals and UV light

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Water treatment systems frequently use strong oxidants or UV light to degrade chemicals that pose human health risks. Unfortunately, these treatments can result in the unintended transformation of organic contaminants into toxic products. We report an unexpected reaction through which exposure of phenolic compounds to hydroxyl radicals (•OH) or UV light results in the formation of toxic α,β-unsaturated enedials and oxoenals. We show that these transformation products damage proteins by reacting with lysine and cysteine moieties. We demonstrate that phenolic compounds react with •OH produced by the increasingly popular UV/hydrogen peroxide (H_2O_2) water treatment process or UV light to form toxic enedials and oxoenals. In addition to raising concerns about potential health risks of oxidative water treatment, our findings suggest the potential for formation of these toxic compounds in sunlit surface waters, atmospheric water, and living cells. For the latter, our findings may be particularly relevant to efforts to understand cellular damage caused by in vivo production of reactive oxygen species. In particular, we demonstrate that exposure of the amino acid tyrosine to •OH yields an electrophilic enedial product that undergoes cross-linking reaction with both lysine and cysteine residues.

reactive transformation products | water treatment | advanced oxidation processes | chemoproteomics | exposome

By 2050, two-thirds of the world's population will be living in cities that are increasingly reliant on drinking water sources affected by agricultural runoff and industrial and municipal wastewater discharges (1, 2). These drinking water sources often contain trace concentrations of phenolic compounds that are widely used in dyes, surfactants, pharmaceuticals, and pesticides, including bisphenol A, triclosan, and nonylphenol-ethoxylates (3, 4). As a result of concerns about adverse health effects from chronic exposure to phenolic compounds, the Environmental Protection Agency and other regulatory agencies require the removal of certain phenolic compounds during water treatment. One approach for treating phenol-containing water involves the oxidation of these compounds with hydroxyl radicals (•OH). •OH-based treatment technologies, such as the use of UV light to photolyze hydrogen peroxide (i.e., the UV/H_2O_2 process), are becoming increasingly common in drinking water treatment, potable water reuse, and remediation of contaminated groundwater at hazardous waste sites. In these processes, •OH transform phenolic compounds and other organic contaminants through a series of reactions that result in addition of oxygen-containing functional groups to the compounds (5, 6). Although the transformation products formed in these reactions are frequently less toxic then the parent compounds and often can be more easily removed in subsequent water treatment processes, oxidation of phenols can also lead to the formation of toxic transformation products such as p- and *o*-benzoquinone (7). Exposure to quinones is a toxicological concern because their electrophilic character leads to cellular damage through reactions with nucleophilic groups in proteins and DNA (8).

Despite the growing recognition that toxic transformation products may be formed during oxidative water treatment (9), the potential health effects of this practice are uncertain. Because thousands of anthropogenic compounds may be present in drinking water sources, assessment of the effects of every compound that might be present is not feasible. Rather, novel approaches are needed to prioritize further investigations of compounds that are inherently toxic or that might be transformed to toxic transformation products during water treatment.

In toxicology, recognition of the importance of molecular interactions of chemicals with biomolecules has led to the development of the adverse outcome pathway concept (10). As a key feature, molecular initiating events (e.g., the formation of covalent adducts by reaction of both endogenous and exogenous electrophiles with proteins and DNA) have been recognized as an important mechanism involved in a variety of adverse health outcomes, including cancer and cardiovascular diseases (11, 12). This has also led to the development of screening tools that allow for the assessment of reactive candidate pharmaceuticals and their metabolites by investigating the formation of covalent adducts formed when test compounds react with amino acids and proteins (13, 14). To assess the potential for toxic products of oxidative water treatment, we adapted this approach to identify reactive electrophiles that are formed during oxidative

Significance

Phenols are common anthropogenic and natural chemicals that contaminate drinking water sources. To reduce exposure to these compounds, hydroxyl radicals are often used as chemical oxidants during water treatment. Although this treatment process removes phenols, we have found that it unexpectedly produces toxic transformation products. We identify these products and simultaneously assess their toxicity with a technique that detects products formed when the transformation products react with amino acids and peptides. Our results highlight the potential risks of using oxidative treatment on alternative drinking water sources, such as contaminated groundwater and recycled municipal wastewater. They also suggest that these reactions produce these toxic transformation products in other situations, including in clouds and sunlit surface waters and within living cells.

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water treatment. To ensure the sensitive detection of oxidation products of toxicological relevance, we targeted adducts produced when the oxidation products reacted with nucleophilic moieties in biomolecules, including primary amine moieties in *N*-α-acetyl-lysine (NAL) and thiol functional groups in glutathione (GSH) and *N*-acetyl-cysteine (NAC).

Results and Discussion

To assess a representative treatment system, we oxidized phenol with •OH produced by the UV/H_2O_2 process. After exposure, we added NAL and detected the formation of an adduct, R-2-(acetylamino)-6-(2,5-dihydro-2-oxo-1H-pyrrol-1-yl)-1-hexanoic acid (*m/z* 255), which increased in concentration as the phenol concentration decreased (Fig. 1*A* and *SI Appendix*, Fig. S1). Similarly, addition of either GSH or an equimolar mixture of NAC and NAL after oxidation of phenol in the UV/H_2O_2 process yielded the adducts *N*-[4-carboxy-4-(3-mercapto-1H-pyrrol-1-yl)-1-oxobutyl]-L-cysteinylglycine cyclic sulfide (*m/z* 356) and *N*-acetyl-S-[1-[5-(acetylamino)-5-carboxypentyl]-1H-pyrrol-3-yl]-L-cysteine adducts (*m/z* 400), respectively. These data indicate that the oxidation of phenol by •OH results in the formation of the α,β-unsaturated dialdehyde 2-butene-1,4-dial (15). The formation of the same *N*-substituted pyrrolin-2-one and pyrrole adducts have been observed previously during the CYP450-mediated metabolism of furan, a compound that is commonly found in heat-treated food, cigarette smoke, and exhaust gas from car and diesel engines (Fig. 1*B*) (16, 17). 2-butene-1,4-dial reacts with protein and DNA nucleophiles, exhibits a strong positive response in the Ames assay, induces strand breaks and cross-links in DNA, and has been related to the in vivo toxicity of furan (16, 18).

Quantitative analysis of 2-butene-1,4-dial by LC/MS/MS indicated that ∼2% of the phenol that was lost had been transformed into the α,β-unsaturated dialdehyde (Fig. 1*C*). In a separate experiment, we also demonstrated that 2-butene-1,4-dial is formed, albeit at lower yields, during direct UV photolysis of phenol by a medium pressure mercury lamp (Fig. 1*C*). Exposure of phenol to light with wavelengths above 290 nm did not result in the degradation of phenol or the formation of 2-butene-1,4-dial. The formation of the transformation product was also observed when a low-pressure mercury lamp was used as light source.

Decades of research have established a pathway for phenol oxidation by •OH or UV light, consisting of initial hydroxylation of the aromatic ring to form hydroquinone, benzoquinone, and catechol, followed by subsequent ring hydroxylation and cleavage to produce short-chain organic acids, such as maleic acid, formic acid, and oxalic acid (7). Direct cleavage of aromatic rings without sequential hydroxylation has been postulated (19), but our findings represent experimental evidence that α,β-unsaturated aldehydes are formed as an initial product of aqueous phase phenol oxidation by exposure to •OH or UV light. The formation of α,β-unsaturated aldehydes, including 2-butene-1,4-dial and 4-oxo-2-pentenal, from the reaction of •OH with benzene and various methylated benzenes has been observed in the gas phase (20–24). The initial step in the gas phase reaction mechanism yields carbon-centered radicals, either by H-abstraction or OH-addition, which subsequently react with dioxygen to produce peroxides. These unstable intermediates can undergo unimolecular ring-closure, yielding bicyclic alkoxy radicals that then break down to produce enedials and oxoenals. In this final step, the original six carbons of the aromatic ring are converted into a four-carbon and a two-carbon compound. In the aqueous phase, the formation of bicyclic alkoxy radicals after the reaction of •OH with benzene and phenol in the aqueous phase has been postulated (25, 26), but our results provide experimental evidence for the occurrence of this process in water.

Toxicologists have found strong evidence that 2-butene-1,4-dial produced by metabolism of furan causes toxicity and cell death by binding to hepatic proteins (27). 2-butene-1,4-dial is a highly reactive electrophile that can potentially react with cysteines in proteins. To better understand the potential toxicity and proteome-wide cysteine reactivity of 2-butene-1,4-dial, we used a chemoproteomic platform termed activity-based protein profiling (ABPP). ABPP uses reactivity-based probes to map proteome-wide reactive, functional, and ligandable hotspots directly in complex proteomes (28, 29). To initially determine whether

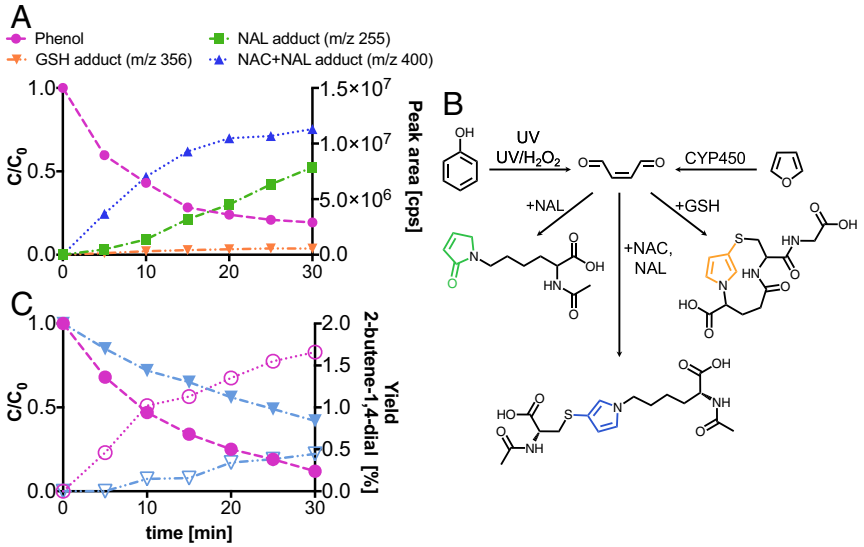

Fig. 1. Formation of 2-butene-1,4-dial during oxidation of phenol in the UV/H_2O_2 process. (*A*) Oxidation of phenol (0.1 mM) in borate buffer (50 mM at pH 8) by UV/H_2O_2 and the reaction of 2-butene-1,4-dial with either *N*-α-acetyl-L-lysine (NAL), glutathione (GSH) or a mixture of NAL and NAC. A medium pressure mercury lamp was used as radiation source. (*B*) Formation of 2-butene-1,4-dial by reaction of phenol with •OH and UV light or by bioactivation of furan by cytochrome P450 (CYP450) in the human body (33) and the resulting adduct formation with NAL, GSH as well as NAC and NAL. (*C*) Oxidation of phenol by UV light in the absence (solid triangles) and presence of H_2O_2 (0.1 mM; solid circles) and quantification of 2-butene-1,4-dial by LC/MS/MS using the standard addition method (open circles and triangles; see *SI Appendix*).

2-butene-1,4-dial had any cysteine reactivity with proteins in mouse liver proteomes, we performed an in-gel ABPP analysis, in which we compared its binding to proteins with that of a cysteine-reactive iodoacetamide alkyne (IAyne) probe (30). We found that 2-butene-1,4-dial exhibits broad cysteine reactivity in a dose-responsive manner, showing more selective reactivity with proteins at lower concentrations (Fig. 2*A*). To map the specific ligandable cysteine hotspots targeted by 2-butene-1,4-dial, we used a more advanced ABPP platform termed isotopic tandem orthogonal proteolysis-enabled ABPP (isoTOP-ABPP) (28). This method uses a biotin-azide tag bearing an isotopically light (for vehicle-treated) or heavy (for 1,4-butene-dial-treated) tag and a tobacco etch virus (TEV) protease recognition sequence that is bound to IAyne-labeled proteins by click chemistry. It thus allows for quantitative proteomic analysis using light to heavy probe-modified peptide ratios. We interpreted those peptides showing >10 ratios as direct targets.

Among more than 600 probe-modified peptides identified, we identified 37 targets of 2-butene-1,4-dial (Fig. 2*B* and Dataset S1). The proteins bound by 2-butene-1,4-dial have diverse functions and include targets involved in protein biosynthesis, energy metabolism, and steroid biosynthesis. Specifically, two contained cysteines that corresponded to an annotated catalytic cysteine, C199 of Nit1 (nitrilase-like protein 1) and C150 of GAPDH (glyceraldehyde 3-phosphate dehydrogenase), suggesting that the function of these enzymes were impaired by 2-butene-1,4-dial. Nit1 is a protein involved in regulation of apoptosis, and thus inhibition of this protein may lead to accelerated proliferation (31). GAPDH is a glycolytic enzyme, and its inhibition likely affects glycolytic metabolism and energetics (32). Consistent with the reactivity with the catalytic C150 of GAPDH, we show that 2-butene-1,4-dial inhibits GADPH function in a substrate activity assay (Fig. 2*C*). Further validation of the IsoTOP-ABPP-determined targets and studies of the in vivo toxicology of 2-butene-1,4-dial could provide insight into the endpoints of concern for human exposure to the products of phenol oxidation in drinking water.

To assess the potential formation of other toxic α,β-unsaturated aldehydes formed during oxidation of common phenol-containing compounds, we simulated the treatment of a suite of methylated phenols by the UV/H_2O_2 process. We relied on the formation of unique pyrrolin-2-one and pyrrole reaction products of enedials

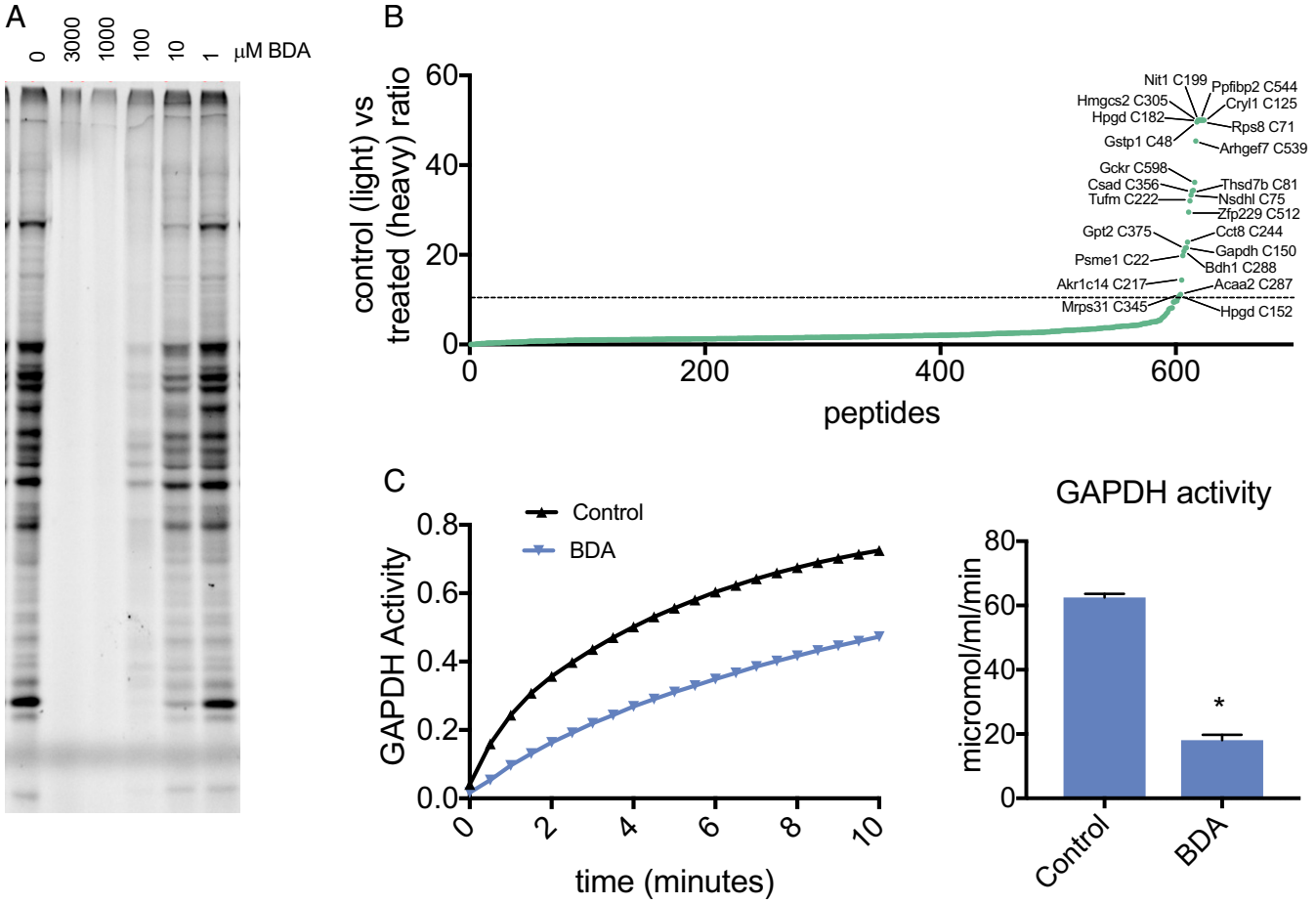

Fig. 2. ABPP analysis of in vitro cysteine reactivity in mouse liver from 2-butene-1,4-dial. (*A*) Mouse liver proteome was exposed to 2-butene-1,4-dial (BDA) at initial concentrations ranging from 1 μM to 3 mM and subsequently labeled with IAyne probe (10 μM), followed by click-chemistry appendage of rhodamine-azide for fluorescence imaging (gel-ABPP) to measure dose-dependent probe displacement. (*B*) IsoTOP-ABPP analysis of mouse liver proteome after exposure 10 μM 2-butene-1,4-dial. Mouse liver proteome was treated with 2-butene-1,4-dial, and labeled with IAyne (100 μM), followed by click-chemistry mediated appendage of a biotin tag bearing a TEV protease cleavage sequence and an isotopically light or heavy valine. Proteomes were combined, avidin enriched, tryptically digested, and modified peptides were isolated by TEV digestion followed by quantitative proteomic analysis. Peptides are arranged in increasing light (control) to treated (heavy) ratios and protein names for peptides showing light-to-heavy ratios >10 are included in the figure. Peptides showing a light-to-heavy ratio >50 are represented as 50. (*C*) Activity assay showing that incubation of pure GAPDH protein with 2-butene-1,4-dial (10 μM) inhibits activity. *$P < 0.05$.

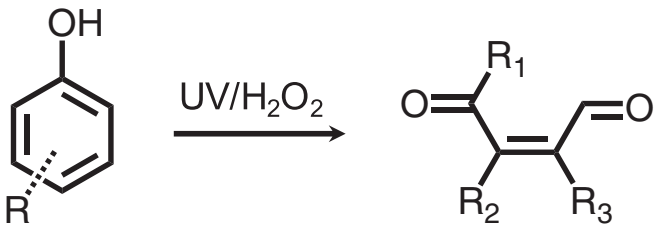

Fig. 3. General reaction mechanism of the oxidation of methylated phenols by UV/H_2O_2 yielding oxoenals and enedials. R_1, R_2, and R_3 indicate the locations of either -H of -CH_3 residues in the C4-dicarbonyl compounds formed during oxidation and depend on the location of the methyl substituents in the investigated phenols (Table 1).

and oxoenals in the presence of NAL or GSH, respectively (Fig. 3) (33). For m-, *o*-, and p-cresol, exposure to •OH produced by the UV/H_2O_2 process led to the formation of 2-butene-1,4-dial and methylated 2-butene-1,4-dial adducts (Table 1 and *SI Appendix*, Figs. S5–S7). The latter were detected in higher abundances for both p- and m-cresol, whereas for *o*-cresol, 2-butene-1,4-dial was the dominant aldehyde transformation product (Table 1). Similarly, oxidation of dimethyl- and trimethylphenols yielded NAL and GSH adducts consistent with the presence of one, two, or three additional methyl groups relative to the transformation product observed when phenol was oxidized (*SI Appendix*, Fig. S10). Thus, our findings demonstrate that oxidation of alkyl-substituted phenols by the UV/H_2O_2 process generally results in the formation of α,β-unsaturated enedials and oxoenals.

Table 1. Products and relative yields from the oxidation of methyl-substituted phenols

Phenol derivatives	-R_1	-R_2	-R_3	Rel. yield,* %
Phenol	**-H**	**-H**	**-H**	**1.0**
p-Cresol	**-H**	**-CH_3**	**-H**	**7.1**
	-H	-H	-H	0.2
m-Cresol	**-H**	**-CH_3**	**-H**	**2.8**
o-Cresol	**-H**	**-H**	**-H**	**0.7**
	-CH_3	-H	-H	0.6
	-H	-CH_3	-H	0.4
2,6-Dimethyl-phenol	**-CH_3**	**-H**	**-H**	**1.4**
	-H	-CH_3	-H	0.2
2,3-Dimethyl-phenol	**-CH_3**	**-CH_3**	**-H**	**2.8**
	-CH_3	-H	-H	0.2
	-H	-CH_3	-H	0.1
2,5-Dimethyl-phenol	**-H**	**-CH_3**	**-H**	**3.7**
3,4-Dimethyl-phenol	**-H**	**-CH_3**	**-CH_3**	**2.1**
	-CH_3	-CH_3	-H	1.7
	-H	-CH_3	-H	0.8
3,4,5-Trimethyl-phenol	**-CH_3**	**-CH_3**	**-CH_3**	**1.3**
	-CH_3	-CH_3	-H	0.6
	-H	-CH_3	-H	0.3
2,4,6-Trimethyl-phenol	**-CH_3**	**-H**	**-CH_3**	**0.9**
	-H	-CH_3	-H	0.1

Positions of substituents (R_1, R_2, R_3) are labeled as shown in Fig. 3. Formed enedials and oxoenals were identified based on their reaction with *N*-α-acetyl-lysine and glutathione. Dominant products are highlighted in bold. Experiments with individual phenols were carried out at an initial concentration of 0.1 mM in the presence of H_2O_2 (0.1 mM) buffered at pH 8 (50 mM borate)

*Relative yields of dicarbonyls were estimated based on chromatographic peak areas of NAL-adducts normalized to that of 2-butene-1,4-dial observed in experiments with phenol.

The ubiquitous presence of •OH in living organisms (34), natural waters (35–37), fog (38), and the atmosphere (39) means that our observations may have potential implications beyond oxidative water treatment. Oxidative stress caused by reactive oxygen species plays an important role in vivo and has been implicated in various adverse health outcomes, including Parkinson's and cardiovascular diseases (34). For example, the reaction of •OH with the phenolic amino acid tyrosine produces reactive intermediates that are capable of reacting with other biomolecules, resulting in protein–protein and protein–DNA cross-links (40). Furthermore, reactions of amino acids, such as tyrosine, with •OH in sunlit surface waters has been linked to the formation of humic-like substances, which play an important role in surface water ecosystems, in particular for the geochemical cycling of trace elements (41). In the troposphere, these reactions also have been implicated in the formation of secondary organic aerosols (42). The formation of reactive tyrosine transformation products from exposure to •OH has thus far been exclusively attributed to the formation of carbon-centered tyrosyl radicals (43). However, our results reveal a previously unknown reaction mechanism. By exposure of a solution of *N*-acetyl-tyrosine to •OH, we demonstrated the production of cross-coupling products by a mechanism analogous to that observed for the substituted phenols via formation of an enedial transformation product (Fig. 4).

Previously published research indicates that dihydroxybenzenes and organic acids account for a significant fraction of the transformation products produced when phenols are exposed to •OH or UV light (5, 6). Our results show that a previously unrecognized group of toxic compounds, enedials and oxoenals, are also formed in this process, albeit at a lower yield than the other products. The toxicity of α,β-unsaturated carbonyl compounds, in particular α,β-unsaturated aldehydes, is attributable to their high reactivity via nucleophilic addition at both the carbonyl-carbon and the β-carbon (44). As a result, α,β-unsaturated aldehydes are much more toxic than their saturated analogs (18, 45).

In addition to phenol, a variety of natural and anthropogenic phenolic compounds are present in the environment. In addition to the presence of phenolic groups in many industrial chemicals, methyl-phenols represent important moieties of aquatic natural organic matter (46). In addition, phenolic compounds are emitted by combustion processes and reach surface waters mainly via wet and dry deposition (47–49). Use of •OH-based and UV treatment technologies may therefore result in formation of many different types of α,β-unsaturated aldehydes that are likely to react with biomolecules (44).

The fate of the dialdehydes in engineered treatment and drinking water distribution systems depend on the water treatment processes that follow the oxidative treatment process. Losses by volatilization from open basins are likely to be small because the dialdehydes are not particularly volatile (e.g., concentrations of a 2-butene-1,4-dial standard decreased by less than 10% after 2 d in an open container stored at room temperature). Because of the polar nature of the dialdehydes, removal by sorption to activated carbon is also likely to be small under conditions used in drinking water treatment plants. The compounds also will not undergo photolysis to an appreciable extent after their formation (e.g., concentrations of 2-butene-1,4-dial did not decrease after most of the phenol was removed in experiments depicted in Fig. 1). Thus, transformation in subsequent stages where water is exposed to microbes (e.g., during sand filtration or biological activated carbon) or in the drinking water distribution system is likely to be the most important sink for the enedials and oxoenals. Additional research is needed to assess the occurrence and rates of these removal processes.

In summary, we show that the formation of adducts between amino acids and proteins can be used as a sensitive means of identifying reactive transformation products that are formed

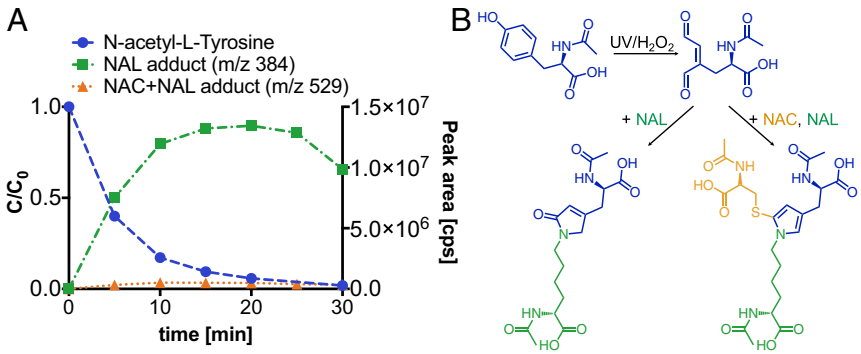

Fig. 4. Oxidation of *N*-acetyl-Tyrosine in the UV/H_2O_2 system. (*A*) Oxidation of *N*-acetyl-tyrosine by •OH and UV light and subsequent reaction of the formed 1,4-enedial with *N*-(α)-acetyl-lysine (NAL) and *N*-acetyl-cysteine (NAC) and NAL (MS^2 spectra of adducts are given in *SI Appendix*, Fig. S12). (*B*) Proposed reaction pathway of reactive enedial *N*-acetyl-tyrosine transformation product and its reaction with NAL and NAC+NAL.

during oxidative water treatment. For the reaction of phenols with •OH and UV light, this has led to the unexpected discovery of oxoenal and enedial transformation products. Sensitive analytical approaches, similar to those described here, offer a powerful approach for identifying reactive electrophiles. This knowledge will allow drinking water treatment plant operators to minimize the potential for human exposure to the compounds. Further studies are necessary to assess the implications of long-term exposure to reactive electrophiles in drinking water (i.e., the drinking water exposome), to assess their fate in drinking water distribution systems and in the aquatic environment, and to evaluate the potential for using pretreatment methods to minimize their formation during oxidative water treatment.

Methods

UV-Photolysis Experiments and Investigation of Adduct Formation with Amino Acids and GSH. Stock solutions of individual phenolic compounds were prepared in ultrapure water (1 or 10 mM). For each sample point, a separate quartz vial was used. Quartz vials (8 mL) were filled with a premixed solution of individual phenols and H_2O_2 (initial concentration: 0.1 mM each) in borate buffer (50 mM at pH 8). To investigate the relevance of •OH, we also conducted experiments in the absence of H_2O_2 (UV only). All photolysis experiments were performed in a merry-go-round photochemical reactor equipped with a medium-pressure mercury lamp (400 W; Ace Glass). The lamp was cooled by a quartz jacket connected to a water tap. At the end of the experiments, 2 mL were taken from each quartz tube and transferred into HPLC vials for determination of phenol removal using HPLC-UV. In addition, 1 mL was transferred into a separate HPLC vial, and 100 μL NAL, GSH, or an equimolar mixture of NAC and NAL was added (final concentration: 0.3 mM). Samples were allowed to react in the dark for 24 h before analysis by positive electrospray ionization (ESI+) LC/MS/MS. Further details on used analytical techniques including HPLC-UV, LC/MS/MS, and high-resolution Orbitrap MS can be found in the *SI Appendix*.

Chemoproteomics Analysis. Reaction of cis-butene-1,4-dial with cysteine residues in mice liver proteome was investigated using gel-based chemoproteomics and isoTOP-ABPP analysis. After incubation of the proteome with cis-butene-1,4-dial, the remaining free cysteine residues were labeled with iodoacetamide alkyne (IAyne) before copper-catalyzed azide-alkyne cycloaddition "click chemistry" to append rhodamine-azide (gel-based chemoproteomics analysis) or isotopically light (control) or heavy (treated) TEV-biotin and click chemistry followed by LC/MS/MS (isoTOP-ABPP analysis). Additional details together with information on the used GADPH enzyme activity assay are provided in the *SI Appendix*.

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