Halogenation of Anilines: Formation of Haloacetonitriles and Large-Molecule Disinfection Byproducts

Zachary T. Kralles[†], Prashant K. Deherikar[†], Christian A. Werner[†], Ximin Hu[‡§], Edward P. Kolodziej[‡§], and Ning Dai[*†]

[†]Department of Civil, Structural and Environmental Engineering
University at Buffalo, the State University of New York, Buffalo, NY, 14260

[‡]Center for Urban Waters
University of Washington-Tacoma, Tacoma, WA 98421

[§]Department of Civil and Environmental Engineering
University of Washington, Seattle, WA, 98195

[*]Corresponding author: Post address: 231 Jarvis Hall, Buffalo, NY 14260
Phone: (716) 645-4015
Email: ningdai@buffalo.edu

Abstract

Aniline-related structures are common in anthropogenic chemicals such as pharmaceuticals and pesticides. Compared with the widely studied phenolic compounds, anilines have received far less assessment of their disinfection byproduct (DBP) formation potential, even though anilines and phenols likely exhibit similar reactivities on their respective aromatic rings. In this study, a suite of 19 aniline compounds with varying *N*- and ring-substitutions were evaluated for their formation potentials of haloacetonitriles and trihalomethanes under free chlorination and free bromination conditions. Eight of the aniline compounds formed dichloroacetonitrile at yields above 0.50%; the highest yields were observed for 4-nitroaniline, 3-chloroaniline, and 4-(methylsulfonyl)aniline (1.6–2.3%). Free bromination generally resulted in greater haloacetonitrile yields, with the highest yield observed for 2-ethylaniline (6.5%). The trihalomethane yields of anilines correlated with their haloacetonitrile yields. Product analysis of aniline chlorination by liquid chromatography–high-resolution mass spectrometry revealed several large-molecule DBPs, including chloroanilines, (chloro)hydroxyanilines, (chloro)benzoquinone imines, and ring-cleavage products. The product time profiles suggested that the reaction pathways include initial ring-chlorination and hydroxylation, followed by the formation of benzoquinone imines that eventually led to ring cleavage. This work revealed the potential of aniline-related moieties in micropollutants as potent precursors to haloacetonitriles and other emerging large-molecule DBPs with expected toxicity.

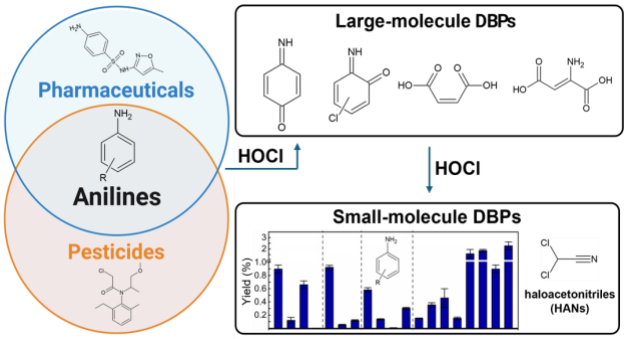
Keywords

disinfection byproduct; haloacetonitrile; aniline; chlorination; bromide; high-resolution mass spectrometry; (halo)benzoquinone imine.

Synopsis

Aniline is a common moiety in anthropogenic chemicals. Upon chlorination, it is a potent, but so far overlooked, precursor to haloacetonitriles and large-molecule disinfection byproducts such as (chloro/hydroxy)anilines and (chloro)benzoquinone imines.

Graphic for Table of Contents



1. Introduction

Disinfection byproducts (DBPs) are compounds formed by the reactions between disinfectants and some natural or anthropogenic compounds present in source waters. [1-7] Epidemiological studies show that DBP exposure can increase the risk of bladder cancer and other health issues. [8, 9] Among the commonly monitored small-molecule DBPs (with 1–2 C atoms), haloacetonitriles (HANs) often contribute the most to the cytotoxicity of finished drinking waters. [10] Recent studies showed that the routinely monitored small-molecule DBPs typically only account for ~30% of the total organic halogen (TOX) [11, 12] and <20% of the total cytotoxicity. [13] Accordingly, large-molecule DBPs (>2 C atoms) were proposed as contributors for the remaining cytotoxicity. [12] Halogen-substituted aromatic DBPs were found to dominate the developmental toxicity of the chlorinated Suwannee River humic acid solutions. [14] Compared with small-molecule DBPs, the occurrence and formation mechanisms of large-molecule DBPs are poorly studied. [12]

Nitrogenous DBPs (N-DBPs) are generally more cytotoxic and genotoxic than those lacking nitrogen, as extensively documented for small-molecule DBPs[15-17] and with emerging evidence for large-molecule (e.g., aromatic) DBPs. [18-22] N-DBP formation potential is higher for nitrogen-rich waters such as wastewater effluents and algal-impacted waters[6, 23, 24]. Some nitrogenous biomolecules can form small-molecule N-DBPs at relatively high-yields. [25] For example, in a recent summary of 34 free amino acid, peptide, and primary amine precursors for dichloroacetonitrile (DCAN) from 11 studies, the 75th percentile of DCAN yields was 0.64%.[26] Anthropogenic chemicals can also form N-DBPs. [5, 27-30] For example, the herbicide isoxaflutole, upon hydrolysis to diketonitrile, can form DCAN at up to 100% yield during chlorination. [27] However, the importance of individual anthropogenic chemicals to the overall DBP formation is not well established, due to their low concentrations (typically ng/L to low μg/L levels). [31] A recent

study reported that in a synthetic mixture of Suwannee River humic acid (2 mg C/L) and ten pharmaceuticals (155 μg C/L total), the pharmaceutical-derived DBPs contributed 9% of the TOX but nearly all of the developmental toxicity. [28] Given the large structural variety of anthropogenic compounds, prioritization of common structural moieties across compound classes is necessary to identify potential DBP precursors.

Aniline is a nitrogen-containing aromatic functional group found in pharmaceuticals and pesticides in forms of primary, secondary, and tertiary amines and amides, such as the antibiotics sulfathiazole and sulfamethoxazole, the herbicides propanil and metalachlor, and the non-steroidal anti-inflammatory drugs acetaminophen and diclofenac. Sulfamethoxazole and acetaminophen respectively have DCAN yields of 0.8–0.9% and 0.6–0.7% (free chlorine 10-fold molar excess, 24–72 h, pH 7), similar to many amino acids. [4, 5, 25] To date, only a few studies have investigated anilines as DBP precursors; they reported substantial formation of HANs, trihalomethanes (THMs), and haloacetic acids. [32-35] Several haloanilines (e.g., 2,4,6-trichloroaniline) were recently detected in finished drinking waters. [36] Given the environmental relevance of aniline and related structures, a dedicated investigation into their potential links to DBPs is warranted.

This study investigated the transformation of anilines upon halogenation. Using a suite of 19 aniline-related compounds with systematically varied *N*- or ring-substitutions, we first evaluated the formation of small-molecule DBPs under free chlorination and free bromination conditions, focusing on HANs and THMs with relevance to driving toxicity and regulatory compliance, respectively. Second, the halogenation products of aniline were examined using liquid chromatography high-resolution mass spectrometry (LC-HRMS) to identify new large-molecule DBPs and shed light on the formation mechanisms of small-molecule DBPs.

2. Materials and Methods

Chemicals and analytical standards are described in Supporting Information Text S1.

2.1 DBP Formation Potential (FP) Experiments

Figure S1 shows the structure of the 19 aniline model precursors assessed in this study, which featured systematic structural variations, including different *N*-substitutions (methyl and carbonyl) and ring substitutions that are electron donating (hydroxyl), near neutral (methyl and ethyl), or electron-withdrawing (acetyl, nitro, sulfonyl, and chlorine), at *ortho*-, *meta*-, or *para*-positions. All DBP-FP experiments were performed in 30 mL solutions containing 30 μM precursor, 10 mM phosphate buffer at pH 7, and 150 μM free chlorine or free bromine. The 5:1 oxidant to precursor molar ratio was previously shown to achieve significant HAN yields without inducing excess HAN decay.[37] Free bromination was used, instead of chlorination in the presence of bromide, to provide direct comparison between chlorination and bromination in terms of HAN yields and reaction mechanisms. Free bromine was prepared as previously described.[38-40] The precursor was added the last to initiate the reactions in both chlorination and bromination experiments. After 24 h, the solutions were quenched with sodium thiosulfate (at 0.75:1 molar ratio to residual chlorine) and then solvent extracted within 10 min to minimize potential DBP decay. Additional experimental details and the DBP analysis method are described in Text S2. Correlation among DBP yields and other parameters were evaluated using the nonparametric Spearman's rank correlation test (Minitab version 21.3.1); the coefficient (ρ) is a measure of monotonicity of the relationship between two variables.

2.2 LC-HRMS Analysis

Samples (10 mL) were prepared with 100 μM aniline, 500 or 1000 μM free chlorine (or free bromine), and 10 mM pH 7 phosphate buffer. Higher precursor concentrations were used to

facilitate the detection of large-molecule DBPs; sample pre-concentration was avoided to minimize the loss of unstable products. At pre-determined time points (5 min and 2, 4, 6, 8, 12, and 24 h), sample vials were quenched with ammonium chloride (see Text S3 for the selection of quenching agents), spiked with 400 μg/L 4-chloroaniline-2,3,5,6-d_4 as an internal standard, and analyzed by LC-HRMS (Thermo Scientific Ultimate 3000 LC with Q-Exactive Focus Orbitrap MS). Experiments were conducted in triplicates. To enable a consistent and short holding time (9–10 min) between sample quenching and LC injection, start times of the triplicate experiments were offset by the instrument run time. To facilitate HRMS data processing, 25 control samples were also analyzed (Table S1). The HRMS was used in positive mode with a scan range of 50–700 m/z and R = 70,000. Discovery mode $ddMS^2$ was employed with a resolution of 17,500, isolation window 3.0 m/z and a stepped collision energy of 10, 20, and 40 eV. Additional LC and MS settings are described in Text S4.

2.3 HRMS Data Processing

The HRMS data were processed in a non-targeted screening workflow using Mass-Suite Version 1.1.2, an open-source Python-based package.[41] All .raw files were converted to .mzML format using ProteoWizard Version 3.0.[42] The data analysis workflow included peak alignment, data reduction, molecular formula assignment, and structure assignment (Figure S2). Table S2 summarizes the values of all parameters used in Mass-Suite data processing. The tuning of the peak alignment parameters is described in Text S5. After alignment, the data was processed with a data reduction function to remove any features also present in control samples based on criteria such as the relative signal intensity between samples and controls or feature occurrence in replicates (see Table S2 for further details). The remaining features were assigned molecular formula using a mass error cutoff of 20 ppm and formula criteria (1) $C_{4\text{-}6}H_{0\text{-}12}O_{0\text{-}5}N_{0\text{-}1}{}^{1}X_{0\text{-}5}{}^{2}X_{0\text{-}5}$

or (2) $C_{12}H_{2\text{-}13}N_{0\text{-}2}O_{0\text{-}5}N_{0\text{-}1}{}^{1}X_{0\text{-}5}{}^{2}X_{0\text{-}5}$, where $^{1}X = {}^{35}Cl$ or ^{79}Br, $^{2}X = {}^{37}Cl$ or ^{81}Br. After molecular formula assignment in Mass-Suite, attempts were made to draw structures after checking the mass error in the specific chromatograms (< 10 ppm). In addition to considering degree of saturation, the halogen isotopic patterns, MS/MS spectra, literature was also consulted related to aniline/phenol oxidation, quinone formation and detection, and reaction mechanisms in environmental organic chemistry. Further details regarding formula and structure assignment are provided in Text S6. Confidence levels followed a modified Schymanski criteria (Text S7). [43]

3. Results and Discussion

3.1 Formation of Small-Molecule DBPs from Aniline Halogenation

3.1.1 Haloacetonitriles

Figure 1a shows the formation of DCAN and DBAN from anilines under free chlorination and free bromination conditions, respectively. Eight of the anilines formed DCAN at yields above 0.50%. 4-Nitroaniline, 3-chloroaniline, and 4-(methylsulfonyl)aniline formed DCAN at the highest yields (1.6–2.3%). When compared with a recent summary of DCAN yields of 34 amino acids, short peptides, and amines under different chlorination conditions[26] (Figure 1b), the DCAN yields of anilines obtained in this study are on par with many nitrogenous precursors tested previously, although not as high as some unique precursors such as free aspartic acid and asparagine. A few anilines have been tested for DCAN yields previously: [32, 33] our results for aniline, 2-hydroxyaniline, and acetanilide were similar to the literature values, but more than 10-fold differences were observed for the 4-nitroaniline and 3-hydroxyaniline (Table S4). These differences may arise from the much greater molar excess of chlorine to precursor used previously (20- or 35-fold) [32, 33] compared with this study (5-fold).

Of the 19 precursors, 16 formed more DBAN during bromination than DCAN during chlorination (Figure 1), similar to that previously observed for tryptophan and indole.[37] Aniline, 3-chloroaniline, 4-chloroaniline, 2-acetylaniline, 2-methylaniline, and 2-ethylaniline all formed DBAN at >2% yields. Across the 19 model precursors, there was an overall positive correlation between DCAN and DBAN (Figure S3). However, some notable exceptions existed: 2-ethylaniline formed DBAN at the highest yield (6.5%) but its DCAN yield (0.30%) was lower than 10 of the compounds tested; 4-(methylsulfonyl)aniline had the highest DCAN yield (2.3%), but its DBAN yield (0.90%) was lower than 11 of the compounds tested. HAN formation involves complex reaction pathways.[26, 37, 44] The overfall greater DBAN yields than DCAN yields may be attributed to the faster reaction of free bromine with aromatic moieties than free chlorine, as previously observed for phenolic compounds.[45, 46] On the other hand, Br incorporation may be more sensitive to steric hindrance due to its larger size than Cl. Depending on the reaction pathway, the halogen initially incorporated into the aromatic moiety may not be present in the final HAN product, as previously observed for tryptophan.[37] Moreover, the lower reduction of HOBr than HOCl (1.34 and 1.48 V, respectively)[47] may also result in different initial products, only some eventually yielding HANs (further discussed below). Future research with mixed halogens (e.g., chlorination in the presence of bromide) can provide further insight into the HAN formation potential of anilines under environmentally relevant conditions.

The HAN yields of the 19 model precursors suggested that both *N*- and ring-substitutions influence HAN formation. Free aniline showed higher HAN yields than *N*-substituted anilines, indicating that *N*-halogenation was involved in some of the HAN formation pathway. Consistent with this, amidification of the free amino group (i.e., acetanilide) completely inhibited HAN formation. For ring-substituted anilines with near neutral or electron donating groups, *ortho*-

substituted compounds had far greater HAN formation than their *meta*- and *para*-substituted analogues. For example, 2-hydroxyaniline formed 7–17 times more HANs than did 3- and 4-hydroxyaniline; 2-methylaniline formed 4–84 times more HANs than 3- or 4-methylaniline. This trend, however, did not apply to anilines substituted with the electron withdrawing acetyl group. Both Hammett constants and the pK_a values of anilines are indicators of the electronic effects of ring substituents, but neither of them correlated with the DCAN/DBAN yields (Text S8), indicating that the influence of ring substituents on the formation of small-molecule DBPs extended beyond the initial ring halogenation. Future research may consider quantifying the kinetic rate constants for the halogenation of anilines and examining their correlation with Hammett constants, similar to the previous assessment for phenols. [48]

The HAN yields obtained in this study can be used to infer the HAN formation potential of environmental contaminants with related structures. For example, the antibiotic sulfamethoxazole, analogous to 4-(methylsulfonyl)aniline with an electron withdrawing substituent *para* to the free amino group, is expected to be a potent DCAN precursor. Indeed, sulfamethoxazole formed DCAN at 0.8-0.9% yields. [4] On the other hand, acetanilide did not form DCAN or DBAN (< 0.003% yield), suggesting that aniline-derived compounds with secondary amide-*N* are unlikely to be potent HAN precursors. However, amide bond is known to be degraded by microorganisms or chemical oxidants to release the free amino group. [49-51] For example, the herbicide propanil (3',4'-dichloropropionanilide), not expected to be a potent HAN precursor itself, can hydrolyze to 3,4-dichloroaniline, [49, 52] which may be a potent precursor based on the DCAN yields of 3-chloroaniline and 4-chloroaniline (1.8% and 0.9%, respectively).

3.1.2 Trihalomethanes

Anilines formed appreciable amounts of chloroform and bromoform, respectively, under free chlorination and bromination conditions (Figure S4). With a free chlorine to precursor molar ratio (Cl:Precursor) of 5 and a reaction time of 24 h, eight of the 19 anilines formed chloroform at molar yields >2%; 2-acetylaniline and 2-methylaniline had the highest yields (4.5% and 3.8%, respectively). These yields are lower than those previously reported for phenolic compounds and (chloro)anilines obtained with much greater Cl:Precursor ratios. [34, 48, 53] For example, with Cl:Precursor = 13–18, the chloroform yields of aniline and chloroanilines were 10–35%;[34] with Cl:Precursor ≥35, phenolic compounds formed chloroform at 1–95% yields. [48, 53] Most THM studies used excess chlorine due to the stability of THMs, in contrast to the concern for HAN decay induced by excess oxidants. [54]

The molar yields of bromoform (0.003–6.5%) for the aniline compounds under bromination conditions are generally within the same range as those of chloroform (Figure S5a), in contrast to the substantially higher yields of DBAN than DCAN (Figure S3a). This may be attributable to the different formation mechanisms for these two DBP groups. As shown in Figure S5, correlation was found between chloroform and bromoform formation, chloroform and DCAN formation, and bromoform and DBAN formation. Interestingly, bromoform and DBAN formed at similar molar concentrations from most of the tested aniline compounds; THM yields did not correlate with Hammett constant.

3.2 Analysis of Aniline Halogenation Products by LC-HRMS

3.2.1 LC-HRMS Data Processing by Mass-Suite

Figure 2a shows the HRMS data analysis workflow using Mass-Suite (chlorination samples): 7675 features were identified during initial peak picking and alignment; 243 features

remained after data reduction based on several filtering criteria, including removing features that were present in samples with less than 100-fold greater abundance than blanks/controls, missing from one or more replicates, and/or with signal-to-noise ratio less than 3 (more details in Table S2). In formula assignment, our workflow focused on the C_{4-6} and C_{12} products from aniline chlorination due to their expected occurrence analogous to the phenol chlorination products[55-57] and their importance for elucidating the pathway for small-molecule DBP formation. Although this workflow will miss the other products (e.g., C_3 and C_{7-11}), it enhances efficiency in the subsequent manual structure assignment (Text S6). As shown in Figure S6, the features after formula assignment (106 C_{4-6} and 39 C_{12} features) spanned 54–420 Da and reflected the entire range of polarity captured by the LC gradient (0.6–11.0 min). Because preliminary analyses identified fewer bromination products and all their counterparts were identified in the chlorination samples, the following section will discuss the chlorination products in detail, followed by a brief summary of bromination products.

3.2.2 Aniline Halogenation Products

Seventeen C_{4-6} products were identified from aniline chlorination (Table 1, and Figure 2b for product time profiles). Of these, 14 products retained the six-membered ring, including 11 ring-substituted products with chloro- and/or hydroxy-functional groups and 3 quinone imines; the remaining 3 were ring-cleavage products. Additionally, six C_{12} dimer products were tentatively identified (Table S6). From preliminary bromination experiments, seven C_{4-6} products were detected (Table S7); all of their chlorinated analogues are included in Table 1.

Ring-substituted products

Two monochlorinated and one dichlorinated anilines (RS-128-1, RS-128-2, and RS-161) were detected, all had RTs (5.5, 8.2, and 9.1 min) longer than aniline (1.9 min), consistent with

the increased hydrophobicity upon chlorine substitution. RS-128-1 and RS-128-2 were identified as 4-chloroaniline and 2-chloroaniline, respectively, with level 1 confidence based on the matching of MS/MS spectra (Figure S7 and S8) and RTs with the standards. Previous studies suggested that ring-chlorination products are prevalent for free aniline. [58-61] Compared with amino acids and alkyl amines that rapidly form *N*-chloramines, [62, 63] aniline's amino group is relatively electron-poor due to the aromatic ring, thus promoting ring halogenation. [58] Although *N*-halogenated intermediates have been observed, they typically rearrange to form ring-halogenated products. [64] For example, when anilines were mixed with free chlorine in non-aqueous solvents, *N*-chlorinated intermediates were detected, but they quickly rearranged to *o*-chloroaniline (majority) and *p*-chloroaniline. [59, 60] Although *N*-chlorosulfamethoxazole and *N*-chlorobenzoquinone imine were found as high-yield products from the free chlorination of sulfamethoxazole (a *para*-substituted aniline), [65] the site of chlorination may have been influenced by the strong electron-withdrawing sulfonyl substituent.

Five (chloro)hydroxyanilines were detected. RS-110-1 with a 1.4 min RT was identified as 4-hydroxyaniline with level 1 confidence based on matching RTs and MS/MS spectra with the standard. Another peak with identical parent mass and MS/MS spectrum (RS-110-2(Q); Figure S9) was detected at RT 7.4 min (same as Q-108; discussed below), much later than 2-, 3-, or 4-hydroxyaniline (1.4, 1.8, and 1.4 min, respectively; via standards). Accordingly, we postulate this product to be *p*-benzoquinone imine that was analytically reduced to 4-hydroxyaniline during ionization. The reduction of quinones has been reported from electrospray ionization (ESI) in both positive[66] and negative modes. [67-69] Other redox-labile compounds can also be reduced during positive ESI, [70-72] and the reduction was promoted by formic acid present in LC mobile phase. [72] Similar diagnostic evidence indicates that RS-144-2(Q), which had similar MS/MS spectra to the monochlorinated hydroxyaniline RS-144-1 but a much longer RT (8.3 and 2.3 min, respectively),

was a reduction product of the corresponding quinone imine during ESI. Indeed, the corresponding benzoquinone imine products Q-108 and Q-142-2 were detected (further discussed below). These observations brought our attention to RS-126(Q), with a mass indicative of a dihyroxyaniline but a RT (7.2 min) much later than monohydroxyanilines (1.4–1.8 min) while similar to the quinone imine products. Therefore, we suspect that RS-126(Q) was a benzoquinone product.

RS-177-1 and RS-177-2 were both dichlorinated hydroxyanilines with Cl, CHO, and CHN fragments (Figure S11). The observed $[C_4H_4Cl]^+$ fragment in both spectra indicated that at least one Cl was on the ring. RS-177-1 was also detected in samples from preliminary experiments quenched with thiosulfate that is known to remove *N*-chloramines, [65] further suggesting that both Cl were on the ring. The last ring-hydroxylated product RS-145 corresponded to a monochlorinated dihydroxybenzene that had lost the amino group.

Most of the (chloro)hydroxyaniline products were detected as early as 5 min (Figure 2b). With Cl:Aniline = 5, most of the products maintained their signal intensity at levels slightly lower than that at 5 min throughout the rest of the 24 h experiment; RS-110-1 and RS-145 showed an overall increasing signal intensity. In contrast, with Cl:Aniline = 10, the signal intensity of most products peaked at 5 min (or 2–4 h) and declined afterwards, suggesting that excess chlorine promoted further transformation.

Benzoquinone imine products

Three benzoquinone imine products were detected from aniline chlorination; in addition, RS-110-2(Q), RS-144-2(Q), and RS-126(Q) were also suspected benzoquinone imines as described above. Q-108 was identified as *p*-benzoquinone imine with level 2b confidence (Figure S12), with observed C=O and C=NH losses consistent with previous spectra reported for benzoquinone. [73] *Ortho*-substituted quinones generally have a prominent $[M+H+2]^+$ peak: [73] the

lack of this peak is the first line of evidence that Q-108 was *p*-benzoquinone imine. Additionally, as discussed above, RS-110-2, the putative reduction product of Q-108 formed during ESI, had MS/MS spectra matching with the 4-hydroxyaniline standard, suggesting *para* ketone and imine groups. Q-142-2 was identified as a chloro-*o*-benzoquinone imine isomer (Figure S13), with a prominent $[M+H+2]^+$ fragment and a $[C_4H_4Cl]^+$ fragment indicative of ring chlorination. In preliminary HRMS experiments with thiosulfate quenching, Q-142-2 was also detected, further supporting that it is ring-chlorinated. Q-142-1 did not trigger $ddMS^2$ but is suspected to have *ortho*-ketone and imine groups, because Q-142-1 is unlikely chloro-*p*-benzoquinone given its almost identical RT as *p*-benzoquinone (7.4 min).

The temporal profiles of benzoquinone imine abundance (Figure 2b) were dependent on chlorine dose. At Cl:Aniline = 10, the abundance of most benzoquinone imine products peaked at 5 min and rapidly declined, with few detected in 12 and 24 h samples. In comparison, with Cl:Aniline = 5, the abundance of several benzoquinone imine products peaked at later times (~4 h); at 24 h, Q-108, Q-142-2, RS-110-2(Q), RS-144-2(Q), and RS-126(Q) were still detected. Cl/Ani =10 samples had overall less abundance of benzoquinone imine products than Cl/Ani = 5 samples.

Ring-cleavage products

Three ring-cleavage (RC) products, including two C_4 products (RC-117 and RC-132) and one C_6 product (RC-179) were detected. Unfortunately, MS/MS spectra were not acquired for these products via $ddMS^2$, likely due to the high background for early eluting products (1.8 min RT for RC-117 and RC-132) and/or their low abundance. The C_4 products likely are dicarboxylic acids without halogens. RC-117 was tentatively assigned as maleic acid based on the matching retention time from a reference standard. RC-132 was likely an amino-modified maleic or fumaric

acid, i.e., a tautomer with the corresponding imine. RC-179 is tentatively assigned as a di-carboxylic acid; other structures are possible including ones with 1-2 terminal aldehyde groups.

Across all detected products from aniline chlorination, the ring-cleavage products show the slowest signal intensity. At Cl/Ani = 10, all three ring-cleavage products were detected: RC-132 and RC-179 were detected at 4 h while RC-117 was detected at 8 h; each maintained relatively stable abundance up to 24 h. At Cl/Ani = 5, RC-132 was the only ring-cleavage product detected, with low abundance and high variability.

Dimer products

Six dimer products were detected, all with two nitrogen and two oxygen atoms and three of them also chlorinated (Table S6, Figure S16). Dimers were likely formed by reactions between hydroxyanilines, [65, 74, 75] hydroxyaniline and benzoquinone imine, [55, 76] or benzoquinone imines. [55] Although dimer formation is not expected at the environmentally relevant concentrations of chlorine and aniline-related precursors, their detection supported the presence of hydroxyaniline and benzoquinone imine intermediates. [55, 65]

Aniline bromination products

When aniline was brominated (Table S7), two dibromohydroxyaniline isomers RS-265-1 and RS-265-2 (Figure S14) and a dibromodihydroxybenzene RS-266 were detected. Based on RT, RS-188(Q) was likely a monobrominated benzoquinone reduced to a hydroxyphenol during ESI. Three benzoquinone (imines) were detected: Q-108 (*p*-benzoquinone imine) with matching MS/MS spectra and RT from that detected in chlorination samples, Q-185 (bromo-*p*-benzoquinone imine; Figure S15), and Q-201. No ring-cleavage products were detected.

Summary of Large-Molecule DBPs from Aniline Halogenation

The products detected by HRMS, including haloanilines, (halo)hydroxyanilines, (halo)benzoquinone imines, and C_4 dicarboxylic acids, represent the large-molecule DBPs that may contribute to the toxicity of disinfected water when aniline-related precursors are present. 4-Chloroaniline and two other mono-/di-chlorinated anilines were detected; although they rapidly decompose in the presence of excess chlorine, their lasting presence in disinfected waters is probable at lower chlorine doses/residual chlorine levels. Indeed, 2,4,6-trichloroaniline was reported in chlorinated drinking water. [36]

Halobenzoquinone imines are a new class of large-molecule DBPs. Benzoquinone imines are soft electrophiles that can react with biomolecules such as proteins and glutathione. [77] Although the toxicity of the specific halobenzoquinone imines detected here remains undefined, several *N*- and ring-chlorinated halobenzoquinone imines showed 1.2–10 times greater cytotoxicity than 2,6-dichlorobenzoquinone, [22] which is the most commonly detected halobenzoquinone and 230 times more cytotoxic than the regulated DBP chloroform. [78] Several benzoquinone imines were recently detected in drinking waters. [22, 79] Future research is warranted to focus on the occurrence and toxicity of halobenzoquinone imines.

The detection of these large-molecule DBPs is consistent with the limited previous reports on the oxidation of aniline-related structures. For example, the chlorination of sulfamethoxazole yielded a hydroxylated sulfamethoxazole, [80] several chloroanilines, (chloro)hydroxyanilines, and even chlorophenols that had lost the amino group. [81] The chlorination of acetaminophen (*N*-acetyl-*p*-aminophenol) forms *N*-acetyl-*p*-benzoquinone imine and *p*-benzoquinone; [76, 82] When aniline is oxidized by chlorine dioxide, *p*-benzoquinone imine and *p*-benzoquinone were detected. [74, 83, 84]

Similarities and differences were observed between phenol and aniline chlorination products. For example, phenol formed 2-chlorophenol and 4-chlorophenol upon chlorination, which fully accounted for the initial phenol transformation; [45, 46, 48] yet the formation of hydroxylation products (e.g., hydroquinone, catechol, etc.) is not commonly reported. While aniline chlorination also formed 4-chloroaniline and other mono-/di-chlorinated anilines, hydroxylation products such as 4-hydroxyaniline were also detected as early as 5 min. Similar to aniline, phenol chlorination also formed benzoquinones and dimers. [45, 56, 85, 86]

3.3 Mechanisms of Aniline Halogenation to Form Large-Molecule DBPs and HANs

The observed products provide insight into the mechanisms of aniline halogenation and the formation of HANs and other small-molecule DBPs. Despite the differences in DCAN and DBAN yields from chlorination and bromination of anilines, similar products were observed, suggesting that the two halogens react with aniline in similar pathways but with different kinetics along the reaction pathways. As shown in Scheme 1a, we propose that aniline reacts initially to form chloroanilines or hydroxyaniline. Chlorohydroxyanilines are then formed from hydroxyanilines or chloroanilines; these further react with free chlorine via a two-electron transfer mechanism to form (chloro)benzoquinone imines. (Chloro)benzoquinone imines then undergo further oxidation to form ring-cleavage products, which further transform into small-molecule DBPs.

A few pathways may be involved in the formation of hydroxyanilines. In the initial stage of the experiments (5 min), aniline may react with free chlorine or free bromine via a single electron transfer to form a radical cation intermediate[87, 88] that can undergo hydrolysis to form hydroxyaniline. Compared with phenols, anilines have lower aqueous oxidation potentials and are therefore more easily oxidized by single electron transfer mechanisms to form radical cations. [89] Consistent with this, aniline and phenol were shown to react differently with triplet state methylene

blue. [90] Alternatively, hydroxyaniline may be formed via singlet oxygen ($^1O_2$), which has been detected by near-IR chemiluminescence spectroscopy[91] and demonstrated by density functional theory calculation for hypochlorite solutions. [92] $^1O_2$ can selectively form a para-hydroxylation product upon reaction with aniline. [93] In the later stage of the experiments (up to 24 h), the continuous detection of 4-hydroxyaniline may involve a mechanism that is analogous to the formation of hydroxyl radical via semiquinone radicals formed by hydroquinone reacting with *p*-benzoquinone: [94] 4-hydroxyaniline (initial product) may react with *p*-benzoquinone imine (further transformation product) to form hydroxyl radicals. Hydroxyl radicals can react with phenol and yield additional hydroxylation to aromatic rings; [95, 96] aniline may similarly form hydroxyaniline. Our findings that chlorination of aniline yields both chloroanilines and hydroxyanilines are consistent with the detection of halogenated and non-halogenated aromatic DBPs upon chlorination of natural organic matter, which can undergo further reactions to form small-molecule DBPs. [97, 98]

The proposed formation of (chloro)benzoquinone imines from (chloro)hydroxyaniline via two-electron transfer was based on the observation of Q-108, Q-142-1, Q-142-2 and other suspected quinone imine products, drawing analogy with the rapid formation of *o*- and *p*-benzoquinone from *o*- and *p*-hydroxyphenols upon reaction with free chlorine via electron transfer. [45] The detection of dimers suggested the presence of radicals and/or quinone (imine) intermediates. [45, 99, 100] Hydroxyl radicals, if formed as described above, can react with aniline to form a *N*-centered radical[101] leading to dimerization. [102] Two of the detected products, benzoquinone imine and 4-hydroxyaniline, can also form dimers. [103] Similarly, aniline readily forms dimers with *o*-benzoquinone. [55]

We proposed two ring-opening pathways to explain the formation of haloacetonitriles and other DBPs (Scheme 1b and 1c). Oxidation of 4-hydroxyaniline would yield *p*-benzoquinone imine (Q-108), which hydrolyzes to the non-nitrogenous *p*-benzoquinone, [104] before forming ring-cleavage products such as RC-117. On the other hand, 2-hydroxyaniline, if formed, can be oxidized to *o*-benzoquinone imine, which then hydrolyzes to RS-126(Q), [105] before ring-cleavage to form RC-132. Previous research established quinones as key intermediates in the formation of small-molecule DBPs from phenols; [56, 106, 107] quinone orientation can influence the formation of ring-cleavage products: for example, hydroquinone and *p*-benzoquinone are oxidized to C_4-dicarboxylic acid, whereas catechol and *o*-benzoquinone are oxidized to the C_6-dicarboxylic acid, muconic acid. [61, 74, 83, 108] From RC-132, a tautomer with the corresponding imine, *N*-chlorination can result in another pair of tautomers, one of which undergoes decarboxylation coupled with chloride loss to form a nitrile, similar to the pathways for amino acids. [44, 62, 109] Dichlorination on the α-carbon of the nitrile leads to DCAN. [26] The proposed DCAN formation through 2-hydroxyaniline was consistent with the DBP-FP results: 2-hydroxyaniline exhibited a higher DCAN yield (0.92%) than 4-hydroxyaniline (0.12%). The higher DCAN yields of 3-chloroaniline, 4-chloroaniline, 4-nitroaniline, and 4-(methylsulfonyl)aniline (0.90-2.3%) may reflect the enhancement in hydroxylation at the 2-position via electronic and/or steric effects of the substituent groups.

4. Environmental Implications

This work is among the first efforts to systematically evaluate anilines as precursors to small- and large-molecule DBPs. The high HAN yields from several anilines implicate the numerous anthropogenic chemicals sharing aniline-like moieties as potential precursors to this group of highly toxic DBPs. While each individual compound at environmentally relevant

concentrations may not form substantial levels of DBPs, the collective pool may contribute significantly. Our findings expand evaluation of HAN precursors beyond amino acids, peptides, and aliphatic amines. A suite of large-molecule DBPs were detected from aniline chlorination, including chloro(hydroxy)anilines and (choro)benzoquinone imines. Mechanisms were proposed for the formation of large- and small-molecule DBPs from aniline chlorination based on the results from HRMS product analysis. Future research is warranted to further elucidate the structural role of anilines in large-molecule DBPs formation potential as well as the toxicity and occurrence of the emerging large-molecule DBPs.

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Supporting Information: Additional text on materials, experimental methods, data analysis workflow, method optimization, and data interpretation; figures for correlation analysis, mass spectra, and additional experimental data; tables for experimental data, analytical instrument parameters, and data processing package parameters

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Figure 1. The molar yields of (a) dichloroacetonitrile (DCAN) and dibromoacetonitrile (DBAN) under free chlorination and free bromination conditions, respectively, from 19 anilines. (b) Comparison of DCAN and DBAN yields observed in this study, as well as with the DCAN yields reported in the literature for amino acids, short peptides, and amines. All experiments in this study were performed with 30 μM initial precursor concentration, at pH 7 (10 mM phosphate buffer), and with 150 μM free chlorine or free bromine for 24 h; samples were quenched by thiosulfate (Text S2). Error bars represent the standard deviation of the results from triplicate experiments. N.D. = non-detect (< 0.1 μg/L). DBP concentration data are shown in Table S3. The literature values for non-aniline amines and amino acids were summarized by Zhou et al.;[26] 64 non-zero records for 34 precursors were plotted. The literature values for DCAN yields from anilines were summarized from two other papers.[32, 33]

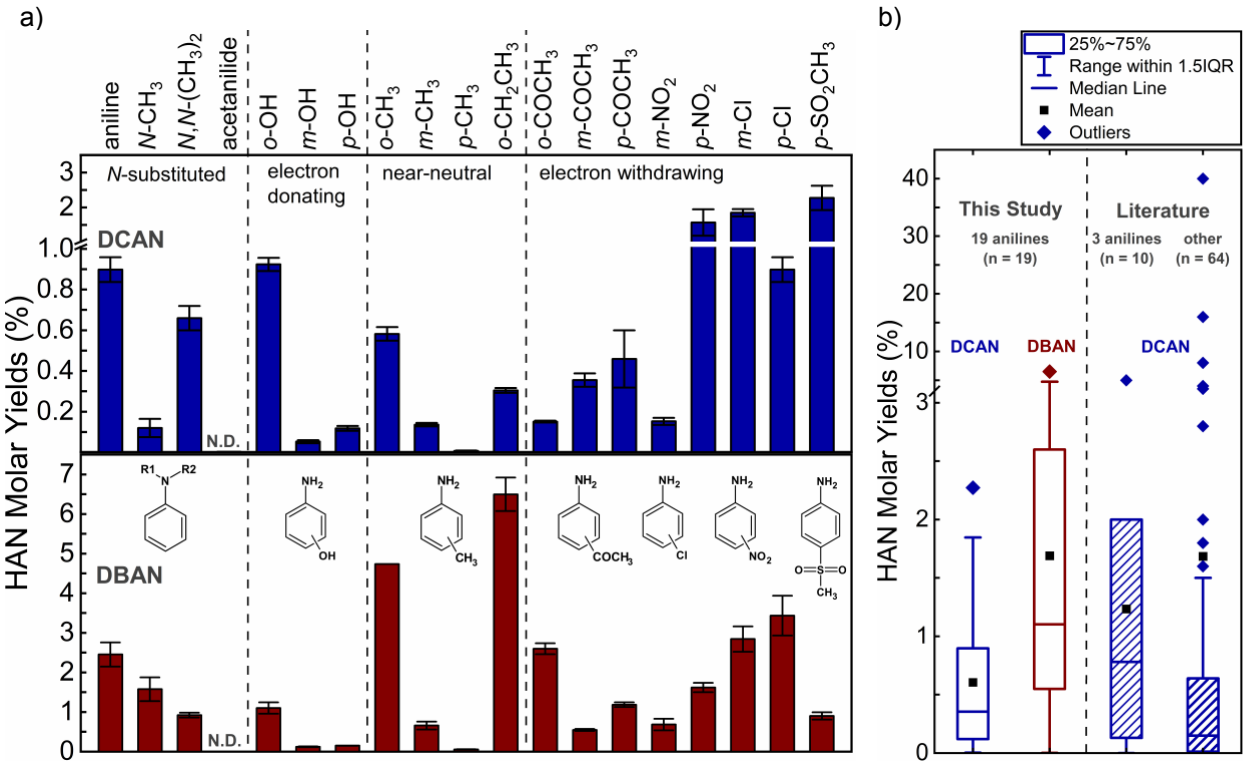


Figure 2. (a) High-resolution mass spectrometry data processing workflow showing the number of features remaining after each step of data processing. (b) Time profiles for the signal intensity of features corresponding to chloroanilines (I), (chloro)hydroxyanilines (II), benzoquinone imines (III), and ring-cleavage products (IV). The postulated structures of these features are shown in Table 1. Peak areas of the features in the chromatogram were normalized by the peak area for the internal standard 4-chloroaniline-2,3,5,6-d_4 (d_4 4-CA) in the sample. Error bars represent the standard deviation from triplicate experimental samples. Samples were from aniline chlorination experiments with an initial chlorine to aniline molar ratio (Cl/Ani) of 5 or 10.

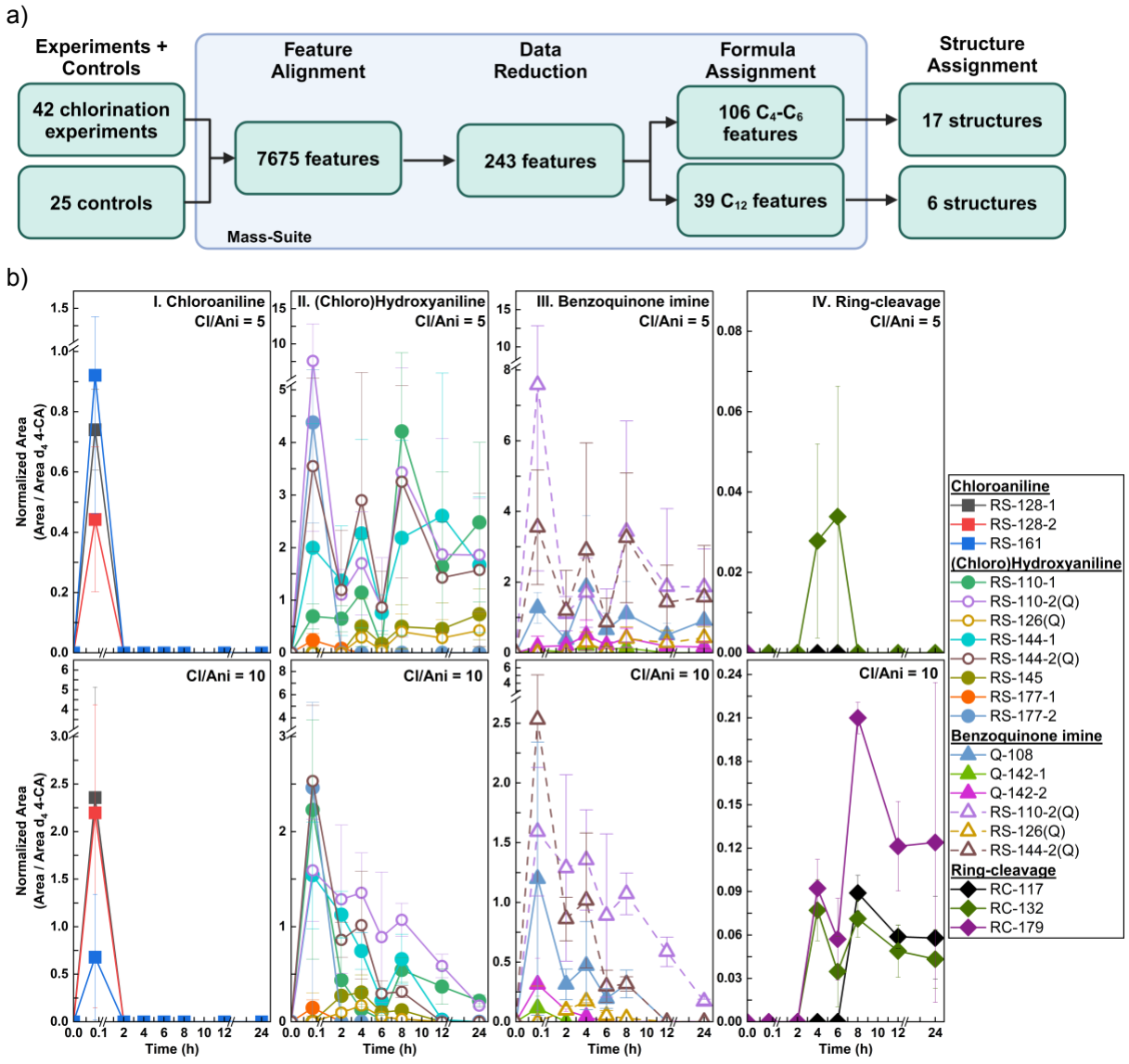
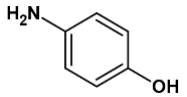
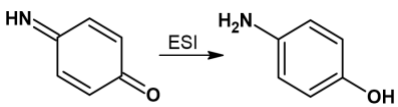
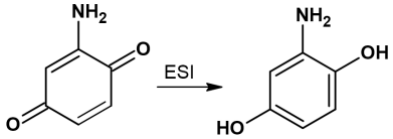
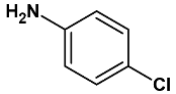
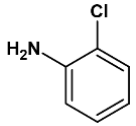
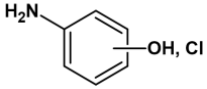
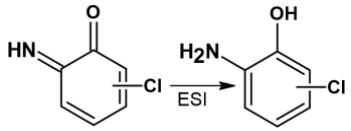
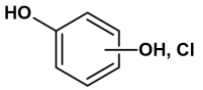
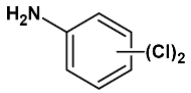
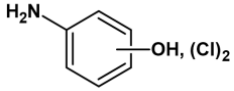
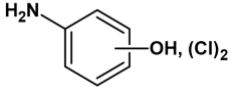
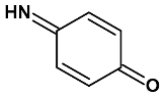
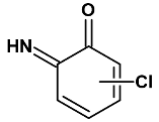
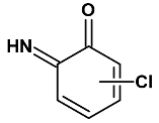
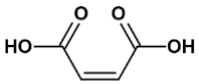
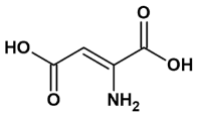
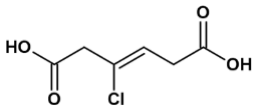


Table 1. The full scan ion and MS/MS fragments (where applicable) for the postulated aniline chlorination products. Products were grouped in three categories: RS = ring-substituted; Q = quinone; RC = ring-cleavage; within each category, products were listed in the order of increasing molar mass and, in the case of identical molar mass, increasing retention time. Experimental conditions: 100 μM aniline, initial free chlorine to aniline molar ratio (Cl/Ani) = 5 or 10, pH 7 10 mM phosphate buffer; samples were quenched by ammonium chloride. The MS/MS spectra for products, when available, are shown in Figures S7-S13. For chlorinated products, the MS/MS spectra displayed are for the most abundant isotope. Confidence level assignment criteria are shown in Text S7; briefly: levels 1, 2a, and 2b are as defined by Schymanski criteria,[43] 3a = matching 2b except that the exact location of the halogen and/or hydroxyl/ketone group cannot be determined; 3b = multiple structures possible, MS/MS spectra not informative or not available.

ID	RT (min)	Full Scan			ddMS^2			Structure	Confidence Level
		m/z	Formula $[M+H]^+$	Error (ppm)	m/z	Formula $[M+H]^+$	Error (ppm)		
RS-110-1 4-hydroxyaniline	1.4	110.0602	C_6H_8NO	1.1	110.0603	C_6H_8NO	2.6		1
					65.0388	C_5H_5	3.0		
					109.0524	C_6H_7NO	1.5		
					80.0495	C_5H_6N	0.1		
					92.0494	C_6H_6N	-0.4		
RS-110-2 [i] (Q-108)	7.4[i]	110.0601	C_6H_8NO	0.8	110.0602	C_6H_8NO	1.6		2b
					65.0387	C_5H_5	2.6		
					109.0524	C_6H_7NO	1.9		
					80.0495	C_5H_6N	-0.3		
					92.0496	C_6H_6N	1.5		
RS-126	7.2	126.0549	$C_6H_8NO_2$	-0.04					3b[ii]
RS-128-1 4-chloroaniline	5.5	128.0264	C_6H_7NCl	1.7	93.0574	C_6H_7N	0.9		1
					128.0263	C_6H_7NCl	1.3		
					75.0229				
					66.0466	C_5H_6	3.0		
					65.0389	C_5H_5	4.4		
RS-128-2 2-chloroaniline	8.2	128.0263	C_6H_7NCl	1.5	128.0262	C_6H_7NCl	0.03		1
					65.0388	C_5H_5	3.4		
					92.0496	C_6H_6N	1.0		
					93.0574	C_6H_7N	1.9		
					75.0229				
RS-144-1	2.3	144.0211	C_6H_7NOCl	0.2	144.0212	C_6H_7ONCl	1.0		3a
					109.0525	C_6H_7ON	2.2		
					80.0495	C_5H_6N	0.2		

RS-144-2 (Q-142-2)	8.3	144.0210	C_6H_7NOCl	-0.2	144.0211 109.0525 80.0495	C_6H_7ONCl C_6H_7ON C_5H_6N	0.2 2.9 0.2		3a
RS-145	4.7	145.0050	$C_6H_6O_2Cl$	-0.8					3b
RS-161	9.1	161.9871	$C_6H_6NCl_2$	-0.7					3b
RS-177-1	7.2	177.9824	$C_6H_6NOCl_2$	1.7	177.9824 143.0135 114.0108 86.9998 78.0340	$C_6H_6ONCl_2$ C_6H_6ONCl C_5H_5NCl C_4H_4Cl C_5H_4N	1.6 1.9 2.3 1.9 1.8		3a
RS-177-2	8.1	177.9821	$C_6H_6NOCl_2$	0.2	177.9823 143.0135 114.1070 142.0055 86.9997	$C_6H_6ONCl_2$ C_6H_6ONCl C_5H_5NCl C_6H_5ONCl C_4H_4Cl	1.0 1.6 1.5 0.9 1.6		3a
Q-108 *p*-benzoquinone imine	7.4	108.0443	C_6H_6NO	-1.1	80.0494 108.0443 53.0389	C_5H_6N C_6H_6ON C_4H_5	-0.7 -0.6 6.1		2b [i]
Q-142-1	7.4	142.0053	C_6H_5NOCl	-0.8					3b
Q-142-2	8.2	142.0054	C_6H_5NOCl	-0.3	144.0211 142.0055 109.0524 114.0108 86.9997	C_6H_7ONCl C_6H_5ONCl C_6H_7ON C_5H_5NCl C_4H_4Cl	0.6 0.9 1.6 2.3 1.6		3a

RC-117 maleic acid	1.8	117.0182	$C_4H_5O_4$	-0.04		3b
RC-132	1.8	132.0291	$C_4H_6NO_4$	-0.5		3b
RC-179	8.0	179.0106	$C_6H_8O_4Cl$	0.2		3b[ii]

[i] RS-110-2 was 4-hydroxyaniline formed via the reduction of *p*-benzoquinone imine (Q-108) during ESI. It has exact mass spectrum match to 4-hydroxyaniline standard, and retention time match to Q-108. This diagnostic evidence led to the assignment of level 2b confidence for RS-110-2(Q) and Q-108.

[ii] Example structure; location of the ketone, imine, and/or chlorine groups are unknown.

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Scheme 1. (a) The proposed overall reaction pathway for the formation of small-molecule DBPs dichloroacetonitrile (DCAN) and chloroform from aniline. The proposed pathway for (b) the formation of ring-cleavage product RC-117 and (c) the formation of DCAN from RC-132.