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Stable isotope labelling to elucidate ring cleavage mechanisms of disinfection by-product formation during chlorination of phenols

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Despite decades of research on the formation of toxic disinfection by-products (DBPs) during water disinfection with chlorine, considerable uncertainties remain regarding the formation mechanism of toxic DBPs from phenolic precursors. Here we report the use of a series of synthesized ethylparabens containing stable isotope (^{13}C) labels at different positions of the molecule to ascertain DBP formation mechanisms from phenols, including those of regulated chloroacetic acids and recently identified α,β-unsaturated dialdehydes and dicarboxylic acids. Our results highlight the involvement of four general ring cleavage pathways. Three of the DBP formation pathways involve carbons originating from the aromatic ring, while the fourth pathway involves the substituent carboxylic ester carbon in the formation of dichloroacetic acid and C_4-dicarboxylic acids. Quantitative comparison of the ^{13}C-labelled DBPs enabled further assessment of the contribution from each of these distinct pathways, providing novel insights into ring cleavage reaction mechanisms that have eluded previous DBP investigations.

Chlorine has been used as a disinfectant for more than a century to inactivate pathogens present in drinking water, municipal wastewater and swimming pools[1,2]. However, the reaction of chlorine with dissolved organic compounds can generate a large number of toxic disinfection by-products (DBPs)[3–5]. Among the DBP precursors, phenols are particularly important due to their high reactivity with chlorine and their widespread occurrence in both natural organic matter (NOM) and anthropogenic compounds[6–8]. Previous studies on the chlorination of phenols have primarily focused on chlorophenols, which are formed via an initial electrophilic substitution mechanism[8–11], contributing to taste and odour problems in drinking water treatment[8,12]. Additionally, attention has been given to the formation of one-carbon-atom C_1-DBPs (trihalomethanes, THMs) and two-carbon-atom C_2-DBPs (haloacetic acids, HAAs)[5,6,13–15]. These compounds are produced from ring cleavage reactions and are the only organic DBPs currently regulated in drinking water in the USA and many other countries[16,17]. Regulation of THMs and HAAs in drinking water is based on their association with bladder cancer and other chronic diseases, as well as their role as surrogates for other unregulated DBPs[18–20]. However, recent studies have indicated that the (cyto)toxicity of chlorinated water can only be partially (~16%) explained by C_1-DBPs and C_2-DBPs[21,22]. These findings suggest the importance of investigating previously unidentified DBPs,

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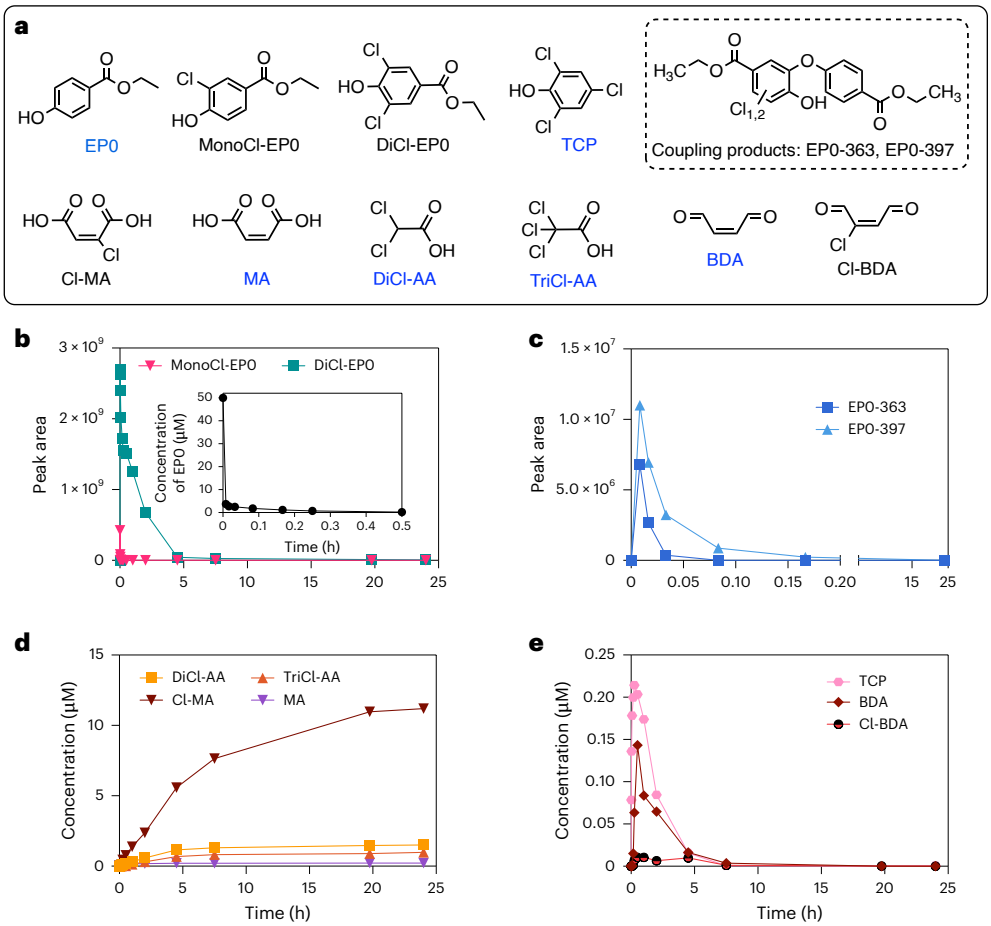

Fig. 1 | Structures of EP0 and its DBPs, along with their time-dependent formation profiles. a, Chemical structures of EP0 and its DBPs detected in reactions with excess free chlorine in borate buffer at pH 8.0. **b**, Evolution of the concentration of EP and the formation of monochloro- (MonoCl-EP0) and dichloro-ethylparaben (DiCl-EP0) over time. As no reference standards were available for MonoCl-EP0 and DiCl-EP0, the peak areas are shown instead of concentrations for the chlorophenol by-products. **c**, The evolution of coupling products EP0-363 and EP0-397 over time. Their detection indicates the formation of phenoxy radicals in the initial phase of the chlorination reaction. As no reference standards were available for these compounds, the peak areas are shown instead of concentrations. **d**, The evolution of the concentrations of C_2-carboxylic acids (DiCl-AA and TriCl-AA) and C_4-carboxylic acids (Cl-MA and MA) over time. **e**, The evolution of the concentrations of TCP and α,β-unsaturated dialdehydes BDA and Cl-BDA over time.

particularly those with more than two carbon atoms[21,23,24]. Motivated by this knowledge gap, recent studies have revisited phenol chlorination and uncovered that, in addition to THMs and HAAs, four-carbon-atom C_4-DBPs are formed, including α,β-unsaturated C_4-dialdehydes and C_4-dicarboxylic acids[23,24]. The α,β-unsaturated C_4-dialdehydes are of particular relevance owing to their cytotoxicity and genotoxicity, resulting from their high reactivity towards bio-nucleophiles, such as proteins and DNA[25–29].

Despite decades of research on the formation of DBPs upon chlorination of phenols, there is a substantial lack of mechanistic understanding of the associated ring cleavage pathways. After the initial identification of THMs as the first-reported DBPs in chlorinated drinking water by Rook in 1974, the same author identified resorcinol as a major THM precursor due to its high chloroform yield[14,30]. Subsequent research conducted by Boyce and Horning in 1983 confirmed via stable isotope labelling that THMs originated from the activated C_2 position of 1,3-dihydroxybenzene[15]. However, for other phenols, chloroform only accounts for a small portion (~10%) of the DBPs generated during the chlorination process[6]. For the C_2-HAAs, dichloroacetic acid (DiCl-AA) and trichloroacetic acid (TriCl-AA) are of particular concern due to their predominance over other halogenated species under typical drinking water conditions[31,32]. TriCl-AA is formed in higher yield (~20%) than DiCl-AA (~2% yield) during chlorination of model phenols[33,34]. TriCl-AA does not readily form from DiCl-AA through further chlorine substitution[16,32,35], suggesting that the co-occurrence of DiCl-AA and TriCl-AA probably arises from different intermediates and reaction pathways which are not well understood[32,36]. For the recently identified C_4-DBPs, C_4-dicarboxylic acids (in particular, 2-chloromaleic acid (Cl-MA)) are the predominant ring cleavage products and exhibit a higher yield compared with chloroacetic acids[24]. Although produced in relatively lower amounts, the α,β-unsaturated C_4-dialdehydes (mainly *cis*-2-butene-1,4-dial (BDA)) are important due to their known toxicity[23,24]. Considering that BDA does not contain chlorine, its formation cannot be explained by the reaction pathways proposed in the existing literature.

To systematically elucidate the ring cleavage pathways leading to the formation of C_2-HAAs and C_4-DBPs during chlorination of phenols, a series of ^{13}C-labelled ethylparabens were utilized as model phenols[37] (Fig. 1a). The choice of ethylparaben allowed for the study of substituent effects on the formation of ring cleavage products. Ethylparaben is of environmental relevance due to its widespread use as an antimicrobial preservative in many products and its frequent detection in the aquatic environment[37–40]. Furthermore, ethylparaben is recognized as an estrogenic disruptor and has been linked with breast cancer and other adverse health outcomes[38–40]. In addition, ethylparaben serves as a model compound for NOM, noting that hydroxy-benzoic acids

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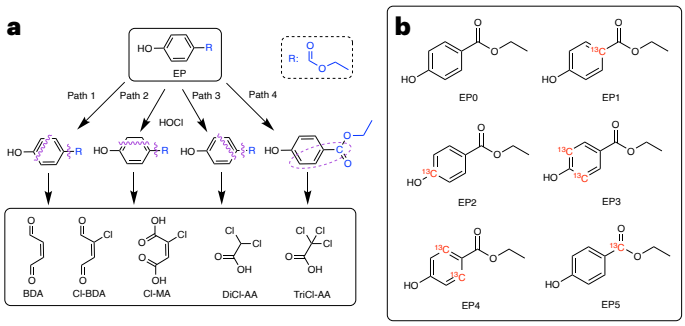

Fig. 2 | Ethylparaben ring cleavage pathways and ^{13}C-labelled ethylparaben species used in this study. a, The potential phenolic ring cleavage pathways leading to the formation of C_2-DBPs and C_4-DBPs in the reactions with free chlorine. **b**, The structures of unlabelled and ^{13}C-labelled ethylparabens (EP, EP0–EP5) investigated in this study. The synthetic routes and characterization of the ^{13}C-labelled ethylparabens are detailed in Methods.

and their esters are common constituents of NOM[41,42]. Thus, the use of ethylparaben also provides insights into DBP formation during chlorination of NOM present in drinking water.

While chlorination of (unlabelled) ethylparaben (EP0) has been studied previously, limited attention has been given to identifying DBPs beyond initially formed chlorinated ethylparaben species[38,40,43]. Most importantly, by employing ^{13}C-labelling at different positions of the ethylparaben molecule, in combination with liquid chromatography–high-resolution mass spectrometry (LC–HRMS) analysis, we tracked the pathways of the different carbon atoms from their phenol precursors into DBPs. Using MS/MS (MS^2) fragment data, we were further able to determine the position of ^{13}C labels in the identified C_2-DBPs and C_4-DBPs DBPs and derive the ring cleavage reaction pathways leading to their formation. Furthermore, by conducting a quantitative comparison of the distinct ^{13}C-labelled C_2-DBPs and C_4-DBPs, we were able to assess the contribution of each pathway to the formation of C_2-DBPs and C_4-DBPs. These findings provide novel insights into the intricate reaction mechanisms and potential intermediates involved in the chlorination of phenols, revealing previously unknown details about the formation of DBPs from phenolic precursors.

Characterization of DBPs

We employed LC–HRMS and a novel reactivity-directed analysis approach to identify DBPs formed upon chlorination of ethylparaben[23,27,44]. In total, 15 DBPs were identified, including the C_2-DBPs and C_4-DBPs of interest (Fig. 1a) (see Supplementary Table 2 and Supplementary Figs. 5–21 for additional mass spectrometry details). Of the 15 DBPs, two chloroacetic acids (DiCl-AA and TriCl-AA), two C_4-dicarboxylic acids (Cl-MA and maleic acid (MA)) and two α,β-unsaturated C_4-dialdehydes (BDA and 2-chloro-BDA (Cl-BDA)) were detected and further confirmed by comparison of LC retention time and mass spectrometry data with commercial reference standards. Analysis of α,β-unsaturated C_4-dialdehydes, which cannot be directly detected by LC–HRMS, was achieved through detection of their corresponding *N*-α-acetyl-lysine adducts, as previously reported[23,24,44,45]. The identity of C_4-dicarboxylic acid Cl-MA was confirmed by comparing its MS^2 spectrum with that of 2-chlorofumaric acid standard (Cl-FA, a *trans* analogue of Cl-MA, not detected as a DBP in this study).

Among the C_2-DBPs and C_4-DBPs, which were the primary focus of this study, the concentration of Cl-MA continuously increased over the course of the 24 h reaction time, while the others reached a plateau around 5 h (Fig. 1d). In contrast, the α,β-unsaturated C_4-dialdehyde BDA reached its peak concentration within the first hour of the reaction, followed by a gradual decline (Fig. 1e). Cl-BDA exhibited a similar formation trend to BDA but at substantially lower concentrations. The distinct temporal trends observed for these C_2-DBPs and C_4-DBPs suggest that they are generated via separate reaction pathways. In terms of the overall mass balance, the maximum molar yields of Cl-MA, BDA, DiCl-AA and TriCl-AA from ethylparaben within a 24 h reaction time were 21.29%, 0.33%, 3.04% and 1.96%, respectively, which collectively accounted for approximately 30% of the molar mass balance. Yields of MA and Cl-BDA were very low (<0.1%). The higher yield of DiCl-AA compared with TriCl-AA in ethylparaben chlorination is consistent with results of methylparaben chlorination observed previously[24]. The yields of Cl-MA and BDA are known to vary significantly depending on the substituent type on the phenolic ring[23,24]. Additional chlorination experiments were conducted with BDA, MA, Cl-FA, DiCl-AA and TriCl-AA to assess the mutual independence of these C_2-DBPs and C_4-DBPs. The results confirm that none of these ring cleavage products were transformed into any of the other DBPs detected in this study (Supplementary Fig. 22).

In addition to the C_2-DBPs and C_4-DBPs, formation of aromatic DBPs was observed, including monochloro-ethylparaben, dichloro-ethylparaben and 2,4,6-trichlorophenol (TCP) (Fig. 1b). Small ion peaks identified as ethylparaben coupling products (EP0-363 and EP0-397), most probably attributable to the formation of phenoxy radicals[24,46,47], were observed only during the first 5 min sampling period (Fig. 1c). Each of these ring-intact compounds exhibited rapid formation within the first few minutes/hours, followed by subsequent decay, indicating further transformation into other DBPs. Apart from these DBPs, in which the phenolic ring remains intact, we observed the presence of oxygen-rich products with six or nine carbons formed via ring cleavage. The MS^2 spectra for these products are shown in Supplementary Figs. 14–18. The temporal trends in the peak area of these products showed an increase over the entire duration of the experiments, which can partially explain the gap in the molar mass balance of ethylparaben's C_2-ring and C_4-ring cleavage products, as illustrated in Supplementary Fig. 23.

Identifying ring cleavage pathways

Considering the symmetrical structure of the C_6-phenolic ring in ethylparaben, we initially hypothesized four ring cleavage pathways that could contribute to the formation of C_2-DBPs and C_4-DBPs (Fig. 2a). Pathways 1–3 result in by-products that only include carbons originating from the aromatic ring of ethylparaben, while pathway 4 also includes the carboxylic ester carbon. For pathway 1, reaction of ethylparaben with chlorine results in the cleavage of the aromatic ring between the OH-substituted carbon and the *ortho* carbon. Similarly, for pathway 2, ring cleavage occurs between the *ortho* carbon and the *meta* carbon. For pathway 3, cleavage occurs between the *meta* carbon and the *para* carbon. Each pathway can conceivably yield C_2-DBPs and C_4-DBPs (Fig. 2a, wavy purple lines). Additionally, the carboxylic ester carbon *para* to the OH-group in ethylparaben could conceivably participate in the formation of C_2-DBPs and C_4-DBPs (Fig. 2a, dotted purple circle), which is indicated by pathway 4.

To further investigate the role of the different ring cleavage pathways in the formation of the C_2-DBPs and C_4-DBPs, we used a series of ^{13}C-labelled ethylparabens (Fig. 2b and Supplementary Table 1). Based on the ^{13}C-labelling of the parent ethylparaben (EP1–5) (Supplementary Table 3), the ^{13}C-labelling of the detected C_2-DBPs and C_4-DBPs was utilized to link their formation to one (or more) of the four hypothesized ring cleavage pathways depicted in Fig. 1b. MS^2 spectra information further allowed us to determine the position of the ^{13}C-labells in the formed DBPs[48,49] (Supplementary Figs. 19–21 and 30–33).

For DiCl-AA, both its unlabelled and ^{13}C-labelled species were observed during the chlorination of all ethylparabens. Notably, ^{13}C-DiCl-AA was found to be the major species in EP5 chlorination, revealing that the ^{13}C-ester carbonyl carbon in the EP5 substituent was involved in the formation of DiCl-AA via pathway 4 (Fig. 1b and Supplementary Fig. 30a). Moreover, the MS^2 spectrum of this ^{13}C-DiCl-AA

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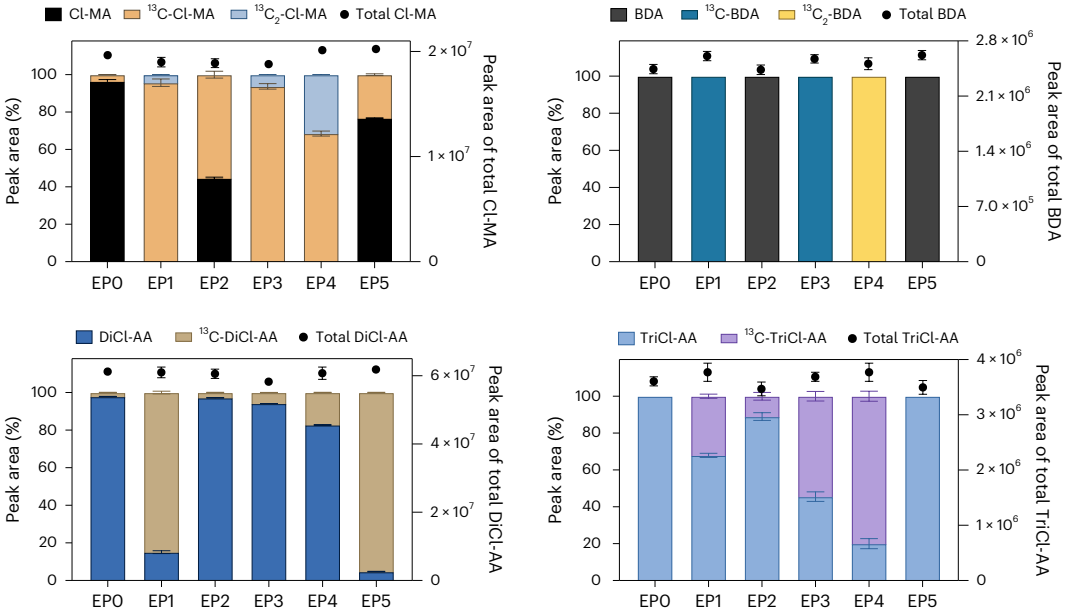

Fig. 3 | Isotopic distribution and consistency of ^{13}C-labelled C_2-DBPs and C_4-DBPs across all ethylparabens. The percentage (left *y* axis) of each ^{13}C-labelled species for Cl-MA, BDA, DiCl-AA and TriCl-AA to the total amount of all species. The total peak areas of all species for each C_2-DBP and C_4-DBP (right *y* axis) show that the amount of each by-product is consistent across all ethylparaben isomers. The data are presented as mean values ± standard deviations of triplicates. It should be noted that the small fraction of ^{13}C-DiCl-AA observed in EP0 chlorination, accounting for 2.1 ± 0.1% of the total, is attributable to the natural abundance of ^{13}C, which is approximately 1.1% per carbon atom (2 × 1.1% for DiCl-AA molecule with two carbons). Likewise, the presence of ^{13}C-DiCl-AA in EP2 chlorination (2.8 ± 0.2%), the small peak of ^{13}C-Cl-MA in EP0 chlorination (3.7 ± 0.2%) and the presence of $^{13}C_2$-Cl-MA in EP1 chlorination (4.2 ± 0.1%) and EP3 chlorination (6.3 ± 0.3%) can be attributed to the natural abundance of ^{13}C in the molecules. The absence of ^{13}C-BDA attributable to ^{13}C natural abundance across all ethylparabens can be explained by the low abundance of BDA. For TriCl-AA, the anticipated formation of ^{13}C-TriCl-AA, resulting from the natural abundance of ^{13}C, was not observed in experiments with EP0 and EP5, which can be attributed to the low abundance of TriCl-AA species in high-resolution mass spectrometry due to ion-source fragmentation. This is further supported by the notably smaller peak area of TriCl-AA compared with DiCl-AA in the standard samples of identical concentration, along with the observation of a significant peak representing an HRMS ion-source fragment of TriCl-AA generated via cleavage of CO_2.

indicates that the ester moiety of ethylparaben is transformed into the carboxylic acid moiety in DiCl-AA (Supplementary Fig. 30a), a mechanism not previously reported. Furthermore, the observation of unlabelled DiCl-AA in EP1 chlorination can be attributed to pathway 1 and/or 2, while the detection of ^{13}C-DiCl-AA in EP4 chlorination can be attributed to pathway 2 and/or 3 (Supplementary Fig. 30b). Thus, pathway 2 consistently emerges as the pathway for DiCl-AA formation, wherein both carbons are derived from the phenolic ring. Overall, pathways 2 and 4 are the two ring cleavage pathways leading to the formation of DiCl-AA during the chlorination of ethylparaben. This conclusion is further supported by the results from EP2 and EP3, as detailed in Supplementary Fig. 30c.

For TriCl-AA, its unlabelled species were observed during the chlorination of all ethylparabens, whereas the ^{13}C-labelled species were observed for all ethylparabens except EP5. The absence of ^{13}C-TriCl-AA in EP5 chlorination indicates that the ester substituent cannot be converted into the carboxylic acid moiety of TriCl-AA via pathway 4. Consequently, pathways 1, 2 and 3 are responsible for the formation of TriCl-AA. Results from EP1–EP4 also corroborate this conclusion (Supplementary Fig. 31).

For Cl-MA, varied combinations of three species containing zero to two ^{13}C-labelled carbon atoms were detected for the different ethylparabens. Similar to DiCl-AA, ^{13}C-Cl-MA was observed as the minor species upon chlorination of EP5, indicating that the ^{13}C-carbonyl carbon in EP5 was involved in the formation of Cl-MA via pathway 4. However, the detection of unlabelled Cl-MA as the major species from EP5 chlorination indicates that other ring cleavage pathways (pathways 1, 2 and 3) also contribute to formation of Cl-MA. To further clarify the role of these pathways, we also examined the results obtained for the other ^{13}C-labelled ethylparabens. For EP4, the formation of $^{13}C_2$-Cl-MA suggests the involvement of pathway 1, while the formation of ^{13}C-Cl-MA as the major species suggests the involvement of pathways 2 and/or 3 (Supplementary Fig. 32a). Furthermore, the MS^2 spectrum of ^{13}C-Cl-MA from EP4 showed the cleavage of unlabelled CO_2 from its precursor, which supports the location of the ^{13}C-label at the alkene moiety rather than at the carboxylic acid group (Supplementary Fig. 32a). Overall, this suggests that pathway 3 is not involved in the formation of ^{13}C-Cl-MA, and its formation is rather attributed to pathways 1, 2 and 4. This conclusion is supported by results from EP1–EP3 (Supplementary Fig. 32b).

For BDA, species containing zero, one or two ^{13}C-labelled carbon atoms were detected for different ethylparabens. For EP5, the absence of ^{13}C-BDA indicates that the carbonyl carbon in the substituent does not participate in the formation of BDA, thus eliminating pathway 4 as a possible pathway. In the case of EP4, the observation of $^{13}C_2$-BDA as the sole species indicates that pathway 1 is the only pathway leading to the formation of BDA. This conclusion is further supported by the results from EP1–EP3 (Supplementary Fig. 33).

Based on the obtained results, we determined the ring cleavage pathways responsible for the formation of the C_2-DBPs and C_4-DBPs (summarized in Supplementary Table 8). To assess the relative contribution of the involved ring cleavage pathways to the formation of the C_2-DBPs and C_4-DBPs, we compared the abundances (that is, peak areas) of the ^{13}C-labelled C_2-DBP and C_4-DBP species observed during the chlorination of each ethylparaben (Supplementary Tables 8–11). Furthermore, for comparative analysis across all ethylparabens, we calculated the percentage of abundance for each ^{13}C-labelled DBP species relative to the total abundance of all DBP species at the peak concentration (Fig. 3 and Supplementary Tables 8–11). Although the relative

Table 1 | Contribution of each pathway to the formation of Cl-MA, BDA, DiCl-AA and TriCl-AA

C_2-DBPs and C_4-DBPs	Average contribution (%)			
	Pathway 1	Pathway 2	Pathway 3	Pathway 4
Cl-MA	22 (20–26)	55 (53–60)	–	23 (21–27)
BDA	100	–	–	–
DiCl-AA	–	10 (4–16)	–	90 (85–96)
TriCl-AA	15 (9–20)	47 (33–60)	38 (31–46)	–

The values presented are average contributions calculated from the results of all five ethylparabens; the numbers in parentheses indicate the range.

abundance of ^{13}C-labelled C_2-DBP and C_4-DBP species varied among different ethylparabens, the total abundance of each ring cleavage product was similar for all ethylparabens (Fig. 3). These results are consistent with the notion that the ionization efficiency of isotope-labelled compounds is unaffected by stable isotope labelling[48,50,51] and further indicate that the introduction of ^{13}C-labels in the phenolic ring, as expected, did not appreciably affect the ring cleavage reactions involved in the formation of the C_2-DBPs and C_4-DBPs. Thus, these percentages represent the contribution of each distinct ring cleavage pathway to the formation of the C_2-DBPs and C_4-DBPs during ethylparaben chlorination (Supplementary Table 13). Additionally, the percentages were adjusted to account for the natural abundance of ^{13}C (Supplementary Table 14). To determine the contribution of each pathway, the results from each ethylparaben can be considered as independent observations. Consequently, the collective results from all ethylparabens established a system of multivariate linear equations. Here, the contributions of each pathway (Fig. 2a) to the formation of Cl-MA, BDA, DiCl-AA and TriCl-AA were treated as variables with binary coefficients of 0 (indicating no contribution) or 1 (indicating contribution), and the relative abundance of ^{13}C-labelled C_2-DBP and C_4-DBP species from each ethylparaben served as the constant. Detailed calculations are provided in Supplementary Text 6. The solutions derived from these equations unveiled the contribution range of each pathway to each C_2-DBP and C_4-DBP (Table 1).

Insights into the mechanism of phenolic ring cleavage

The quantitative assessment of the contributions of different ring cleavage pathways to the formation of C_2-DBPs and C_4-DBPs during ethylparaben chlorination, combined with the detection of the 11 larger molecular weight DBPs (Supplementary Figs. 13–18 and Supplementary Table 2), allowed us to gain insights into the underlying ring cleavage reaction mechanisms. For DiCl-AA, approximately 90% of the total yield of DiCl-AA in ethylparaben chlorination can be attributed to pathway 4, and the remaining 10% is from pathway 2 (Table 1). In contrast, for TriCl-AA, the contributions of pathways 1, 2 and 3 to its total yield were approximately 15%, 47% and 38%, respectively, whereas the contribution from pathway 4 was negligible. These results support the critical role of the carboxylic ester group in ethylparaben for DiCl-AA formation via pathway 4. To further validate this finding, we performed additional chlorination experiments with ^{13}C-labelled 4-hydroxybenzoic acid, ^{13}C-4HBA (^{13}C-labelling at the carboxylic acid carbon). We observed that only unlabelled DiCl-AA was formed, which indicates that pathway 4 is only relevant for phenols with an ester functional group as the *para* substituent (for example, parabens) but not for the corresponding carboxylic acid (4HBA). It is also worth noting that significantly higher yields of TriCl-AA compared with DiCl-AA have been observed during chlorination of many other phenolic compounds with diverse *para* substituents[33,34]. This contrasts with the higher yield of DiCl-AA compared with TriCl-AA for ethylparaben (and methylparaben) chlorination[24]. The findings presented here provide an explanation for this discrepancy and highlight the importance of pathway 4 for the formation of DiCl-AA during chlorination of parabens. In contrast, chlorination of the carboxylic acid analogue 4HBA proceeds via sequential chlorination of the aromatic ring leading to the formation of TCP followed by ring cleavage via pathways 1–3. This is also supported by the low TCP yields (<0.5%) during ethylparaben chlorination observed in this study.

For DiCl-AA, previous studies have identified the β-dicarbonyl acid ester moiety (for example, ethyl acetoacetate) as an important precursor, in which one of the carbonyl groups is a ketone and the other is an ester group[33]. Our results demonstrate that DiCl-AA formed via pathway 4 consists of the *para* carbon with two attached chlorine atoms and the carboxylic ester moiety that undergoes conversion into the carboxylic acid group. Hence, for DiCl-AA formation through pathway 4, the intermediate probably contains a β-dicarbonyl acid ester structure, with a ketone group at a carbon atom associated with the *meta* position before ring cleavage (for example, intermediate 15 in Fig. 4). Conversely, the chlorination of *para*-substituted phenols is known to be initiated by electrophilic substitution on one of the *ortho* carbons, resulting in the formation of mono- and di-chlorophenols. Therefore, for pathway 2, in which the *ortho* carbon and *meta* carbon are transformed into DiCl-AA, the *ortho* carbon is bonded to two chlorine atoms while the *meta* carbon is converted into a carboxylic acid group, producing intermediates resembling structure 13 in Fig. 4 (ref. 52).

For TriCl-AA, the only currently known formation mechanisms during phenol chlorination, to our knowledge, is derived from the chlorination of resorcinol[15,53]. Thus, this study provides novel insights into the formation mechanisms of TriCl-AA from (*para*-)substituted phenols. Based on our findings, the intermediate leading to TriCl-AA probably contains a ketone group positioned *meta* to the phenolic hydroxy group. This observation suggests the possibility of a 1,3-diketone structure, which could potentially serve as an intermediate and undergo further ring cleavage (for example, 8 → 9 and 12 → 14; Fig. 4)[13,33], thus forming TriCl-AA via pathway 1. Furthermore, as established above, TriCl-AA formed via pathway 2 consists of the *meta* carbon in phenol with three attached chlorine atoms and the *ortho* carbon that undergoes conversion into a carboxylic acid group. This indicates oxidation of the *ortho* carbon, resulting in the formation of intermediates resembling structure 5 (ref. 52) (Fig. 4). Regarding the TriCl-AA formation from pathway 3, both the *meta* and *para* carbons can either be attached to three chlorine atoms or be part of the carboxylic acid group. This corresponds to the oxidation of the *meta* carbon, resulting in intermediates resembling structure 14 and the oxidation of the *para* carbon, resulting in intermediates resembling structure 4, respectively.

As for Cl-MA, the contributions of pathways 1, 2 and 4 were 22%, 55% and 23%, respectively. Although Cl-MA is a major product of TCP oxidation by H_2O_2 in catalytic systems[54,55], its formation under chlorination conditions has rarely been reported[24,56], and the underlying mechanism remains largely unknown. Our results indicate that, similar to DiCl-AA, pathway 4 also contributes to the formation of Cl-MA during ethylparaben chlorination. This observation indicates that oxidation occurs at the *ortho* carbon in intermediate 2 (Fig. 4), converting it into a carboxylic acid group. Furthermore, for Cl-MA formation via pathway 1, both the *ortho* carbon and its non-adjacent *meta* carbon can be converted into carboxylic acid groups, indicating intermediates resembling structures 8 and 10 (refs. 57,58) (Fig. 4). As for pathway 2, the OH-attached carbon and *para* carbon undergo conversion into the carboxylic acid groups, indicating intermediates resembling structure 17 (refs. 57,58) (Fig. 4).

For BDA, its formation can only be attributed to pathway 1, consistent with oxidation of both the *ortho* carbon and its non-adjacent *meta* carbon into aldehydes through intermediates resembling structure 9 in Fig. 4. Several of the proposed intermediates were not detected, probably due to their instability and/or incompatibility with LC–HRMS detection. The structures of

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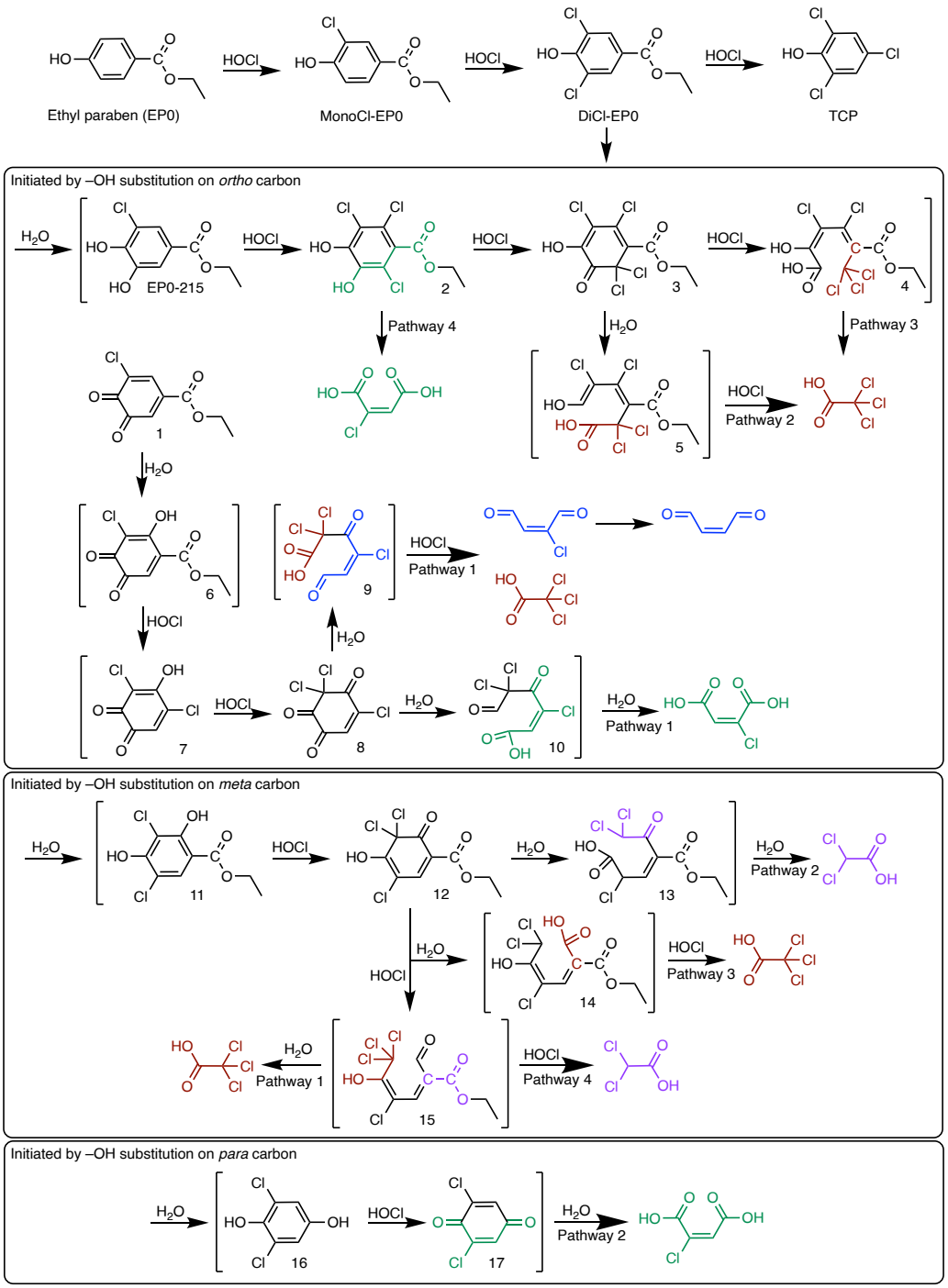

Fig. 4 | Proposed ring cleavage pathways including postulated intermediates for chlorination of ethylparaben. The larger arrows indicate the dominant pathways for DBP formation. Further details on the reaction pathways can be found in Supplementary Text 7.

these postulated intermediates share similarities with the C_6- and C_9-oxygen-rich ring cleavage products detected in this study. Notably, the analysis revealed EP0-215 (Fig. 4) as a 1,2-dihydroxybenzene, susceptible to rapid oxidation yielding a 1,2-benzoquinone (structure 1 in Fig. 4) under the employed conditions[59,60]. To further validate the role of benzoquinone as an intermediate, we conducted additional chlorination experiments with 3,5-dichloro-1,2-dihydroxybenzene and 2,6-dichloro-1,4-benzoquinone. We used 3,5-dichloro-1,2-dihydroxybenzene instead of 3,5-dichloro-1,2-benzoquinone, as the latter was unavailable as a reference standard. Moreover, the 3,5-dichloro-1,2-dihydroxybenzene probably undergoes oxidation, thus resulting in the formation of 3,5-dichloro-1,2-benzoquinone[59,60]. Chlorination of both compounds produced DiCl-AA, TriCl-AA and Cl-MA, which supports the notion that 1,2-benzoquinones and 1,4-benzoquinones serve as intermediates in the formation of these ring cleavage products during chlorination of phenols (Supplementary Fig. 34). More importantly, a high yield of BDA was observed only during chlorination of 3,5-dichloro-1,2-dihydroxybenzene, which supports our proposal that 1,2-benzoquinone is an important intermediate for BDA formation through pathway 1. While recent studies have identified the formation of 2,6-dichloro-1,4-benzoquinone as a DBP during the chlorination of phenols, formation of the more

unstable DBPs, specifically 3,5-dichloro-1,2-benzoquinone, has not been reported in previous studies. This study represents the first evidence of 3,5-dichloro-1,2-benzoquinone as an important intermediate for the formation of α,β-unsaturated C_4-dialdehyde BDA and other C_2-DBPs and C_4-DBPs.

Conclusion

For decades, researchers have struggled to determine how aromatic compounds, particularly phenols, are transformed into lower-molecular-weight DBPs through ring cleavage mechanisms, including regulated HAAs. This study provides a detailed assessment of the pathways by which phenolic compounds form C_2-DBPs and C_4-DBPs, including DBPs of toxicological concern. The identification of four ring cleavage pathways provides a more comprehensive understanding of the fundamental mechanisms underlying the formation of C_2-DBPs and C_4-DBPs during phenol chlorination. Our findings provide direct evidence that DiCl-AA and TriCl-AA form through distinct reaction pathways, confirming previous inferences drawn from observations of differing formation patterns of these two DBPs during phenol chlorination[13,53,61,62]. The relevance of pathway 4 (transformation of an ester group into a carboxylic acid, yielding DiCl-AA and Cl-MA) highlights the significant impact of substituents on the ring cleavage reactions. Lastly, while recent studies have primarily investigated the formation of chlorinated 1,4-benzoquinones as DBPs during the chlorination of phenols, this study demonstrates that chlorinated 1,2-benzoquinones are probably key intermediates leading to the formation of α,β-unsaturated C_4-dialdehydes. Discerning these reaction pathways can assist in recognizing relevant precursors, such as NOM, which are critical to understanding the formation of toxic DBPs. Collectively, these insights underscore the intricate interplay between phenolic compound transformations and DBP formation in chlorinated waters.

To more fully understand the formation of toxic DBPs, future studies should focus on characterizing the (trans)formation of postulated intermediates, such as chlorinated 1,2-benzoquinones. Our results also highlight the need to look beyond the currently regulated HAAs and THMs to determine the potential environmental and human health effects associated with exposure to C_4-DBPs and other non-regulated DBPs. For some of the DBPs, such as α,β-unsaturated aldehydes that are difficult to detect via existing methods, novel analytical approaches should be pursued. Solid-phase reactivity-directed extraction (SPREx)[63] as well as mixed-mode or HILIC columns for LC–HRMS separation[64], in combination with gas chromatography–mass spectrometry, show great potential in addressing existing analytical limitations. Lastly, future research should expand the use of isotopically labelled compounds (including ^{13}C-labelled phenols) to further elucidate by-products formed during disinfection and other oxidative treatment approaches aiming at the removal of organic contaminants.

Methods

Synthesis of ^{13}C-labelled ethylparabens

The ^{13}C-labelled ethylparabens EP1–EP3 were synthesized in the laboratory with detailed procedures described in our previous publication[37]. Synthesis procedures for EP4, EP5 and the ^{13}C-labelled 4-hydroxybenzoic acid are shown in Supplementary Fig. 1, with elaboration provided in Supplementary Text 1. The identities of these synthesized ^{13}C-labelled compounds were confirmed by both nuclear magnetic resonance (NMR) and LC–HRMS analysis. The characterization data for EP1–EP3 were previously published. The NMR spectra for EP4, EP5 and ^{13}C-4HBA are provided in Supplementary Figs. 2–4, and the corresponding LC–HRMS data can be found in Supplementary Tables 2 and 3.

Chlorination experiments

The experiments were performed in amber vials on a magnetic stirring plate at room temperature (20 ± 3 °C). To identify the complex ring cleavage products and reveal the fate of the carbons in phenolic ring, chlorination experiments of both the unlabelled and ^{13}C-labelled ethylparabens with fixed initial molar concentrations of HOCl and ethylparaben at 500 μM and 50 μM, respectively, were conducted. The experiments were performed in ultrapure water buffered at pH 8.0 ± 0.2 using 10 mM borate.

For all these experiments, before initiating the experiment by adding HOCl to the mixed solution, an aliquot (1 ml) was transferred into an HPLC vial containing sodium thiosulfate as quencher (2 μl, final concentration 2.5 mM). This control sample was used to determine the initial concentration of ethylparaben and to validate that the identified transformation products were only attributable to the reaction of HOCl with ethylparaben. After addition of HOCl, aliquots were collected in regular intervals over 24 h and were treated the same as the control sample. For the analysis of α,β-unsaturated C_4-dialdehydes, 5 μl of *N*-α-acetyl-lysine was added (final concentration 500 μM, equivalent to ten times the initial concentration of ethylparaben)[23,44], and the samples were incubated for 24 h at room temperature before LC–HRMS analysis. All other transformation products were directly analysed by LC–HRMS. To assess the mutual independence of the C_2-DBPs and C_4-DBPs, the same chlorination experiments were conducted using BDA, MA, Cl-FA, DiCl-AA and TriCl-AA as a parent compound, respectively. Additionally, to substantiate the proposed mechanisms, the same chlorination experiments were conducted using ^{13}C-4HBA, 3,5-dichlorocatechol and 2,6-dichloro-1,4-benzoquinone as parent compound, respectively.

LC–HRMS analysis

Chromatographic separation was achieved using a Phenomenex Synergi Hydro-RP column (4 μm, 80 Å, 1 × 150 mm) with eluents of ultrapure water containing 0.1% (v/v) formic acid (A) and methanol (B). The gradient method was 0–3 min, 90% A; 3–12 min, linear decrease from 90% to 5% of A; 12–14 min, 5% A; 14.1 min, 90% A, total run time 20 min. The flow rate was 75 μl min^{-1}, and the column oven temperature was set to 25 °C. The sample volume was set to 10 μl. Negative ESI mode was used for all compounds as preliminary experiments revealed no additional TPs in positive mode. The ESI source parameters were set as follows: sheath gas flow rate of 20 a.u., auxiliary gas flow rate of 10 a.u., spray voltage of 2.5 kV for negative and 3.8 kV for positive, capillary temperature of 250 °C, S-lens RF level of 60 and auxiliary gas heater temperature of 100 °C.

Data-dependent acquisition was used to conduct MS^2 experiments as follows: a full scan (50–750 *m*/*z*, resolution >120,000; negative mode) was performed followed by data-dependent MS^2 for the ten most intense ions with resolution of >60,000. Collision-induced dissociation with stepped normalized collision energy of 10%, 30% and 50% was used for fragmentation with an isolation window of 1.0 *m*/*z*.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the paper and its Supplementary information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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Author contributions

C.P. conceived and supervised the project. Z.Z. performed the chlorination experiments, with the help of L.Z., as well as all laboratory analyses. K.P.R., N.O., P.D.G. and M.V. synthesized the ^{13}C-labelled ethylparabens and performed NMR analysis of synthesized compounds. C.P., J.D.S., K.P.R. and D.L.M. were involved in the interpretation of data. The paper was written by Z.Z. with contributions from C.P., J.D.S., K.P.R. and D.L.M.

Competing interests

The authors declare no competing interests.

Additional information

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Data collection	Data was collected using high-resolution mass spectrometry as described in the methods section
Timing and spatial scale	Laboratory experiments conducted over the duration of up to 24h
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Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.