Supporting Information for:

Novel Chlorination Byproducts of Tryptophan: Initial High-Yield Transformation Products Versus Small Molecule DBPs

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Text S1: Materials

N-Acetyltryptophan (99%), N-acetylalanine (99%), sodium phosphate monobasic dihydrate (99%), sodium phosphate dibasic (99%), methyl tert-butyl ether (99.5%), L-ascorbic acid (≥ 99%), and sodium thiosulfate (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hypochlorite (~6%, w/v), methanol (≥ 99.9%, Optima™ grade), acetonitrile (≥ 99%, Optima™ LC/MS grade), and formic acid (88%) were purchased from Fisher Scientific (Waltham, MA, USA). Deionized water (electrical resistivity >18.0 MΩ cm; Milli-Q purification system; Millipore) was used in all experiments.

Text S2: Analytical details for NacTrp and initial byproducts

NacTrp and its initial chlorination byproducts were measured by low-resolution LC/MS (Agilent 1260 HPLC system coupled to a 6460 triple quadrupole mass spectrometer) using a 5 μL injection volume and separation on an Agilent Poroshell 120 EC-C18 column (3.0×50 mm, 2.7 μm) with 0.1% v/v formic acid (FA) in deionized water as eluent A and 90% acetonitrile/10% deionized water with 10 mM FA as eluent B. The overall flowrate was 0.4 mL/min. The elution profile (15 minutes total) was 98% solvent A and 2% solvent B held for 2 minutes, a linear gradient to 10% solvent A and 90% solvent B over 4 minutes and held for 3 minutes, a linear gradient to 98% solvent A and 2% solvent B over 2 minutes and held for 4 minutes. Mass spectrometry was conducted in single ion monitoring (SIM) negative ion mode with the nebulizing gas pressure set to 45 psi. The sheath gas temperature and flow were set to 250 °C and 9 L/min, respectively. The nozzle voltage was 500 V. The mass spectrometer capillary voltage was set to 3.5 kV. The drying gas temperature and flow rate were 300 °C and 7 L/min, respectively.

Purification of standards was conducted using an Agilent 1260 Infinity HPLC system equipped with a Clipeus C18 5 μm, 250 × 10 mm preparatory column (Higgins Analytical, Inc.) at 3 mL/min using deionized water with 0.1% formic acid and acetonitrile with 0.1% formic acid as mobile phases and detection at 230 nm; Table S1 provides the conditions employed to purify standards. The elution profile (50 minutes total) was 98% solvent A and 2% solvent B held for 6 minutes, a linear gradient to 10% solvent A and 90% solvent B over 24 minutes and held for 10 minutes, and a linear gradient to 98% solvent A and 2% solvent B over 5 minutes and held for 5 minutes.

Table S1. Purification conditions for novel DBPs

Byproduct	**261**	**277-1**	**277-2**	**277-3**	**295**	**329**
Chlorine:NacTrp[a]	1:1	3:1	3:1	3:1	3:1	3:1
RT (min)[b]	22-24	12-14	12-14	16-18	28-30	38-40
Impurity (%)[c]	5.60	3.38	3.38	7.50	8.83	5.63
R^2 [d]	0.9774	0.9932	0.9932	0.9938	0.9913	0.9972

[a] Molar ratio during chlorination conducted with concentrated NacTrp (5 mM) for 24 h contact
[b] Retention time interval for fraction collection from the preparatory HPLC system
[c] Percent impurity based on area counts measured by low-resolution LC/MS
[d] R^2 value for the linearity of standard curves

Text S3: Small molecule DBP analyses

Aliquots (50 mL) were treated with ascorbic acid to quench residual chlorine, and analyzed by gas chromatography mass spectroscopy for the chlorinated analogues of small molecule DBP classes, including one THM (trichloromethane), two haloacetonitriles (dichloroacetonitrile and trichloroacetonitrile), two haloacetamides (dichloroacetamide and trichloroacetamide), one haloacetaldehyde (trichloroacetaldehyde), one halonitromethane (trichloronitromethane), and two haloketones (1,1-dichloropropanone and 1,1,1-trichloropropanone) by modified US EPA Method 551.1 with ~0.2 μg/L method reporting limits, and three HAAs (chloroacetic acid, dichloroacetic acid and trichloroacetic acid) by modified US EPA Method 552.3 with ~0.2 μg/L method reporting limits.

Small molecule DBPs other than haloacetamides were analyzed using an Agilent 6890N gas chromatograph interfaced with an Agilent 6890 mass spectrometer in the electron ionization mode. Injections (2 μL) were splitless at an injection port temperature of 170 °C onto an Agilent HP-5MS column (30 m × 0.25 mm, 0.25 μm thickness). The oven was initially held at 60 °C for 2 min, ramping to 200 °C at 10 °C/min, then ramping to 280 °C at 40 °C/min and holding at 280 °C for 2 min. Haloacetamides were analyzed using an Agilent 7890A gas chromatograph interfaced with an Agilent 240 Ion Trap mass spectrometer in the methanol chemical ionization mode. Injections (2 μL) were splitless at an injection port temperature of 225 °C onto an Agilent DB-1701 column (60 m × 0.25 mm, 1.0 μm thickness). The oven was initially held at 65 °C for 4 min, ramping to 205 °C at 10 °C/min, holding for 7 min, then ramping to 280 °C at 40 °C/min and holding at 280 °C for 2 min.

For haloacetic acids, water samples (40 mL) were acidified with 1 mL of concentrated sulfuric acid to pH < 0.5, salted with ~12 g of oven-dried (105 °C for 2 h) anhydrous sodium sulfate, and extracted vigorously by hand for 2 min with 3 mL of MtBE containing 300 μg/L 1,2-dibromopropane as the internal standard. The recovered MtBE extracts were mixed with 3 mL of acidic methanol (with 10% v/v concentrated H_2SO_4). The mixtures were incubated at 55 °C for 2 h in a thermostatted water bath, washed with 5 mL of 150 g/L Na_2SO_4 solution, and were neutralized with 2 mL of saturated $NaHCO_3$ solution. The solvent layers were transferred and further concentrated to a final volume of ~0.5 mL with a nitrogen blowdown concentrator. Methylated haloacetic acids were analyzed on the Agilent 6890N GC and 5793N MS system described above. The oven was initially held at 35 °C for 10 min, ramping to 120 at 5 °C/min, then ramping to 280 at 59 °C/min and holding at 280 ºC for 2 min. Additional analytical details were provided previously (Chuang et al., 2019).

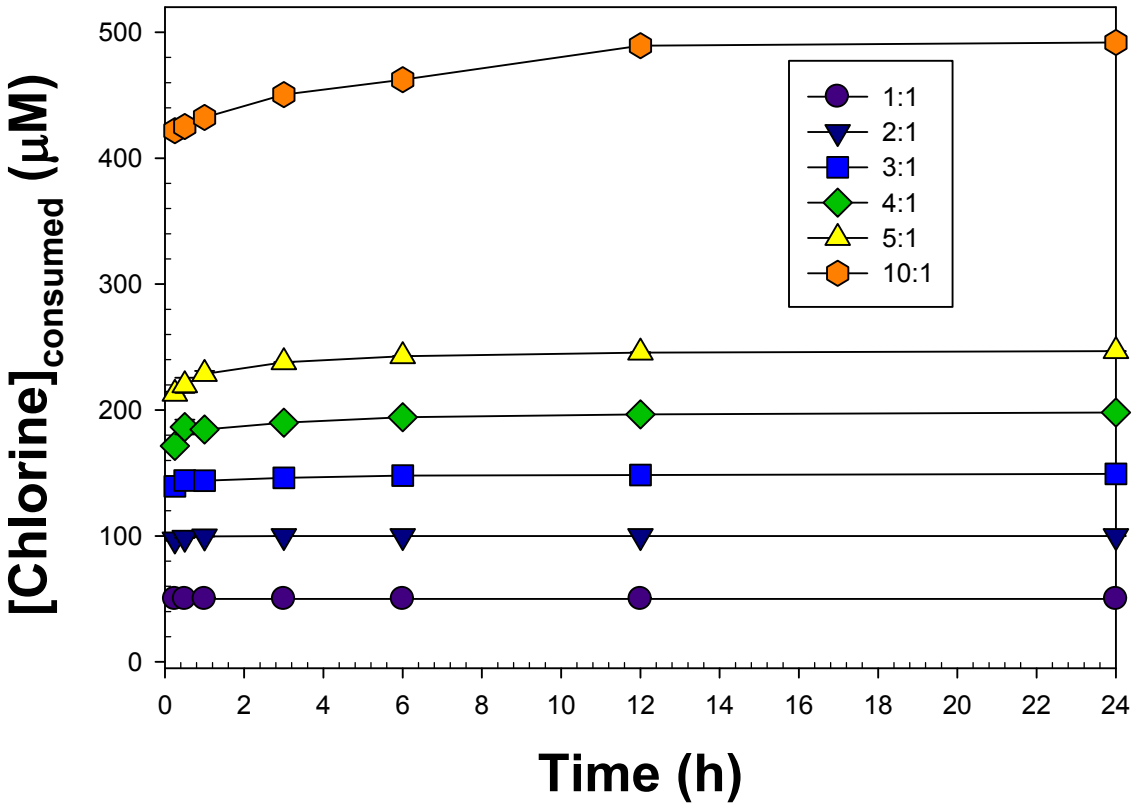


Figure S1: Consumption of the free chlorine residual between 15 min and 24 h after treatment of 50 µM NacTrp at pH 7.4 (10 mM phosphate) with chlorine at chlorine:NacTrp molar ratios of 1:1 up through 10:1.

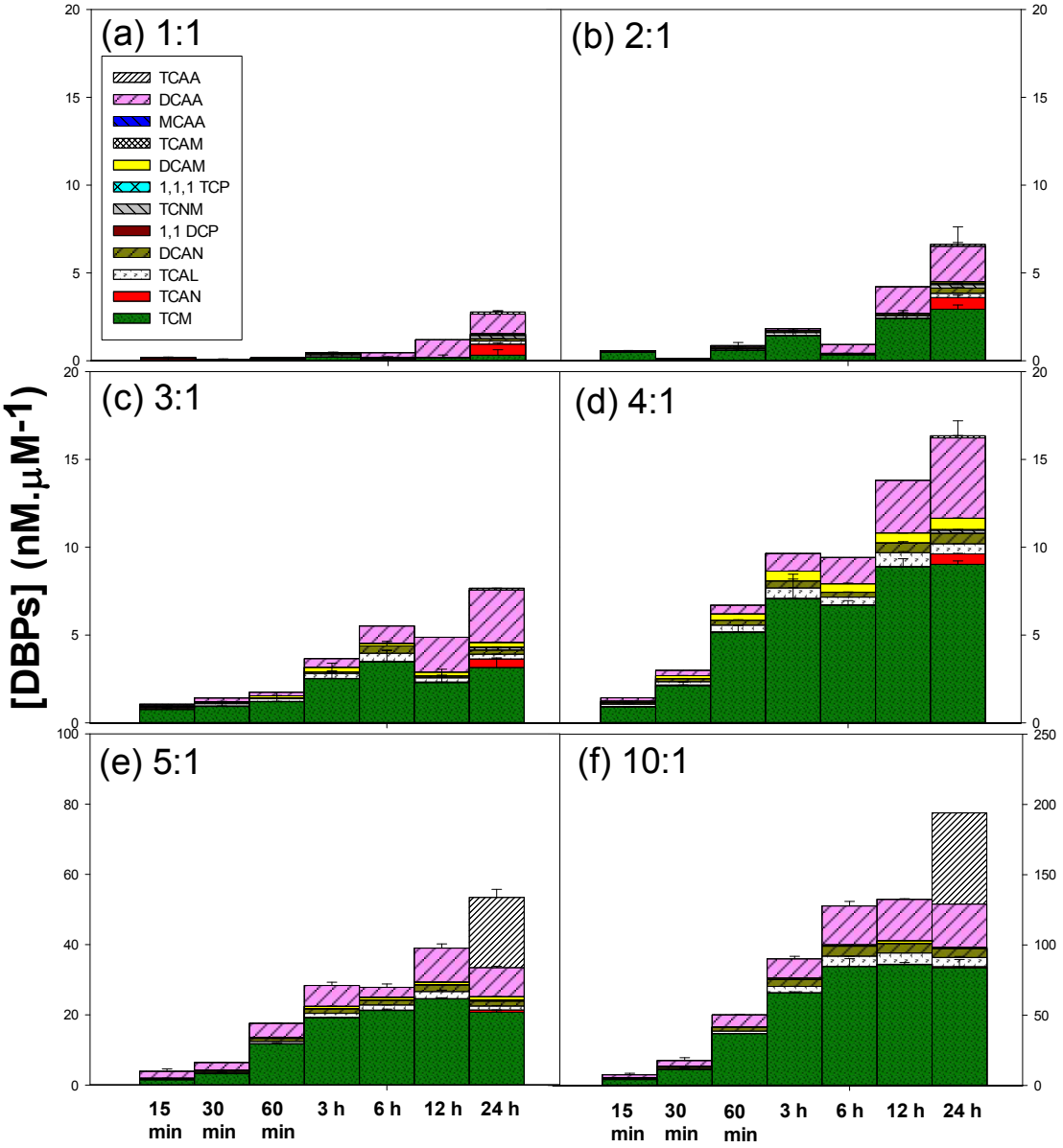


Figure S2: Molar yields of individual small molecule DBP species measured at various times after treatment of 50 μM NacTrp with chlorine at chlorine:NacTrp molar ratios of A) 1:1, B) 2:1, C) 3:1, D) 4:1, E) 5:1 and F) 10:1 at pH 7.4 in 10 mM phosphate buffer. Error bars indicate the range of experimental duplicates. TCAA = trichloroacetic acid, DCAA = dichloroacetic acid, MCAA = monochloroacetic acid, TCAM = trichloroacetamide, DCAM = dichloroacetamide, 1,1,1-TCP = 1,1,1-trichloropropanone, TCNM = trichloronitromethane (chloropicrin), 1,1-DCP = 1,1-dichloropropanone, DCAN = dichloroacetonitrile, TCAL = trichloroacetaldehyde (chloral hydrate), TCAN = trichloroacetonitrile, TCM = trichloromethane (chloroform).

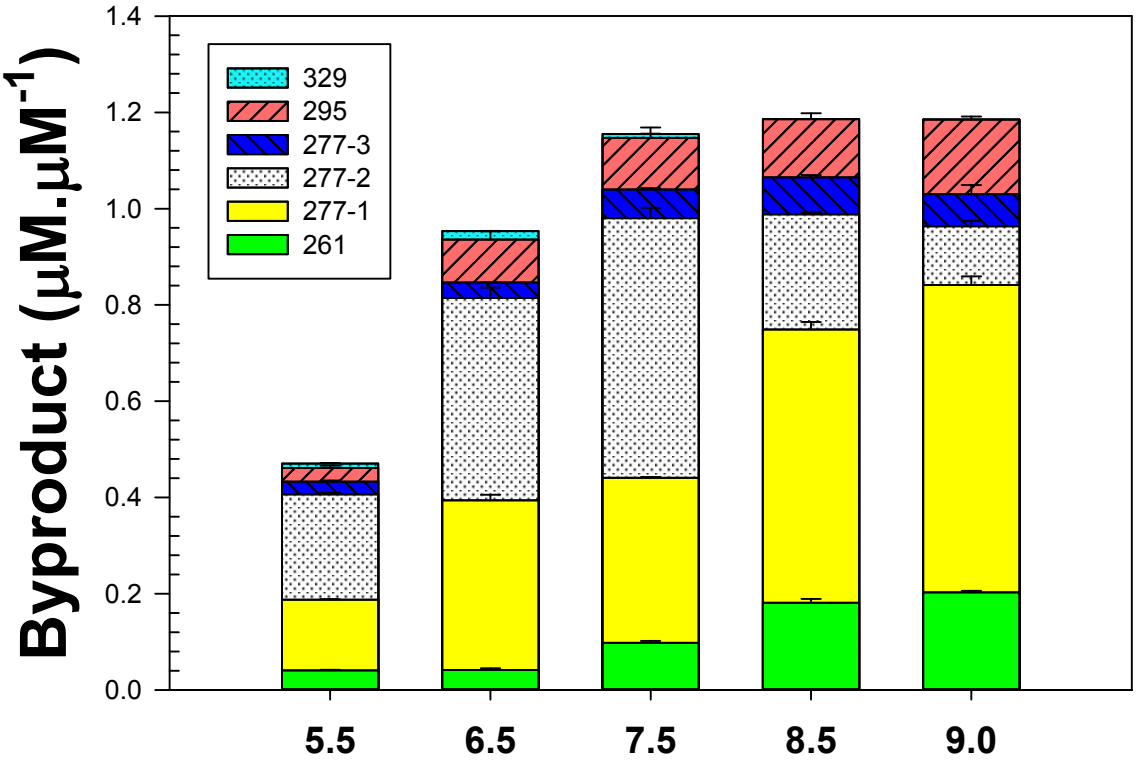


Figure S3: Molar yields of DBPs measured 24 h after treatment of 50 μM NacTrp at a 3:1 chlorine:NacTrp molar ratio at pH 5.5-9.0 in 10 mM phosphate buffer. Error bars indicate the range of experimental duplicates. Product 261 represents the sum of two isomers resolved by high-resolution LC/MS, but not by the low-resolution LC/MS analysis used for quantification. Note the y-axis break.

Text S4: High resolution MS analysis of novel DBPs

Analysis: High resolution HPLC-MS/MS analysis was used to characterize the structures of the 6 purified products detected via low-resolution MS analysis. Separation was achieved using an Agilent Zorbax Eclipse Plus C18 RRHD column (2.1×100 mm, 1.8 μm) held at 30 °C on an Agilent 1290 HPLC. Elution was at 0.4 mL/min with 30% acetonitrile and 70% deionized water. Products were detected using an Agilent 6560 Ion Mobility Q-TOF system operated in negative ion mode with electrospray ionization. Selected analyses were performed with the drift tube activated to achieve ion mobility separation in 4.2 torr of N_2 gas. The masses of purified products were low, preventing further characterization by NMR in all cases, and hindering high-resolution MS/MS analysis in several cases. While we were able to resolve two products from the initial 261 m/z product characterized by low resolution MS analysis, we were unable to characterize the third product at 277 m/z (277-3) by high-resolution MS due to the low mass of purified product.

Product 261 m/z: While only one 261 m/z product was resolved and purified by low-resolution HPLC-MS analysis, high-resolution MS analysis of this same material resolved two products at 261.0891 m/z at 0.506 min and 0.760 min using the Agilent Zorbax Eclipse Plus C18 RRHD column (2.1×100 mm, 1.8 μm) at 0.4 mL/min with the 30% acetonitrile:70% deionized water eluent. The elemental formulae for both products were proposed to be $C_{13}H_{13}N_2O_4^-$ (exact mass 261.0891 m/z; mass difference 3.89 ppm). In addition to the slight difference in retention times, these peaks also exhibited slight differences in drift times in the QTOF of 18.27 ms for the first peak (0.506 min retention time) and 18.13 ms for the second peak (0.760 min retention time), indicating slight differences in structure. Due to the low mass of purified products, MS/MS analysis revealed only weak fragment ions; these fragments included ions at 58.0298, 77.0388, and 131.0378 m/z for both products, estimated to be C_2H_4NO (exact mass 58.0293; 8.6 ppm mass difference), C_6H_5 (exact mass 77.0391; -3.9 ppm mass difference), and C_8H_5NO (exact mass 131.0371; mass difference 5.3 ppm), respectively.

Figure S4 provides structures of 2-acetamido-3-(2-oxoindolin-3-yl)-propanoate and 2-acetamido-3-(2-hydroxy-3H-indol-3-yl)-propanoate, proposed as products consistent with these parent and fragment masses. These products are tautomers that could form from initial chlorine attack on the indole ring by anti-Markovnikov addition, wherein a carbocation is located on the secondary carbon. Another isomer (structure A in Figure 3) could form from initial chlorine attack on the indole ring by Markovnikov addition, wherein the carbocation would be located on a tertiary carbon. Markovnikov addition might be favored due to the stabilization of the carbocation by this tertiary carbon. We were unable to purify sufficient mass to distinguish among these structures using NMR. However, we suggest that the two observed 261.0881 m/z isomers correspond to the two tautomers (structures C and D in Figure 3 and Figure S4) for several reasons. First, we suspect that while the isomer resulting from Markovnikov addition (structure A in Figure 3) does form, it should be more reactive with chlorine than the anti-Markovnikov products, proceeding more rapidly to the formation of the monochlorinated product (295.0491 m/z; structure E in Figure 3). The indole nitrogen should promote ring chlorination. However the proximity of the oxygen to the indole nitrogen in structures B, C and D would deactivate the nitrogen (e.g., resulting in an amide moiety in structure C), hindering ring chlorination and enabling accumulation of the 261.0881 m/z isomers as intermediates. Second, structure B should mainly be replaced by a more stable tautomeric structure (structure C in Figure 3) equilibrating with another lactim tautomer (structure D in Figure 3). Further support for the two tautomers as the identities of the two 261.0881 products is provided by the fact that dilution of the purified product from 50% acetonitrile:50% water ten-fold in water promoted a relative increase in the first peak at the expense of the second (Figure S5). As the zwitterionic lactam form will be favored in polar, hydrogen bonding solvent,[1] this behavior concurs with the conversion of the lactim tautomer (proposed as the second peak) to the lactam tautomer (proposed as the first peak) with the increasing dielectric constant of the solvent.

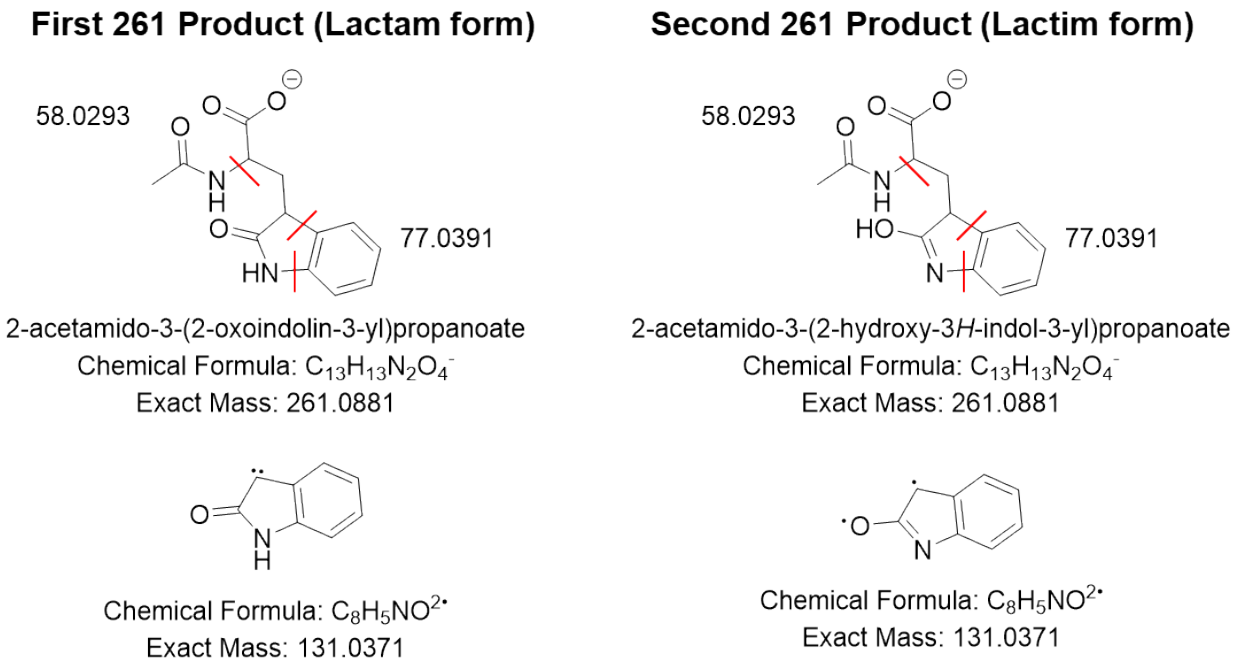


Figure S4: Structures of parent and fragment ions for two proposed products with 261.0881 m/z that could form by anti-Markovnikov addition to the indole ring. Blue lines provide fragmentation cleavage points to produce fragment ions. The fragments below each structure correspond to the proposed structures of fragments with exact mass 131.0371 Da.

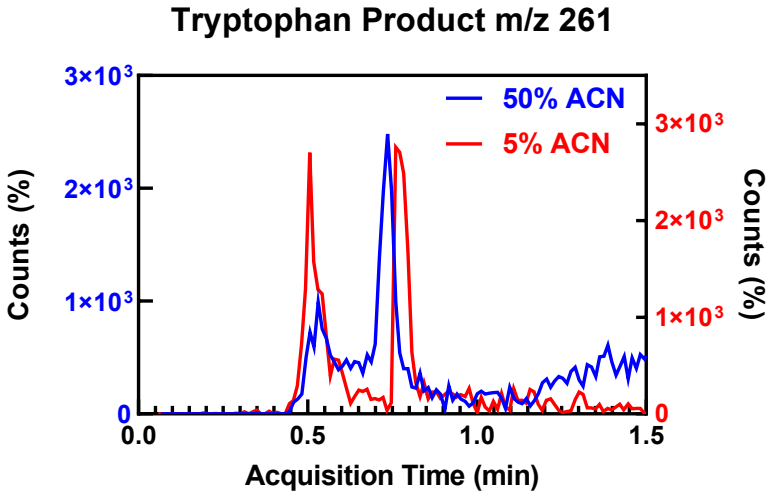


Figure S5. Dilution of the 261 m/z products from 50% acetonitrile/50% water ten-fold in water increases the relative importance of 261-1 vs. 261-2, suggesting that the increase in the solvent dielectric constant promotes conversion of the lactim tautomer to the lactam.

Products 277 m/z: The three 277 m/z products isolated by low resolution MS analysis were separated during high resolution MS/MS analysis using the Agilent Zorbax EclipsePlus C18 RRHD column (2.1×100 mm, 1.8 μm) with 30% acetonitrile and 70% deionized water at 0.430 min, 0.589 min, and 1.157 min retention times; however, the mass of purified material for the product resolved at 1.157 min retention time was insufficient for further characterization.

The measured masses for the first two products (corresponding to 277-1 and 277-2) were 277.0810 m/z, corresponding to elemental formulae of $C_{13}H_{13}N_2O_5^-$ (exact mass 277.0830 m/z; mass difference -7.2 ppm). The measured drift times for these two products in the QTOF were 18.95 ms and 18.94 ms, respectively; the similar drift times indicate similar structures. MS/MS analysis revealed fragment ions at 58.0300, 84.0450, 92.0504, and 144.0447 m/z for both products, proposed to represent C_2H_4NO (exact mass 58.0293; mass difference 12.1 ppm), C_4H_6NO (exact mass 84.0449; mass difference 1.2 ppm), C_6H_6N (exact mass 92.0500; mass difference 4.3 ppm), and C_9H_6NO (exact mass 144.0449; mass difference -1.4 ppm), respectively. The first product featured additional fragments at 120.0445, 131.0371, and 148.0401 m/z, proposed to represent C_7H_6NO (exact mass 120.0449; mass difference -3.3 ppm), C_8H_5NO (exact mass 131.0371; mass difference 0 ppm), and $C_8H_6NO_2$ (exact mass 148.0399; mass difference 1.4 ppm), respectively. The second product featured an additional fragment at 77.0394 m/z, proposed to represent C_6H_5 (exact mass 77.0391; mass difference 3.9 ppm).

Figure S6 provides structures of 2-acetamido-3-(3-hydroxy-2-oxoindolin-3-yl)-propanoate and 2-acetamido-3-(2,3-dihydroxy-3H-indol-3-yl)-propanoate, proposed as products consistent with these parent and fragment masses. These products are also consistent with the proposed mechanism involving initial chlorine attack on the indole ring by either Markovnikov addition, wherein the carbocation is stabilized on a tertiary carbon, or by anti-Markovnikov addition, wherein the carbocation is located on a secondary carbon (Figure 3). Note that these two products are tautomers (Figure 3). We propose that these two structures correspond to products 277-1 and 277-2, due to the similarity in their structures and the small difference in retention times for these two products. A slow interconversion rate is expected between two tautomers at neutral pH,[2,3] and the zwitterionic lactam form, as well as the intermolecular hydrogen bonds in lactam form, will be favored in polar, hydrogen bonding solvent.[1,2] Support for this suggestion is provided by the fact that dilution of the purified products from 50% acetonitrile:50% water ten-fold in water promoted a relative increase in the first peak at the expense of the second (Figure S7). This behavior concurs with the conversion of the lactim tautomer (proposed as the second peak) to the lactam tautomer (proposed as the first peak) with the increasing dielectric constant of the solvent.

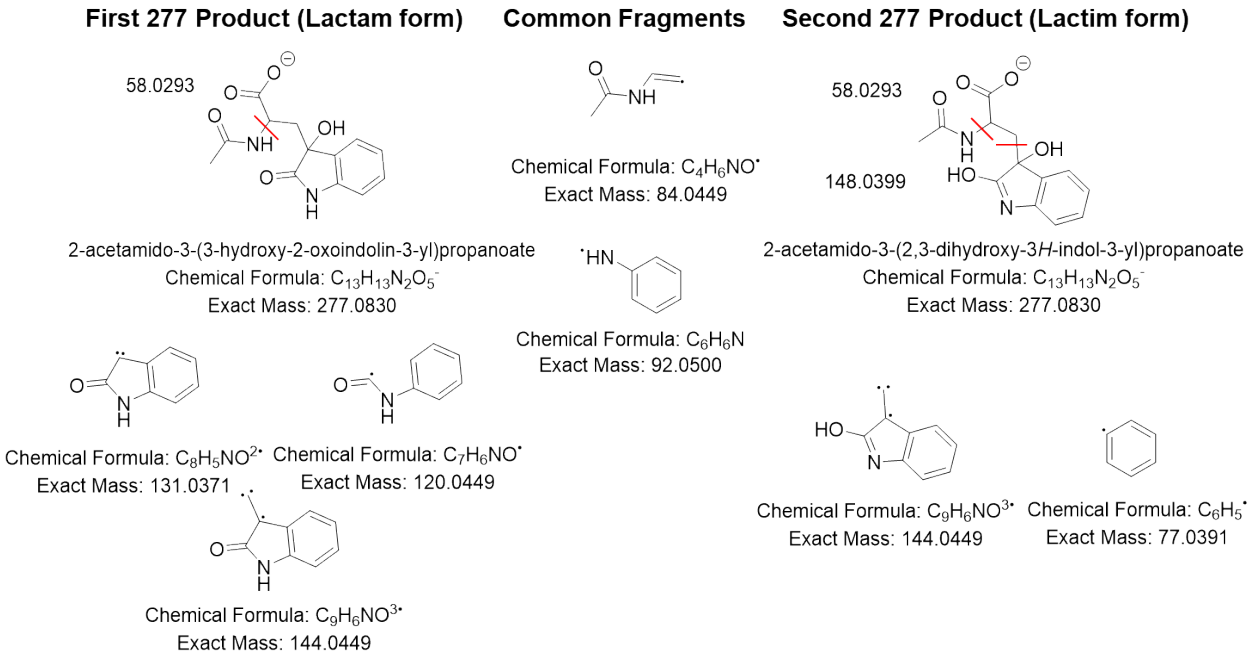
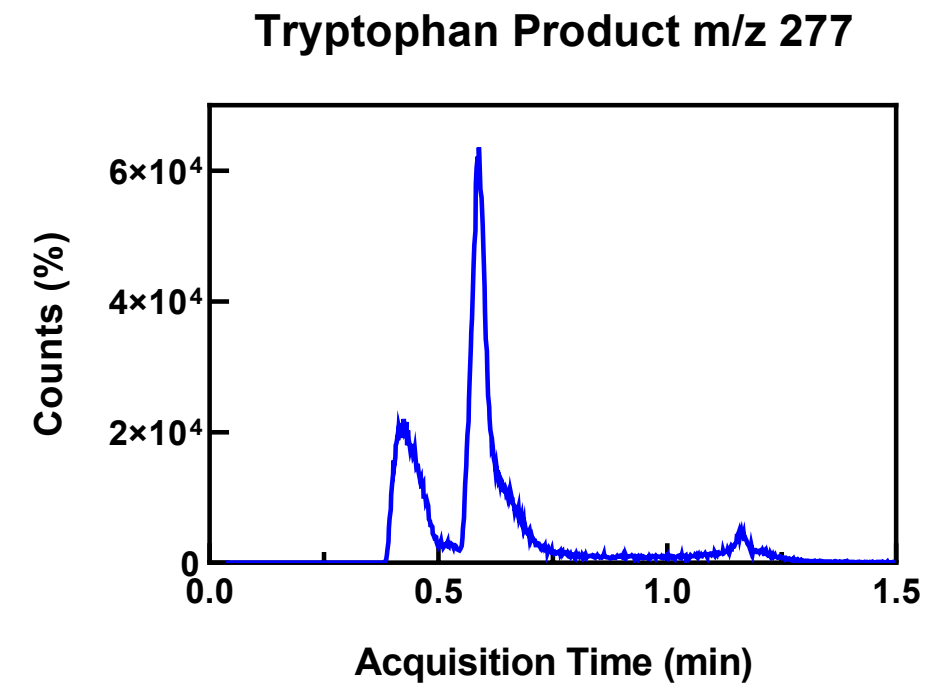


Figure S6: Structures of parent and fragment ions for two proposed products with 277.0380 m/z. Blue lines provide fragmentation cleavage points to produce fragment ions. The fragments below each structure correspond to the proposed structures of fragments with different exact masses detected during MS/MS analysis of each product. The two fragments in the center correspond to the proposed structures of fragments with exact masses that were detected during fragmentation of both parent compounds.



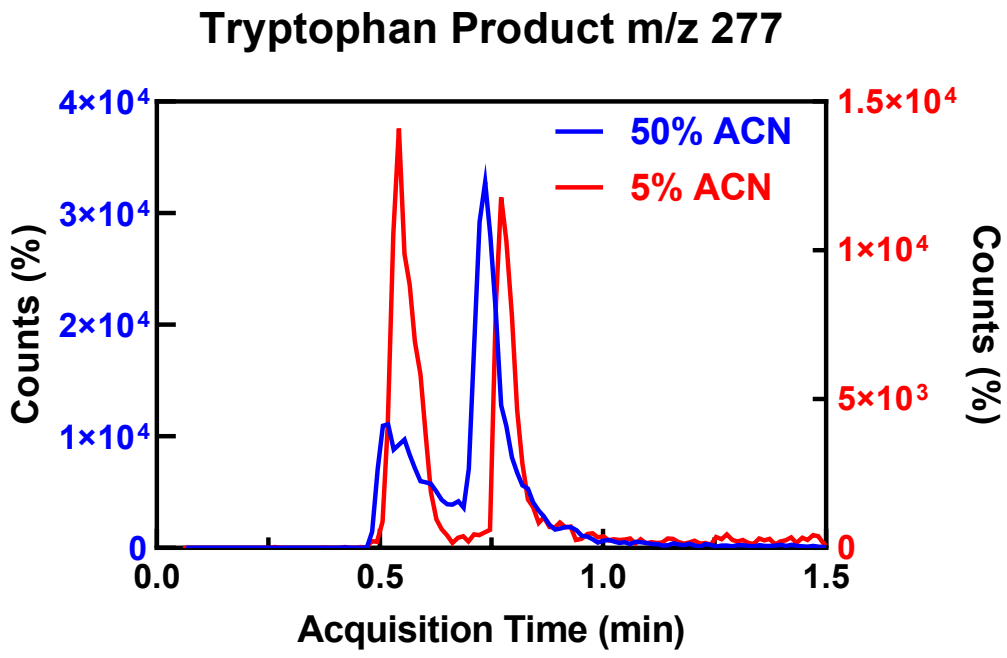
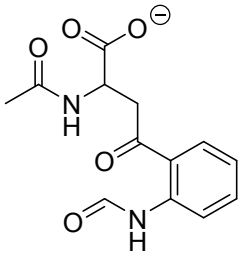


Figure S7: Dilution of the 277 m/z products from 50% acetonitrile/50% water ten-fold in water increases the relative importance of 277-1 vs. 277-2, suggesting that the increase in the solvent dielectric constant promotes conversion of the Lactim tautomer to the Lactam.

There was insufficient mass to conduct a thorough MS/MS characterization of the product corresponding to 277-3. However, we propose this structure to be 2-acetamido-4-(2-formamidophenyl)-4-oxobutanoate (Figure S8) based upon a proposed alternative reaction pathway (structure K in Figure 3). The structural differences between this compound and the two tautomers (277-1 and 277-2) may have resulted in a longer column retention time; for example, the two tautomers feature a hydroxyl group that would increase their polarity, lowering their retention times. Purification of higher masses of these products would be needed to validate these proposed structures by additional analyses by mass spectrometry and NMR.



2-acetamido-4-(2-formamidophenyl)-4-oxobutanoate
Chemical Formula: $C_{13}H_{13}N_2O_5^-$
Exact Mass: 277.0830

Figure S8: Structure of 2-acetamido-4-(2-formamidophenyl)-4-oxobutanoate, proposed to be the parent compound for the product with 277.0380 m/z, corresponding to 277-3.

Product 295 m/z: One product with a parent mass of 295.0511 m/z was resolved at a retention time of 1.049 min with the Agilent Zorbax Eclipse Plus C18 RRHD column (2.1×100 mm, 1.8 μm). The drift time of the compound in the QTOF instrument was 19.1 ms. Fragments produced during MS/MS analysis and the proposed structure are discussed in the main text. Consistent with the discussion of the proposed identities of the 261 m/z products, we suggest that the 295 m/z product results from ring chlorination of the 261 m/z product formed by initial Markovnikov addition of chlorine to the indole functional group, as opposed to chlorination of the anti-Markovnikov addition products (structure G in Figure 3).

Product 329 m/z: One product with a parent mass of 329.0127 m/z was detected using the Agilent Zorbax Eclipse Plus C18 RRHD column (2.1×100 mm, 1.8 μm) with 30% acetonitrile and 70% deionized water as eluent at a retention time of 1.736 min. The drift time in the QTOF was 19.87 ms. The parent compound was proposed to have the elemental formula of $C_{13}H_{11}Cl_2N_2O_4^-$ (exact mass 329.0101; mass difference 7.9 ppm). MS/MS fragments were detected at 58.0300, 84.0458, 115.0293 and 198.9595 m/z, proposed to represent C_2H_4NO (exact mass 58.0293; mass difference 12.1 ppm), C_4H_6NO (exact mass 84.0449; mass difference 10.7 ppm), $C_4H_5NO_3^-$ (exact mass 115.0275; mass difference 15.6 ppm), and $C_8H_3Cl_2NO$ (exact mass 198.9592; mass difference 0.9 ppm).

Figure S9 provides the structure of 2-acetamido-3-(5,7-dichloro-3-hydroxy-3H-indol-3-yl)-propanoate, proposed as a product consistent with these parent and fragment masses. This product is also consistent with the proposed mechanism, produced by chlorine attack on the

monochlorinated 295 product (Figure 2 and structure F in Figure 3). The first chlorine addition to the ring would deactivate the ring towards further chlorination, resulting in lower yields of the dechlorinated product relative to the monochlorinated product.

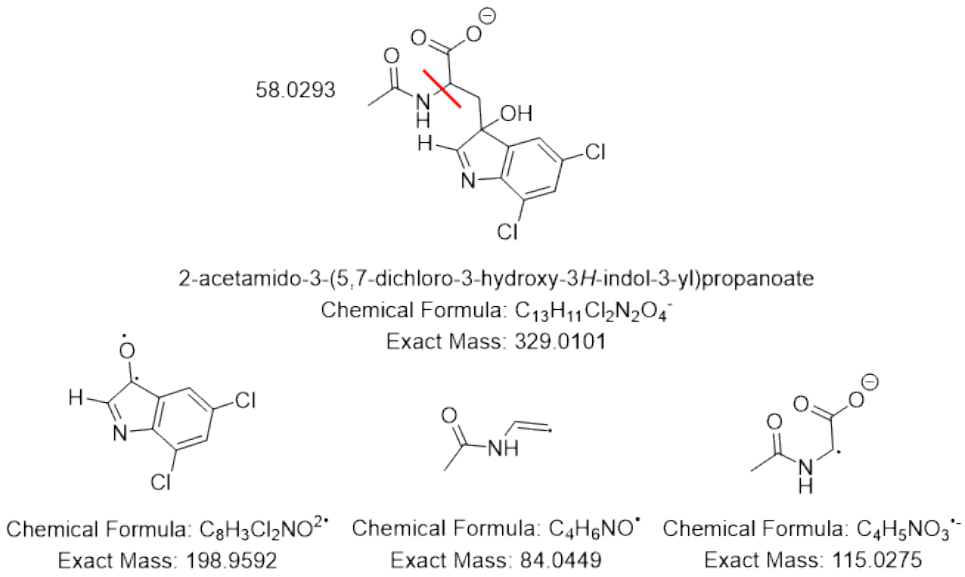


Figure S9: Structures of parent and fragment ions for the proposed product with 329.0101 m/z. Blue lines provide fragmentation cleavage points to produce fragment ions. The fragments adjacent to the structure correspond to the proposed structures of fragments with different exact masses detected during MS/MS analysis of the product.

References

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