Supporting Information

Kinetics of 3-Chlorotyrosine Formation and Loss Due to Hypochlorous Acid and Chloramines

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Experimental Procedures

Materials. **Synthesis of N-acetyl-3-chlorotyrosine**. 3-Chloro-L-tyrosine (1 g, 4.6 mmol) was acetylated with excess acetic anhydride (4 ml, 42 mmol) in 100 ml acetone at room temperature. The crude product containing N,O-diacetyl-3-chlorotyrosine was formed after reacting overnight and is soluble in acetone. The acetone was removed using rotary evaporation after filtering off any remaining insoluble impurities. Remaining acetic anhydride was hydrolyzed by addition of water. The phenol acetate ester was rapidly hydrolyzed for one hour by addition of 1M NaOH until the solution pH was between 8 and 9 resulting in the formation of N-acetyl-3-chlorotyrosine (Ac3ClTyr) in high purity (> 95% pure). The aqueous solution was acidified to a pH < 2 with concentrated hydrochloric acid and then added to a SupelClean, LC-18 packing, solid phase extraction (SPE) column (Supelco, Bellefonte, PA). Following washing with aqueous 0.1% trifluoroacetic acid (TFA), the desired compound was eluted with 5-10% methanol, 0.1% TFA solution. Fractions of ≥ 98% purity, as determined by analytical HPLC, were dried by vacuum centrifugation and stored for later use. The compound gave the expected UV/Vis absorbance maximum at 279 nm (Reference 51). ^{1}H NMR (500 MHz, DMSO-d_6), δ: 9.79 (s, 1H, –O*H*), 8.17 (d, *J* = 8.1 Hz, 1H, N*H*), 7.16 (d, *J* = 1.8 Hz, *H*2), 6.98 (dd, *J* = 1.8, 8.3 Hz, 1H, *H*6), 6.86 (d, *J* = 8.3 Hz, 1H, *H*5), 4.33 (m, 1H, *J* = 5.0, 8.3, 9.5 Hz, *H*α), 2.92 (dd, 1H, *J* = 5.0, 13.9 Hz, *H*β), 2.72 (dd, *J* = 9.5, 13.9 Hz, *H*β), 1.79 (s, 3H, CH_3).

Synthesis of N-acetyl-3,5-dichlorotyrosine. The procedure of Allevi et al. (Reference 49) was used to synthesize 3,5-dichlorotyrosine (Cl_2Tyr) from tyrosine, which was then used without further purification. Cl_2Tyr was acetylated as described above for the acetylation of 3ClTyr. The desired compound eluted from the C18 SPE column with a solution consisting of 20% methanol and 0.1% TFA. The UV/Vis spectrum in 0.1 M HCl showed the expected absorbance maximums at 281 and 287 nm (Reference 51). ^{1}H NMR (500 MHz, DMSO-d_6), δ: 9.94 (s, 1H, –O*H*), 8.15 (d, *J* = 8.2 Hz, 1H, N*H*), 7.21 (s, 2H, *H*2,6), 4.36 (m, *J* = 5.0, 8.2, 9.5 Hz, 1H, *H*α), 2.94 (dd, *J* = 5.0, 13.8 Hz, 1H, *H*β), 2.73 (dd, *J* = 9.5, 13.8 Hz, *H*β), 1.78 (s, 3H, CH_3).

Synthesis of Ac-KGNYAE-NH_2. A small peptide, Ac-KGNYAE-NH_2, was synthesized using solid phase synthesis and fast Fmoc chemistry on an Applied Biosystems (Foster City, CA) 433A peptide synthesizer. The sequence of this peptide is identical to residues 36 to 41 of human histone H2A. We used an Fmoc-amide resin and Fmoc-protected amino acids with the lysine side-chain protected by a Boc group. After synthesis, the N-terminal Fmoc-group was removed with piperidine and acetylated by acetic anhydride and triethylamine in dimethylformamide. The peptide was cleaved from the resin and purified by HPLC as previously described (Reference 43). Following purification, the peptide was characterized by MALDI-TOF in positive-ion mode

and determined that the peptide monoisotopic mass ($M+H^+$) was 722.339 m/z; the expected monoisotopic mass is 722.347 m/z. The purity of the peptide was determined to be $\geq$ 97% by analytical HPLC.

Methods. HPLC Measurement of Chlorination Products. The concentration of products and reactants in kinetics samples were measured using a ThermoFinnigan (Waltham, MA) Surveyor HPLC system containing a MS Pump, Autosampler, and PDA detector. Standard curves were generated using pure stocks ($\geq$ 98% pure) of AcTyr, Ac3ClTyr, and $AcCl_2Tyr$ ($r^2 \geq 0.999$). Separation of each analyte was accomplished by using a Restek Ultra IBD column (C18, 3μm, 150 x 2.1 mm). The initial solvent mix was 95% mobile phase A (0.1% aqueous TFA) and 5% mobile phase B (0.85% TFA in acetonitrile) for 5 minutes followed by a linear gradient to 50% B over 20 minutes at a flow rate of 200 μL/min. The limit of detection (S/N $\geq$ 3) was $\approx$ 0.5 μM while the limit of quantitation was 100 - 200 μM depending on the analyte. Kinetics samples were diluted as necessary to achieve a final concentration of analyte less than the limit of quantitation. The Qual browser module of Xcalibur, ver. 1.3 (Thermo-Finnigan, Ontario, Canada) was used to analyze the HPLC chromatogram. Standard curves were obtained periodically to ensure that measured concentrations were accurate.

Characterization of Peptide Products by MALDI-TOF. Fractions corresponding to peaks were collected manually during HPLC analysis of products following the reaction of Ac-KGNYAE-NH_2 with HOCl. These fractions were dried down and resuspended in 20 μL water. MALDI samples were prepared using the dried-droplet method. α-Cyano-4-hydroxycinnamic acid (10 mg/ml) or 2,5-dihydroxybenzoic acid (10 mg/ml) in 0.1 % aqueous TFA/acetonitrile (1:1, v/v) was used as the matrix for positive or negative ion modes, respectively. Each fraction was analyzed using a Bruker (Billerica, MA) Autoflex MALDI-TOF in both positive- and negative-ion reflectron modes. The accelerating voltage was 20 kV, the mirror to accelerating voltage ratio was 0.3, and the low mass gate was 400 Da. Spectra (80 shots) were acquired using the FlexControl 2.4 software. The mass-to-charge ratio was calibrated using a commercial peptide solution containing angiotensin I and II, substance P, bombesin, clipped ACTH residues 1-17 and 18-39, and somatostatin (Bruker, Billerica, Ma).

Results

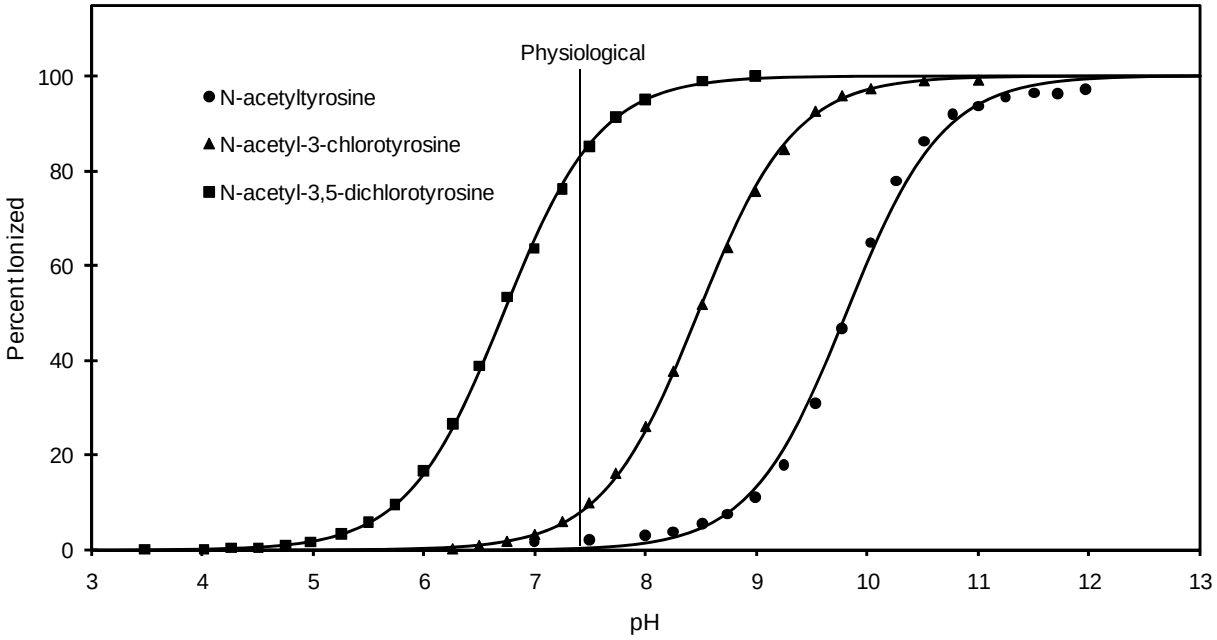

Figure S1. Chlorination of tyrosine lowers the pKa value of the phenol hydroxyl. The curves plot the function, percent ionized = $(100 \cdot 10^{(pH - pKa)})/(1 + 10^{(pH - pKa)})$, where the pK_a value was fitted to the experimental data. The 100% ionized and 0% ionized absorbance values were fitted to the experimental values at 242, 244, and 246 nm for N-acetyltyrosine, N-acetyl-3-chlorotyrosine, and N-acetyl-3,5-dichlorotyrosine, respectively. The calculated pK_a values for N-acetyltyrosine, N-acetyl-3-chlorotyrosine, and N-acetyl-3,5-dichlorotyrosine are 9.8, 8.5, and 6.7, respectively.

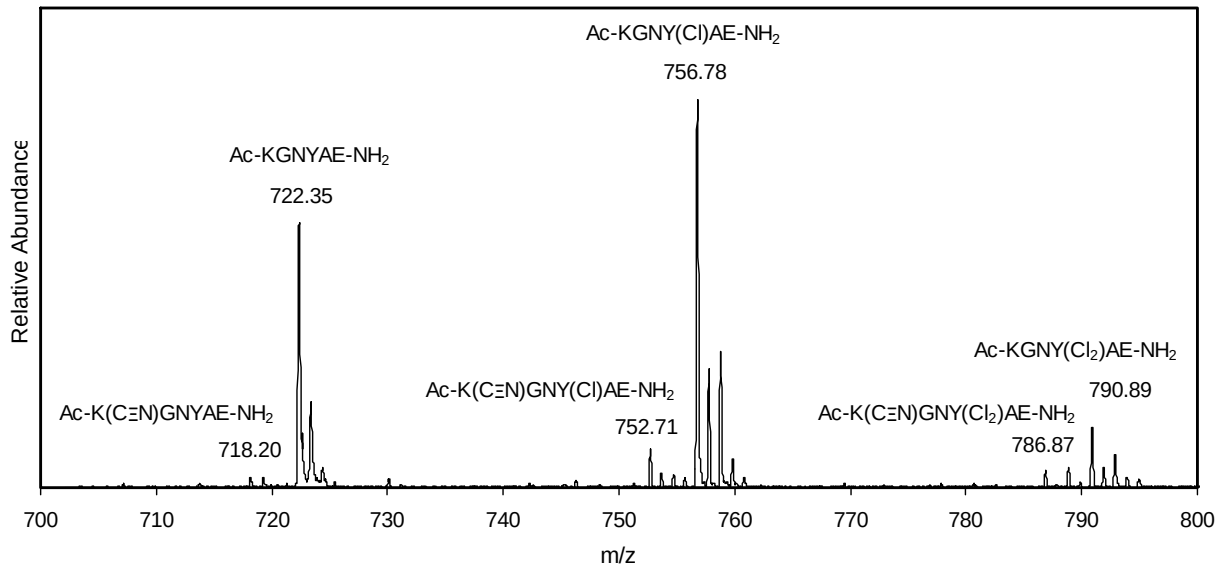

Figure S2. MALDI-TOF was used to identify products of the reaction of Ac-KGNYAE-NH_2 with HOCl. MALDI samples were prepared using the dried-droplet method and 2,5-dihydroxybenzoic acid as the matrix. Samples were analyzed in negative-ion reflectron mode by a Bruker Autoflex MALDI-TOF. The mass-to-charge ratio was calibrated using a commercially-available peptide solution containing angiotensin I and II, substance P, bombesin, clipped ACTH residues 1-17 and 18-39, and somatostatin. Due to the weak signal of these standards in negative-ion mode, we also added the previously characterized peptide Ac-KGNYAE-NH_2 (MH^- ion at 722.3468 m/z) to the standard mix to achieve a more accurate standard curve. This shows the final products when 260 μM HOCl reacts with 125 μM Ac-KGNYAE-NH_2 at 37 °C for 1 hr. Peaks at 722.4, 756.8, and 790.9 m/z correspond to the MH^- ions of the peptide containing tyrosine, 3-chlorotyrosine, and 3,5-dichlorotyrosine, respectively. Peaks at 718.2, 752.7, and 786.9 m/z correspond to the lysine nitrile form of the peptide with tyrosine, 3-chlorotyrosine, and 3,5-dichlorotyrosine, respectively.