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Selective Inactivation of Serine Proteases by Nonheme Iron Complexes

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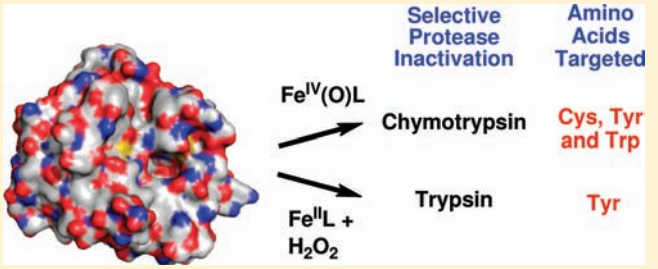
Supporting Information

ABSTRACT: Oxidative inactivation of the serine proteases trypsin and chymotrypsin by nonheme iron complexes is described. The nonheme ligands N4Py (**1**) and derivative 3CG-N4Py (**2**), which contains a pendant guanidinium group, were used as ligands for iron. Ferryl ($Fe^{IV}O$) species derived from these ligands, $[Fe^{IV}(O)(N4Py)]^{2+}$ (**7**) and $[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ (**8**), inactivate trypsin and chymotrypsin by the oxidation of amino acid side chains. Ferryl **8** is most effective with chymotrypsin (IC_{50} value of 26 μM for **8** vs 119 μM for **7**). IC_{50} values of 71 and 54 μM were obtained for trypsin with **7** and **8**, respectively. Amino acid analysis confirmed that residues cysteine, tyrosine, and tryptophan are oxidized under these conditions. Trypsin is inactivated preferentially over chymotrypsin under catalytic conditions, where the enzyme was pulsed with H_2O_2 in the presence of ferrous complexes $[Fe^{II}(OH_2)(N4Py)]^{2+}$(**5**) and $[Fe^{II}(Cl)(3CG\text{-}N4Py)]^{2+}$ (**6**). Control experiments support the action of a unique oxidant, other than ferryls or hydroxyl radicals, under these conditions, where tyrosine residues are targeted selectively.



INTRODUCTION

Enzyme inhibitors play a pivotal role in life sciences today, as reagents for chemical biology and as therapeutics for the treatment of human diseases. To achieve inhibition, molecules that bind selectively to targets and diminish enzyme activity are delivered. This inhibition can be reversible or irreversible. In the case of reversible inhibition, the inhibitor molecule binds tightly to the target using noncovalent interactions. Although this strategy is most common, it is reversible and can require high concentrations of inhibitor to achieve the desired effect, which can lead to off-target activities. Irreversible inactivation of the target enzyme, or suicide inhibition, involves the formation of a covalent bond between the inhibitor molecule and target enzyme. Because of issues with toxicity and immunogenicity, suicide inhibition is less common in pharmaceuticals. Importantly, both of these strategies require a stoichiometric amount of the inhibitor. To circumvent these issues, many have recognized that catalytic inactivation of enzymes would be an ideal approach for halting protein function because it can lead to irreversible inactivation of the target protein with low concentrations of inhibitor.[1−6]

Metal catalysts are ideal for inactivating proteins because of their reactivity. These catalysts can be classified as hydrolytic complexes, which attack the amide bonds of the peptide backbone, or oxidative complexes, which can cleave the peptide backbone or oxidize amino acid side chains. The general strategy for either approach is the same and involves the tethering of metal catalysts to protein-affinity ligands for achieving target specificity. Examples of hydrolytic complexes include copper(II) and cobalt(III) complexes, which hydrolyze amide bonds near physiological pH,[3,7−12] and palladium(II) complexes, which cleave peptides and proteins adjacent to histidine residues.[13−16] Metal complexes that inactivate proteins by oxidation can be separated into two classes: those that generate diffusible reactive oxygen species (ROS), such as $^1O_2$, superoxide, or hydroxyl radicals, and those that generate metal-based oxidants, where the oxygen atom is bound to a metal center and does not diffuse from the catalytic center.

Damage to proteins by ROS has been well studied because of its significance in aging, neurodegenerative diseases, and radiation therapy.[17−21] Early examples of catalysts that relied on the formation of ROS included Fe^{III}EDTA-based reagents for targeting calmodulin[1] and streptavidin[2] as well as a copper(I) complex of 1,10-phenanthroline for targeting carbonic anhydrase.[22] Later work with chromophore-assisted light inactivation showed that proteins can be inactivated selectively using $^1O_2$ generated by the metal-based chromophore $[Ru^{II}(bpy)_3]^{2+}$,[23,24] which showed an advantage over more traditional organic photosensitizers,[25−27] which are susceptible to photobleaching. Appending $[Ru^{II}(bpy)_3]^{2+}$ to peptoid derivatives provided a straightforward way to increase potency without resorting to extensive optimization of the inhibitor structure.[24] Importantly, selective targeting of intra- and extracellular proteins was demonstrated with this approach.

When compared with ROS, inactivation of proteins by metal-based oxidants represents an underexplored area of chemistry. Nickel(II) and copper(II) complexes derived from the tripeptide Gly−Gly−His have proven applications in protein oxidation,

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where control experiments indicate that the action of a metal-based oxidant is responsible for the inactivation event. However, the identities of the metal-based oxidants have not been established. With nickel(II) complexes, site-specific cleavage of calmodulin was demonstrated,[28,29] whereas with copper(II) complexes, inactivation of angiotensin-converting enzyme, endothelin-converting enzyme 1[4], and human carbonic anhydrase-I[29] occurred by oxidizing the amino acid side chains of the target proteins rather than cleaving the peptide backbone.

Considering that there were only a few examples of targeting proteins with metal-based oxidants, we found interest in applying iron complexes developed for small-molecule oxidation toward this purpose. Since studies with iron complexes first began, ethylenediaminetetraacetic acid (EDTA) has been the ligand of choice, especially for footprinting applications.[30−35] However, others established that moving from carboxylate-rich ligands, such as EDTA, to more nitrogen-rich ligands resulted in better control and higher levels of selectivity in the oxidation of small molecules[36] because of the action of metal-based oxidants rather than ROS, specifically HO$^{\bullet}$ through the Fenton reaction.[37] Work in this area proved that iron-based oxidants, such as the well-characterized high-valent iron(IV) oxo species $[Fe^{IV}(O)(N4Py)]^{2+}$,[38−40] have spectacular applications in the selective oxidation of organic molecules[38,40−47] and in mimicking non-heme protein active sites found in nature,[48−50] including enzymes that are used to oxidize amino acids.[51,52] We were further motivated to investigate these complexes as protein-targeting reagents because they mimic the action of bleomycin, an example of an iron-based catalytic drug that has been used for decades in cancer chemotherapy, which selectively oxidizes and cleaves DNA catalytically using bioavailable oxidants such as O_2 or H_2O_2.[53−61] Yet, the targeting of proteins with these reagents remained unexplored.

As a starting point to investigate protein targeting with iron-based oxidants, our group demonstrated that the well-characterized ferryl $[Fe^{IV}(O)(N4Py)]^{2+}$ oxidizes side chains and cleaves the backbone of protected amino acids.[62,63] However, questions remained regarding the reactivity of iron-based oxidants toward full proteins, where the reactivities of residues could be dependent on the local protein structure and could differ from the model systems. Would a ferryl, which is typically more selective than HO$^{\bullet}$, be reactive enough to inactivate a protein, and if so, could this be done selectively? Could the parent iron(II) complexes oxidize and inactivate enzymes in a catalytic and selective fashion using bioavailable oxidants, and if so, would protein cleavage or side-chain oxidation be observed? In order to address these questions, the reactivity of two ferryl complexes, the known ferryl $[Fe^{IV}(O)(N4Py)]^{2+}$ and a derivative containing an acylpropylguanidinium group for protein binding named $[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ for 3-carbon guanidinium (see Figure 1 for ligands), were evaluated for their ability to inactivate serine proteases trypsin and chymotrypsin under single-turnover conditions. In addition, the starting ferrous complexes were combined with enzyme and treated with oxidants to determine if catalysis was possible. Results disclosed herein prove that ferryls can act as selective reagents for inactivation of serine proteases and that selectivity can be observed under catalytic conditions, although evidence supports that a metal-based oxidant other than ferryl is at play. These results confirm for the first time that the new class of iron complexes that have extensive applications in the oxidation of small molecules can be applied to the selective targeting of proteins.

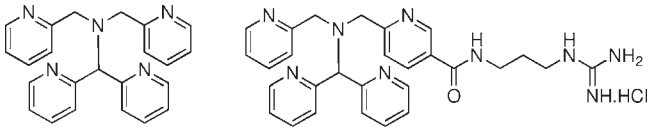

Figure 1. Ligands N4Py (**1**) and derivative 3CG-N4Py (**2**) containing a propylguanidinium group.

EXPERIMENTAL SECTION

General Considerations. All reagents were purchased from commercial suppliers and used as received. NMR spectra were recorded on a Varian FT-NMR Mercury 400 MHz spectrometer. Mass spectrometry (MS) spectra were recorded on a Micromass Quattro LC Triple-Quad mass spectrometer using an electrospray ionization source. IR spectra were recorded on a Nicolet FT-IR spectrophotometer. Trypsin from bovine pancreas and its substrate, *N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride, were obtained from Sigma and Acros Organics, respectively, and were used as received. Chymotrypsin and its substrate *N*-succinyl-Ala-Ala-Pro-Phe-pNA were obtained from Sigma. Absorbance data were collected on a GENios Pro, microplate reader, TECAN, using 96-well microplates. The compounds 1,1-dipyridin-2-yl-*N*,*N*-bis(pyridin-2-ylmethyl)methanamine (N4Py, **1**),[55] methyl 6-[[(dipyridin-2-ylmethyl)(pyridin-2-ylmethyl)-amino]methyl]nicotinate (**3**),[61] and *N*-acetyltyrosine (**9**)[64] were synthesized using literature procedures.

Ligand Synthesis. *Preparation of tert-Butyl 1-{6-[[(Dipyridin-2-ylmethyl)(pyridin-2-ylmethyl)amino]methyl]pyridin-3-yl}-11,11-dimethyl-1,9-dioxo-10-oxa-2,6,8-triazadodecan-7-ylidenecarbamate (**4**).* A solution of *N*-(3-aminopropyl)-6-[[(dipyridin-2-ylmethyl)(pyridin-2-ylmethyl)-amino]methyl]nicotinamide (100 mg, 0.21 mmol) in a mixture of tetrahydrofuran (THF; 0.3 mL) and H_2O (20 μL) was treated with a solution of 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (61.8 mg, 0.21 mmol) in THF (0.2 mL) dropwise at room temperature. The reaction mixture was heated to 50 °C for 1 h and then concentrated in vacuo. The resulting crude reaction mixture was partitioned between $CHCl_3$ (5 mL) and a saturated aqueous solution of $NaHCO_3$ (5 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated to obtain **4** as a pale-yellow solid (131 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 11.50 (s, 1H), 9.07 (d, *J* = 1.6 Hz, 1H), 8.55−8.52 (m, 3H), 8.48 (d, *J* = 4.0 Hz, 1H), 8.29−8.26 (m, 1H), 8.16 (dd, *J* = 8.1 and 2.4 Hz, 1H), 7.71−7.56 (m, 7H), 7.24−7.07 (m, 3H), 5.29 (s, 1H), 3.99 (s, 2H), 3.93 (s, 2H), 3.47−3.41 (m, 4H), 1.74−1.72 (m, 2H), 1.48 (s, 9H), 1.28 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 165.5, 163.2, 162.9, 159.8, 157.5, 153.2, 149.3, 149.1, 148.4, 136.4, 136.2, 135.5, 128.4, 123.9, 122.7, 122.2, 122.1, 121.9, 83.5, 79.7, 71.3, 57.2, 56.9, 36.9, 35.5, 30.3, 28.2, 28.0. IR (cm^{-1}): 3324, 3055, 2978, 2931, 2359, 2239, 1723, 1642, 1589, 1569, 1474, 1433, 1368, 1325, 1223, 1135, 1049, 1026, 995, 912, 855, 732, 645, 616. HRMS (ESI). Calcd for $C_{38}H_{47}N_9O_5$ [(M + Na)$^+$]: *m*/*z* 732.3598. Found: *m*/*z* 732.3589.

*Preparation of 6-[[(Dipyridin-2-ylmethyl)(pyridin-2-ylmethyl)amino]-methyl]-N-(3-guanidinopropyl)nicotinamide Hydrochloride (**2**).* Compound **4** (100 mg, 0.141 mmol) was maintained with 4 M HCl in 1,4-dioxane (2 mL) for 3 h at room temperature. The reaction mixture was concentrated to furnish **2** (100 mg) as a hydroscopic pale-yellow solid in quantitative yield as its hydrochloride salt. Stock solutions of **2** in H_2O (4 mM) were prepared from this solid for the following studies based on the quantitative yield in the conversion of **4** to **2**. ^{1}H NMR (DMSO-d_6): δ 9.18 (s, 2H), 8.82 (d, *J* = 4.1 Hz, 1H), 8.62 (d, *J* = 4.1 Hz, 2H), 8.41 (d, *J* = 7.3 Hz, 1H), 8.27 (t, *J* = 7.3 Hz, 1H), 8.18−8.14 (m, 2H), 8.01 (br s, 1H), 7.92 (d, *J* = 8.1 Hz, 2H), 7.82 (d, *J* = 7.3 Hz, 2H), 7.69−7.62 (m, 4H), 6.06 (s, 1H), 4.44 (s, 2H), 4.26 (s, 2H), 3.37−3.33 (m, 2H), 3.23−3.21 (m, 2H), 1.76−1.73 (m, 2H). ^{13}C NMR (CD_3OD): δ 167.3, 161.4, 161.2, 161.1, 156.1, 156.0, 148.7, 147.5, 146.6, 143.3, 143.0, 138.5, 131.0, 127.8, 127.3,

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126.7, 126.4, 124.8, 118.7, 115.8, 58.1, 55.9, 40.0, 38.2, 29.8. IR (cm^{-1}): 3105, 2359, 1674, 1539, 1469, 1435, 1320, 1200, 835, 798, 721. HRMS (ESI). Calcd for $C_{28}H_{32}N_9O$ $[(M + H)^+]$: m/z 510.2730. Found: m/z 510.2733.

Metal Complexation and Ferryl Generation. The iron−ligand complexes $[Fe^{II}(OH_2)(N4Py)]^{2+}$ (**5**) and $[Fe^{II}(Cl)(3CG\text{-}N4Py)]^{2+}$ (**6**) were prepared by adding 1 equiv of $Fe^{II}(ClO_4)_2 \cdot xH_2O$ (100 mM stock solution in H_2O) to H_2O solutions of **1** and **2**, respectively. Ferryls $[Fe^{IV}(O)(N4Py)]^{2+}$ (**7**) and $[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ (**8**) were generated by adding 8 μL of a 100 mM solution of peracetic acid in H_2O (2 equiv) to solutions of **5** and **6** (200 μL, 2 mM).

Magnetic Susceptibility Measurements and NMR Studies. The solution-phase magnetic moments of iron complexes **5** and **6** were determined by ^{1}H NMR in a D_2O solvent following Evan's method[65] using *tert*-butyl alcohol as the reference. A small capillary tube (closed at one end) was filled with a solution of D_2O and *tert*-butyl alcohol [50−75 μL, 7:1 (v/v)] and sealed. Solutions of **5** (4 mM, 0.016 mmol, 4 mL) and **6** (5 mM, 0.015 mmol, 3 mL) were made by mixing ligands **1** and **2** with 1 equiv of $Fe(ClO_4)_2$, respectively, in D_2O containing *tert*-butyl alcohol. The sealed capillary tubes were placed inside the NMR tubes, iron complex solutions (800 μL) were added, and ^{1}H NMR spectra were recorded. For solution NMR studies, 10 mM solutions of ligands **1** and **2** were prepared in a D_2O solvent. Their iron complexes **5** and **6** were generated in situ by mixing 800 μL of ligand solutions **1** and **2**, with 10 μL of a 0.8 M $Fe(ClO_4)_2$ solution (203.8 mg, 0.8 mmol, 1 mL, 1 equiv) in D_2O, respectively. The samples were analyzed by ^{1}H NMR spectroscopy after the addition of ligand, iron, and CH_3CN (21 μL, 50 equiv; Figures S16−S19 in the Supporting Information).

Enzyme Inactivation Assay. The enzymatic assays were initiated by adding the substrate (*N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride for trypsin and *N*-succinyl-Ala-Ala-Pro-Phe-pNA for chymotrypsin) to the solutions of enzyme containing varied concentrations of inhibitor (0−1000 μM). The initial velocities, obtained from the A_{405} versus time plot, were converted to the percentage of enzyme activity (% enzyme activity) with respect to the control reaction containing no inhibitor. For each assay, % enzyme activity was computed using the average of three runs, with the error equal to the standard deviation, and plotted as a function of the inhibitor concentration. Data were fit using a sigmoidal equation to obtain the IC_{50} value.

Pulse Experiments with H_2O_2. The enzyme (1 μM) was incubated with $Fe^{II}(ClO_4)_2$, or **5** or **6** (20 μM) (total volume 600 μL), and H_2O_2 (5 × 40 μM) was added every 10 min. Enzyme activities were determined on aliquots removed from the solution as described above, 10 min after each addition of H_2O_2. Activities were adjusted for dilution prior to their conversion to % enzyme activity, with 100% activity equal to the activity of the blank reaction in the absence of inhibitor at $t = 0$. In control experiments, ROS scavengers D-mannitol, imidazole, and NaN_3 (see Figure 8) were added as aqueous solutions before the addition of H_2O_2.

Liquid Chromatography/Mass Spectrometry (LCMS) Analysis of Protein Samples. LCMS was performed on a Micromass QuattroLC triple quadrupole mass spectrometer with an electrospray/APCI source and Waters Alliance 2695 liquid chromatograph. Approximately 100 pmol of the sample was injected onto a Jupiter 5 μm C18 300 Å 50 × 2.0 mm column (Phenomenex) using an autosampler. Separation was achieved using a linear gradient of 20−30% (trypsin) and 20−40% (chymotrypsin) CH_3CN in water and a 0.1% HCO_2H solution in water. A total of 80 μL of H_2O (blank) was injected after each sample to minimize any carryover from the previous sample. High-performance liquid chromatography (HPLC) was carried out at a flow rate of 0.6 mL min^{-1} with the column heated to 45 °C.

Carbonyl Assay. The carbonyl contents of the oxidized enzyme samples were determined using the following literature method.[66] Enzymes (20 μM) were treated with **5** or **6** (50 μM) and pulsed with varying amounts of H_2O_2 (100−500 μM, 100 μM per pulse). After pulsing, protein samples were precipitated with 20% (w/v) trichloroacetic acid, centrifuged (5 min, 25 °C, 6600 rpm), the supernatant was removed, and the precipitate was treated with 2,4-dinitrophenylhydrazine in 2 M HCl (10 mM, 500 μL). The samples were allowed to stand at room temperature for 1 h with vortexing every 10−15 min. Enzymes were precipitated with 20% trichloroacetic acid (500 μL) and centrifuged (5 min, 25 °C, 6600 rpm), the supernatant was discarded, and the pellet was washed three times with 1 mL of ethanol/ethyl acetate (1:1) to remove any free reagent. The sample was allowed to stand 10 min before centrifugation (5 min, 25 °C, 6600 rpm), and the supernatant was discarded each time. The precipitated enzyme was dissolved in 0.6 mL of a 6 M guanidine solution (pH = 2.3, 20 mM potassium phosphate), and the carbonyl contents were deteremined by UV−vis spectroscopy using the known molar extinction coefficient, $\varepsilon_{360} = 22\,000\ M^{-1}\ cm^{-1}$.[66]

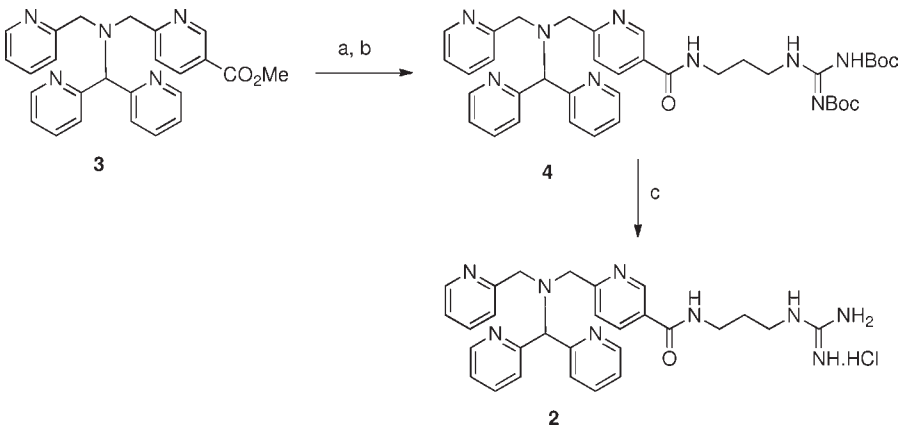
Synthesis of 10. A solution of Ac-Tyr-OH (**9**; 56 mg, 0.25 mmol) and $[Fe^{II}(N4Py)(CH_3CN)](ClO_4)_2$ (17 mg, 25 μmol) in a 10 mM acetate buffer (25 mL, pH = 6.0, 150 mM NaCl) was pulsed with H_2O_2 (50 μL × 5, 10 mM total, 2 mM per pulse) every 10 min over the course of 1 h. The solution was frozen and lyophilized. The lyophilized powder was dissolved in water and filtered through Dowex 50WX4-100 ion-exchange resin to remove ligand and iron. The filtrate was frozen and lyophilized. The product **10** was purified by HPLC (HPLC column Zorbax XDB-C18, 21.2 × 150 mm, 5 μm equipped with a guard column Zorbax XDB-C18, 21.2 mm, 5 μm, 0.1% TFA/MeOH 87:13, flow rate = 20 mL min^{-1}; T_R = 6.24 min); ^{1}H NMR and MS data of isolated **10** matched the literature data for the known compound.[67]

RESULTS

Synthesis of Ligand 2. The N4Py derivative **2**, which contains a pendant acylpropylguanidinium group, was synthesized in three steps starting from the known ester **3** (Scheme 1).[61] Following a literature procedure, refluxing the methanolic solution of **3** and 1,3-diaminopropane in the presence of a catalytic amount of NaCN under a N_2 atmosphere gave the aminated product in 77% yield.[61] The resultant primary amine was treated with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in THF/H_2O at 50 °C for 1 h, furnishing **4** in 88% yield. Compound **4** was deprotected by treatment with 4 M HCl in 1,4-dioxane for 3 h at room temperature, giving the ligand 3CG-N4Py (**2**) as a hydrochloride salt in quantitative yield.

Generation and Characterization of Ferryl Species. The ferrous complexes **5** and **6** were generated in situ by adding 1 equiv of $Fe^{II}(ClO_4)_2$ to aqueous solutions of ligands **1** and **2**, respectively. The orange complexes **5** and **6** were characterized by UV−vis and ^{1}H NMR spectroscopies and MS. UV−vis spectroscopy indicated similar data for **5** and **6** ($\varepsilon_{380} = 1700\ M^{-1}\ cm^{-1}$ with a shoulder at 460 nm and $\varepsilon_{375} = 1700\ M^{-1}\ cm^{-1}$ with a shoulder at 470 nm for **5** and **6**, respectively).[68] ^{1}H NMR spectra for **5** and **6** in D_2O were consistent with the complexes bearing high-spin $S = 2$ Fe^{II} centers. Broad resonances ranging from 150 to 20 ppm, well outside of the diamagnetic region, were observed for **5**. Complex **6** showed resonances within the same region, except that downfield resonances were split in two, which is consistent with the lower symmetry of **6** (C_1 symmetry) with respect to **5** (C_s symmetry) because of the acylpropylguanidinium group on the ligand 3CG-N4Py. Magnetic moments for **5** and **6** in D_2O were 4.4 and 4.5 μ_B, respectively, as determined by Evan's method, which are close to the expected spin-only values for a high-spin iron(II) complex.[69] Interestingly, treatment of **5** or **6** in D_2O with excess CH_3CN (50 equiv) caused a spin transition from high to low spin, as judged by ^{1}H NMR spectroscopy, in which resonances between 200 and 20 ppm were no longer observed. In addition, a sharp drop in the magnetic moment,

Scheme 1. Synthesis of Ligand 2[a]



[a] (a) 1,3-Diaminopropane, MeOH, NaCN, reflux, 24 h; (b) 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, THF/H_2O, 50 °C, 1 h; (c) 4 M HCl in 1,4-dioxane, 3 h.

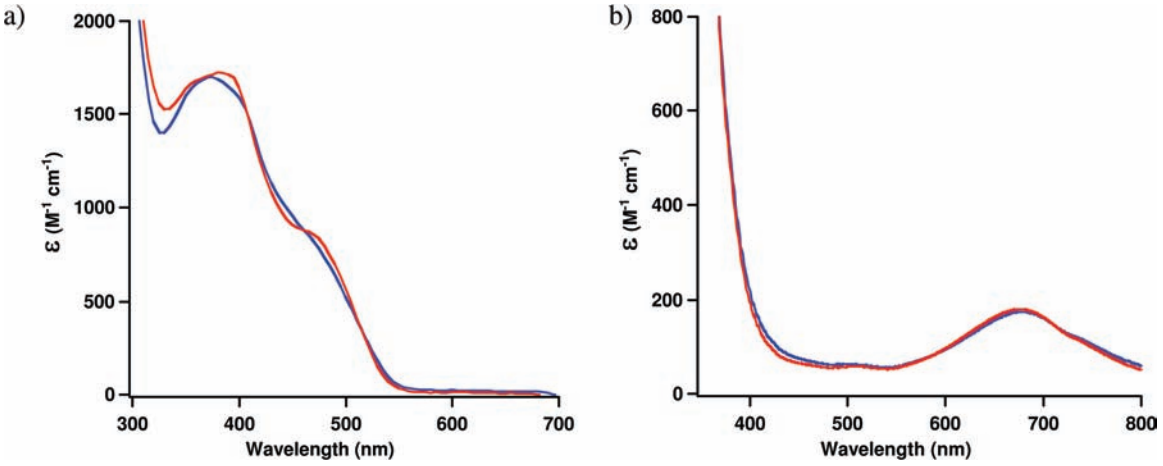


Figure 2. UV−vis spectra of ferrous and ferryl complexes in H_2O: (a) **5** (red) and **6** (blue); (b) **7** (red) and **8** (blue).

<0.2 μ_B for both samples, was observed by Evan's method. MS data for **5** in H_2O matched the formula $[Fe^{II}(OH_2)(N4Py)]^{2+}$ as described previously,[70] and data for **6** in H_2O showed a prominent molecular ion at m/z 300.5860, along with an isotope pattern that agreed with the molecular formula $[Fe^{II}(Cl)(3CG\text{-}N4Py)]^{2+}$ (Figure S3 in the Supporting Information), where chloride presumably binds to the iron center in place of H_2O because it is present as the counterion to the guanidinium salt. Treatment of **5** with peracetic acid at 25 °C gave a pale-green species, $[Fe^{IV}(O)(N4Py)]^{2+}$ (**7**), in ~20 min with a maximum absorption wavelength at λ_{max} = 680 nm (ε = 170 M^{-1} cm^{-1}), which agrees with the published data.[71] A similar procedure was used to prepare the ferryl $[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ (**8**; λ_{max} = 675 nm, ε = 170 M^{-1} cm^{-1}; Figure 2) from **6**, where it took ~10 min for complete generation. Ferryl **8** was characterized by high-resolution electrospray ionization MS (ESMS), which displayed a prominent molecular ion at m/z 194.0682, along with a suitable isotopic pattern, consistent with a trication derived from the molecular formula $[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ (Figure S1 in the Supporting Information).

Enzyme Inactivation with Ferryl Compounds. In order to evaluate the ability of the ferrous and ferryl complexes **5**−**8** to inactivate trypsin, the enzyme activities were determined in the presence of varied concentrations of these reagents along with controls, and IC_{50} values were calculated (Table 1). In these studies, trypsin (1 μM) was treated with each reagent (0−1000 μM) in a acetate buffer (pH = 6.0, 150 mM NaCl) at room temperature. After incubation for 1 h, the enzyme activities were determined by addition of the chromogenic substrate BAPNA. These experiments indicated that ligands **1** and **2** inhibit trypsin in the mid-micromolar range (entries 1 and 2). Ligand **2** containing the propylguanidinium group was the more potent inhibitor. Despite the fact that $Fe^{II}(ClO_4)_2$ had no effect on the trypsin activity, the ferrous complexes **5** and **6** showed more potent inhibition than ligands alone (entries 3 and 4). Although peracetic acid inhibited trypsin, ferryls **7** and **8** were more potent oxidants (entries 5−7). Between the ferryls (Figure 3a), **8** (IC_{50} = 54 μM) was found to be a better oxidant than **7** (IC_{50} = 71 μM).

When inactivation studies were performed on chymotrypsin, different results were obtained (Table 1). Neither the ligands **1** and **2** nor the ferrous complexes **5** and **6** (entries 1−4) inhibited chymotrypsin in the range from 0 to 1000 μM, with the exception of **6**, which inhibited chymotrypsin only at very high concentrations (IC_{50} = 790 μM). This observation is consistent with the loss of binding at the active site because the S_1 pocket of chymotrypsin prefers hydrophobic groups as opposed to the charged propylguanidinium group of **2**. Despite the fact that ligands **1** and **2** and their iron complexes show only weak

inhibition of chymotrypsin, both ferryls **7** and **8** (entries 6 and 7) inactivate chymotrypsin in a concentration-dependent fashion (Figure 3b). Between the two ferryls, again **8** was found to be a better oxidant, and a higher level of selectivity was observed between the ferryls with chymotrypsin versus trypsin. Binding of ferryl **8** near the active site is not likely because the S_1 pocket prefers hydrophobic groups. Instead, the lower IC_{50} value of **8** compared to **7** may be attributed to favorable electrostatic interactions between the charged ferryl **8** and carboxylates on the surface of chymotrypsin, which are present in higher abundance than with trypsin (Figure S4 in the Supporting Information).[72]

Characterization of Products. To gain insight into the mode of inactivation, enzyme products were analyzed by SDS-PAGE (Figure 4), which confirmed that trypsin was oxidized but not cleaved extensively after treatment with ferryls **7** and **8**. Incubation of the protein alone for 1 h resulted in a major loss of intensity for the parent band at 23 kD and the formation of new bands with lower molecular weight, consistent with cleavage of the protein by autoproteolysis (Figure 4a). In contrast, enzyme samples treated with peracetic acid or ferryls **7** and **8** showed little or no cleavage of the protein, consistent with an inactivation event being faster than autoproteolysis of the enzyme. In the case of trypsin treated with peracetic acid (lane 3), a sharp, well-defined band was observed at 23 kD, whereas samples treated with the ferryl complexes showed a broad, smeared band (lanes 4 and 5) near the trypsin region (~23 kD), indicating a distribution of molecular weights near the parent mass of the enzyme.[73]

Similar results were obtained with chymotrypsin. In the control sample with enzyme alone, fragments with lower molecular weights were observed, which is consistent with autoproteolysis (Figure 4b, lane 2). Autoproteolysis was inhibited by peracetic acid, but the enzyme appeared as a sharp band (not shown). In contrast, broad smeared bands near the 25 kD region were observed for chymotrypsin treated with ferryls **7** and **8** (lanes 3 and 4). Fragments of lower molecular weight were not observed in lanes 3 and 4, indicating that inactivation of the enzyme was rapid and complete before autoproteolysis began. Because chymotrypsin is composed of three subunits, α (1254 kD), β (13 924 kD), and γ (10 067 kD), which are linked by disulfides, the same three samples were treated with dithiothreitol (DTT) to determine if internal cross-links had formed between the subunits. Lane 5 with enzyme plus DTT showed three major fragments, plus new bands that were expected because of autoproteolysis. Only three bands were observed in samples treated with ferryls **7** and **8** followed by DTT (lanes 6 and 7), which are consistent with the expected molecular weights for the subunits (β and γ) plus an additional band resulting from incomplete cleavage of the disulfide bond between the α and β subunits. Although the band at 14 kD could represent a covalent cross-link that was not cleaved by DTT, the sample of enzyme only treated under the same conditions shows the same pattern, suggesting that incomplete reduction of the disulfide bond between the α and β subunits occurred. However, bands at 14 and 11 kD in samples treated with ferryls **7** and **8** appear broad with respect to the same bands observed in lane 5, as would be expected if the individual subunits were oxidized at the side chains and present as a distribution of masses near the parent region.

LCMS chromatograms of trypsin (20 μM, not incubated), and trypsin incubated with the ferryls **7** and **8** (1000 μM) for 1 h at room temperature, show different retention times and product distributions for enzyme (control) versus enzymes treated with ferryls **7** and **8** (Figure 5, left panels). Integration of the peaks and deconvolution of the MS spectra gave a single peak for the

Table 1. IC_{50} Values (μM) for the Inhibition of Trypsin and Chymotrypsin by Ligands 1 and 2 and Their Respective Ferrous (5 and 6) and Ferryl (7 and 8) Complexes

entry	compound	trypsin[a]	chymotrypsin[a]
1	N4Py (**1**)	493	>1000
2	3CG-N4Py (**2**)	354	1024
3	$[Fe^{II}(OH_2)(N4Py)]^{2+}$ (**5**)	301	>1000
4	$[Fe^{II}(Cl)(3CG\text{-}N4Py)]^{2+}$ (**6**)	217	787
5	peracetic acid	236	128
6	$[Fe^{IV}(O)(N4Py)]^{2+}$ (**7**)	71	119
7	$[Fe^{IV}(O)(3CG\text{-}N4Py)]^{3+}$ (**8**)	54	26
8	$Fe^{II}(ClO_4)_2$	>1000	>1000

[a] The % enzyme activities at different concentrations were determined as the average from three independent experiments, with 100% activity equal to the activity of the blank reaction in the absence of inhibitor and the error equal to the standard deviation of the data set. Activities were plotted against log [inhibitor] and fit to a sigmoidal curve to calculate the IC_{50} values. The enzyme (trypsin or chymotrypsin) concentration was 1.0 μM; the trypsin substrate *N*-α-benzoyl-DL-arginine-4-nitroanilide hydrochloride and chymotrypsin substrate *N*-succinyl-Ala-Ala-Pro-Phe-pNA concentrations were 1.0 mM. The reactions were conducted at pH = 6.0 in a 10 mM acetate buffer containing 150 mM NaCl.

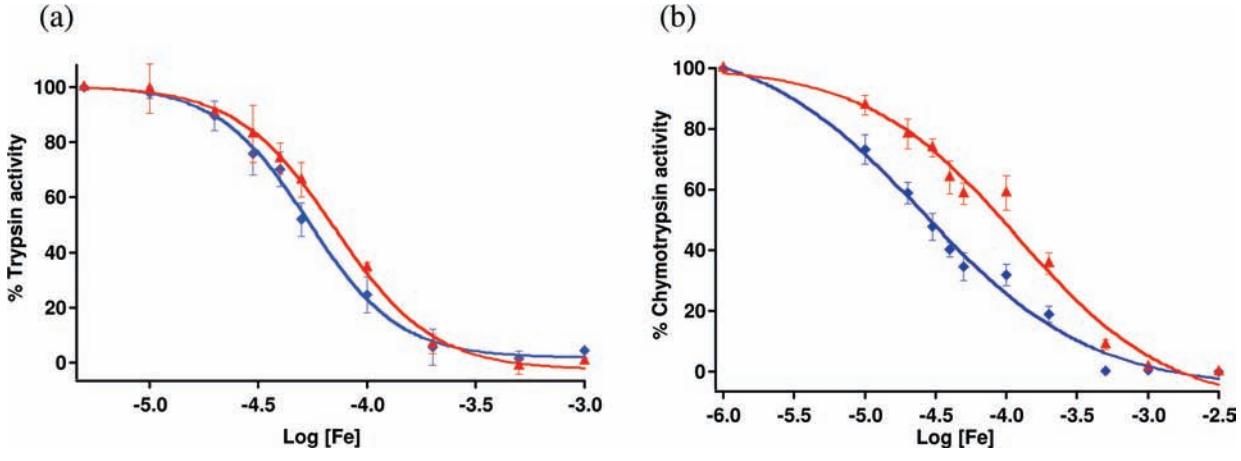


Figure 3. IC_{50} plots with ferryl species **7** (red ▲) and **8** (blue ◆) for trypsin (a) and chymotrypsin (b). See Table 1 footnote a for conditions and other details.

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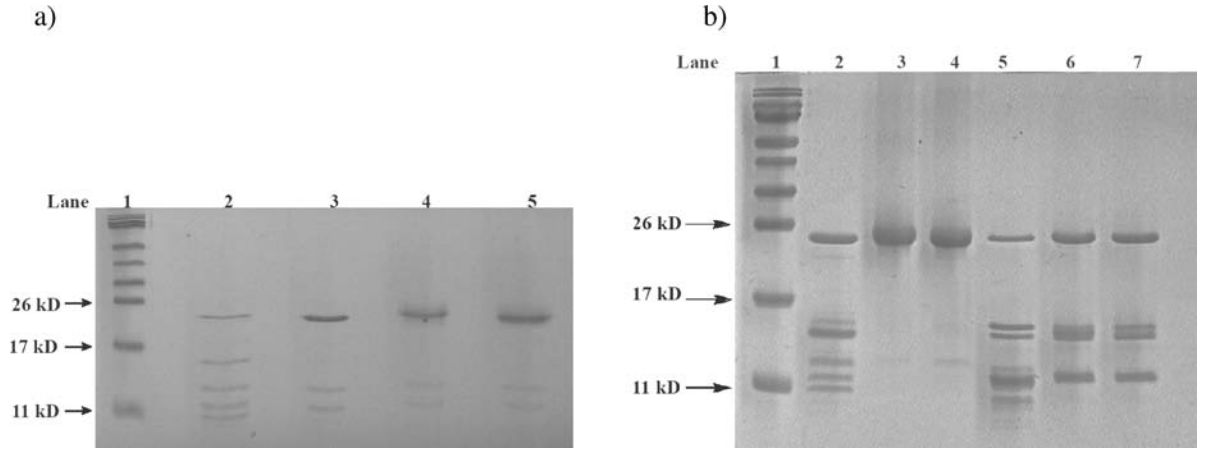

Figure 4. (a) SDS-PAGE analysis (16%), stained with Coomassie blue, of trypsin (20 μM) incubated at room temperature for 1 h at pH = 6.0, in a 10 mM acetate buffer containing 150 mM NaCl. A smeared band near 23 kD in lanes 3−5 showed oxidized enzyme. Lane 1: MW marker. Lane 2: enzyme only. Lane 3: peracetic acid (1000 μM). Lane 4: **7** (1000 μM). Lane 5: **8** (1000 μM). (b) SDS-PAGE analysis (16%), stained with Coomassie blue, of chymotrypsin (20 μM) incubated at room temperature for 1 h at pH = 6.0, in a 10 mM acetate buffer containing 150 mM NaCl. A smeared band near 25 kD in lanes 3 and 4 showed oxidized enzyme. Lane 1: MW marker. Lane 2: enzyme only. Lane 3: **7** (500 μM). Lane 4: **8** (500 μM). Lane 5: enzyme with DTT (100 mM). Lane 6: **7** (500 μM) with DTT (100 mM). Lane 7: **8** (500 μM) with DTT (100 mM). Equal amounts of protein were loaded in each lane.

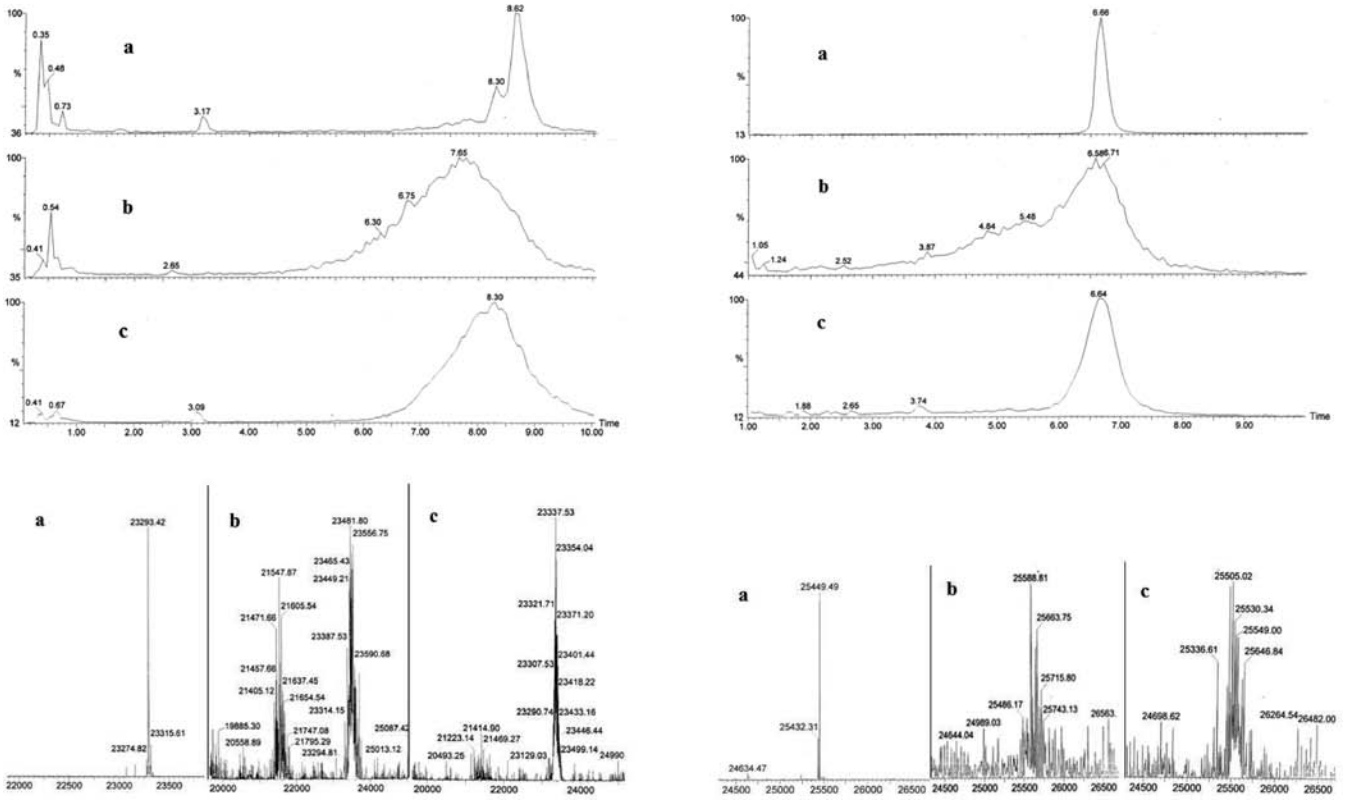

Figure 5. Left panels: LC chromatograms (top) and deconvoluted MS spectra (bottom) of trypsin (spectrum a) and after treatment with **8** (spectrum b) or **7** (spectrum c). Reactions were conducted at room temperature by incubating trypsin (20 μM) with **7** or **8** (1000 μM) for 1 h before desalting and subjecting the samples to ESMS analysis. Right panels: LCMS chromatograms (top) and deconvoluted MS spectra (bottom) of chymotrypsin (spectrum a) and after treatment with **8** (spectrum b) or **7** (spectrum c). Reactions were conducted at room temperature by incubating chymotrypsin (20 μM) with **7** or **8** (1000 μM) for 1 h before desalting and subjecting the samples to ESMS analysis.

control sample with enzyme only, which corresponds to the MW of bovine trypsin (MW = 23 293) and complex spectra for both samples treated with ferryls. For the treated samples, a distribution of molecular weights larger than that of trypsin was observed, consistent with oxidation of the enzyme by the addition of multiple oxygen atoms to the protein, as evidenced by the difference between masses corresponding to 16 units. In the case of the sample treated with ferryl **8** (left panel, spectrum b), a second group of

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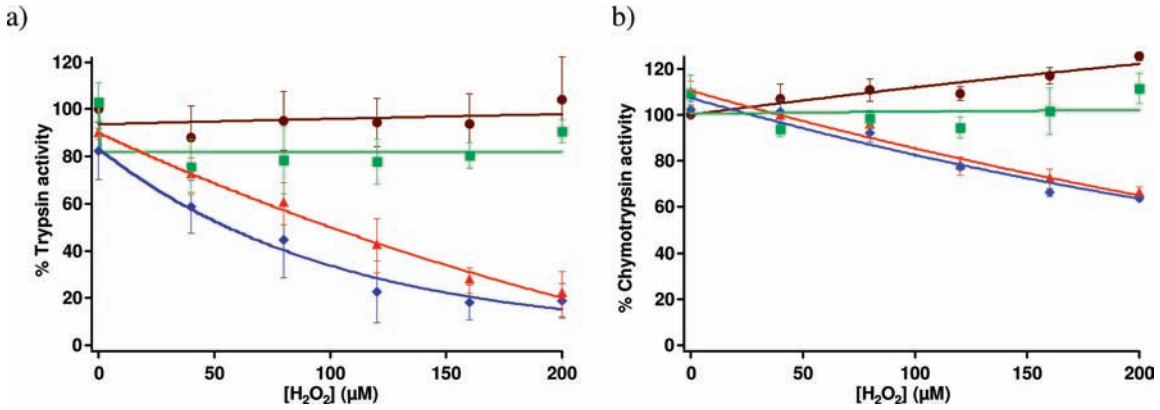

Figure 6. Trypsin (graph a) and chymotrypsin (graph b) activities as a function of added H_2O_2. Samples containing enzyme only (brown ●,1 μM) and enzyme treated with $Fe^{II}(ClO_4)_2$ (green ■), **5** (red ▲), or **6** (blue ◆) (20 μM) were pulsed with H_2O_2 every 10 min (40 μM per pulse). The reaction was performed in an acetate buffer of pH = 6.0 containing 150 mM NaCl. The activities were converted to % enzyme activity, with 100% activity equal to the activity of the blank reaction in the absence of inhibitor at t = 0. Data points are averages from three independent experiments, where errors are reported as standard deviations.

peaks was observed below the parent mass of trypsin, which is consistent with oxidation of trypsin and cleavage of a small fragment, approximately 2 kD in weight, most likely from the N terminus (vide infra). Similar results were observed for chymotrypsin. LCMS chromatograms of chymotrypsin (20 μM) incubated with the ferryls **7** and **8** (1000 μM) for 1 h at room temperature are shown in Figure 5 (right panels). The sample with enzyme only showed a sharp peak that was integrated and deconvoluted to a mass of 25 449, whereas broad peaks were observed for samples treated with ferryls **7** and **8**. Again integration of these peaks gave complex spectra with peaks higher in masses than the chymotrypsin itself consistent with oxidation of protein.

Enzyme Inactivation with Ferrous Complexes and H_2O_2. After it was established that trypsin and chymotrypsin could be inactivated selectively with ferryls **7** and **8**, experiments were performed in which the enzymes were treated with ferrous complexes **5** and **6** prior to the addition of oxidant. These conditions were examined to determine if catalytic inactivation of the proteases was possible. When enzymes were incubated in the presence of **5** or **6** under an aerobic atmosphere, the enzyme activity did not change within reasonable time periods (<6 h). Similar results were obtained in the presence of the reductant ascorbate or DTT, which did not accelerate enzyme inactivation. Thus, these results confirm that enzyme inactivation of trypsin and chymotrypsin using O_2 as the oxidant, either in the presence or absence of reductant, is a relatively slow process with these iron complexes.[74]

More promising results were obtained when H_2O_2 was used as the oxidant. For these experiments, solutions of trypsin (1 μM) and several iron species [$Fe^{II}(ClO_4)_2$, **5** or **6**, 20 μM] were pulsed with H_2O_2 (40 μM per pulse). Enzyme activities were determined by removing aliquots after each pulse (10 min between pulses) and plotted against the total amount of peroxide added (Figure 6a). Negligible changes in the activities were observed with trypsin alone and trypsin treated with $Fe^{II}(ClO_4)_2$. In the case of the sample treated with $Fe^{II}(ClO_4)_2$, the trypsin activity dropped immediately by ~15% after the first addition of H_2O_2 but did not drop further after the addition of more H_2O_2, which is consistent with a Fenton reaction carried out by an iron species that was no longer viable after the first addition of the oxidizing reagent. In contrast, enzyme activities were lost in a dose-dependent fashion with respect to H_2O_2 when pulsing was performed in the presence of **5** or **6**. In both cases, the activities were diminshed by roughly 80% after the addition of 200 μM H_2O_2, 10 equiv with respect to the iron complexes. These data confirm that **6**, which contains the propylguanidinium group, inactivates trypsin more effectively than **5**. For example, after three additions of H_2O_2 (120 μM total), the trypsin activity dropped by 80% with **6** compared to 60% with **5**. The more efficient inactivation of trypsin with **6** compared to **5** confirms that selectivity can be gained between two iron complexes and is consistent with the protein-affinity group (propylguanidinium) of **6** directing the complex toward the protein, presumably the S_1 pocket where Asp 189 may form a salt bridge with the guanidinium group.

When chymotrypsin was treated with H_2O_2 under the same conditions, controls with enzyme alone or enzyme plus $Fe^{II}(ClO_4)_2$ showed no significant changes after the addition of 200 μM H_2O_2 (Figure 6b). Again, the enzyme activity was lost in a dose-dependent fashion when chymotrypsin was pulsed with H_2O_2 in the presence of **5** and **6**. However, the enzyme activities dropped only by ~35% in both cases after the addition of 200 μM H_2O_2, as opposed to 80% with trypsin. Selectivity was not observed between **5** and **6**. These data confirm that selective inactivation of trypsin over chymotrypsin can be observed with the iron complexes **5** and **6** in the presence of H_2O_2. Differences in reactivity for **5** and **6** with trypsin versus chymotrypsin parallel data from inhibition studies (Table 1, entries 3 and 4), where the complexes **5** and **6** show more a potent inhibition of trypsin than chymotrypsin. The trend observed is consistent with the active reagents derived from **5** or **6** and H_2O_2 possessing a higher effective molarity for trypsin over chymotrypsin and causing more efficient oxidative damage.

Analysis by LCMS and SDS-PAGE was used in order to characterize the enzyme products from trypsin and chymotrypsin. SDS-PAGE analysis of the enzymes (1 μM), pulsed with H_2O_2 (40 μM per pulse) every 10 min, in the presence of **6** showed an increase in the smearing of the band near the parent trypsin band (23 kD, Figure 7a) and the chymotrypsin band region (25 kD, Figure 7b) with added H_2O_2, although smearing was less extensive than in the samples treated with ferryls **7** and **8**. This observation is consistent with a distribution of molecular weights after oxidation with **6** and H_2O_2. Because other bands were not observed in either case, the oxidation with **6** and H_2O_2

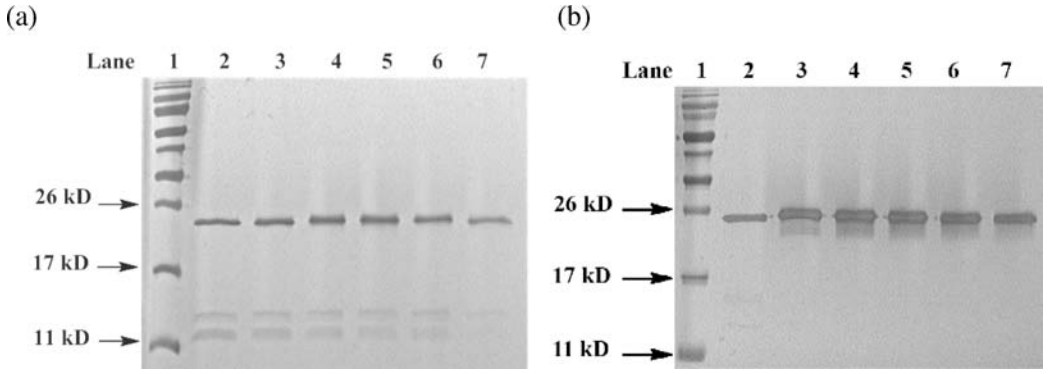

Figure 7. SDS-PAGE analysis (16%), stained with silver, of (a) trypsin and (b) chymotrypsin (1 μM), pulsed with H_2O_2 (40 μM) every 10 min in the presence of **6** (20 μM) at room temperature at pH = 6.0, in a 10 mM acetate buffer containing 150 mM NaCl. Smeared bands near 23 kD in part a and 25 kD in part b in lanes 3−7 showed oxidized enzymes. Lane 1: MW marker. Lane 2: enzyme only. Lane 3: H_2O_2 (40 μM). Lane 4: H_2O_2 (80 μM). Lane 5: H_2O_2 (120 μM). Lane 6: H_2O_2 (160 μM). Lane 7: H_2O_2 (200 μM).

does not lead to cleavage of the enzymes. The chromatograms of trypsin (1 μM) and chymotrypsin pulsed with H_2O_2 (40 μM per pulse) in the presence of **5** or **6** (20 μM) at room temperature show different retention times and product distributions for enzyme (control) versus treated samples (Figure S5 in the Supporting Information). Integration of the peaks and deconvolution of the MS spectra gave single peaks for the control samples with enzyme only, while complex spectra were obtained for samples where enzymes were pulsed with H_2O_2 in the presence of **5** or **6**, confirming that oxidation of the proteins occurred.

Mechanistic Studies. Additional experiments were carried out to gain further insight into the nature of protein inactivation under single-turnover (ferryls **7** and **8**) and catalytic conditions (**5** or **6** plus H_2O_2). In particular, the identity of the active oxidant under catalytic conditions was in question. Both $HO^{\bullet}$ (Fenton chemistry) and iron-based oxidants have been implicated in oxidation reactions when ferrous complexes such as **5** are treated with H_2O_2, and the outcome varies according to the ligand set and the conditions of the experiment (vide infra).[75−77] To distinguish between Fenton chemistry ($HO^{\bullet}$) and iron-based oxidants, control experiments were performed in the presence of reagents that scavenge ROS. When the inactivation of trypsin with **6** was performed in the presence of D-mannitol, a potent hydroxyl radical scavenger, inactivation was not inhibitited with 0.1 mM D-mannitol and was only slightly inhibited with 20 mM D-mannitol, confirming that $HO^{\bullet}$ can act as only a minor component of the inactivation pathway if at all (Figure 8 for trypsin; see Figure S6 in the Supporting Information for chymotrypsin). Thus, another oxidant was likely at play. Similar results were obtained in the presence of imidazole, a scavenger of the hydroxyl radical and $^1O_2$. Interestingly, inactivation was inhibited somewhat in the presence of NaN_3 (0.1−1 mM), again in a concentration-dependent fashion. Although azide is a scavenger of $^1O_2$, it is known to inhibit tyrosine nitration catalyzed by the heme enzyme myeloperoxidase.[78] In this case, rather than quenching $^1O_2$, competitive binding of azide to the peroxidase iron center may poison the heme by blocking access to H_2O_2.[79] In support of this mode of inhibition, the binding of azide to **6** was confirmed by UV−vis titration of **6** with NaN_3 (Figure S7 in the Supporting Information), which suggests that the action of peroxide with the iron catalyst can be inhibited by ligands that bind competively to the sixth site of the iron center. Together, these results indicate that an oxidant other than $HO^{\bullet}$ or $^1O_2$ is responsible for the

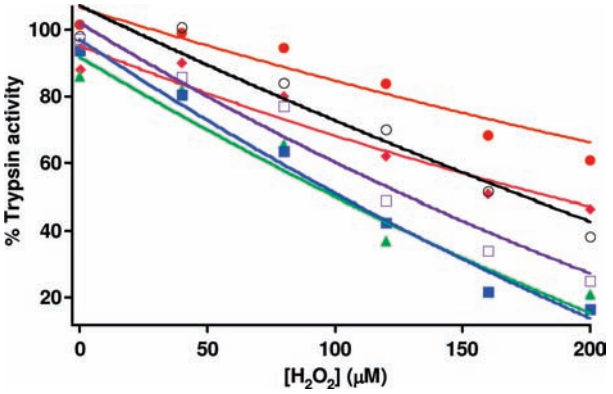

Figure 8. Inactivation of trypsin in the presence of ROS scavangers (azide, D-mannitol, and imidazole). The reactions were conducted at pH = 6.0, in a 10 mM acetate buffer containing 150 mM NaCl. Trypsin (1 μM) with **6** (20 μM) was pulsed with H_2O_2 (40 μM) in the presence of a buffer as a blank (blue ■) and ROS scavengers D-mannitol (0.1 mM, green ▲; 20 mM, black ○), imidazole (0.1 mM, purple □), and NaN_3 (0.1 mM, maroon ◆; 1.0 mM, red ●). Enzyme activities, where 100% activity is equal to the activity of the blank reaction in the absence of inhibitor at t = 0, were determined by removing aliquots before each pulse (10 min between pulses) and plotted against the total amount of peroxide added. Data points are averages from three independent experiments; errors were ±5%.

inactivation of trypsin and chymotrypsin in the presence of **5** or **6** and H_2O_2.

The presence of ketones and aldehydes in proteins, known as the carbonyl content, is a key indicator of oxidative damage observed in vitro and in vivo during aging and under conditions of oxidative stress.[66] Because normal proteins do not contain ketone or aldehyde carbonyls that react electrophilically with hydrazine nucleophiles, the reagent 2,4-dinitrophenylhydrazine can be used to determine the overall protein carbonyl content resulting from protein oxidation. Following a literature method,[66] the amount of carbonyl groups formed with trypsin and chymotrypsin (20 μM) was determined as a function of the pulsed H_2O_2 concentration in the presence of **6** (50 μM; Figure 9). Similar results were obtained with **5** (not shown). In the case of both enzymes, an increase in the carbonyl content was observed that plateaued at 500 μM H_2O_2 to approximately 20 nmol/mg of protein. Because nearly identical results were

obtained with trypsin and chymotrypsin, these data indicate that the formation of protein carbonyls is not coupled to the inactivation event imparted by **6** and H_2O_2 (Figure 6a,b). In fact, using the molecular weight of the proteins and the observed content of protein carbonyls, one can estimate that only four or five carbonyls are formed per protein out of 223 (trypsin) and 241 (chymotrypsin) residues when the proteins (20 μM) are treated with **6** (50 μM) and 500 μM H_2O_2. Assuming that a single carbonyl is formed per oxidized residue, this modification accounts for less than 3% of all residues.

The aforementioned results confirm that a unique oxidant must give preferential inactivation of trypsin over chymotrypsin under catalytic conditions. Attempts to analyze the oxidized proteins by LCMS/MS analysis were inconclusive due to low sequence coverage because the oxidized proteins did not respond well to digestion. Although LCMS/MS analysis can reveal information about the location of oxidized residues in proteins, it is difficult to obtain quanitative measurements of protein oxidation using this method. Therefore, amino acid analysis was performed on the oxidized samples, in order to determine which types of residues were affected by treatment of trypsin and chymotrypsin under the single-turnover (ferryls **7** and **8**) and catalytic conditions (ferrous complexes **5** and **6** plus H_2O_2) (Figure 10). Strikingly, catalytic conditions produced a major modification in the levels of the amino acid tyrosine with respect to the control, while leaving other amino acid levels unchanged within the margin of error. Different results were obtained with the ferryls, where the levels of tyrosine, cysteine, and tryptophan were diminished significantly with respect to controls. These data are consistent with published model studies that indicated that cysteine, tyrosine, and tryptophan were the most reactive amino acids with **7**.[63] Results obtained in the single-turnover and catalytic conditions were not highly dependent on the ligand structure, suggesting that only a modest level of directablility was achieved by attaching the propylguanidinium group to the N4Py ligand. These results confirm that catalytic conditions result in a milder form of oxidation than oxidation by the ferryls and support the hypothesis that an oxidant other than ferryl is acting in the presence of H_2O_2.

In order to gain insight into the reaction of the iron catalysts with tyrosine in the presence of H_2O_2, the oxidation of **9** was examined. Under the same conditions as those used in the enzyme experiments, the catechol product Ac-DOPA-OH (**10**) was observed as a major product by HPLC analysis (Scheme 2 and Figure S8 in the Supporting Information). Dityrosine, a common product of tyrosine oxidation resulting from oxidative coupling of the two phenol rings, was detected by fluorescence analysis of the crude reaction mixture but not by ESMS, suggesting that this product was present in only trace amounts. Importantly, chlorotyrosine was not detected by ESMS or HPLC analysis of the crude reaction mixture, which confirmed that

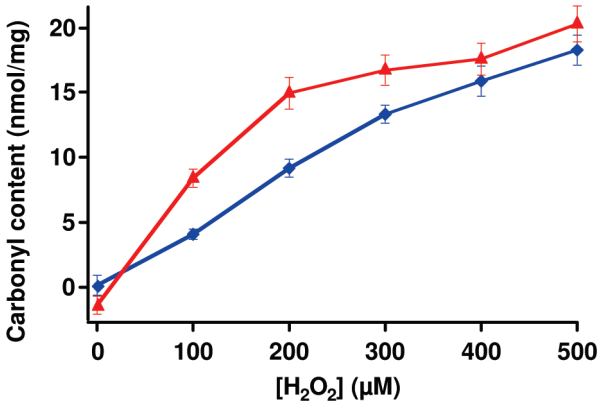

Figure 9. Carbonyl content (nmol/mg of protein) as a function of pulsed H_2O_2 (0−500 μM) for enzymes (20 μM) trypsin (blue ●) and chymotrypsin (red ▲) in the presence of **6** (50 μM). Data points are averages from three independent experiments, where the errors are reported as standard deviations.

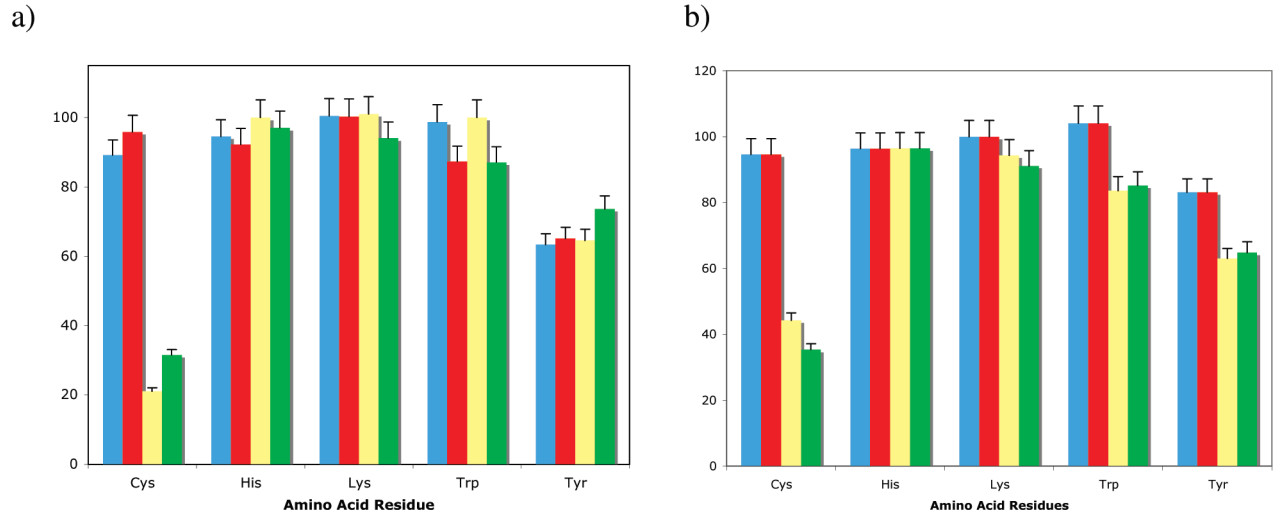

Figure 10. Bar graphs showing the compositions of five natural amino acids present in the oxidized proteins samples isolated from single-turnover (ferryls **7** and **8** in green and gold, respectively) and catalytic conditions (**5** or **6** plus H_2O_2 in red and blue, respectively) for trypsin (a) and chymotrypsin (b). Compositions are reported as percentages normalized with respect to the control experiments with no iron or oxidant added. Enzymes trypsin and chymotrypsin (20 μM) were treated with ferryl **7** or **8** (1000 μM) or with **5** or **6** (50 μM) and pulsed with H_2O_2 (500 μM total, 100 μM per pulse). Standard errors are 10%. The results indicate that tyrosine was modified selectively in the case of catalytic conditions with **5** or **6** plus H_2O_2, whereas cysteine, tyrosine, and tryptophan were modified by ferryls **7** and **8**. Full tables of calculated and experimental compositions for all 20 natural amino acids and cystic acid are presented in the Supporting Information (Tables S1−S4).

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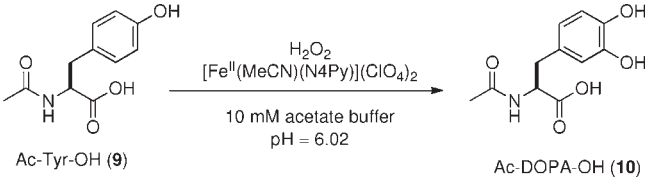
Scheme 2



oxidation of **9** was not due to the action of HOCl generated from H_2O_2 and chloride ion.[80,81] Taken together, these observations are diagnostic and significant because catechol products are not formed when tyrosine derivatives are treated with the ferryl **7**, confirming that a different mechanism of action takes place when H_2O_2 is used as the oxidant. In other words, ferryl **7** was not the active oxidant. To probe this further, the reaction was monitored by UV−vis spectroscopy. When the ferrous complex **5** was treated with 0.5 equiv of H_2O_2 under these conditions, bleaching occurred, which consumed the signature absorbances for Fe^{II} at 380 and 464 nm (Figure S9 in the Supporting Information). However, ferryl **7** was not observed, suggesting that a ferric complex was formed instead (vide infra). When this mixture was treated with **9**, the yellow color of the starting ferrous complex returned and reached completion after approximately 10 min, as judged by UV−vis spectroscopy. If this reaction solution was treated again with peroxide, bleaching occurred again, and after ca. 10 min, the UV spectrum returned to its original intensity, suggesting that turnover was possible without significant loss of the starting ferrous complex (Figure S10 in the Supporting Information). In conclusion, these results are consistent with the phenol ring of tyrosine being oxidized by an oxidant other than ferryl **7**, which is discussed in the following section.

DISCUSSION

Our results confirm that ferryls are potent and selective oxidants that inactivate serine proteases. The ferryls **7** and **8** were more powerful oxidants than peracetic acid. Selectivity was observed for a single ferryl between two enzymes (trypsin and chymotrypsin; Table 1, entry 7 vs entry 8) but also for a single enzyme between two ferryls (Table 1, entries 7 and 8, trypsin vs chymotrypsin). Ferryl **8** was most effective in the inactivation of chymotrypsin with an IC_{50} value of 24 μM. With both serine proteases, the oxidation of protein side chains occurred. Major cleavage products were not observed by SDS-PAGE or LCMS, confirming that cleavage of the protein backbone is a slow process relative to side-chain oxidation. In the oxidation of trypsin by **8**, LCMS analysis indicated a minor cleavage product consistent with a loss of approximately 2 kD, presumably at a glysine residue located near the active site, 20 residues from the N terminus of the enzyme (Figure S11 in the Supporting Information). Therefore, these results are in good agreement with model studies that focused on protected amino acids,[63] which confirmed that activation of the α-CH bond of amino acids and resultant oxidative cleavage of the backbone are slow relative to side-chain oxidation with ferryl complexes. Futhermore, the results from the amino acid analysis of enzyme samples oxidized by the ferryls prove a direct correlation between the most reactive amino acids (cysteine, tyrosine, and tryptophan) in the model studies with Ac-AA-NHtBu substrates and the residues modified in the proteins trypsin and chymotrypsin. These results have relevance to biology because, under conditions of oxidative stress, ferryls such as ferrylmyoglobin can oxidize proteins.[21,82−88] Observations reported herein reveal useful information about how proteins are modified and inactivated by ferryls. For example, when tyrosine and trytophan residues are oxidized, as our results confirm, this type of oxidation is often irreversible.[17] In contrast, oxidative modification of cysteine residues can be reversed if disulfides are formed.[89] However, when higher oxidation states of sulfur are obtained, such as sulfenic and sulfonic acid derivatives, oxidative damage becomes irreversible. The levels of cystic acid did not change in these studies between control and treated samples of enzyme, consistent with the oxidants presented herein forming products other than cystic acid.

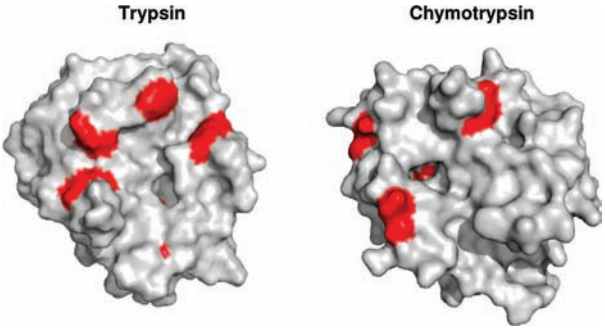


Figure 11. Surface models of trypsin and chymotrypsin with tyrosine residues near the active site highlighted in red. Trypsin contains four residues near the active site, whereas chymotrypsin contains three.

Resonable levels of selectivity were observed in the oxidation of trypsin and chymotrypsin catalyzed by **5** and **6** in the presence of H_2O_2. However, as opposed to the single-turnover conditions where ferryl **8** was the most effective at inactivating chymotrypsin, catalytic conditions provided the most efficient inactivation of trypsin with both **5** and **6**. The advantage of incorporating iron and oxidant with the inhibitors **1** and **2** was clear. Upon addition of these two reagents, a ligand concentration of 20 μM becomes more effective than 500 μM inhibitor alone, which amplifies the effects of the ligand by almost 2 orders of magnitude. The results obtained from amino acid analysis prove that tyrosine residues of both proteins were selectively targeted under these conditions, with residues of trypsin being diminished to a greater extent (60% of control) than chymotrypsin (80%). These results suggest that iron complexes derived from N4Py mimic the action of myeloperoxidase, a heme enzyme produced by neutrophil granulocytes (white blood cells) that is known to oxidize tyrosine residues of proteins using H_2O_2 as the oxidant.[90]

The selective inactivation of trypsin observed under catalytic conditions may be due to the active iron reagent obtained with H_2O_2, giving a higher effective molarity with trypsin than with chymotrypsin. Alternatively, this selectivity could be due to an inherent sensitivity of trypsin over chymotrypsin to oxidation of its Tyr residues. Both enzymes have numerous tyrosine residues on the surface near the active site (Figure 11). The fact that ligands **1** and **2** were more effective at inhibiting trypsin than chymotrypsin, in the absence and presence of $Fe^{II}(ClO_4)_2$, supports the hypothesis that the active reagents derived from **5** and **6** bind tighter to trypsin than chymotrypsin. Furthermore, previous studies performed with $^1O_2$ confirmed that trypsin and chymotrypsin have similar sensitivity to oxidation, although the types of residues targeted in this case were likely different and were not confirmed.[91] More studies in this area are needed, specifically over a larger group of enzymes such as the family of

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serine proteases, to discern between these two possibilities. Importantly, these studies suggest that incorporating protein affinity groups that bind tighter to enzyme targets and place iron catalysts in the vicinity of tyrosine residues has the potential to achieve protein inactivation at low concentrations.

The results with iron complexes and H_2O_2 indicate that a unique oxidant was responsible for the selective protease inactivation. Control experiments ruled out ROS such as $HO^{\bullet}$ (Fenton chemistry) or $^1O_2$. The action of ferryls under these conditions is also unlikely because ferryls were not observed when the ferrous complexes **5** or **6** were treated with peroxide.[92] Furthermore, studies in Table 1 prove that chymotrypsin is inactivated at lower ferryl concentrations than trypsin, so the opposite trend in selectivity would be expected under catalytic conditions if ferryls were operating. The action of a different oxidant is also supported by the fact that catechol is observed in the oxidation of Ac-Tyr-OH **9** with H_2O_2. Catechols are not observed when tyrosine derivatives are treated with ferryl **7**; instead, hydrogen-atom transfer occurs to generate phenoxyl radicals that decompose, presumably by polymerization.[63] Taking into account our results and data in the literature,[93] we can conclude that another oxidant is likely to be generated under catalytic conditions. However, further mechanisitc studies in this area are needed to differentiate between the potential pathways and to determine if there is an analogy between the chemistry reported herein and the action of the nonheme iron enzyme tyrosine hydroxylase[52,94,95] or the heme enzyme myeloperoxidase.[90] In any case, the effect of the ligand N4Py is clear in the inactivation experiments because $Fe^{II}(ClO_4)_2$ did not give the same level of dose-dependent inactivation with H_2O_2.

CONCLUSIONS

Nonheme iron complexes inactivate serine proteases selectively. Ferryls are potent oxidants that inactivate trypsin and chymotrypsin by oxidation of amino acid side chains rather than backbone cleavage, where the residues cysteine, tyrosine, and tryptophan are the most susceptible to oxidation. Oxidation of the proteases by ferrous complexes in the presence of hydrogen peroxide leads to the preferential inactivation of trypsin over chymotrypsin. In this case tyrosine residues are targeted, and data support action of a pathway unique from that of an Fe^{II}/Fe^{IV} cycle involving ferryl compounds. Importantly, these results suggest a promising future direction for the method presented herein where complexes that cycle between accessible oxidation states are used to target proteins. Although the scope of such a method may be limited with respect to ROS for attack on tyrosine residues, there are many proteins, including important medicinal targets, that contain crucial tyrosine residues near their active sites.

ASSOCIATED CONTENT

Supporting Information. Amino acid analysis data, UV−vis spectra, HRMS and LCMS data, HPLC chromatograms, ^1H and ^{13}C NMR data, and three-dimensional figures of trypsin and chymotrypsin. This material is available free of charge via the Internet at http://pubs.acs.org.

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