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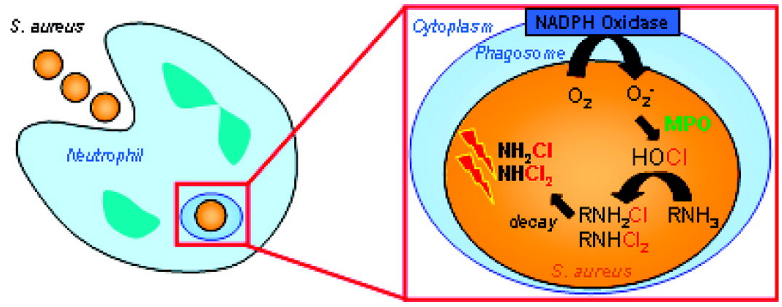

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Pathways for the Decay of Organic Dichloramines and Liberation of Antimicrobial Chloramine Gases

Melanie S. A. Coker,*,† Wan-Ping Hu,‡,§ Senti T. Senthilmohan,‡ and Anthony J. Kettle†

Free Radical Research Group, Department of Pathology, University of Otago, Christchurch, P.O. Box 4345, Syft Technologies Ltd., P.O. Box 28-149, and Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

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When neutrophils phagocytose bacteria, they generate the cytotoxic agent hypochlorous acid (HOCl). The specific role that HOCl plays in bacterial killing is unclear. In the phagosome, it should react with neutrophil proteins to form protein chloramines and dichloramines. We investigated the stability of model dichloramines that are likely to be formed on *N*-terminal amino acids and Lys residues of proteins contained within phagosomes. Dichloramines were much more unstable than their analogous monochloramines. The stability was affected by substituents on the α-carbon. Amino acid dichloramines were extremely unstable, indicating that an α-carboxyl group facilitated decomposition. In general, the absence of a substituent enhanced stability. The carboxyl group on *N*-terminal Glu residues favored break down, but this effect was not apparent with Asp residues. Unstable dichloramines that contained a substituent on their α-carbon were cytotoxic and killed 50% of 10^5 *Staphylococcus aureus* (LD_{50}) at a dose of approximately 2.5 nmol. Their cytotoxicity declined with time. The dichloramines of *N*-α-acetyl Lys and taurine were not bactericidal up to 10 nmol per 10^5 *S. aureus*. None of the analogous monochloramines were cytotoxic at this dose. Dichloramines decomposed to yield chlorimines, aldehydes, and the inorganic gases ammonia monochloramine (NH_2Cl) and ammonia dichloramine ($NHCl_2$). The LD_{50} values were determined for NH_2Cl (0.37 ± 0.14 nmol), $NHCl_2$ (0.08 ± 0.02 nmol), and HOCl (0.14 ± 0.04 nmol). Stable products formed during the breakdown of dichloramines were not bactericidal. We propose a potential antimicrobial mechanism that explains in part how HOCl can react mainly with neutrophil components but still promote killing of phagocytosed bacteria. HOCl produced in phagosomes will react with amine groups on neutrophil proteins to form unstable dichloramines that will liberate cytotoxic NH_2Cl and $NHCl_2$. These gases will contribute to killing of ingested bacteria.

Introduction

Phagocytosis of bacteria by white blood cells was first observed by Metchnikoff in the 1880s (*1*). Since then, numerous investigations have been undertaken to establish the mechanisms responsible for bacterial killing by neutrophils, which are the most abundant white blood cells. When neutrophils ingest bacteria into a phagosome, they undergo an oxidative burst (*2*). The NADPH oxidase assembles on the phagosomal membrane and transfers electrons from NADPH in the cytosol to oxygen in the phagosome to form superoxide. The oxidative burst is required for optimal antimicrobial function by neutrophils as patients with an inactive oxidase have a predisposition to bacterial and fungal infection (*3*). During the respiratory burst, granule proteins are discharged into the phagosomal space to an estimated concentration of 500 mg/mL (*4*). One of these proteins, myeloperoxidase, dismutates superoxide and uses the resulting hydrogen peroxide to oxidize chloride to the potent bactericidal agent hypochlorous acid (HOCl) (*5, 6*). Myeloperoxidase constitutes about 25% of all granule protein, and several studies have demonstrated that myeloperoxidase is required for the majority of oxygen-dependent killing of bacteria (*7*−*9*). HOCl is the major candidate for the lethal agent released by myeloperoxidase. However, its mechanism of action is uncertain.

Although HOCl modifies bacterial proteins inside the phagosome, the majority of protein chlorination occurs on neutrophil rather than bacterial proteins (*10*). This unexpected finding is supported by kinetic modeling that suggests that due to their high concentrations, neutrophil proteins should be a major target for HOCl in the phagosome (*6*). HOCl reacts rapidly with amino acid side chains and the *N*-terminal α-amino groups of proteins (*11*). Cysteine (Cys) and methionine (Met) residues are kinetically preferred sites of oxidation (*11*). Amines, such as the lysine (Lys) side chain or *N*-terminal α-amino groups, react with HOCl to produce mono- and dichloramines (*12*). The formation of amino acid chloramines decreases the initial antibacterial activity of HOCl (*13, 14*). However, chloramines retain some of the oxidizing activity of HOCl (*15*). It has been demonstrated that the latent oxidizing ability of monochloramines is released on addition of ammonium ions via formation of lipophilic and bactericidal ammonia monochloramine (NH_2Cl) (*14, 16*).

$$RNHCl + NH_3 \rightarrow RNH_2 + NH_2Cl$$

NH_2Cl is capable of penetrating hydrophobic cell membranes and oxidizing intracellular components. This is one way that chloramine derivatives could mediate the antimicrobial action of HOCl.

Little attention has been focused on the contribution that dichloramines may make to oxidative killing by neutrophils.

* To whom correspondence should be addressed. Tel: +64(3)364 0567. Fax: +64(3)364 1083. E-mail: melanie.coker@otago.ac.nz.
† University of Otago.
‡ Syft Technologies Ltd.
§ University of Canterbury.

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However, dichloramines are more potent antimicrobial agents than monochloramines (*17*). Thomas et al. showed evidence of dichloramine formation in isolated neutrophil granules treated with hydrogen peroxide (*18*). This suggests that dichloramine formation is likely to occur in neutrophil phagosomes. Therefore, we have investigated the stability and bactericidal activity of monochloramines and dichloramines of small peptides. We show that dichloramines with substituents on their α-carbon are much more unstable and cytotoxic than monochloramines. They liberate the highly toxic gases NH_2Cl and ammonia dichloramine ($NHCl_2$). We present a potential antimicrobial mechanism that explains in part how HOCl can react mainly with neutrophil components but indirectly kill phagocytosed bacteria.

Experimental Procedures

Materials. HOCl was purchased as commercial chlorine bleach from Household and Body Care (Auckland, New Zealand). Amino acids and peptides were from Sigma-Aldrich (St. Louis, MO), Pierce (Rockford, IL), and Merck (Darmstadt, Germany). Dihydrorhodamine was purchased from Fluka (Steinheim, Germany). *Staphylococcus aureus* ATCC 27217 (502a) was obtained from the New Zealand Communicable Disease Centre (Porirua, New Zealand). All other chemicals were from Sigma-Aldrich.

Chloramine Preparation. The concentration of HOCl stock solutions was determined by measuring its absorbance at 292 nm (pH 12, $\epsilon_{292} = 350\ M^{-1}\ cm^{-1}$) (*19*). Amino acid and peptide monochloramine species (1 mM) were prepared by adding HOCl dropwise to the respective amino compounds while vortexing at a molar ratio of amine:HOCl of at least 5:1 in 10 mM phosphate buffer, pH 7.4. Dichloramine species (10 mM) were prepared similarly, with a molar ratio of amine:HOCl of 1:2 in 100 mM phosphate buffer, pH 7.4. The formation of organic dichloramine was confirmed by observation of the characteristic dichloramine absorbance spectra with an absorbance maximum at 304 nm. There was no evidence of a shoulder at 252 nm, indicating that there was no detectable monochloramine present ($\epsilon_{304} = 370\ M^{-1}\ cm^{-1}$) (*15*). For mass spectrometry analysis, when higher concentrations of HOCl were used, the pH of HOCl was adjusted to 7.4 before reacting with the amine. NH_2Cl and $NHCl_2$ were prepared by addition of HOCl (at a molar ratio of amine:HOCl of 10:1 and 1:2, respectively) to 10 mM ammonium chloride and 100 mM NaCl in 50 mM phosphate buffer, pH 7.4 and 5.0, respectively. The formation of ammonia chloramine and dichloramine was confirmed by observation of the characteristic absorbance spectra as for the organic chloramines and measuring the absorbance at 252 ($\epsilon_{252} = 429\ M^{-1}\ cm^{-1}$) (*15*) and 294 nm ($\epsilon_{294} = 272\ M^{-1}\ cm^{-1}$) (*20*), respectively. Solutions were checked for unreacted HOCl by demonstrating that the solutions could not oxidize dihydrorhodamine (DHR) unless iodide was present (*21*). All of the HOCl had reacted to form chloramines before the solutions were used to kill bacteria.

Assessment of Chloramine Stability. Monochloramines and dichloramines were prepared as described above and incubated in 10 or 100 mM phosphate buffer, pH 7.4, respectively. Dichloramines were diluted 10-fold in 10 mM phosphate buffer before analysis. The stability of the chloramines was assessed immediately after preparation by monitoring their spectral changes. UV spectra were monitored in the range between 190 and 400 nm for 30 min at 20−22 °C (room temperature) in an Agilent 8453 UV−visible diode array spectrophotometer. The stability of monochloramines and dichloramines was followed at 254 ($\epsilon_{254} = 429\ M^{-1}\ cm^{-1}$) and 304 nm ($\epsilon_{304} = 370\ M^{-1}\ cm^{-1}$) (*15*), respectively. Initial yields of monochloramines ranged between 92 and 111%. Yields of dichloramines were calculated to range between 83 and 107%, except for those which decayed so rapidly that it was not possible to calculate the initial yield (hence, their decomposition was compared to theoretical values). A stopped-flow apparatus (SFA-12 Rapid Kinetic Accessory, Hi-Tech Scientific Ltd., Salisbury, United Kingdom) was attached to the spectrophotometer to measure the decay kinetics of Glu-amide dichloramine. The stability of the chloramines was determined over 30 min by reacting them with yellow 5-thio-2-nitrobenzoic acid (TNB; $\epsilon_{412} = 13100\ M^{-1}\ cm^{-1}$) to form colorless 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (*19*). Half-lives of chloramines were calculated with linear or exponential (two or three parameter) fits to decay curves using SigmaPlot 7.0 software.

Assessment of *S. aureus* Viability after Treatment with HOCl and Chloramines. *S. aureus* were cultured on Columbia sheep blood agar plates (Fort Richard, Auckland, New Zealand). Single colonies were used to inoculate nutrient broth, which was incubated overnight at 37 °C, with shaking. *S. aureus* was pelleted by centrifugation at 1700*g* and washed twice with 10 mM phosphate buffer, pH 7.4. The concentration of bacteria was determined by absorbance at 550 nm (0.12 absorbance units corresponded to approximately 1×10^8 colony forming units/mL). Bacteria were then diluted in 10 mM phosphate buffer, pH 7.4. *S. aureus* (10^5) at a concentration of approximately 10^6/mL were mixed by vortexing with varying concentrations of HOCl or chloramines and then incubated at 37 °C for 1 h. After incubation, bacteria were diluted and plated to assess exact cell numbers for untreated bacteria and the viability of those that were treated with oxidants.

Selected Ion Flow Tube Mass Spectrometry (SIFT-MS). The SIFT-MS instrument used is known as the Voice100 (Syft Technologies Ltd., Christchurch, NZ) and has been described previously (*22, 23*). Monochloramine (1 mM) and dichloramine (10 mM) species of amino acids and small peptides were prepared as described above with continuous mixing in a volume of 5 mL and sealed in 125 mL screw-top vials with TFE/silicone liners. The volatile substances ammonia (NH_3) (17 *m/z*), NH_2Cl (51, 53 *m/z*), and $NHCl_2$ (85, 87, 89 *m/z*) were monitored continuously by SIFT-MS from the headspace above the liquid samples at 22 °C, as previously described (*24*), prior to addition of HOCl and for a minimum of 10 min after the addition of HOCl. The sample was mixed continuously by stirring. In the SIFT-MS, volatile compounds in air were identified by soft chemical ionization reactions occurring in a flow tube reactor. O_2^+ and H_3O^+ reagent ions were selected using a quadrupole mass filter into the flow tube and then reacted with volatile compounds introduced from the headspace of samples at approximately 1.94 Torr L s^{-1}. Reagent and product ions were carried along the reactor by a stream of helium and argon against a pressure gradient by utilizing a venturi orifice (*25*). The ion reagents and products were analyzed by a second quadrupole mass filter and counted by a continuous dynode type particle multiplier. Henry's law constant (distribution coefficient) was used to calculate the concentration of NH_2Cl {[kH/(mol L^{-1} atm^{-1})] = 94} and $NHCl_2$ {[kH/(mol L^{-1} atm^{-1})] = 29} in solutions containing the chlorinated amino acids and peptides (*26*).

Detection of Chloramine Decay Products by LC/MS. The products of the decay of Glu-Val-Phe dichloramine were separated on a Thermo Corp. Hypercarb column (particle size 5 μm, 100 mm × 2.1 mm) (Phenomenex, Torrance, CA) using a Surveyor HPLC Pump (Thermo Corp., San Jose, CA). The column was maintained at 30 °C. The products were eluted at a flow rate of 200 μL/min using a linear gradient of two solvents: solvent A (0.1% formic acid) and solvent B (50% acetonitrile, 50% isopropanol, and 0.1% formic acid). The gradient was as follows: 0−20 min, increased solvent B from 0 to 100%; 20−21 min, maintained solvent B at 100%; 21−30 min, decreased solvent B to 0%. The injection volume was 20 μL.

Breakdown products of Asp-Phe methyl ester dichloramine were separated on a Jupiter proteo-90A column (particle size 4 μm, 150 mm × 2.0 mm) (Phenomenex, Torrance, CA) using a Surveyor HPLC Pump (Thermo Corp., San Jose, CA). The column was maintained at 30 °C. The products were eluted at a flow rate of 200 μL/min using an isocratic elution with 35% acetonitrile and 0.1% formic acid. The injection volume was 10 μL.

The HPLC was coupled to an ion-trap mass spectrometer (ThermoFinnigan LCQ Deca XP Plus, Thermo Corp.) equipped with an electrospray ionization source. The mass spectrometer was operated with positive ionization using full scan mode (scan range, 100−1000 *m/z*) and selected ion monitoring. The electrospray

voltage was set at 5.0 kV. The capillary temperature was 275 °C. Nitrogen, the sheath gas, was 47 in units. Selected ion monitoring was performed with collision energy of 35%. Breakdown products of Glu-Val-Phe dichloramine (10 mM) were also analyzed by direct infusions of the solutions into the mass spectrometer. The solutions were diluted 100-fold and injected at a flow rate of 5 μL/min into a 200 μL/min stream of 50% acetonitrile and 0.1% formic acid.

Results

Chemical Stability of Chloramines. We investigated the stability of the chloramines of four classes of amino acids as models of the *N*-terminal residues of major neutrophil proteins that are discharged into neutrophil phagosomes during bacterial killing. Alanine (Ala) was selected because it is the *N*-terminal amino acid of the neutrophil defensin HNP-1 (*27*) and resembles that of the alkyl amino acids that are present on the *N*-termini of myeloperoxidase valine (Val) (*28*) and neutrophil elastase isoleucine (Ile) (*29*). Aspartate (Asp) is the *N*-terminal amino acid of HNP-3 (*27*). Glutamate (Glu) and glycine (Gly) were chosen to assess whether subtle changes in the α-carbon substituent of Asp and Ala, respectively, impacted dichloramine stability. *N*-α-acetyl Lys was chosen as a model of protein Lys residues, while taurine was investigated because it is present in high concentrations within the cytoplasm of neutrophils.

The chemical stability of the chloramines and dichloramines was determined by measuring the loss in their reactivity (Figure 1A) and by monitoring the UV maxima of the chloramines and dichloramines, as well as the absorption spectra between 190 and 400 nm (Figure 1B,C). Half-lives for chloramine reactivity are given in Table 1 and are generally in agreement with previous studies (*12, 30, 31*). In general, the stability of the chloramine chromophores matched that of chloramine reactivity (data not shown). We observed exceptions to this with Ala-Phe and Gly dichloramine (Figure S1A,B, Supporting Information). With some compounds it was not possible to compare chemical stability with the absorbance changes because products of the decay interfered with the UV spectra of the chloramines. For example, asparagine (Asn) monochloramine (Figure S1C, Supporting Information) showed a small increase in absorbance at 252 nm, due to formation of a product that absorbed maximally at 273 nm. Therefore, we measured the decomposition of Asn monochloramine by its loss in chloramine reactivity. The stability of the monochloramines and dichloramines varied widely. In all cases, dichloramines were less stable than the corresponding monochloramines.

Monochloramines of amino acids decayed much faster than their analogues with a blocked C terminus, which is in agreement with previous studies (*30*). This phenomenon was also observed with dichloramine derivatives. For example, the dichloramines of Asp and Asn were at least 2 orders of magnitude less stable than those of Asp-amide and Asp-Phe methyl ester. These results indicated that the α-carboxyl moiety is directly involved in the decomposition of amino acid monochloramines and dichloramines.

The presence of a substituent on the α-carbon also decreased the stability of the dichloramines. For example, the dichloramines of Ala, Asp, and Glu were much less stable than Gly dichloramine. The most stable dichloramines were derived from taurine and *N*-α-acetyl-Lys, which lack both an α-carboxyl group and another substituent on the α-carbon.

Blocking the carboxyl group on the α-carbon of the compounds that contained Asp (Asp-amide and Asp-Phe methyl ester) dramatically increased the stability of both their chloramines and dichloramines. However, conversion of the carboxyl group on the Asp substituent to an amide (Asn) had no affect on the stability of either chloramine. These results indicated that the carboxyl moiety in the side chain of Asp did not influence chemical stability. In contrast, the carboxyl group on the Glu substituent did affect the stability of the dichloramines of *N*-terminal Glu compounds. This is apparent from the equal half-lives for Glu-amide and glutamine (Gln), which were an order of magnitude less than that for Glu.

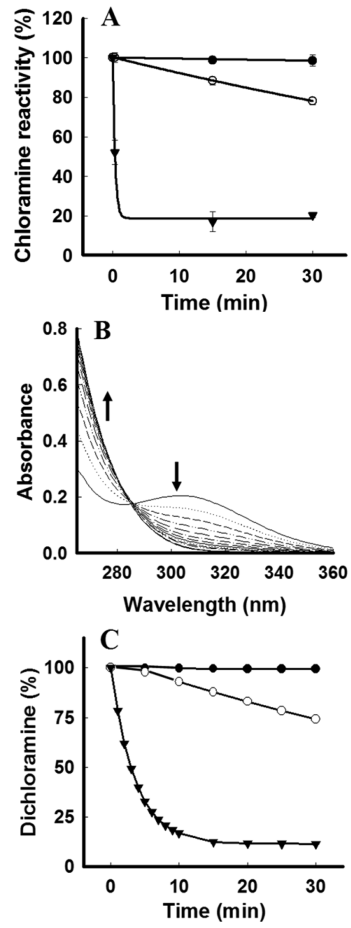


Figure 1. Decay of dichloramines. Dichloramines of taurine (●), Asp-Phe methyl ester (○), and Glu-Val-Phe (▼) were prepared at 10 mM and diluted 10-fold into 10 mM phosphate buffer, pH 7.4, and their stability was recorded at 22 °C. (A) Residual chloramine reactivity was followed by determination of their ability to bleach TNB or (B) monitoring the changes in their UV absorption spectrum as shown for 1 mM Glu-Val-Phe dichloramine, with spectra recorded every minute for 10 min or (C) monitoring the absorbance maximum for each of the dichloramines.

From these results, we conclude that a carboxyl group on the α-carbon of dichloramines has the greatest affect on destabilizing dichloramines. However, alkyl groups also destabilize dichloramines, as does the carboxyl group on *N*-terminal Glu residues.

Comparative Toxicities of Monochloramines and Dichloramines Against *S. aureus.* We then assessed the ability of chloramines and dichloramines on *N*-terminal peptides and amino acids amides, as well as *N*-α-acetyl Lys and taurine, to kill *S. aureus*. The unreacted amines alone showed no bactericidal activity (data not shown). None of the monochloramines had bactericidal activity up to 10 nmol per 10^5 *S. aureus.* This dose corresponded to 100 μM oxidant added to 10^5 bacteria in 100 μL. The majority of dichloramine species were bactericidal (Figure 2A and Table 2). Dichloramines killed 50% of the bacteria (LD_{50}) at a dose of 2−7 nmol per 10^5 *S. aureus* (20−70

Table 1. Stabilities of Monochloramines and Dichloramines of Amino Acid and Small Peptides[a]

amine	monochloramine half-life (min)	dichloramine half-life (min)
Ala	61.2 ± 3.9	<0.3
Ala methyl ester	90.8 ± 12.4	5.3 ± 2.0
Ala-Phe	≫120	36.3 ± 0.4
Asp	14.2 ± 0.4	<0.3
Asp-amide	≫120	34.9 ± 3.4
Asp-Phe methyl ester	≫120	68.0 ± 3.0
Asn	11.0 ± 0.8	<0.3
Glu	37.5 ± 3.5	<0.3
Glu-amide	53.0 ± 1.6	3.6 ± 0.2
Glu-Val-Phe	84.9 ± 21.9	0.3 ± 0.03
Gln	36.6 ± 0.8	3.0 ± 0
Gly	≫120	13.0 ± 6.9
Gly-Asp	≫120	85.0 ± 9.1
Gly-Phe	≫120	29.7 ± 1.5
N-α-acetyl Lys	≫120	≫120
Taurine	≫120	≫120

[a] Monochloramine and dichloramine stabilities were assessed by reaction with TNB. Monochloramines and dichloramines were incubated at 22 °C in 10 or 100 mM phosphate buffer, pH 7.4, respectively. Half-lives ($t_{1/2}$) of chloramines were calculated from their decay curves and presented as means ± SD ($n = 3$).

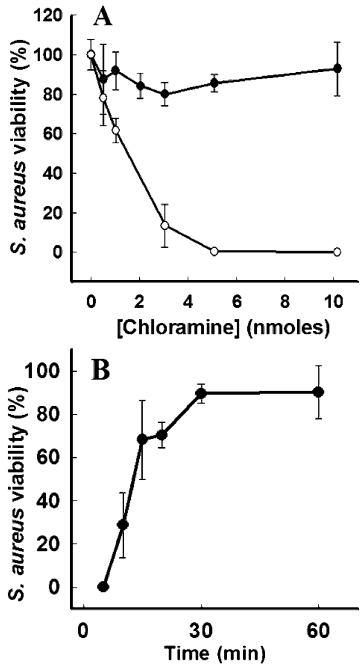


Figure 2. Bactericidal toxicity of Asp-Phe methyl ester chloramines. (A) *S. aureus* (10^5) were incubated with varying concentrations of Asp-Phe methyl ester monochloramine (●) and dichloramine (○) for 1 h at 37 °C. Aliquots for each sample were diluted and plated to assess viability. (B) Asp-Phe methyl ester dichloramine was incubated for various times at 22 °C, and then, 50 μM (●) was added to *S. aureus* (10^5) in 100 μL. After an hour at 37 °C, bacteria were diluted and plated to assess viability. Data are means and standard deviations of two experiments carried out in duplicate.

μM). At these concentrations, some of the bacterial colonies were smaller than colonies of untreated bacteria, indicating short-term growth arrest. These stasis colonies were counted and included in the data that we have presented. However, this stasis effect implies that the organic dichloramines had additional toxicity to that required to kill 50% of the bacteria under our conditions. All of our killing assays were conducted at a fixed concentration of bacteria, and it cannot be assumed that a higher concentration of bacteria would require a proportionally higher dose of oxidant.

For comparison, the toxicities of HOCl, NH_2Cl, and $NHCl_2$ were 0.14 ± 0.04, 0.37 ± 0.14, and 0.08 ± 0.02 nmol per 10^5 *S. aureus*, respectively. Thus, the toxicities of organic dichloramines were considerably less than these inorganic reactive chlorine species. Residual HOCl present with organic dichloramines could not have been responsible for killing the bacteria, as free HOCl was undetectable as assessed by its ability to promote oxidation of dihydrorhodamine (*21*) (data not shown).

The bactericidal activity of the dichloramines was dependent upon the presence of a substituent on the α-carbon adjacent to the terminal amine group. Dichloramines containing a proton in this position, such as taurine and *N*-terminal Gly peptides, did not kill the bacteria. The dichloramine of *N*-α-acetyl Lys was also not bactericidal up to 10 nmol per 10^5 *S. aureus* (Table 2). These results suggest that direct reaction of the dichloramines with the bacteria is unlikely to explain their toxicity. This also confirms that any residual HOCl was not responsible for killing.

The longevity of the bactericidal activity of dichloramines was investigated by incubating Asp-Phe methyl ester dichloramine at 22 °C for up to 2 h before adding it to the bacteria. At a dose of 5 nmol per 10^5 *S. aureus*, Asp-Phe methyl ester dichloramine progressively lost its bactericidal activity over 30 min (Figure 2B).

Analysis of Products Formed from the Decomposition of Dichloramines. To assess how dichloramines killed bacteria under our reaction conditions, we investigated the kinetics of their decay and analyzed the products formed. The dichloramines of Glu-amide and Gln lost their UV absorbance much faster than their chloramine reactivity. Unlike Gly dichloramine (Figure S1B, Supporting Information), their spectral changes did not indicate that they decayed to their respective monochloramines (Figure 3A). To accurately measure the half-life of Glu-amide dichloramine, its UV decay was analyzed by stopped-flow kinetics. Even when the dichloramine absorbance had disappeared completely, there was still approximately 50% chloramine reactivity remaining (Figure 3B). These results suggested that the dichloramine decayed to liberate another chloramine species but not Glu-amide monochloramine.

Other possible chloramine species formed in the decay of dichloramines are NH_2Cl and $NHCl_2$. To establish the existence of these volatile compounds, we examined the headspace above solutions of several amino acid and small peptide chloramines during their decay using SIFT-MS (*25*). Initially, we confirmed that $NH_2Cl(g)$ could be detected in the headspace above a 1 mM solution of this inorganic chloramine. Upon sampling the headspace, there was a rapid increase in the signal for $NH_2Cl(g)$, which was constant for at least 5 min. This indicated that the $NH_2Cl(aq)$ was stable and rapidly equilibrated with the gas phase. The signal was directly proportional to the concentration of $NH_2Cl(aq)$ formed in solution (Figure S3A, Supporting Information). When we prepared solutions of $NHCl_2(aq)$, we also detected $NHCl_2(g)$ in the headspace above this inorganic chloramine. However, its signal reached a maximum after a few seconds of sampling and then declined exponentially. This suggests that $NHCl_2(aq)$ was unstable and therefore could not equilibrate with the gas phase. The instability of $NHCl_2(aq)$ is consistent with observations of previous studies (*32*). The maximum signal obtained for $NHCl_2(g)$ was directly proportional to the initial concentration formed in solution (Figure S3B, Supporting Information). We next analyzed the headspaces over solutions containing chloramines of taurine, Glu, and Asp

Table 2. Antimicrobial Activity of Monochloramines, Dichloramines, and HOCl[a]

	chloramine LD_{50} values (nmol/10^5 *S. aureus*)[b]		
amine	monochloramine	dichloramine	α-carbon substituent
Ala-Phe	>10	2.7 ± 0.2	$-CH_3$
Asp-amide	>10	4.9 ± 3.6	$-CH_2-COOH$
Asp-Phe methyl ester	>10	2.2 ± 1.3	$-CH_2-COOH$
Glu-amide	>10	2.3 ± 0.4	$-CH_2-CH_2-COOH$
Glu-Val-Phe	>10	7.4 ± 6.0[c]	$-CH_2-CH_2-COOH$
Gly-DL-Asp	>10	>10	−H
Gly-Phe	>10	>10	−H
N-α-acetyl Lys	>10	>10	−H
Taurine	>10	>10	−H
HOCl	0.14 ± 0.04[d]	NA[e]	NA
NH_2Cl	0.37 ± 0.14	NA	NA
$NHCl_2$	NA	0.08 ± 0.02[d]	NA

[a] *S. aureus* (10^5) were incubated with varying concentrations of HOCl or chloramines for 1 h at 37 °C. After incubation, bacteria were diluted and plated to assess viability. Results are presented as means ± ranges. [b] Results are for two experiments done in duplicate, unless otherwise stated. [c] Results are for three experiments done in duplicate. [d] Results are for five experiments done in duplicate. [e] Not applicable.

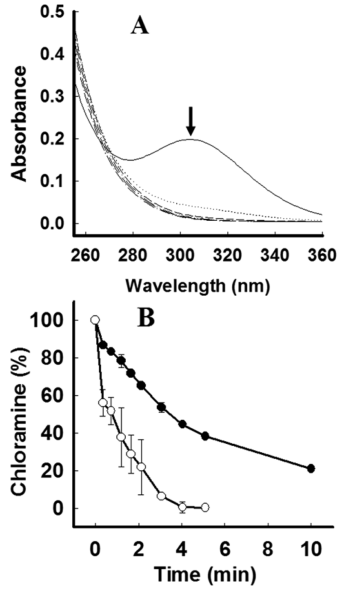


Figure 3. Decay of Glu-amide dichloramine. The dichloramine of Glu-amide was prepared as described in Figure 1, and its decay was monitored at 22 °C by (A) following the changes in its UV absorption spectrum, which were recorded every min for 10 min. (B) Its decay was also followed by measuring the loss in dichloramine absorbance at 304 nm, as measured by stopped-flow (○) or by measuring residual chloramine reactivity using TNB (●). Results are means and standard deviations of triplicate experiments.

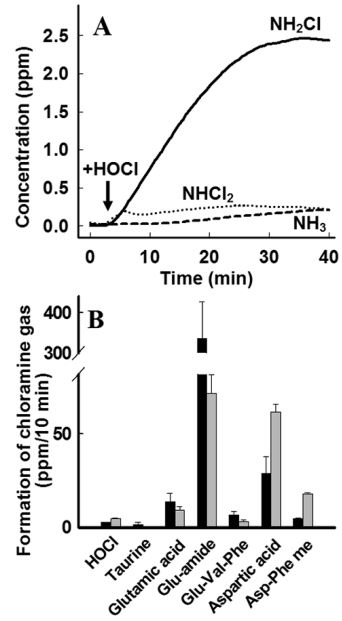
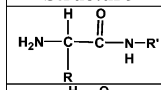
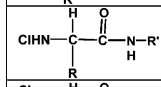
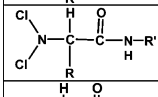
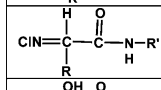
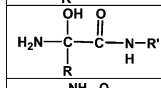
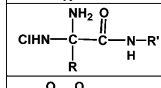
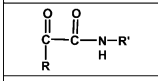
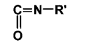


Figure 4. Liberation of NH_2Cl and $NHCl_2$ from amino acid dichloramines. The volatile substances NH_3 (17 *m/z*), NH_2Cl (51 and 53 *m/z*), and $NHCl_2$ (85, 87, and 89 *m/z*) were monitored continuously by SIFT-MS in the headspace above the liquid samples at 22 °C prior to addition of HOCl and for a minimum of 10 min after the addition of HOCl. (A) Real-time liberation of NH_3 (dashed line), NH_2Cl (solid line), and $NHCl_2$ (dotted line) from Glu-amide dichloramine. The arrow indicates the addition of HOCl. (B) Total amount of volatile NH_2Cl (black bars) and $NHCl_2$ (gray bars) detected into the headspace above solutions of amino acid dichloramine derivatives over 10 min at 22 °C.

derivatives. Amino acids alone did not produce any volatile NH_3 derivatives (data not shown). Small short-lived signals for $NH_2Cl(g)$ and $NHCl_2(g)$ were detected in the headspace above the buffer to which HOCl was added. This could be explained by the presence of trace amounts of NH_3 dissolved in the buffer. None of the monochloramines produced significant amounts of $NH_2Cl(g)$ and $NHCl_2(g)$ (data not shown). Dichloramines of Glu and Asp derivatives produced both $NH_2Cl(g)$ and $NHCl_2(g)$. Continuous formation of $NH_2Cl(g)$ was observed from Glu-amide dichloramine (Figure 4A). The concentration of $NH_2Cl(g)$ detected in the headspace above 10 mM Glu-amide dichloramine reached a plateau at approximately 2.5 ppm, which was equivalent to that liberated from a 400 μM standard solution of NH_2Cl. Figure 4B shows the total amount of volatile chloramines liberated into the headspace during 10 min for each amino acid derivative. Glu-derived dichloramines liberated predominantly $NH_2Cl(g)$ into the headspace, while Asp-derived dichloramines favored release of $NHCl_2(g)$. Taurine dichloramine, which was found to be highly stable (see above), did not liberate either NH_2Cl or $NHCl_2$ (Figure 4B). These results demonstrate that unstable dichloramines can break down to liberate NH_2Cl and $NHCl_2$. We did not determine the yields of their formation because of their potential reactivity with other components in solution and the instability of $NHCl_2$.

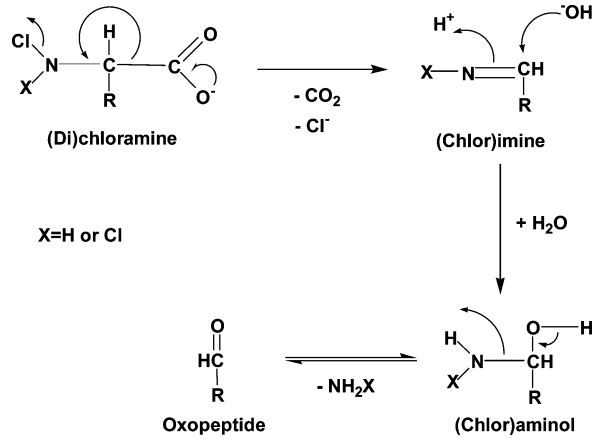
We also analyzed solutions of chloramines using infusion mass spectrometry, HPLC, and LC/MS to identify additional decomposition products. Characterization of decomposition products was necessary to identify other possible cytotoxic species and assist in deducing the pathways for decay of chloramines. We chose to examine chloramines of Glu-Val-Phe and Asp-Phe methyl ester because they were most amenable to analysis by liquid chromatography. The chemical structures of their observed breakdown products and their molecular masses are listed in Table 3. The correct mass of Glu-Val-Phe monochloramine was confirmed by infusion mass spectrometry. Using LC/MS, we observed slow formation of the Glu-Val-Phe aminol, which suggested that the monochloramine decom-

Table 3. Chemical Structures, Designations, and Masses of Monochloramines, Dichloramines, and the Products of Their Decomposition[a]

Chemical Structure	Designation	Asp-Phe methyl ester		Glu-Val-Phe	
		H^+	Na^+	H^+	Na^+
	Parent Peptide	**295.0**	**317.0**	**394.0**	**416.2**
	Monochloramine	**329.0 (3)**[b] **331.0 (1)**[b]	351 (3) 353 (1)	428 (3) 430 (1)	**450.1 (3)** **452.1 (1)**
	Dichloramine	**362.9 (9)** **364.9 (6)** **366.9 (1)**	**385.0 (9)** **387.0 (6)** **389.0 (1)**	462 (9) 464 (6) 466 (1)	484 (9) 486 (6) 488 (1)
	Chlorimine	**326.9 (3)** **328.9 (1)**	**349.0 (3)** **351.0 (1)**	**426.0 (3)** **428.0 (1)**	**448.1 (3)** **450.1 (1)**
	Aminol	311	333	**409.9**	432
	Monochloramine diamine	**343.8 (3)** **345.8 (1)**	366 (3) 368 (1)	**442.8 (3)** **444.8 (1)**	465 (3) 467 (1)
	Oxopeptide	294	316	**393.0**	**415.2**
	Isocyanopeptide	192	214	**291.0**	313

[a] Observed masses are indicated in bold font. [b] These masses were observed but not referred to in the text. Numbers in parentheses indicate expected chlorine isotope ratios.

Scheme 1. Reaction Mechanism for Decay of Chloramines and Dichloramines of α-Amino Acids[a]



[a] Decay of (di)chloramines containing a α-carboxyl group occurs via decarboxylation to yield an unstable (chlor)imine, which undergoes rapid hydrolysis via an aminol (or chloraminol) with subsequent loss of NH_3 (or NH_2Cl) to form an oxopeptide.

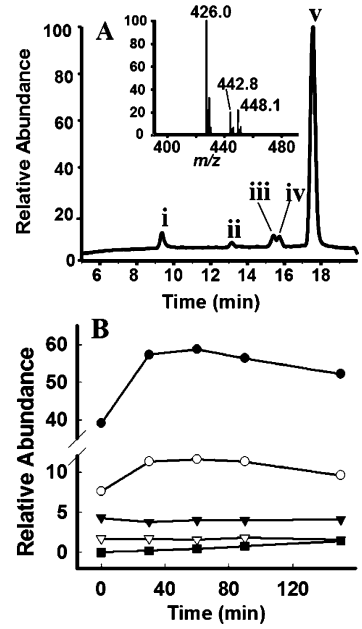
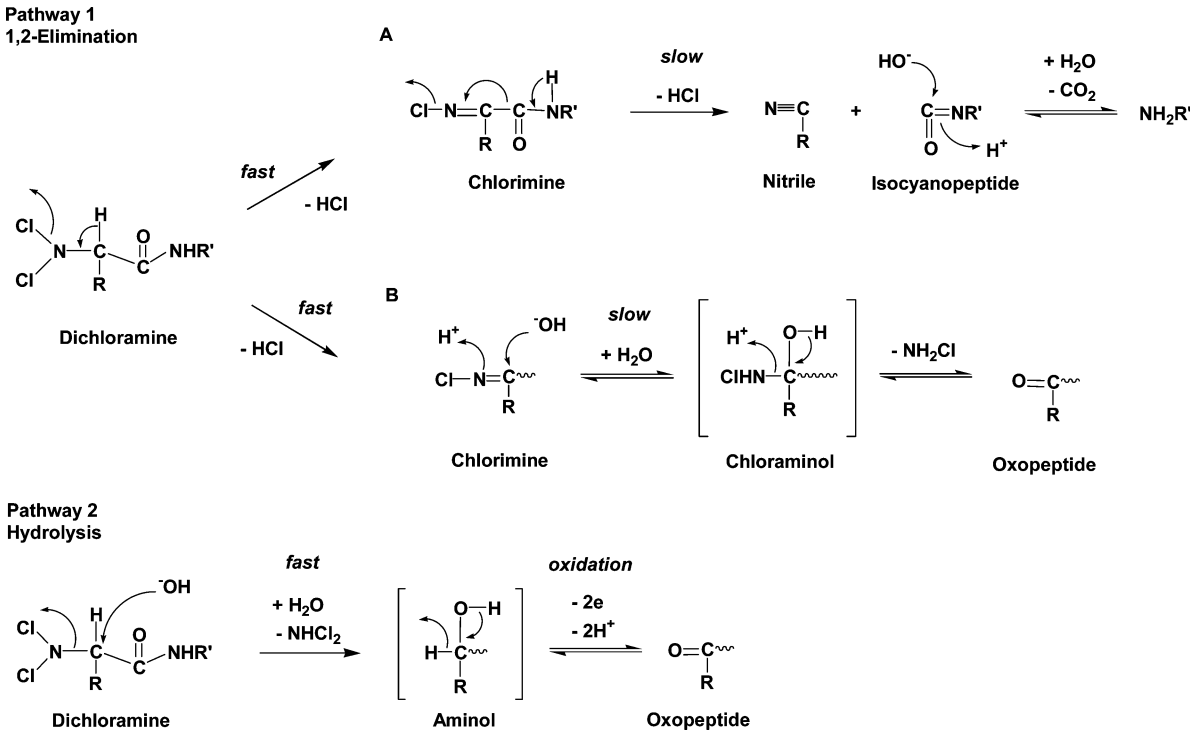


Figure 5. Identification of the products formed during decay of Glu-Val-Phe dichloramine. Glu-Val-Phe dichloramine was prepared by reacting a 5 mM concentration of the parent peptide with 10 mM HOCl in 50 mM phosphate buffer, pH 7.4. It was diluted 100-fold, then separated, and analyzed by ESI-LC/MS. (A) Products in the peaks of the total ion chromatogram present 60 min after forming the dichloramine included (i) unreacted Glu-Val-Phe, (ii) unidentified peak, (iii) Glu-Val-Phe oxopeptide and hydrolyzed Glu-Val-Phe imine, (iv) Glu-Val-Phe isocyanopeptide, and (v) Glu-Val-Phe chlorimine and Glu-Val-Phe monochloramine diamine. The inset shows the mass spectrum of peak v. (B) The relative abundances of Glu-Val-Phe chlorimine (●), Glu-Val-Phe monochloramine diamine (○), Glu-Val-Phe oxopeptide (▼), Glu-Val-Phe aminol (▽), and Glu-Val-Phe isocyanopeptide (■) were followed as the parent dichloramine decayed. Peak areas were determined from the extracted masses. Results are representative of at least three experiments.

posed by the initial loss of HCl to form the imine and subsequently hydrolyzed to the aminol (Figure S4, Supporting Information). We also observed slow formation of the aldehyde, Glu-Val-Phe oxopeptide, which suggests that the aminol broke down to release NH_3 and the resultant aldehyde (Scheme 1).

The highly unstable Glu-Val-Phe dichloramine was analyzed by LC/MS. At 60 min after the formation of the dichloramine, the total ion chromatogram showed five predominant peaks (Figure 5A). Peaks i, ii, and iii were the same as those seen in the monochloramine system: that is, (i) unreacted peptide, (ii) a constant unknown peak, and (iii) Glu-Val-Phe oxopeptide and a mass consistent with the Glu-Val-Phe aminol (data not shown). Peak v contained molecular ions attributable to the protonated and sodiated adducts of Glu-Val-Phe chlorimine (Figure 5A, inset). These species had the expected 3:1 ratio for a chlorine-containing compound. The ion with *m/z* 442.8 corresponds to the product of the addition of NH_3 to the chlorimine, Glu-Val-

Scheme 2. Reaction Mechanism for Decay of *N*-Terminal Dichloramines of Model Peptides[a]



[a] Dichloramines can decompose via two pathways. Rapid 1,2-elimination (pathway 1) results in the formation of the chlorimine, which can slowly decompose to the nitrile and isocyanopeptides (pathway 1, A), or slowly hydrolyze to a chloraminol, which can liberate NH_2Cl resulting in the oxopeptide (pathway 1, B). Rapid hydrolysis (pathway 2) would result in the release of $NHCl_2$ and formation of the related alcohol, which upon oxidation would form the oxopeptide.

Phe monochloramine diamine (the monochloramine derivative of the geminal diamine). This was deduced from fragmentation of the ion (data not shown). The major ion in peak iv was attributable to the protonated ion of the isocyanopeptide of Glu-Val-Phe (not shown), which indicates decomposition of the chlorimine and loss of HCl to produce the corresponding nitrile and isocyanopeptide (Scheme 2). We did not detect any evidence of the nitrile. Glu-Val-Phe dichloramine was not detected by LC/MS. This was not surprising as this dichloramine decays rapidly with a $t_{1/2}$ of 0.3 min.

Analysis of the products of the decomposition of Glu-Val-Phe dichloramine every 30 min after formation revealed that Glu-Val-Phe chlorimine had the largest peak area via HPLC (258 nm) and greatest signal via LC/MS (however, absolute amounts of each product were not quantified). At the initial time point, the chlorimine was already at 70% of it maximal concentration. The chlorimine reached its maximum after 30 min and gradually declined thereafter (Figure 5B). Glu-Val-Phe monochloramine diamine followed the same pattern as the chlorimine. This suggested that the ammoniated product could be derived from the chlorimine. Once the chlorimine reached its maximal concentration, the formation of the Glu-Val-Phe isocyanopeptide could be detected. This suggested that this product was formed from further decomposition of the chlorimine. Both the Glu-Val-Phe oxopeptide and the Glu-Val-Phe aminol formed rapidly and did not decompose during the time of analysis. This is in contrast to the monochloramine system where their concentrations continually increased as the monochloramine decayed. The formation of these products from the dichloramine was considerably less than from the monochloramine.

Asp-Phe methyl ester dichloramine, which decayed much less rapidly than Glu-Val-Phe dichloramine (Table 1), gave additional information about the decomposition of dichloramines. Asp-Phe methyl ester dichloramine was detected using an isocratic HPLC gradient (Figure 6A; peak iii). This was assigned from its absorbance maxima (λ_{max} = 308.9 nm) (*15*) and mass confirmation by LC/MS (Figure 6B). Peak ii (λ_{max} = 250.9 nm)

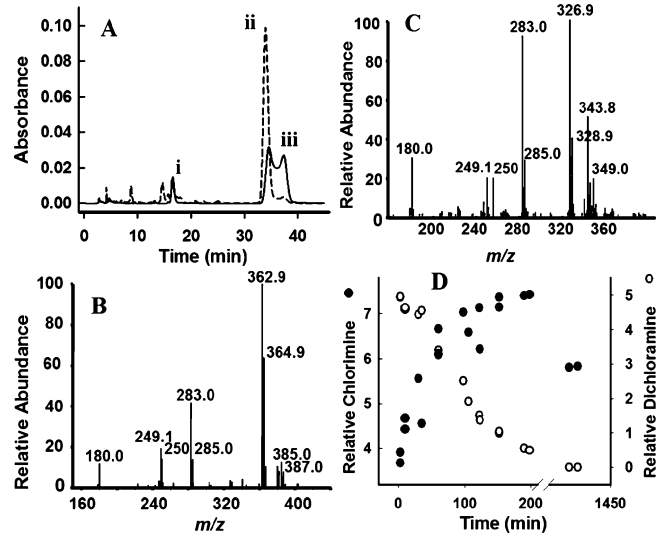


Figure 6. Identification of the products formed during decay of Asp-Phe methyl ester dichloramine. The dichloramine of Asp-Phe methyl ester was prepared by adding 20 mM HOCl to 10 mM peptide in 100 mM phosphate buffer, pH 7.4, at 22 °C. (A) Asp-Phe methyl ester dichloramine and its decay products were separated by HPLC 3 (solid) and 200 min (dashed) after formation and monitored at 258 nm. (B) The mass spectrum of peak iii is attributed to Asp-Phe methyl ester dichloramine. (C) The mass spectrum of peak ii is attributed to Asp-Phe methyl ester chlorimine and Asp-Phe monochloramine diamine. (D) The decay of Asp-Phe methyl ester dichloramine (○) and formation of chlorimine (●) were followed by measuring their peak areas at 258 nm over time. Results are typical of at least three experiments.

was identified as Asp-Phe methyl ester chlorimine via mass confirmation by LC/MS (Figure 6C). Within peak ii was a mass consistent with the product of the addition of NH_3 to Asp-Phe methyl ester chlorimine (monochloramine diamine) as seen with Glu-Val-Phe chlorimine. Other masses in both peaks ii and iii were consistent with in source decomposition products of the chlorimine (see the Supporting Information). Peak i was identified as the monochloramine by its absorbance (λ_{max} = 257.9 nm) and its retention time, which was consistent with the monochloramine (data not shown).

To determine the time course for decomposition of the dichloramine and formation of the chlorimine, we analyzed the relative peak areas of the Asp-Phe methyl ester dichloramine and the chlorimine by integration of the chromatograms at their absorption maxima. The time courses for loss of dichloramine and formation of chlorimine were offset by about 30 min due to their elution time from the HPLC column. The decay of the dichloramine was mirrored by the formation of the chlorimine, which reached its maximum concentration after 200 min. After 24 h at 22 °C, approximately 80% of the chlorimine still remained. Collectively, these results indicated that the decay of the dichloramine proceeded via a pathway that generated the relatively stable chlorimine, but other routes were available for its decomposition. This conclusion is also supported by the additional finding that as the dichloramine decayed numerous peaks were separated by HPLC (Figure S5, Supporting Information). Most of these species could not be confidently identified using LC/MS (see the Supporting Information).

Discussion

In this investigation, we have shown that dichloramines of *N*-terminal amino acids containing a substituent on their α-carbon are unstable and decay to generate species that are bactericidal. Not only is instability required for toxicity to *S. aureus*, but the chemical structure of dichloramines is important. Specifically, a substituent on the α-carbon is required to favor the release of NH_2Cl and $NHCl_2$. These gases were the only compounds that we identified that could kill bacteria. These simple inorganic gases had similar cytotoxicity to HOCl. Stable dichloramines and monochloramines of peptides were not toxic under the conditions of our experiments. The progressive loss of bactericidal activity of dichloramines, as shown for Asp-Phe methyl ester dichloramine in Figure 2B, indicates that the microbicidal activity of dichloramines is transient, and any stable decomposition products were not responsible for killing the bacteria. We propose that when HOCl is generated inside neutrophil phagosomes, it will react with amine residues on neutrophil proteins to form dichloramines. These will then break down to release NH_2Cl and $NHCl_2$, which will form a vital component of the neutrophil's antimicrobial arsenal.

The mechanism for decomposition of monochloramines of amino acids is well-established. Mechanistic studies were first initiated by Zgliczynski and co-workers who proposed that decarboxylation and deamination of amino acids may be relevant to the physiological function of myeloperoxidase (*33*). Our finding that a carboxyl group on the α-carbon enhanced the decomposition of monochloramines is consistent with data from previous groups (*12, 30, 31*). Decay of monochloramines containing a carboxyl group on their α-carbon occurs via decarboxylation to yield an unstable imine, which undergoes rapid hydrolysis with subsequent loss of NH_3 to form an aldehyde (*33*) (Scheme 1). α-Amino acids generate aldehydes via decarboxylation when treated with the myeloperoxidase−H_2O_2−Cl system (*30, 34*) and also when taken up by phagocytosis (*35*). Aldehydes and oxopeptides have also been detected from the decomposition of monochloramines formed on the *N*-terminal amino group of small peptides (*36, 37*). Our data for the half-lives of chloramines were generally consistent with published reports, except for Asn monochloramine. A previous study reported that the half-life for Asn monochloramine was greater than 60 min, as determined by monitoring at a single wavelength of 249 nm (*12*). We observed an increase in absorbance at this wavelength due to interfering products, which absorbed between 265 and 273 nm (Figures S1C and S2A,B, Supporting Information). By measuring chloramine reactivity, we showed that the half-life of Asn monochloramine is significantly shorter than previously published at approximately 11 min. We also confirmed that a product of decay was consistent with the imine of Asn (Figure S2C, Supporting Information), which is consistent with previous literature (*38*).

As compared with monochloramines, much less is known about the stability and decay of dichloramines (*38*). Recent findings suggest that decay of dichloramines occurs much more rapidly than monochloramines (*39*), and our results supported this observation. Earlier work demonstrated that chlorination of dipeptides gives *N*,*N*-dichloropeptides (*40*) and that these decay to chlorimines and nitriles (*41*). Previous work has also shown that Asn dichloramine can release NH_2Cl and $NHCl_2$ as detected by membrane introduction mass spectrometry (*42*). In this current study, we showed that dichloramines of amino acid derivatives decayed by competing pathways to give an array of products. As with monochloramines, the presence of a carboxyl group on the α-carbon promoted the rapid decay of dichloramines to chlorimines via decarboxylation (Scheme 1). The facile decay of dichloramines of *N*-terminal peptides to give chlorimines is consistent with a classical 1,2-elimination or E2 reaction (Scheme 2, pathway 1) (*43*). The rapid decay of the dichloramines with a Glu residue suggests that its carboxyl group catalyzes 1,2-elimination through an ionic interaction between the carboxylate anion and the positive dipole of the α-carbon. This interaction would stabilize the positive charge and optimize the release of $NHCl_2$. A precedent for this type of intramolecular elimination reaction has been proposed for the decay of *N*-chloro amino alcohols (*38*). The carboxyl group on the Asp residue did not facilitate decay of dichloramines, presumably because it would not interact favorably with the hydrogen on the α-carbon.

Chlorimines eventually break down to give a nitrile and an isocyanopeptide (*37, 41*) (Scheme 2, pathway 1A). We observed the isocyanopeptide upon decomposition of Glu-Val-Phe dichloramine, and its rate of formation mirrored the loss of the Glu-Val-Phe chlorimine. However, we did not detect the corresponding nitrile. Chlorimines may also slowly hydrolyze in an analogous fashion to imines to give a chloramine with an α-hydroxyl group, which should then undergo a second elimination to liberate NH_2Cl and the oxopeptide (Scheme 2, pathway 1B).

We propose that hydrolysis of dichloramines competes with 1,2-elimination. This would result in the release of $NHCl_2$ and formation of the related alcohol, which upon oxidation would form the oxopeptide (Scheme 2, pathway 2). This mechanism is supported by our finding that Glu-Val-Phe dichloramine produced approximately 70% of the maximal amount of oxopeptide almost immediately after its formation. The oxopeptide did not appear to be a product of the decomposition of the chlorimine but formed via a different pathway (Figure 5B). The $NHCl_2$ detected could not be a

product of dismutation of NH_2Cl because this requires a pH of less than 5.5 (*44*). Hydrolysis is likely to occur by an S_N1 mechanism and thereby require initial formation of a carbocation on the α-carbon. Its stabilization would promote release of $NHCl_2$ (Scheme 2, pathway 2). Thus, dichloramines attached to secondary carbons would undergo hydrolysis more readily than those attached to primary carbons. This is consistent with our findings that dichloramines of secondary amino acid derivatives liberated chloramine gases and killed bacteria, whereas those of taurine, Gly, and *N*-α-acetyl Lys did not. Hydrolysis via a S_N2 mechanism could not have occurred because the most $NHCl_2$ would have been released from taurine dichloramine due to its less sterically hindered α-carbon (*43*). Drawing this distinction between the possible nucleophilic substitution reactions is important because stabilization of a carbocation within the tertiary structure of proteins may facilitate other reactions of dichloramines that favor release of $NHCl_2$.

It is conceivable that the chloramine gases were responsible for bacterial killing. This is because only 2% of the organic chloramines would have to decay via the hydrolysis pathway to liberate sufficient $NHCl_2$ for killing to occur. Indeed, Glu-amide dichloramine (10 mM) decayed to liberate approximately 400 μM NH_2Cl, which is 4% of the original oxidant (Figure 4A). This may be a lower limit because approximately 50% of the chloramine activity of Glu-amide dichloramine remained after all of the Glu-amide had decayed (Figure 3B). $NHCl_2$ is reactive and unstable. In the presence of NH_3, it decays to NH_2Cl, but in the presence of trace amounts of HOCl, it will form trichloramine (NCl_3) (*32*). NH_2Cl and $NHCl_2$ could also form via chlorine-transfer reactions between organic chloramines and ammonium ions in solution. The likelihood of this reaction is low as only trace levels of NH_3 would have been present and the transhalogenation reaction with taurine dichloramine barely occurs over an hour (*16*).

It is possible that other unstable toxic products were produced along with NH_2Cl and $NHCl_2$. We observed the formation of oxopeptides from the decomposition of both monochloramines and dichloramines. Aldehydes and ketones are reactive, particularly with sulfhydryl, histidyl, and lysyl residues (*45*). Aldehydes and ketones generated from amino acids have previously been proposed to be toxic to bacteria (*33, 46, 47*) and suggested to be toxic products generated by myeloperoxidase (*11, 30, 34, 48*). However, Klebanoff reported that aldehydes formed by HOCl-dependent deamination were relatively nontoxic to bacteria (*49*). In support of this, we showed that monochloramines decayed to produce significantly more oxopeptide than the related dichloramines, yet were not toxic to *S. aureus* up to 100 μM. Thus, aldehydes and ketones formed from amino acids with a blocked C terminus do not appear to be responsible for the bactericidal activity of dichloramines in our study. Similarly, it follows that imines and aminols, products that were formed in greater yields during the decomposition of monochloramines, must also be benign at the levels produced. Other products formed from the decomposition of dichloramines include chlorimines, nitriles, and isocyanopeptides (*36, 50*). These are also unlikely to be toxic in our system, because they were either stable or formed after the peptide dichloramines had lost most of their bactericidal activity. These results do not rule out the possibility that oxidation products formed from free amino acid chloramines and dichloramines may be toxic.

In conclusion, we have shown that cytotoxic NH_2Cl and $NHCl_2$ are formed from the decomposition of dichloramines of *N*-terminal amino acids and are the probable perpetrators of the cytotoxicity observed against *S. aureus*. Given the enormous flux of HOCl generated inside neutrophils phagosomes, it is plausible that dichloramines will be formed on *N*-terminal amino acid residues and release NH_2Cl and $NHCl_2$. These oxidizing species are likely to contribute to innate immune defense and explain, at least in part, how HOCl can react mainly with neutrophil components but still indirectly kill phagocytosed bacteria. Dichloramines formed on Lys residues may not liberate the toxic gases. However, they are likely to be susceptible to reductive homolysis by metal-catalyzed reactions of superoxide to form radicals (*51*). In addition to their bactericidal activity, lipophilic NH_2Cl and $NHCl_2$ may exacerbate inflammation because they will diffuse freely from neutrophils and damage bystander cells of the host.

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Supporting Information Available: Further information on the chemical stability of chloramines: UV spectra of chloramines with unusual decay spectra (Ala-Phe dichloramine, Gly dichloramine, and Asn monochloramine), detection of NH_2Cl and $NHCl_2$ standard solutions by SIFT-MS, HPLC trace and LC/MS spectrum of products of the decay of Glu-Val-Phe monochloramine, HPLC trace and LC/MS spectrum of the decay of Asn monochloramine, and further analysis of Asp-Phe methyl ester dichloramine decay products. This material is available free of charge via the Internet at http://pubs.acs.org.

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