ELSEVIER

Journal of Chromatography A, 898 (2000) 153–165

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Degradation pathway of homoserine lactone bacterial signal molecules by halogen antimicrobials identified by liquid chromatography with photodiode array and mass spectrometric detection

James J. Michels[a,*], Eric J. Allain[a], Scott A. Borchardt[a], Peifeng Hu[b], William F. McCoy[a]

[a]*Global Research, Nalco Chemical Company, One Nalco Center, Naperville, IL 60563, USA*

[b]*Corporate Research and Technical Services, Baxter Healthcare Corporation, Route 120 and Wilson Road, Round Lake, IL 60073, USA*

Received 12 April 2000; received in revised form 8 August 2000; accepted 11 August 2000

Abstract

The degradation pathway of acylated homoserine lactone bacterial signaling molecules by oxidizing hypochlorite and stabilized hypobromite antimicrobials has been characterized. A reversed-phase HPLC separation using a cyano column was developed to detect the parent lactones, lactone-hydrolysis products, and halogenation products. Elucidation of the structures of the reaction products was done with the aid of online photodiode array UV spectroscopy and atmospheric pressure chemical ionization mass spectrometry. Quantitative output of the HPLC method was also used to estimate the kinetics of the degradation pathway. The results of this work found that only β-keto-amide signal molecules are halogenated, where normal amide signals are not, and may represent one possible mechanism for control of industrial biofilms.

Keywords: Bacterial signaling molecules; Halogen antimicrobials; Biofilms; Quorum sensing; Homoserine lactone

1. Introduction

Recent research has made great progress in the area of bacterial cell-to-cell communication [1]. It is now believed that many different types of bacteria are able to produce and respond to various hormone-like ''signal molecules'' that allow the organisms to act in a coordinated manner to accomplish various tasks. A particular subset of these signaling molecules (acylated homoserine lactones, or AHLs) are used by a wide variety of Gram-negative microorganisms to detect the relative number of bacteria that are within their own population. This type of cell density monitoring is called quorum sensing. Bacteria use quorum sensing to regulate various functions in a cell density dependent manner.

It has recently been discovered that AHL signal molecules are involved in biofilm formation in the

*Corresponding author. Tel.: +1-630-305-2318; fax: +1-630-305-2921.

E-mail address: jmichels@nalco.com (J.J. Michels).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00849-9

Gram-negative bacterium *Pseudomonas aeruginosa* [2]. Biofilms are communities of surface attached microorganisms found in a wide variety of aqueous environments. Biofilm accumulation in an industrial water system can cause numerous problems such as heat transfer resistance, under deposit corrosion, reduced flow, and product contamination.

Control and treatment of biofilms in industrial water is commonly done with oxidized halogen antimicrobials such as hypochlorite (chlorine bleach) and stabilized hypobromite (Stabrex brand antimicrobial). To date, no work has been reported determining the chemical and biological effects of oxidized halogen antimicrobials on the AHL signaling molecules and their impact on the mechanism of biofilm control. Therefore, an interdisciplinary study was done to examine this possible phenomenon. Microbiological aspects of the project are discussed in another publication [3]. This report will focus on the elucidation of the reaction pathway of AHL with chlorine bleach or stabilized hypobromite using HPLC. Multi-dimensional detection was done with UV photodiode array (DAD) spectroscopy and atmospheric pressure chemical ionization mass spectrometry (APCI-MS) to aid the characterization of reaction products.

2. Experimental

2.1. Chemicals

Acylated homoserine lactones were either obtained commercially or synthesized in-house. The 3-oxo-hexanoyl (OHHL), 3-oxo-dodecanoyl (ODHL), and hexanoyl (HHL) homoserine lactones were purchased from Quorum Sciences (Coralville, IA, USA) and used as received.

The nonanoyl homoserine lactone (NHL) was synthesized from 2-amino-4-butyrolactone (Fluka, Milwaukee, WI, USA) and nonanoic acid (Aldrich, Milwaukee, WI, USA) using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Aldrich) as a coupling agent. Briefly, a reaction mixture consisting of 2 g 2-amino-4-butyrolactone hydrobromide, 1 ml nonanoic acid, 1 g EEDQ and 0.1 ml triethylamine (Aldrich) in 40 ml methylene chloride (EM Industries, Gibbstown, NJ, USA) was stirred overnight at room temperature. Purification involved two extractions with an equal volume of 1 *M* HCl (EM) followed by three extractions with 1 *M* Na_2CO_3 (EM). Production of nonanoyl homoserine lactone was verified via ^1H NMR and FTIR spectroscopy, and HPLC–APCI-MS.

Model compounds for the DPD (*N*,*N*-diethylphenylenediamine) halogen demand experiments were purchased commercially. Acetoacetamide, 2,4-pentanediketone, 2-pentanone, and methyl acetoacetate (Aldrich), *N*-methylvaleramide and methyl butyrylacetoacetate (Lancaster Synthesis, Windham, NH, USA), and *N*-methylacetoacetamide (TCI America, Portland, OR, USA) were all used as received.

Halogen antimicrobials were purchased commercially. Stabrex brand antimicrobial (Nalco Chemical, Naperville, IL, USA) and chlorine bleach (Clorox, Oakland, CA, USA) were titrated for active halogen by the iodometric I titration [4] prior to preparing a stock solution for a given experiment.

Stock solutions of the homoserine lactones and model compounds were made to concentrations of roughly 500 ppm (w/w) in 10 or 50% ethanol (EM) in water. Stock solutions of the antimicrobials were made to concentrations of 7.1 m*M* (roughly 500 ppm w/w as Cl_2) in deionized water.

Aqueous buffers for the halogenation reactions were prepared from NaH_2PO_4, Na_2HPO_4, concentrated H_3PO_4, $CaCl_2$, $MgSO_4$, and $NaHCO_3$ (EM). The buffers at pH 3 and 6 were made to concentrations of 0.1 *M* of the mono and dibasic sodium phosphate salts, respectively, and adjusted to the desired pH with concentrated H_3PO_4. The buffer at pH 8 was artificial cooling water (1.5 m*M* $CaCl_2$, 0.8 m*M* $MgSO_4$, 2.2 m*M* $NaHCO_3$). All reagents for the DPD test for halogen determination were purchased from Hach Company (Loveland, CO, USA).

HPLC analyses were done with HPLC grade water (Millipore, Bedford, MA, USA) and acetonitrile (EM). Mobile phase pH was acidified with either concentrated H_3PO_4 at 0.005 *M* for DAD or trifluoroacetic acid (Aldrich) at 0.01% for APCI-MS. Mobile phases were filtered through a 47 mm, 0.45 μm HV membrane (Millipore).

2.2. DPD halogen-demand assay

Reaction between halogen antimicrobials and the test compounds was followed using the DPD

colorimetric test for total oxidized halogen determination [4]. A solution of 10 ppm (as Cl_2) halogen antimicrobial was prepared in 10 ml of either 0.1 *M* sodium phosphate (pH 6 or 3), or artificial cooling water (pH 8). A 1 ml aliquot was removed, diluted ten fold in deionized water and assayed for total halogen to determine the level at time = 0. Signal molecule or analog was then added to a final concentration of 0.05 m*M*. Halogen level was determined over time by diluting 1 ml aliquots ten or five fold in deionized water prior to analysis.

2.3. HPLC–DAD

Quantitative liquid chromatography was performed using a Waters (Milford, MA, USA) 600E gradient pump, a Rheodyne (Cotati, CA, USA) 7125 injector with a 100 μl sample loop, a Waters 996 photodiode array UV detector, and a Waters Millennium 2010 data system.

Specific HPLC conditions consisted of a Zorbax (Hewlett-Packard, Wilmington, DE, USA) 5 μm SB-CN column (250×4.6 mm, plus SB-CN guard), premixed isocratic mobile phases in the range 5 to 40%(v/v) acetonitrile in 0.005 *M* phosphoric acid, and a flow-rate of 1.5 ml/min. Screening gradients were done by pump-blending a given premix with pure acetonitrile up to a ratio 90/10 (v/v) of acetonitrile-to-premix at a rate of 3%/min. Quantitative detection was done at 200 nm and UV spectra were acquired at a rate of 1 spectrum/second with a resolution of 3.6 nm over the range of 195 to 400 nm.

2.4. HPLC–APCI-MS

LCMS experiments were carried out on a Hewlett-Packard (Avondale, PA, USA) 1090 HPLC and a Fisons VG (Manchester, UK) Quattro triple quadrupole MS. The HPLC conditions were identical to those used for DAD except the mobile phase was pump-blended in the range 5 to 40% (v/v) acetonitrile-to-0.1% trifluoroacetic acid. The LC flow to the mass spectrometer was limited to approximately 10% of the overall flow-rate by a polyetheretherketone (PEEK) tubing of 1.5 m length and 0.635 mm I.D. Atmospheric pressure chemical ionization (APCI) was used to couple the HPLC and the mass spectrometer. The APCI probe was operated at 500°C and the ion source temperature was maintained at 120°C. The cone voltage was set to 25 V so that the protonated molecular species were observed. For MS/MS experiments, argon was used as the collision gas. The collision gas pressure was adjusted to obtain a precursor beam suppression of approximately 90%. Collision energy was set to 30 V.

3. Results and discussion

3.1. Structures of acylated homoserine lactones

Two types of acylated homoserine lactone signal molecules that have been observed in the area of industrial biofilms are studied in this work. The structures of these compounds are shown in Fig. 1 [5] and differ only in the fatty acid group *N*-acylating the homoserine lactone. One type is the 3-oxo-alkanoyl, or β-keto-amide type, and the other is the *N*-alkanoyl, or normal amide. The hydrocarbon chain of the acid of either type can range in length from two to twelve units and may even possess some degrees of unsaturation. Most gram-negative bacterial signal compounds known to exist in nature possess these root structures.

3.2. DPD analysis of AHL/halogen reactions

Before any chromatographic work started, an initial study was done to determine if a reaction occurs between the AHLs and halogenated antimicrobials utilizing a wet chemical test for oxidized halogen compounds. By colorimetrically monitoring antimicrobial levels during a reaction, any evidence of reaction and approximate rate would be observed. The wet test involved the DPD (*N*,*N*-Diethylphenylenediamine) method of halogen analysis [4].

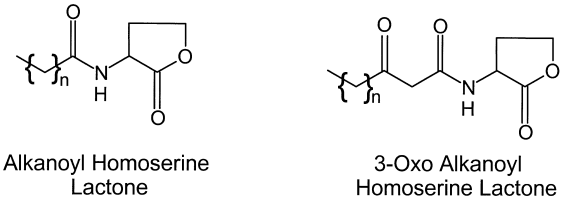

Fig. 1. The general chemical structures of the acylated homoserine lactone (AHL) bacterial signal molecules studied in this work.

For DPD work, the compounds employed were the hexanoyl-, nonanoyl-, and dodecanoyl-acylated homoserine lactones and other normal and β-keto-type analogs. These test compounds were chosen based on previously documented studies [1,2] and compound availability. The antimicrobials used were chlorine bleach and stabilized hypobromite. Reactions were performed at pH 3, 6, and 8 and done below 50 ppm of test compound to simulate industrial water concentrations. Antimicrobials were dosed at an equal ppm concentration (in units of ppm Cl_2), or approximately a threefold molar excess of test compound.

The DPD data collected are shown in Table 1. The speed of the reaction was estimated by the percentage of halogen consumed at 1-min reaction time. At all pH's tested, both bleach and stabilized hypobromite reacted only with the β-keto-type species. At pH 6 and 8, reactions with these compounds were almost completed at 1 min. For the single test done at pH 3, reaction occurred at a much slower rate. For compounds not containing the β-keto moiety, no significant reaction above the blank was noticed in as long as 30 min.

From these experiments, it was concluded that for a bacterial signal compound to significantly react beyond a blank, the molecule required the presence of the β-keto-amide functionality. Reactivity was independent of substitution of the homoserine lactone with a ketone, methylamide, or methyl ester. One hypothesis for explaining the observed reactivity was the keto-enol tautomerism of the β-keto class of compounds. Tautomerism would expose the α carbon to electrophilic attack by the hypohalite, which is acknowledged as an electrophilic reagent for halogenation of aromatic compounds [6]. If this were true, the resulting material would be singly or doubly halogenated at the α position. Chromatographic analysis was then needed to determine the species produced.

3.3. HPLC of AHLs

An HPLC method was developed to analyze the AHLs and their potential reaction products. One reported methodology separated multiple analogs of AHLs using a C8 column coupled with acetonitrile gradient elution [7]. Since a more rapid analysis was desired that could accommodate a wider range of analyte size and polarity, a cyano column was utilized that would still adequately retain the fatty materials as a function of their hydrophobicity. With

Table 1
DPD halogen-demand results for both β-keto (β-K) and normal (*N*) acylated homoserine lactones and related test compounds reacted with chlorine bleach (Cl) and stabilized hypobromite (Br) for a 1-min reaction period

Test compound	Type	Antimicrobial	pH	% Halogen consumed
HHL	*N*	Cl	6	<5
HHL	*N*	Cl	8	<5
HHL	*N*	Br	6	<5
HHL	*N*	Br	8	<5
OHHL	β-K	Cl	3	15
OHHL	β-K	Cl	6	76
OHHL	β-K	Cl	8	70
OHHL	β-K	Br	6	66
OHHL	β-K	Br	8	73
NHL	*N*	Cl	6	<5
ODHL	β-K	Cl	6	77
2,4-Pentanedione	β-K	Cl	6	84
2-Pentanone	*N*	Cl	6	<5
Acetoacetamide	β-K	Cl	6	61
N-Methylacetoacetamide	β-K	Cl	6	75
N-Methylvaleramide	*N*	Cl	6	<5
Methylacetoacetate	β-K	Cl	6	84
Methylbutyrylacetate	β-K	Cl	6	81

the more polar column, a lower acetonitrile level was needed to elute not only the original AHLs, but their multiply halogenated products as well. An acidic mobile phase pH was also necessary for lactone-hydrolyzed AHL analogs that would require protonation for reversed-phase retention. Analytical detection of the AHLs had to be done at low UV wavelengths for maximum sensitivity since these molecules possessed no significant chromophore. To reduce the possibility of misleading ghost or system peaks, all primary analyses were run in isocratic mode, but initial screenings were done by gradient elution to detect later-eluting reaction products. If impurities were detected using gradients, confirming analyses were done isocratically at the appropriate acetonitrile percentages.

The AHL analogs in the HPLC study were the 3-oxohexanoyl-(OHHL) and hexanoyl-(HHL) homoserine lactone signal compounds. This pair was chosen based on compound availability and because OHHL has been documented to be a biofilm enhancer [2]. Fig. 2 shows a chromatogram of the two AHLs plus their lactone hydrolysis products using the cyano column and an isocratic mobile phase of 5% acetonitrile. If multiple halogenations of the AHLs were to occur, the high polarity column allowed mobile phase conditions to be flexible enough to allow elution of such molecules.

Differences in the parent lactones could be observed by photodiode array detection. Embedded in Fig. 2 are overlaid normalized UV spectra of OHHL and HHL which show a slight spectral red shift upon addition of the 3-oxo functional group to the HHL molecule. No changes were observed in the UV spectra of either AHL upon lactone hydrolysis.

Identification of the AHLs and hydrolysis products was also confirmed by APCI-MS. The LCMS analysis of OHHL and hydrolyzed OHHL (stacked spectra presented in Fig. 3) yielded $(M+H)^+$ ions at the anticipated m/z values 214 and 232, respectively.

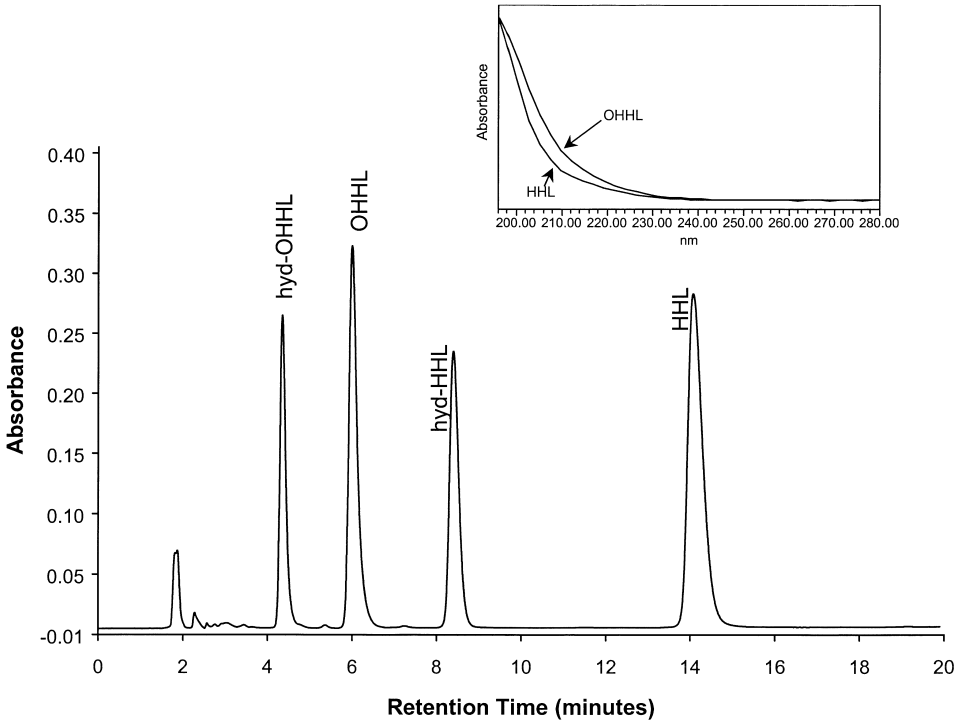

Fig. 2. The HPLC chromatogram of the acylated homoserine lactone bacterial signal molecules and their lactone hydrolyzed products, along with the UV spectra of the parent lactones. Analytical column: Zorbax SB-CN 25 cm×4.6 mm plus guard column. Mobile phase: acetonitrile–water (5:95, v/v), 5 m*M* H_3PO_4. Flow rate: 1.5 ml/min. Injection volume: 100 μl. Detection: 200 nm.

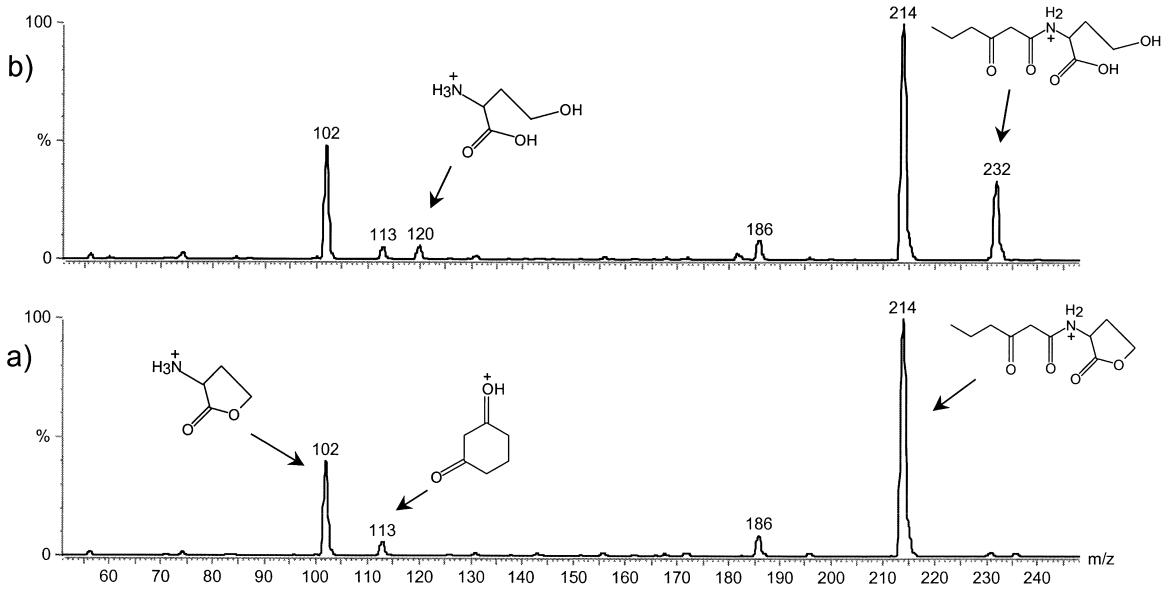

Fig. 3. The APCI-MS spectra of (a) OHHL and (b) lactone hydrolyzed OHHL.

Both molecules showed a fragment ion at m/z 113 for the diketone group. OHHL expressed a fragment at m/z 102 for the homoserine lactone group, while the hydrolysis product possessed an ion at m/z 120 for the hydrolyzed homoserine lactone.

3.4. HPLC analysis of reactions at pH 8

Once the HPLC analysis had been established, reaction studies were commenced using both hypochlorite and stabilized hypobromite. A pH of 8 was done first since industrial cooling water is typically at that pH. The experiments found halogenation of OHHL had occurred but did not produce the expected mono or dihalogenated keto-amide. Instead it was determined that after halogenation of the α carbon, an α–β hydrolysis event severed the molecule to yield a dihalogenated ethanoyl homoserine lactone and butyric acid. While similar results were obtained for both chlorine and bromine antimicrobials, evidence for these conclusions will be presented using the stabilized hypobromite reaction as an example. Figs. 4–6 illustrate chromatographic, DAD, MS and MS–MS data for the newly discovered reaction product α,α-dibromoethanoyl homoserine lactone (abbreviated DBEHL) and its lactone hydrolysis product α,α-dibromoethanoyl-2-(4-hydroxy)butanoic acid (abbreviated hyd-DBEHL). While aqueous halogenation of β-diketone compounds is well-documented [8,9], no reports exist for the observed α–β scission after halogenation.

Fig. 4 is a chromatogram of the pH 8 stabilized hypobromite reaction with OHHL at 2 min reaction time. Rapid reaction of OHHL to DBEHL (at 5.2 min retention) was observed, with DBEHL in turn slowly degrading to the lactone hydrolysate. Comparison of OHHL and DBEHL can be seen in the normalized UV spectra embedded in Fig. 4, where a large spectral red shift is noticed with the bromination product. Such a red shift in the UV spectra of halogenated carbonyl compounds from the spectrum of the parent would be expected [10].

Fig. 5 contains stacked APCI-MS spectra of the products in Fig. 4. The $(M+H)^+$ ion of DBEHL (seen in Fig. 5a) was found at m/z 300/302/304. Isotope ions with a 2 amu spacing were found to have a relative abundance ratio of approximately 1:2:1, indicating the presence of two bromines in its structure [11]. From the observed cluster of peaks representing the $(M+H)^+$ and supporting fragmentation, the structure of DBEHL embedded in Fig. 5a was deduced. This structure was supported by the

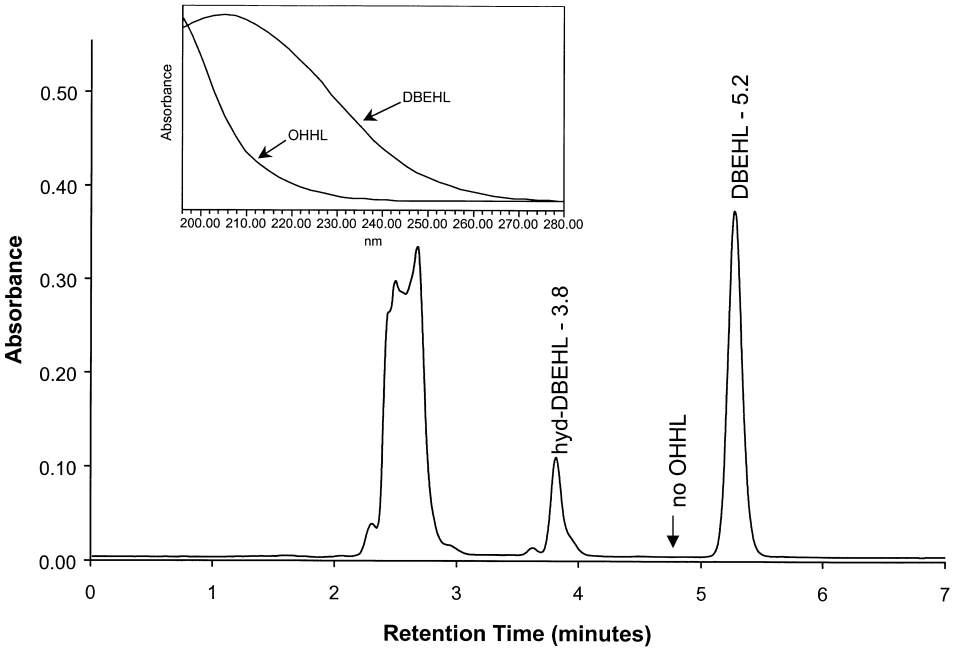

Fig. 4. The HPLC chromatogram of the pH 8 OHHL-stabilized hypobromite reaction, along with the UV spectra of OHHL and the identified product DBEHL. Mobile phase: acetonitrile–water (10:90, v/v), 5 m*M* H_3PO_4.

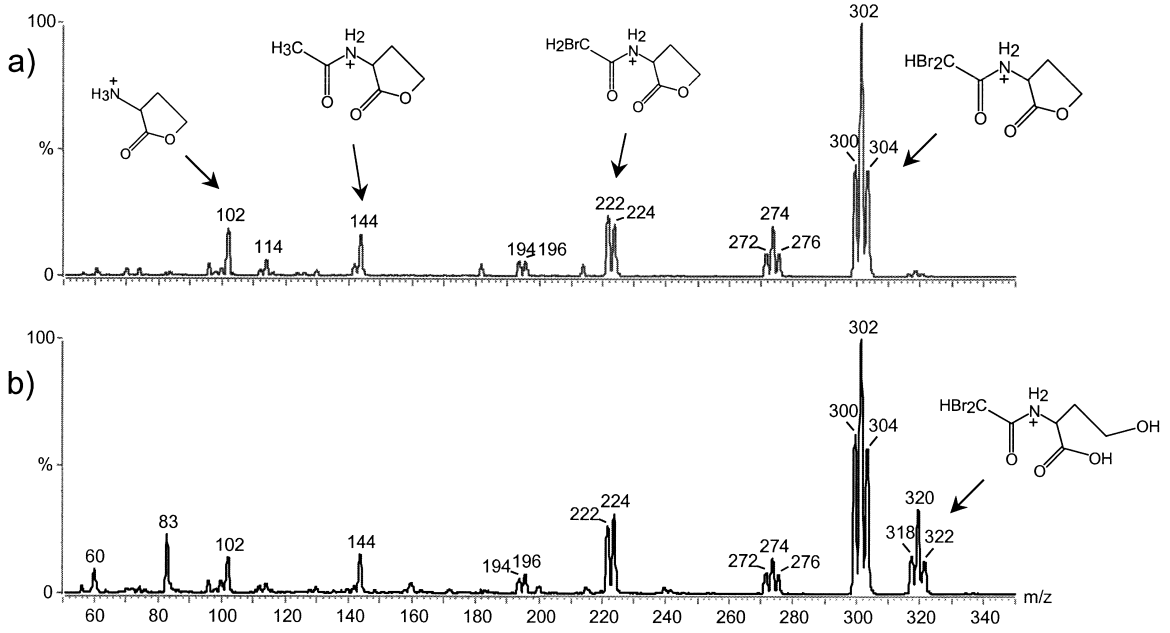

Fig. 5. The APCI-MS spectra of (a) DBEHL and (b) lactone hydrolyzed DBEHL.

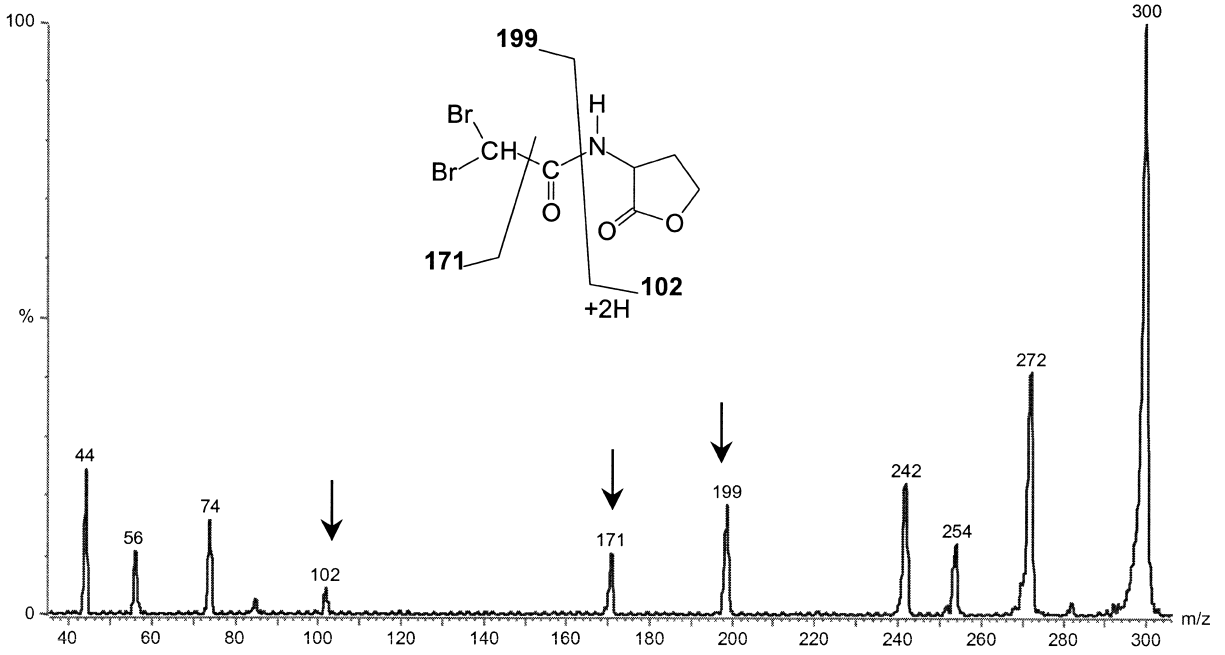

Fig. 6. The MS–MS spectrum of the m/z 300 ion of DBEHL.

observance of the ion at m/z 102 for the homoserine lactone, and the ion at m/z 144 resulting from the loss of two bromine atoms. The ion cluster at m/z 222/224 had isotope ions with a 2 amu spacing and relative abundance ratio of 1:1, indicating the presence of one bromine. Based on these data, it was concluded that OHHL was doubly halogenated at the α carbon prior to forming DBEHL, but further experimental confirmation was needed to substantiate the final structure.

Supplemental gas chromatographic analysis of a completed OHHL/pH 8 stabilized hypobromite reaction was performed to verify the HPLC data. GC analysis not only detected butyric acid, but found (by external standardization) the theoretical amount based on the initial quantity of OHHL, thus confirming the proposed reaction pathway.

Tandem mass spectrometry (MS–MS) was also done on the pH 8 reaction mixture to further verify the proposed structure of DBEHL. The collisionally activated dissociation (CAD) spectrum of the m/z 300 ion is seen in Fig. 6. Formation of the m/z 102 ion indicates the lactone part of the molecule was not brominated. A molecule was formed that had the bromines substituted on the α-carbon of OHHL accompanied by concurrent cleavage of the α-β bond. The m/z 199 and 171 ions lend further support to the structure assignment.

Fig. 5b is the spectrum of the DBEHL hydrolysate (3.8 min retention in Fig. 4) by LCMS. The spectrum revealed a protonated molecular species clustered at m/z values of 318/320/322 (1:2:1), 18 amu higher than the ion cluster of DBEHL. This clearly shows that the lactone hydrolysis product of DBEHL is formed.

Halogen antimicrobial reactions were also attempted with the normal amide HHL at pH 8. As expected by the DPD results in Table 1, no halogenation occurred and only slow, base-catalyzed hydrolysis of the lactone ring was observed.

3.5. HPLC analysis of reactions at acidic pH

The pH of the OHHL and stabilized hypobromite reaction was then adjusted to more acidic levels in an attempt to slow the reaction rate and detect the mono and dihalogenated OHHL precursors to DBEHL. Fig. 7 is overlaid chromatograms of the reactions performed at pH 6 and 3. At pH 6 in Fig. 7a, a later-eluting component at 9.5 min attributed to

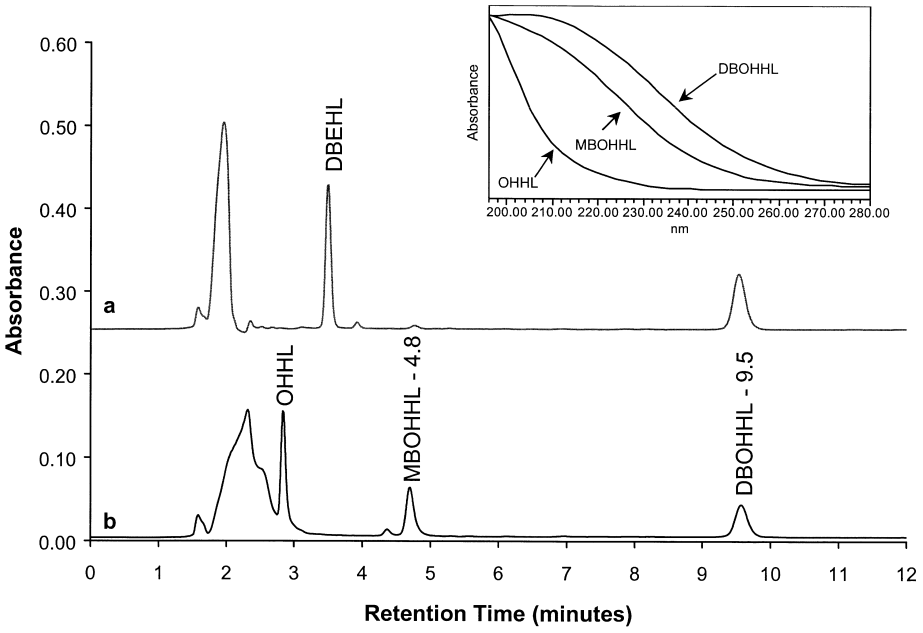

Fig. 7. The HPLC chromatograms of the (a) pH 6 and (b) pH 3 OHHL-stabilized hypobromite reactions, along with the UV spectra of OHHL and the identified products MBOHHL and DBOHHL. Mobile phase: acetonitrile–water (20:80, v/v), 5 m*M* H_3PO_4.

dibrominated OHHL (DBOHHL) was observed that upon increasing reaction time, appeared to degrade to DBEHL.

Reaction at pH 3 detected both dihalogenated and monohalogenated products. The chromatogram in Fig. 7b is a 2 min reaction profile and simultaneously shows OHHL, monobromo-OHHL (MBOHHL) at 4.8 min retention, and DBOHHL. MBOHHL had

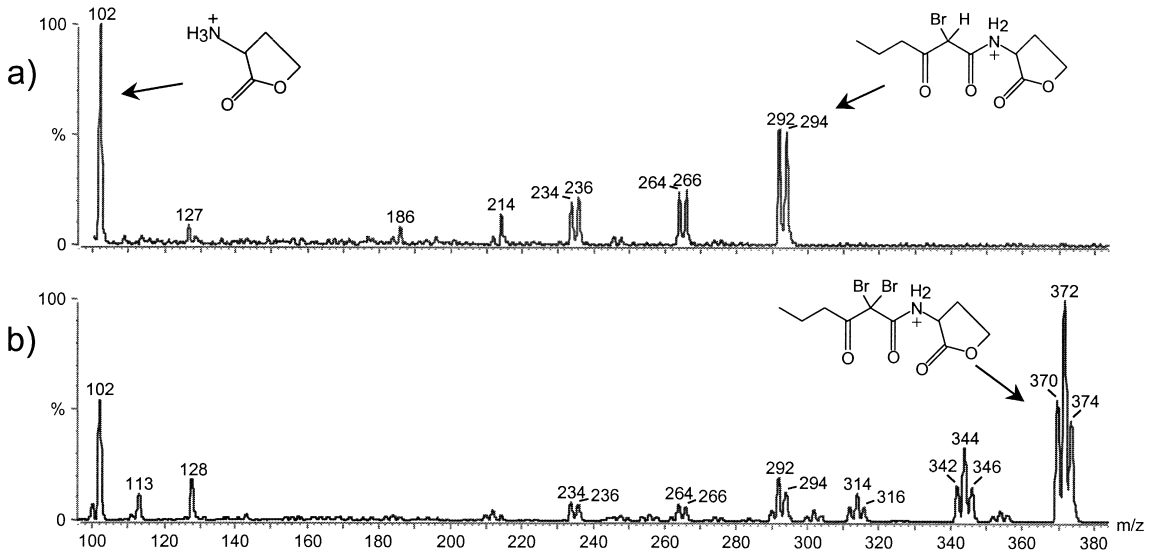

Fig. 8. The APCI-MS spectra of (a) MBOHHL and (b) DBOHHL.

disappeared by 5 min of reaction, while DBOHHL achieved a steady-state concentration after complete loss of its two precursors. Due to the low solution pH, DBOHHL did not degrade to DBEHL by base-catalyzed hydrolysis.

Photodiode array spectra expressed evidence of multiple bromination of OHHL. As expected, the embedded normalized UV spectra in Fig. 7 show that upon going from OHHL, to MBOHHL, to DBOHHL, the spectra became increasingly red shifted.

Mass spectrometry confirmed the identities of the two new compounds in Fig. 7 as the α-mono- and α,α-dibrominated analogs of OHHL. The spectrum in Fig. 8a is for the monobrominated species (MBOHHL) and the 1:1 isotope peak pattern starting at m/z 292 for the protonated molecule verified the addition of one bromine atom to OHHL. Fig. 8b is the spectrum of the dibrominated product (DBOHHL), where the 1:2:1 isotope peak pattern starting at m/z 370 is consistent with the addition of two bromines. The presence of the m/z 102 ion in each APCI spectrum again suggests bromination was not associated with the lactone ring.

3.6. Reaction pathway and kinetics

With the information obtained by HPLC, a proposed pathway is illustrated in Fig. 9 for the reaction of OHHL with oxidizing hypochlorite or stabilized hypobromite antimicrobial. The entire scheme is constructed of four distinct reactions that can be termed α-mono-halogenation, α,α-di-halogenation, α–β hydrolysis, and lactone hydrolysis. These four reactions yield the products MXOHHL (II), DXOHHL (III), DXEHL (V), and hydrolyzed-DXEHL (VI). Each reaction can be described by a pseudo first-order rate constant whose magnitude is pH dependent. While this pathway possesses four unique events, at a given pH the pathway can be simplified to only two as in a Series First Order Model [12]. After loss of OHHL (I) directly to intermediates II, III, or V, the second observed event was then formation of the final products III, V, or VI. This simplification can be done because at the various pH conditions, some of the reactions are too fast to be a unique measurable event or are not significantly detected. For example, at pH 8, the

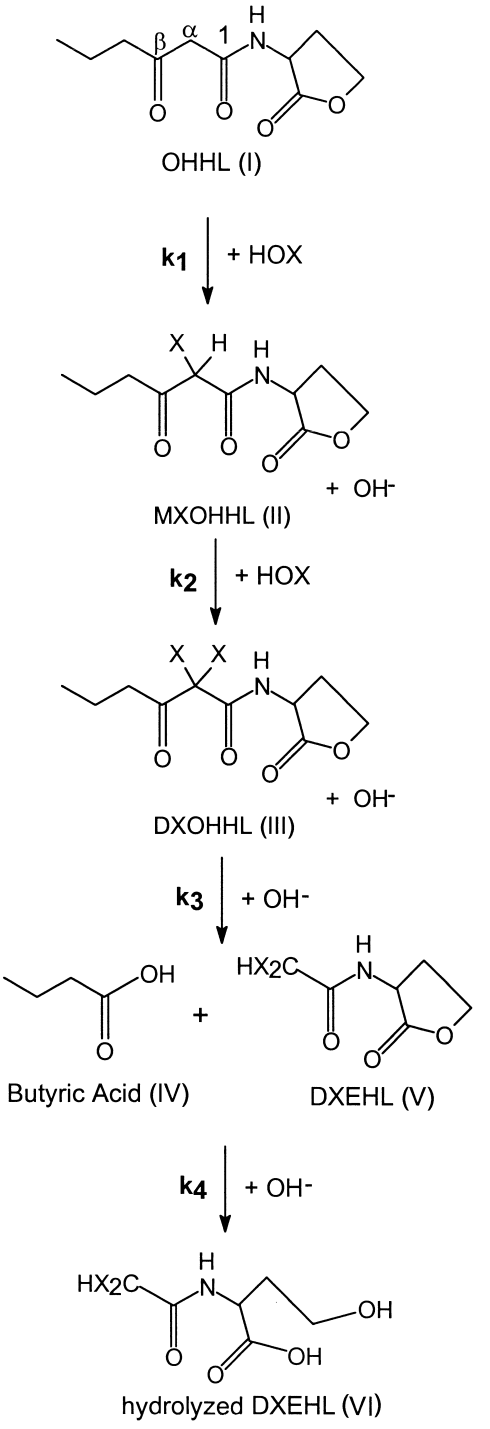

Fig. 9. The complete pathway of the reaction between OHHL and halogen antimicrobial (HOX, hypochlorite or stabilized hypobromite).

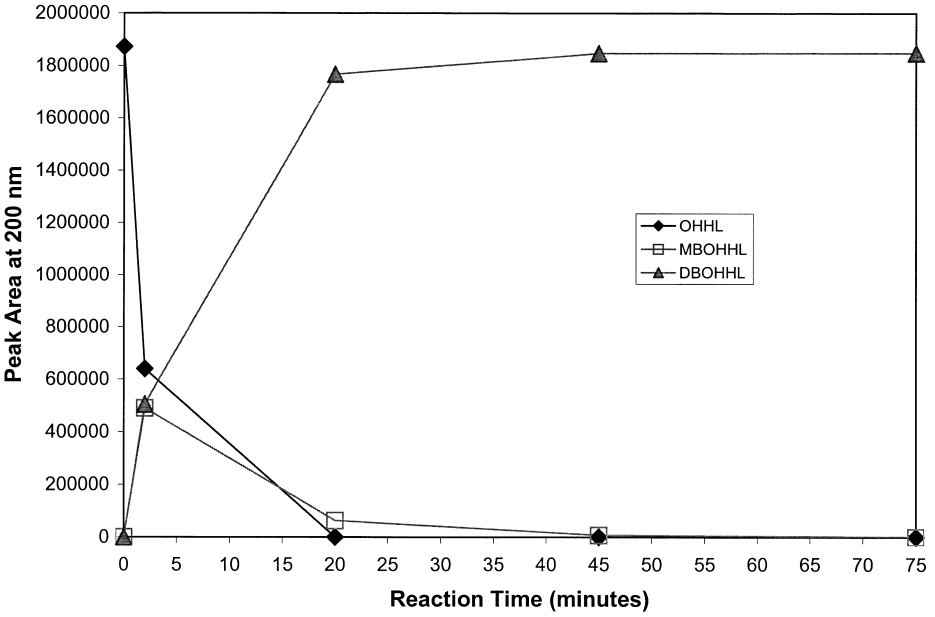

Fig. 10. The peak area versus pH 3 reaction time profiles for the OHHL-stabilized hypobromite Series First Order reaction.

halogenated OHHL products (II and III) are not detected such that DXEHL (IV) is formed instantaneously. Therefore, rate constants k_2 and k_3 are too large to be measured and are described in k_1, leaving the pathway characterized only by k_1 and k_4. Conversely at pH 3, only products II and III are detected,

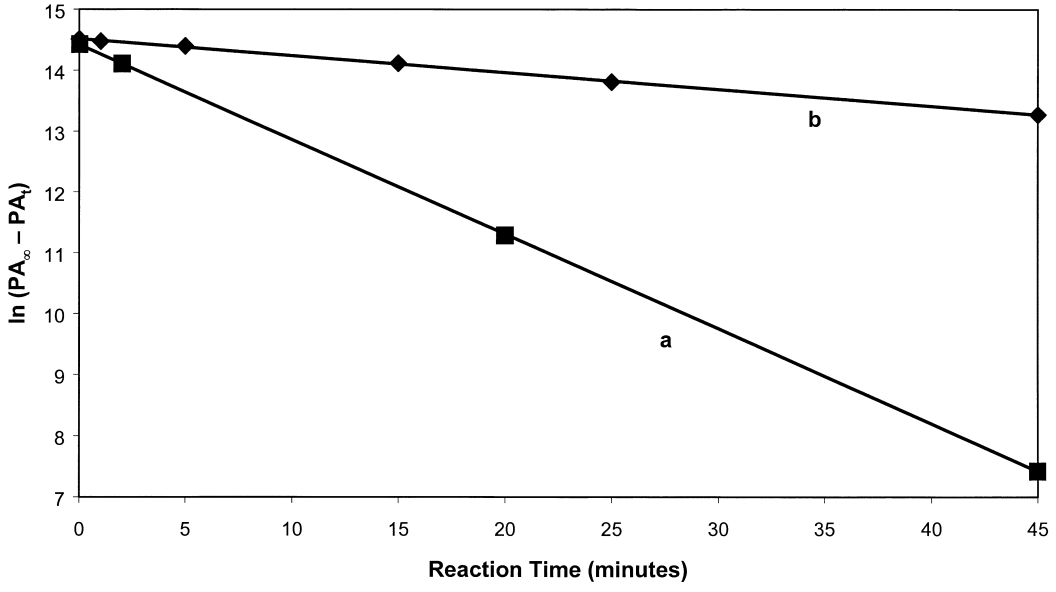

Fig. 11. Pseudo first-order kinetics plots based on the model of Jensen and Lenz [13] for the third component of the OHHL-stabilized hypobromite Series First Order reactions done at (a) pH 3 for DBOHHL where the slope equals k_2 and (b) pH 6 for DBEHL where the slope equals k_3.

Table 2
Estimated pseudo first-order rate constants (units of min^{-1}) for the degradation pathway of 3-Oxo-hexanoyl homoserine lactone (OHHL) by hypochlorite (Cl) and stabilized hypobromite (Br) antimicrobials using the model of Jensen and Lenz [13]. A table cell marked with an ''×'' signifies the rate constant was too large to be measured and was incorporated into k_1, while a cell marked with ''N/A'' denotes the reaction step was not applicable

Reaction	$-k_1$	$-k_2$	$-k_3$	$-k_4$
Br, pH 3	0.5	0.2	<0.001	<0.001
Cl, pH 3	0.2	0.2	<0.001	<0.001
Br, pH 6	>10	×	0.02	<0.001
Cl, pH 6	>10	×	0.06	<0.001
Br, pH 8	>10	×	×	0.016
Cl, pH 8	>10	×	×	0.005
Control, pH 8	N/A	N/A	N/A	0.003

making the pH 3 pathway governed only by k_1 and k_2, with k_3 and k_4 not detected.

To further show the utility of the HPLC method for characterizing the pathway, the individual rate constants for each reaction were estimated from the peak areas of the chromatographic data. The first rate constant, k_1, was measured directly by the loss of OHHL. Based on the model of Jensen and Lenz [13], the second rate constant was estimated from the steady-state peak area of the final product (PA_∞) and its peak area at a given timepoint (PA_t). Fig. 10 presents a plot of the 200 nm peak areas for the three compounds involved at pH 3 versus reaction time, which is a prototypical Series First Order Reaction profile. When the data for compound III at pH 3 and compound V at pH 6 were plotted by the Jensen and Lenz model in Fig. 11, linear pseudo-first order kinetics plots were obtained from which the slopes were equal to the second rate constants k_2 and k_3.

Kinetics treatments were done for OHHL reactions using both the bleach and stabilized hypobromite antimicrobials at all three pH conditions. Calculated rate constants are presented in Table 2 for all six antimicrobials plus an OHHL pH 8 lactone-hydrolysis control. For the six antimicrobial reactions, the halogenation events described by the rate constants k_1 and k_2 increase by over an order of magnitude with higher pH. As the pH increases, though, the hydrolysis events expressed by the rate constants k_3 and k_4 are measurable and are found to be an order of magnitude or more lower than halogenation. This type of information is highly useful for researching the effects of using halogenated antimicrobials for biofilm control.

4. Conclusions

The degradation pathway of acylated homoserine lactone signaling molecules by oxidizing halogenated antimicrobials has been elucidated. An HPLC separation was developed to not only detect the parent lactones and their hydrolysis products, but also their α-halogenation products. Photodiode array UV spectroscopy and atmospheric pressure chemical ionization mass spectrometry were used online with the separation to aid in the identification of degradants. The results of this work show that a chemical interaction occurs between the halogenated antimicrobials and the β-keto-amide signal molecules, but not the normal amide signals. Degradation of the β-keto-amides by the reported pathway may represent one possible mechanism for control of industrial biofilms. The HPLC methodology described in this work could easily be used for studying bacterial signal molecules in biological matrices or examining fundamental reactions of hypohalites and β-diketone compounds.

Acknowledgements

The authors would like to thank Dr. T.P. McGinnis of Nalco Chemical and Dr. B.F. Michels of BP Amoco for their helpful comments during the preparation of this manuscript.

References

[1] G.M. Dunny, S.C. Winans (Eds.), Cell–Cell Signaling in Bacteria, ASM Press, Washington, D.C, 1999.
[2] D.G. Davies, M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, E.P. Greenberg, Science 280 (1998) 295.
[3] E.J. Allain, S.A. Borchardt, W.F. McCoy, J.J. Michels, Appl. Env. Microbiol., submitted for publication.
[4] A.E. Greenberg, L.S. Clesceri, A.D. Eaton, M.A.H. Franson (Eds.), Standard Methods for the Examination of Water and Wastewater, 18th edition, American Public Health Association, Washington, D.C, 1992, pp. 4–37, method 4500-Cl.

[5] J. Zhu, J.W. Beaber, M.I. More, C. Fuqua, A. Eberhard, S. Winans, J. Bacteriol. 180 (1998) 5398.
[6] K. Smith, M. Butters, W.E. Paget, B. Nay, Synthesis (1985) 1155.
[7] M. Camara, M. Daykin, S.R. Chhabra, Meth. Microbiol. 27 (1998) 319.
[8] J. Kosmrlj, M. Kocevar, S. Polanc, Synth. Commun. 26 (1996) 3583.
[9] H.J. Cowe, P.J. Cox, R.A. Howie, R.H. Thomson, J. Chem. Res., Synop. (1987) 266.
[10] H.H. Willard, L.L. Merritt, J.A. Dean, in: Instrumental Methods of Analysis, 5th ed, D. Van Nostrand,, New York, 1974, p. 109.
[11] F.W. McLafferty, F. Turecek, in: Interpretation of Mass Spectra, 4th ed, University Science Books, Sausalito, CA, 1993, p. 22.
[12] J.W. Moore, R.G. Pearson, in: Kinetics and Mechanism, 3rd ed, John Wiley, New York, NY, 1981, p. 290.
[13] J.L. Jensen, P.A. Lenz, J. Am. Chem. Soc. 100 (1978) 1291.