

Reaction of *N*-Acylhomoserine Lactones with Hydroxyl Radicals: Rates, Products, and Effects on Signaling Activity

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Chemical communication in bacteria, sometimes called quorum sensing, is a fundamental microbial process that is based on the exchange of molecular signals between cells. The signaling molecules involved in this process are thermodynamically unstable in some environments and their degradation affects microbial communication. This work reports the oxidation of a series of substituted *N*-acylhomoserine lactones (AHLs, a class of quorum sensing signals) by hydroxyl radicals. The corresponding bimolecular rate constants were obtained and correlated positively with the length of the acyl side chain (C, in numbers of carbon atoms) ranging from $2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ to $9.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (C4- to C10-AHL), $2.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for 3-*oxo* C6-AHL, and $2.94 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for 3-*oxo* C8-AHL. Liquid chromatography–mass spectrometric techniques were applied to qualify the identity and quantify the yields of the hydroxyl radical oxidation products of C6-AHL (aldo, keto, and hydroxylated C6-analogues identified). The biological activity of C6-AHL and associated products was determined using the *Vibrio harveyi* bioluminescence bioassay. Oxidation resulted in a net increase in assay response indexed against the starting AHL. This result suggested that the application of HO•-based technologies such as advanced oxidation processes for biofilm control may result in unintended quorum sensing responses by microbial communities.

Introduction

N-Acylhomoserine lactones (AHLs) and their derivatives are a class of molecules that play important roles in intercellular microbial signaling and can induce the development of bacterial colonies and biofilms (1–8). The common structural features of the AHLs are a five-membered lactone linked to an acyl chain through an amide (Figure 1). Their uptake by cells results in changes in gene expression and consequent biosynthesis at the cellular and community level (2, 9, 10). The outcomes of this process include biofilm formation,

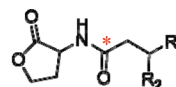
protection and dispersal; horizontal transfer of DNA within the film, production of virulence factors, antibiotics, etc. (11). Biofilm formation has been linked to degradation of water quality in water distribution systems, fouling of industrial and transport equipment, potential transfer of organic contaminants and nanoparticles from the water column to the food web and the persistence of disinfection resistant pathogens (12–16). The microbial activities regulated by AHLs relate to both their specific structures and concentrations. The effects of natural attenuation on the persistence of microbial signals are unknown (17, 18).

Sharp microscale (e.g., micrometer) redox gradients have been shown to occur in biofilms in water pipes, condensers, photosynthetic microbial mats, and marine sediments (e.g., conversion from aerobic to sulfate-reducing communities) (19). A net result is that molecules diffusing through biofilms can be subjected to a wide range of oxidative attenuation processes or destabilizing conditions in a very small volume. These changes may be particularly important to biofilms growing in oxidizing environments such as the surfaces of sunlit metal oxide particles at beaches; drinking water distribution systems with secondary disinfectants or food surfaces during electron beam treatment, etc (20).

The hydroxyl radical ($E^\circ = 2.80 \text{ V vs NHE}$) is a strong oxidant that reacts at nearly diffusion controlled rates with most organics (21, 22). This radical occurs naturally in sunlit natural waters and is purposefully generated in the application of several oxidative remediation technologies (23–25). In this work we report the application of a competitive kinetic strategy to determine the bimolecular rate constant for the oxidation of a series of AHLs by the hydroxyl radical and the application of LC-MS techniques to identify their oxidation products. The bimolecular rate constants varied positively across the series in proportion to the molecular weight of the AHL. Hydroxyl radicals were an unselective oxidant under our conditions and generated a suite of products, including the corresponding AHL ketones, aldehydes, and alcohols. The biological activity of C6-AHL and its HO•-derived oxidation products were investigated using the *V. harveyi* bioluminescence bioassay for signaling activity.

Materials and Methods

N-Hexanoyl-DL-homoserine lactone (C6-AHL), *N*-heptanoyl-DL-homoserine lactone (C7-AHL), *N*-octanoyl-DL-homoserine lactone (C8-AHL), *N*-decanoyl-DL-homoserine lactone (C10-AHL), *N*- β -ketocaproyl-DL-homoserine lactone (*oxo*-C6-AHL), and *N*-(β -keto-octanoyl)-DL-homoserine lactone (*oxo*-C8-AHL) were purchased from Sigma-Aldrich (97+% purity). Sodium nitrate (99.1%), sodium bicarbonate (100%), benzoic acid (99.5%), 2-propanol (99.9%), sodium phosphate monobasic (99%), and sodium phosphate dibasic (99%) were obtained from Fisher. Tetrahydrofuran (THF) (99.5%) was received from EM Science. Methylene chloride (HPLC grade) was purchased from Mallinckrodt Chemicals. All materials were used without further purification. All aqueous solutions were prepared using $18 \text{ M}\Omega \cdot \text{cm}$ water (Barnsted E-Pure). An Orion 410A meter with a Cole Parmer 05997-10 Ag/AgCl



$R_1 = -\text{H}, = \text{O}, -\text{OH}; R_2 = -\text{C}_n\text{H}_{2n+1}, -\text{C}_n\text{H}_{2n}$

FIGURE 1. The molecular structure of *N*-acylhomoserine lactones (* indicates C1 for numbering purposes).

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electrode was used for pH measurement. The AHL indicator strain *V. harveyi* BB120 was purchased from American Type Cell Culture (ATCC, Rockville, MD) (26).

Experimental Procedure. For kinetics, a reference solution (pH 7.2) was prepared of 1 mM benzoate and 6 mM sodium nitrate (27). Individual AHL stock solutions were prepared in methylene chloride, then calculated aliquots were added to 40.0 mL vials, and residual methylene chloride was removed through evaporation. The contents were reconstituted with 1.0 mL aqueous solution, stirred, transferred to 2 mL screw top borosilicate vials, and irradiated with polychromatic light (300 nm–800 nm). Seventy-two vials were prepared, in triplicate, for each AHL (0.1 mM) including a reference control solution without added AHL. Solutions stored in the dark exhibited no oxidation and less than 1.0% hydrolytic losses over the time scale of the experiments. Samples prepared for product isolation were initially 3.0 mM phosphate buffer (pH 5.0), 12 mM NaNO₃ and 100 μM [AHL]₀, then illuminated under the same conditions.

Irradiation. Hydroxyl radicals were generated by the photolysis of nitrate (27–33). Samples were irradiated in a Suntest XLS+ Solar Simulator equipped with a 2200 W Xe vapor lamp (Atlas Material Testing Solutions). Light intensity was calibrated with an internal radiometer and determined spatially with a nitrate actinometer (27–33). Light intensity was set to 655 W/m² (300 nm–800 nm). Black standard surface temperature was maintained at 26 °C ± 2 °C. Photoreactors were perpendicular to the incident radiation on the illuminated surface during all experiments (34, 35). The lamp was allowed to equilibrate for at least 60 min before sample irradiation. During the photolysis period, three vials from each set of samples were removed from the reaction chamber at 70 min time intervals and either stored frozen at –80 °C until analysis (LCMS) or analyzed immediately (fluorescence), *vide infra*.

Analysis. A SpectraMax M5 microplate reader (Molecular Devices Corporation) was used for all fluorescence measurements. Fluorescence intensity was measured for each sample. Vial contents were transferred to a 96 well plate for salicylic acid quantification at the end of the experiment. Average integrated fluorescence intensity was measured at an excitation wavelength of 305 ± 7.5 nm and an emission of 410 ± 7.5 nm (27). Intensity was correlated against known salicylate calibration standards, and the slope of benzoate oxidation was determined to generate the experimental rate constant for the various AHLs (*k*_{obs}). The method is selective for measuring HO because a unique product, salicylic acid, is the analyte rather than the loss of benzoate.

Quality Assurance. The bimolecular rate constants for the reaction of the hydroxyl radical with THF and 2-propanol were determined using the same experimental procedure described above as a method validation step. The results were compared with the reported rate constants for 2-propanol (1.91 ± 0.31 × 10⁹ L mol⁻¹ s⁻¹) and THF (4.0 × 10⁹ L mol⁻¹ s⁻¹) to verify that the method was in fact measuring the rates of HO reactions and not those of possible secondary radicals (36–38). The rate constants obtained from this work were 1.5 ± 0.1 × 10⁹ (L mol⁻¹ s⁻¹) for 2-propanol and 6.0 ± 0.9 × 10⁹ L mol⁻¹ s⁻¹ for THF, which agreed with the published data within the 95% confidence interval using a two-tailed Student's *t* test.

Mass Spectrometric Analysis. AHLs and their oxidation products were resolved using HPLC-MS techniques (39). Full scan spectra were initially acquired to obtain retention times of target fragments. Fragment peaks were manually collected based on retention time. Fractions were flash frozen with liquid nitrogen and stored at –80 °C. Frozen samples were concentrated by centrifugation/evaporation using a vacuum centrifuge from ThermoSavant (SPD121P).

TABLE 1. Measured Bimolecular Rate Constants for the Reaction of AHLs with Hydroxyl Radical^a

substrates	average <i>k</i> /10 ⁹ M ⁻¹ s ⁻¹	std error
AHL-C4	2.4	0.1
AHL-C6	7.2	0.3
AHL-C7	7.7	1.6
AHL-C8	8.2	1.4
AHL-C10	9.4	2.1
3-oxo-AHL-C6	0.2	0.5
3-oxo-AHL-C8	2.9	0.9
2-propanol	1.5	0.1
THF	6.0	0.9

^a Based on eq 2. Measured rates for 2-propanol and THF oxidation are included for quality assurance (21, 37, 59).

HPLC Product Separation. Analysis of C6-AHL and its corresponding oxidation products was conducted using high pressure liquid chromatography (HPLC; Agilent 1100 series) with an acetonitrile/water mobile phase (0.1% (v/v) formic acid_(aq) and 0.1% formic acid in acetonitrile) on an AquaSep reverse phase column (150 × 2.1 mm, 5 μm; ES Industries). The mass of the products was determined using a Micromass Quattro triple-quadrupole tandem mass spectrometer that was coupled to the HPLC system (Waters, Milford, MA, USA) with an electrospray ionization source in positive ion mode. After 2 min of isocratic flow at 5% acetonitrile the ratio was increased over 18 min at a constant rate to 60% acetonitrile. The column was then returned to the initial condition and allowed to equilibrate for 5 min prior to the analysis of the next sample. Full scan spectra were initially acquired to obtain retention times of the products. Product peaks were manually collected based on retention time. Product assignment was based on previously reported work (39). Fractions were flash frozen with liquid nitrogen and stored at –80 °C. Mass spectra of all analytes are reported in the Supporting Information (Figures S1–S3). No optical characterization or detection of the products was attempted.

Luminescence Assay. The signaling activity of the acyl-homoserine lactones and their individual oxidation products were tested using the *V. harveyi* luminescence bioassay. *V. harveyi* BB120 is commonly used for detection of AHLs and is sensitive to the structures of AHLs used in this study (40). The indicator strain was cultured in aerated Luria–Bertani Media (21) broth (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) at 28 °C for 16–18 h and then diluted 100 fold in fresh broth (41). The resulting culture was distributed into wells of a Costar 96-well black microplate (Corning, USA). All HPLC fractions were brought to dryness, their masses determined, then reconstituted in 500 μL of deionized water. Triplicates of test fractions (10 μL) and deionized water (10 μL) were added to the assigned wells. The assay plate was incubated at 28 °C for 5 h, then the luminescence intensity of each well was read using a Veritas Microplate Luminometer (Turner Biosystems, USA) in accordance with established screening methods for AHLs and analogues (9, 42–44). Relative Luminescence Units (RLU) were calculated by dividing the luminescence intensity by the optical density measured at 600 nm using a Power Wave 200 Microplate Scanning Spectrophotometer (Bio-Tek Instruments, USA) (26, 45).

Results and Discussion

Rates. All AHLs in the study oxidized upon irradiation in the presence of nitrate. Oxidation was first order in AHL for all cases. The bimolecular rate constants for AHL oxidation by HO (*k*_{AHL}) were determined by comparison of the experimentally obtained oxidation rate (*k*_o) obtained in the presence of varying amounts of a known HO scavenger, benzoic acid

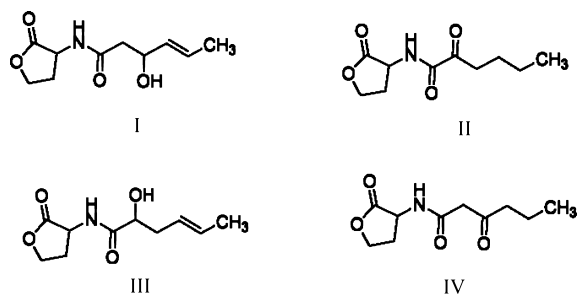


FIGURE 2. Nominal structures of C6 AHL oxidation products (I–IV) determined by LCMS in this study. The position of the carbon–carbon double bond on III is indeterminate with this technique (C3–C4 vs C4–C5).

(Table 1) (13, 46). The fraction of HO that reacted with benzoic acid (Fr_{OH-BA}) was estimated (1)

$$Fr_{OH-BA} = \frac{k_{BA}[OH][BA]}{k_{BA}[OH][BA] + k_{AHL}[OH][AHL]} \quad (1)$$

where k_{BA} ($5.9 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) was the bimolecular rate constant for benzoate by HO (21, 28). Rearranging eq 1 and accounting for $[HO]_{ss}$ yielded an expression for obtaining the bimolecular rate constant for the reaction of hydroxyl radicals with a given AHL (k_{AHL}) (2)

$$k_{AHL} = \frac{k_{BA} \times [BA] \times [1 - (k_x/k_0)]}{(k_x/k_0) \times [AHL]} \quad (2)$$

where k_0 was the experimentally determined rate of benzoate oxidation in the absence of AHL, and k_x was the experimentally determined rate of benzoate oxidation in the presence of AHL. All rates were obtained using the method of initial rates to avoid interference from oxidation products (i.e., less than 20% of the initial AHL was oxidized). The AHLs reacted with HO with bimolecular rate constants of $10^9 \text{ M}^{-1} \text{ s}^{-1}$ or greater, in agreement with the order of magnitude expected for most organic molecules that are not perhalogenated (Supporting Information, Figure S4). The two 3-oxo AHLs (C6 and C8) reacted more slowly than the corresponding

saturated analogues, an observation consistent with the bimolecular rates for the oxidation of other alkane/ketone pairs such as n-butane ($2.9 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) (47) and butanone ($6.6 \times 10^8 \text{ L mol}^{-1} \text{ s}^{-1}$) (48) or pentane ($5.4 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) (49) and 2-pentanone ($1.9 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) (50) or 3-pentanone ($1.4 \times 10^9 \text{ L mol}^{-1} \text{ s}^{-1}$) (50).

AHL Oxidation Products and Bioassays. The oxidation of C6-AHL and corresponding product evolution was followed by HPLC-MS operating in full scan mode (Figures 2 and 3). Products were identified according to their fragmentation patterns and high resolution mass spectra (39). Product detection and quantification was limited by the operational window of the HPLC-MS, which had poor sensitivity for products with a molecular weight <150 Da. Product yield peaked at approximately 15 h of illumination (Σ product yield of ~20% on a per mole AHL consumed basis) with a concurrent mass balance of approximately 40%.

The initial oxidation of the AHL could have occurred by HO attack at several different locations on the molecule; however, secondary alkyl radical centers are known to migrate to positions β or γ to carbonyls (51). Products isolated from AHL oxidation were consistent with this pattern of rearrangement, followed by peroxidation to generate the corresponding structures I–IV, with the highest yield in this study obtained for I (Figure 3) (51, 52). There is uncertainty in the placement of the double bond generated during rearrangement or fragmentation of the initial radical (I and III); however, the solution pH would not have supported the development of stable enols under our conditions. Given the high concentration of AHL still left in the solution at the time of sample isolation ($t = 15 \text{ h}$, Figure 3) it is unlikely any of the detected products were the result of successive oxidation steps.

This result is significant because many of the *lux*-based response systems (*V. harveyi*, *P. aeruginosa*, *V. cholera*, etc.) are broadly responsive to AHLs and their structural analogues (53). All of them are sensitive to structural variations within a particular class; i.e. those resulting from partial oxidation of the alkyl side chain, and some of the synthesized AHL analogues elicit stronger responses from the assays than the naturally occurring structures (54–57).

The signaling activity coefficient of the isolated AHL degradates (collected at $t = 15 \text{ h}$ to maximize yield) was

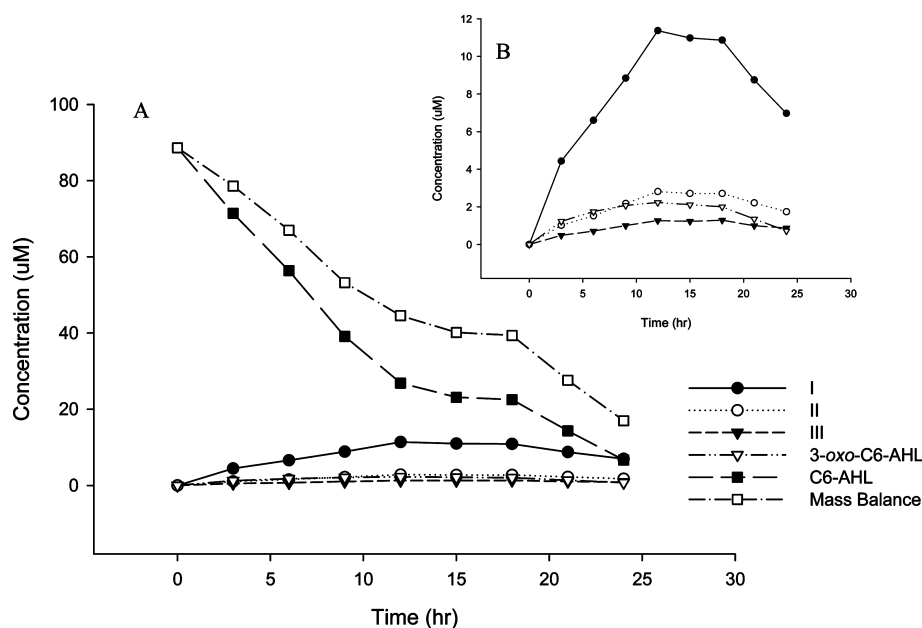


FIGURE 3. The oxidation of C6-AHL and formation of corresponding oxidation products (I–IV) with mass balance during protracted oxidation (A). An expanded view of the product profile with time is inset (B). Samples were isolated from the $t = 15 \text{ h}$ point for product isolation and signal activity assays. Experimental conditions: 12.0 mM NaNO_3 , 3.0 mM phosphate buffer pH = 5.0, light intensity of 655 W/m^2 (300 nm–800 nm), and $T = 26 \pm 2 \text{ }^\circ\text{C}$.

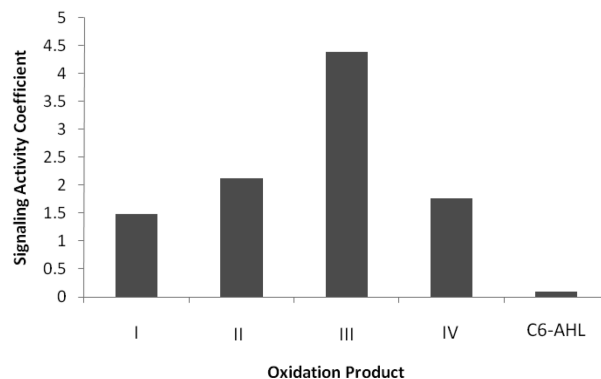
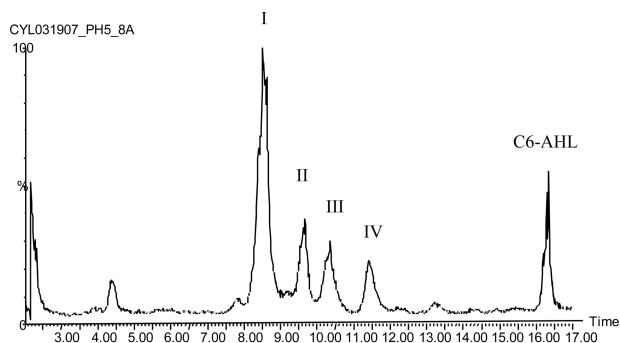


FIGURE 4. The reaction mixture was resolved using HPLC techniques (see Methods) with MS detection. Fractions were collected, dried, and analyzed for signaling activity by the *V. harveyi* bioluminescence assay. Detected products were uniformly more effective signaling agents as indexed against the starting material.

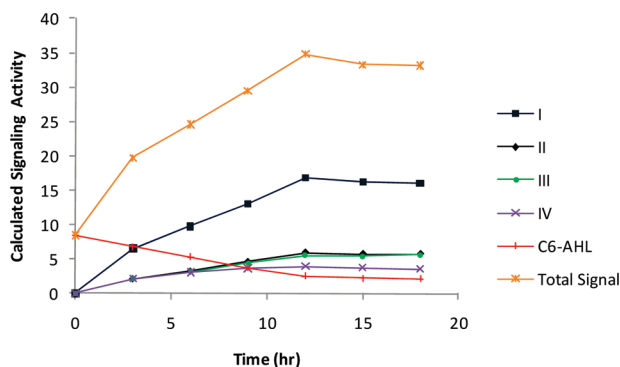


FIGURE 5. The calculated signaling activity (RLU) of C6-AHL and oxidation products (I–IV) over time based on the *V. harveyi* assay.

quantified on a per mole basis by the *V. harveyi* BB120-based signal bioluminescence assay (Figure 4) (58). Signaling activity (in relative luminescence units, RLU) was estimated for the system as a function of time based on the signaling activity coefficient. The coefficient was obtained by normalizing the RLU of the blank against that of the sample on a per mole basis. Multiplying these coefficients by the concentration of products and starting material over time allowed an estimation of the whole sample assay response over the course of the oxidation (Figure 5). Based on this estimate the aggregate assay response increased by roughly a factor of 4, even though approximately 70% of the starting material was consumed. It is important to note that “aggregate” is used in the additive sense here and does not exclude the possibility that the starting mixture could have elicited a synergistic response. Although it is reasonable to speculate that the harsh conditions corresponding to this much oxidation would partially depopulate the biofilm, these data indicate the remaining microbes could exist in a signal rich environment even if their population fell below the

threshold value for signal induction (7). It should be noted that currently there is no standardized method accepted by the community for reporting the response of this assay on a per-mole basis, nor is the quantitative structure–activity relationship (QSAR) for the AHLs and this assay fully mapped. Similarly, other bioassays for AHLs exist that presumably have different QSAR functions. The observation that the oxidation of AHLs in microbial communities may yield products that differentially affect the community relative to the parent signal is nonetheless significant.

Acknowledgments

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Supporting Information Available

Data sets describing the rate of salicylate formation as a function of added AHL. The bimolecular rate constant for the reaction between various AHLs and HO was obtained from these. The LC-MS/MS data used to quantify C6-AHL and its oxidation products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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