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Technical Note

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MALDI-MS-Based Quantitative Analysis for Ketone Containing Homoserine Lactones in *Pseudomonas aeruginosa*

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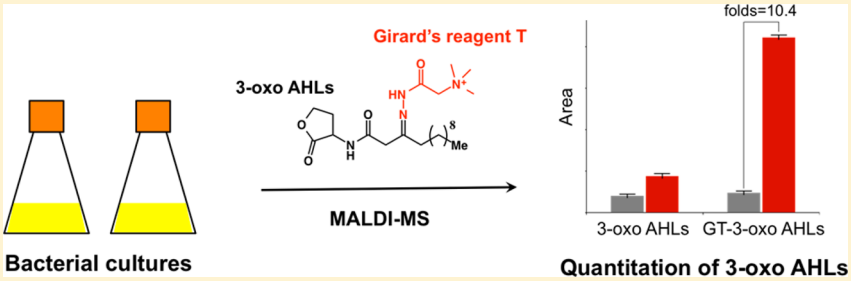
Supporting Information



ABSTRACT: *N*-Acyl homoserine lactones (AHLs), quorum sensing molecules produced by Gram-negative bacteria, are used as important secondary metabolites for antibacterial drug development and cell-to-cell communication. Although various analytical techniques have been developed for detection and quantitation of AHLs from more complex bacterial culture media, only a few methods have been applied to AHL identification in physiological samples. Here, we developed a highly sensitive and reliable MALDI-based 3-oxo AHL quantitation method by employing Girard's reagent T (GT) to produce a permanent cationic charge state $[M]^+$ at the ketone group of AHLs. After extracting AHLs from the supernatant of bacterial cultures using ethyl acetate, the extracts were subsequently derivatized with GT without any additional purification or desalting steps. The chemical derivatization of 3-oxo AHLs dramatically enhanced sensitivity (up to 60 000 times) by lowering the limit of detection (LOD, ~0.5 fmol)/limit of quantitation (LOQ, ~2.5 fmol). Additionally, the GT-derivatized 3-oxo AHLs allowed more accurate quantitative analysis from the *Pseudomonas aeruginosa* PAO1 culture supernatants. This method may be applied for developing high-throughput and sensitive detection methods of quorum sensing signal molecules in biofilm-related clinical applications such as virulence factor characterization and antibacterial drug development.

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Publication Date (Web): January 8, 2015 | doi: 10.1021/ac5039362

Quorum sensing molecules (QSMs), which are involved in bacterial communication, are utilized as representative extracellular signaling molecules to regulate various biological functions such as virulence factor expression, biofilm formation, and cell population density monitoring.[1−6] For example, bacteria use the small molecules to coordinate gene expression in response to their own population density. As the population of bacteria reaches a "quorum", the concentration of signal molecules increases to a threshold level and QSMs trigger the expression of various types of phenotype genes.[1,5,7,8] Gram-positive bacteria that participate in quorum sensing typically use oligopeptides that are actively secreted from cells, whereas Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as signal molecules in bacterial communication processes.[9−11] Although the fundamental purpose of QSMs in Gram-positive and Gram-negative bacteria is identical, the signal molecules, signaling receptors, and signaling mechanisms utilized by Gram-negative bacteria are different from those of Gram-positive bacteria.[9]

AHLs in Gram-negative bacteria are composed of a conserved aromatic ring and amide (N)-linked acyl side chains located between C_8 and C_{14}.[10] The activation of specific quorum sensing transcriptional regulators and the resulting precise regulation of gene expression rely on their corresponding AHLs.[12] *Pseudomonas aeruginosa* (*P. aeruginosa*), one of the Gram-negative bacteria, produces *N*-3-oxododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl homoserine lactone (C_4-HSL) as two major QSMs.[13] These QSMs interact with transcription regulators in the bacteria and express various phenotypes that can modulate bacterial behaviors.[14,15] For example, 3-oxo-C_{12}-HSL forms a complex with lasR to activate virulence factors such as elastase, alkaline protease, and exotoxin A,[16,17] whereas C_4-HSL binds to rhlR to express

Received: October 21, 2014
Accepted: December 19, 2014
Published: December 19, 2014

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DOI: 10.1021/ac5039362
Anal. Chem. 2015, 87, 858−863

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pyocyanin, cyanide, and lipase.[17,18] Consequently, QSMs have been the main targets in antibacterial drug development[19] and drug resistance mechanism studies.[20] To monitor the biological activity of bacteria and the progress of disease, however, highly sensitive and quantitative QSM analysis tools are required.

Conventional methods for quantitative analysis of AHLs have relied on thin-layer chromatography (TLC) to separate a mixture of AHLs.[21−23] However, the AHLs are usually produced at very low concentrations. For this reason, it is very hard to precisely quantify them using TLC. In addition, TLC cannot distinguish between QSMs that have the same chain lengths but different moieties, like 3-oxo and 3-hydroxy analogs which have similar mobility.[22] AHLs are challenging compounds to detect by liquid chromatography (LC) using UV detectors because they have absorbance maxima at low wavelengths, increasing the difficulty of detection when coupled with the high background absorbance of conventional solvents.[23] To overcome the problems related to detecting AHLs, LC in conjunction with mass spectrometry (LC-MS/MS) has been used for qualitative and quantitative analysis for subnanomole quantities of AHLs.[24−26] Although gas chromatography−mass spectrometry (GC/MS) allows simultaneous identification and highly sensitive quantification of AHLs,[15] the obvious disadvantage of the method is that it will not quantitatively detect heat-labile AHLs.[25,27] Recently, the MALDI-MS imaging (MALDI MSI) approach has been applied to obtain a chemical map showing the distribution of QSMs such as rhamnolipids and quinolones in bacterial biofilm. This method enabled one to detect and visualize the variety of bioactive secondary metabolites in an *in situ* manner, but it still was inevitable that the complicated mix of proton or sodium adducts of the QSMs was generated in the MALDI MSI analysis, which may interfere with the precise quantitative QSM analysis.[28,29]

In this study, we present the development of a robust MALDI-MS-based method for quantitative analysis of AHLs. MALDI-MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) is extremely sensitive, fast, and suitable for the analysis of complex mixtures without prior chromatographic purification. However, it is rarely employed in quantitative analysis due to the complicated mix of protonated $[M + H]^+$ and sodium $[M + Na]^+$ that originate from the salts in the sample. To simplify the multiple adduct ions, we introduced a permanent cationic charge by using Girard's reagent T (GT) on ketone-containing AHLs (i.e., 3-oxo-C_{12}-HSL and 3-oxo-C_{14}-HSL). The chemical derivatization dramatically increased the signal-to-noise ratio, reproducibility, and linearity for quantitative work compared with their underivatized counterparts.[30−33] Moreover, the cultivation supernatant of *P. aeruginosa* PAO1 was used directly for quantification of 3-oxo-C_{12}-HSL. Therefore, this novel method will provide more precise quantitative information on AHLs to monitor bacterial behaviors such as biofilm formation, virulence, and antibiotic resistance.

MATERIALS AND METHODS

Chemicals. *N*-(3-Oxododecanoyl)-L-homoserine lactone, Girard's reagent T, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich (MO, USA). *N*-(3-Oxotetracanoyl)-L-homoserine lactone was purchased from Cayman chemical company (MI, USA). *Pseudomonas aeruginosa* PAO1 (1637) was obtained from Korean Collection for Type Culture (Daejeon, South Korea) and nutrient broth was obtained from BD Difco (MD, USA). All HPLC grade solvents such as methanol, water, and ethyl acetate were purchased from Fisher Scientific (PA, USA), and analytical solvents were purchased from Junsei (Tokyo, Japan).

Girard's Reagent T Derivatization. The 3-oxo-C_{12}-HSL and C_4-HSL were, respectively, dissolved with 70% methanol/30% water [v/v]. Ten μL of authentic homoserine lactone solution was mixed with an equivalent volume of Girard's reagent T (10 mg/mL in 1% acetic acid/99% methanol [v/v]). The mixture was incubated at room temperature for 2 h. After the reaction, the mixture was prepared for MALDI-MS analysis without any purification steps.

MADLI-TOF MS Analysis. One μL of the GT-derivatized samples (20 μL) was mixed with 1 μL of α-CHCA solution (10 mg/mL in 70% acetonitrile/30% water [v/v]). One μL of the mixture was deposited on a stainless-steel MALDI plate and allowed to dry at room temperature. Microflex MALDI-TOF MS with a 337 nm nitrogen laser from Bruker Daltonics (Bruker, Bremen, Germany) was used to obtain mass spectra. The analysis parameters were as follows: positive ion and reflectron mode, detector gain = 3.9, and laser power = 25%. A total of 1200 shots from 6 different spots were scanned to obtain a mass spectrum. The area from the first to the third isotopic peak of each ion was integrated for quantification.

Structural Analysis and Quantification of HSL by LC-MS. To identify the chemical structure of GT-derivatized 3-oxo AHLs, tandem mass spectrometry analysis was performed using a LCQ Deca XP ion trap mass spectrometer (Thermo Electron, San Jose, CA) in positive-ion mode at the spray voltage of 5 kV and the nitrogen sheath gas at flow rate of 20 arbitrary units. The heated capillary was maintained at 200 °C. Three μL of sample was injected and carried into the spray needle by a capillary LC system (Agilent, Santa Clara, CA) at a flow rate of 3 μL/min. For the structural analysis, MS/MS and MS^n analysis of GT-derivatized 3-oxo AHLs was performed manually, inducing collision energy from 15 to 37 eV. The maximum ion collection time was set to 50 ms, and 3 Da of isolation width was used. All LC/MS data were visualized by Xcalibur software (Thermo Electron, San Jose, CA).

To quantify the homoserine lactones, 20 μL of HSL was applied for analysis using UPLC/triple quadrupole mass spectrometry (UPLC/QQQ-MS). Liquid chromatography was performed on the Accela 1250 UPLC system (Thermo-Fisher scientific, USA) with Thermo Fisher Hypersil Gold C_{18} column (2.1 × 100 mm, 1.8 μm, ThermoFisher scientific, USA). The temperatures of the column oven and autosampler were maintained at 30 and 10 °C, respectively. The mobile phases were (A) HPLC grade water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The linear gradient program was initiated at 10% (B) for 10 min and was increased to 90% (B) over 15 min before being returned to the initial conditions at 10% (B), which were maintained for 5 min. The total analysis time was 20 min with a flow rate of 200 μL/min and an injection volume of 10 μL using partial-loop mode. Mass analysis and quantification of AHLs were performed using a TSQ quantum Access Max QQQ mass spectrometer (ThermoFisher scientific, USA). Ionization was performed in the positive and negative switching and heated-electrospray (HESI) mode. Scan time for SRM transition (dwell time) was 5 ms; each discharge current value was 4 μA between positive and negative polarity. The conditions used for the ESI source were as follows: capillary voltage, 4.0 kV; vaporizer temperature, 40 °C; source temperature, 270 °C; and desolvation temperature,

DOI: 10.1021/ac5039362
Anal. Chem. 2015, 87, 858−863

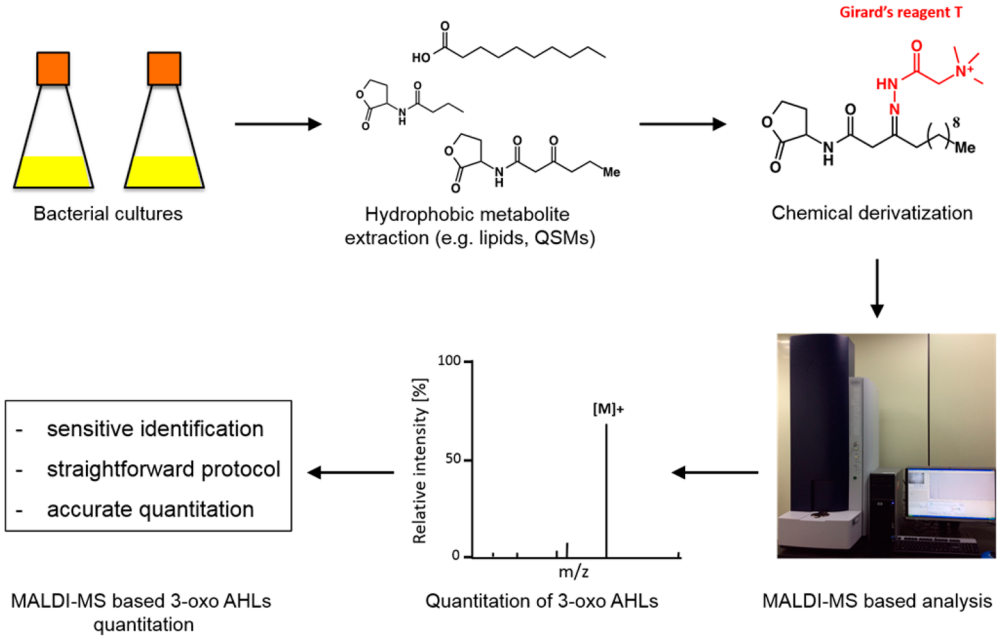

Figure 1. Overall strategy of MALDI-based 3-oxo AHLs quantitation.

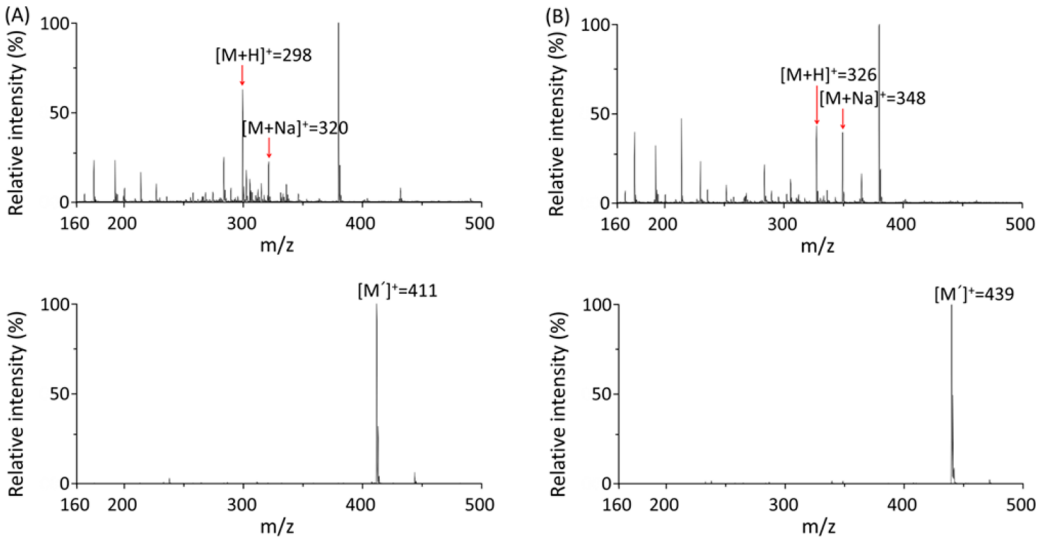

Figure 2. (A) MALDI spectra of 3-oxo-C_{12}-HSL and GT derivatized 3-oxo-C_{12}-HSL; M, 3-oxo-C_{12}-HSL; M′, GT derivatized 3-oxo-C_{12}-HSL. (B) MALDI spectra of 3-oxo-C_{14}-HSL and GT derivatized 3-oxo-C_{14}-HSL; M, 3-oxo-C_{14}-HSL; M′, GT derivatized 3-oxo-C_{14}-HSL.

350 °C. Nitrogen was used as the sheath gas with a pressure of 30 psi. ThermoFisher Xcalibur interface was used to control mass spectrometry. To evaluate the structure of the GT-derivatized 3-oxo AHL, Mass Frontier 7.0 software (Thermo-Fisher, USA) was used.

Bacterial Cultivation and Extraction of Homoserine Lactone. *Pseudomonas aeruginosa* PAO1 (Korean Collection for Type Culture 1637, South Korea) was cultured in nutrient broth (BD Difco, MD, USA) at 37 °C with agitation at 150 rpm. Six volumes of bacterial culture (30, 50, 300, 1000, 1500, and 3000 mL) were obtained when the optical density of the solution at 600 nm (OD_{600}) reached 1.5, respectively. The cultured bacteria were centrifuged at 4500 rpm for 30 min, and then, the supernatant was carefully transferred into a new glass tube. The equal volume of ethyl acetate (EA) with 0.5% of acetic acid was mixed with the supernatant in a separating funnel. The funnel was vigorously agitated and was allowed to stand to completely separate water from the organic solvent. The upper phase EAwas collected and dried by a rotary evaporator (Buchi, Switzerland) at 37 °C rotating at 30 rpm for 30 min. Then, 100 μL of 70% methanol/30% water [v/v] solution was added to dissolve the hydrophobic extracts. Ten μL of the dissolved extracts was mixed with an equivalent volume of Girard's reagent T (10 mg/mL in 1% acetic acid/99% methanol [v/v]). The mixture was allowed to react at room temperature for 2 h. The reactant was prepared for UPLC/QQQ-MS and MALDI-MS analysis.

RESULTS AND DISCUSSION

Quantitative analysis of QSMs is very important to characterize the functions of the molecules according to their concentrations in bacterial culture and provide comprehensive information to investigate their roles in regulatory mechanisms, biological activities, and biosynthesis. However, the analysis of

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QSMs has been difficult due to low concentrations. It was also hard to quantify the target QSMs in a rich complex culture medium using conventional methods (i.e., TLC or bioassays). In this study, MALDI-MS was used as a quantitative analytical tool to identify chemically derivatized AHLs (Figure 1) for the first time. The total lipid extracts from bacterial culture medium were directly treated with Girard's reagent T and then analyzed by MALDI-MS without any further purification steps. This presents a drastic improvement in experimental time and labor compared with other conventional analytical methods. For example, a large number of assays take at least several days to manually execute the extraction, purification, and chromatographic separation for LC-MS/MS analysis. Our straightforward method facilitated simple sample preparation and rapid characterization of the target compounds.

To validate the chemical derivatization process, authentic 3-oxo-C_{12}-HSL and 3-oxo-C_{14}-HSL were individually derivatized with Girard's reagent T and then directly analyzed by MALDI-MS. All of the expected mass spectra of the derivatized homoserine lactones were detected successfully at high resolution (Figure 2). The MALDI spectra of the unlabeled 3-oxo-C_{12}-HSL shows both the $[M + H]^+$ ions at 298 m/z and the $[M + Na]^+$ ions at 320 m/z, whereas the $[M]^+$ ion was observed only at 411 m/z, corresponding to the derivatization product of 3-oxo-C_{12}-HSL by Girard's reagent T. Furthermore, GT-derivatized 3-oxo-C_{14}-HSL was observed only at 439 m/z as the $[M]^+$ ion. As expected, no proton, sodium, or potassium adducts were detected after introducing the single cationic charge on both AHLs. The quality of the AHL mass spectra in MALDI-MS was also dramatically improved, although we did not perform additional purification.

To evaluate linearity and reproducibility of the GT derivatization method, seven concentrations (from 0.5 to 400 pmol) of 3-oxo-C_{12}-HSL were tested. As shown in Figure S1, Supporting Information, the standard calibration curve presents good linearity according to the amount of 3-oxo-C_{12}-HSL. The R^2 value from three repeated samples was greater than 0.99. The linearity and sensitivity were quite comparable to LC-MS/MS (Figure S2, Supporting Information), which means the utilization of MALDI-MS allows us to quantify AHLs at levels similar to those quantified by LC-MS/MS analysis. Moreover, we could assess the limit of detection (LOD) and limit of quantitation (LOQ) for the positive-mode MALDI assay of GT-derivatized 3-oxo-C_{12}-HSL. This chemical derivatization approach remarkably improved the detection limit of underivatized 3-oxo-C_{12}-HSL 6×10^4-fold, from 30 pmol to 0.5 fmol. In addition, a reliable quantitation of the GT-derivatized 3-oxo-C_{12}-HSL was attainable from 2.5 fmol on the MALDI plate (Table 1 and Figure S4, Supporting Information). Therefore,

Table 1. LOD and LOQ Analysis of Natural and GT Derivatized 3-oxo-C12-HSL

HSL	LOD	LOQ
3-oxo-C_{12}-HSL	30 pmol	40 pmol
GT derivatized 3-oxo-C_{12}-HSL	0.5 fmol	2.5 fmol

these results suggest that the GT derivatization method significantly improves sensitivity and provides reliability for qualitative and quantitative analysis of AHLs using MALDI-MS.

To characterize the structure of the parent $[M]^+$ ion at m/z 411.5, which corresponds to the GT-derivatized 3-oxo-C_{12}-HSL, tandem mass spectrometry analysis was performed (Figure 3). In the MS/MS analysis, abundant fragment ions were detected at m/z 100.3, 209.1, 310.4, and 352.0 that were mainly generated by cleavage of the amide bonds. The abundant ions at 310.4 corresponded to elimination of the amino butyrolactone moiety and were selected for the MS^3 study to identify the GT-labeling position. As shown in Figure 3, the abundant fragment ions at m/z 223.2, 251.1, 268.2, and 282.3 arise from the m/z 310.4 parent ion. Among the fragment ions, m/z 223.2 and 268.2 were characteristic ions that demonstrate the coupling of GT onto the ketone group of 3-oxo-C_{12}-HSL. These results describe the chemical structure of GT-derivatized 3-oxo-C_{12}-HSL, and therefore, other 3-oxo AHLs from different biological sources may be able to be derivatized with GT in the same manner.

Once the approach was developed, we validated the method to detect and quantify 3-oxo-C_{12}-HSL from the culture supernatant of *P.aeruginosa* PAO1. All hydrophobic molecules in the cell culture supernatant were extracted by adding an equal volume of ethyl acetate. The dried extracts were directly used to detect natural 3-oxo-C_{12}-HSL and GT-derivatized 3-oxo-C_{12}-HSL using MALDI-MS without any additional purification steps. For example, the MALDI spectrum obtained from 50 mL of culture media shows that the $[M + H]^+$ ion at m/z 298 corresponding to natural 3-oxo-C_{12}-HSL was detected with very low intensity and even the $[M + Na]^+$ ion was identified. LC-MS/MS analysis was performed to characterize AHL from the bacterial culture media (Data not shown). GT-derivatized 3-oxo-C_{12}-HSL was clearly identified at m/z 411 as only a $[M]^+$ ion in the MALDI spectrum.

As we expected, natural C_4-HSL was also detected in both MALDI spectra (Figure 4A), but the underivatized AHL displayed similar peak intensity as the natural form. As for the sensitivity, we were able to quantify GT-derivatized 3-oxo-C_{12}-HSL from ~1 mL of cell culture medium. These results demonstrate that we could identify AHLs from cell culture medium and that chemical derivatization heightens the peak intensity in MALDI-MS analysis. In addition, to evaluate the quantitative relevancy of GT derivatization for AHLs, we compared the relative quantities of AHLs extracted from 30 and 300 mL of *P. aeruginosa* PAO1 culture media. Interestingly, the GT derivatization method produced significantly better AHL analysis quantitative results depending on the volume ratio. After GT derivatization of the AHL, peak areas of 3-oxo-C_{12}-HSL from 300 mL of culture media were ~10-fold larger than those from 30 mL (Figure 4B and Figure S3, Supporting Information). When we calculate the amount of 3-oxo-C_{12}-HSL from the standard calibration curve, it was 0.28 (from 300 mL culture media) and 0.027 nmol (from 30 mL culture media), respectively. Singh et al. demonstrated that *P. aeruginosa* uses secretory and extracellular quorum sensing molecules to coordinate biofilm formation according to the ratios of the acyl homoserine lactones (C_4-HSL and 3-oxo-C_{12}-HSL).[34] Moreover, they showed that the reliable and sensitive profiling method for quorum sensing molecules from specific bacterial strains might provide a biomarker in screens to identify potential drug candidates that interrupt biofilm generation. Taken together, the MALDI-based 3-oxo AHL quantitation method by employing GT could provide more precise quantitative data and will be an attractive avenue for highly sensitive quantitation of AHLs in biofilm-related clinical applications such as virulence factor characterization and antimicrobial drug development.

DOI: 10.1021/ac5039362
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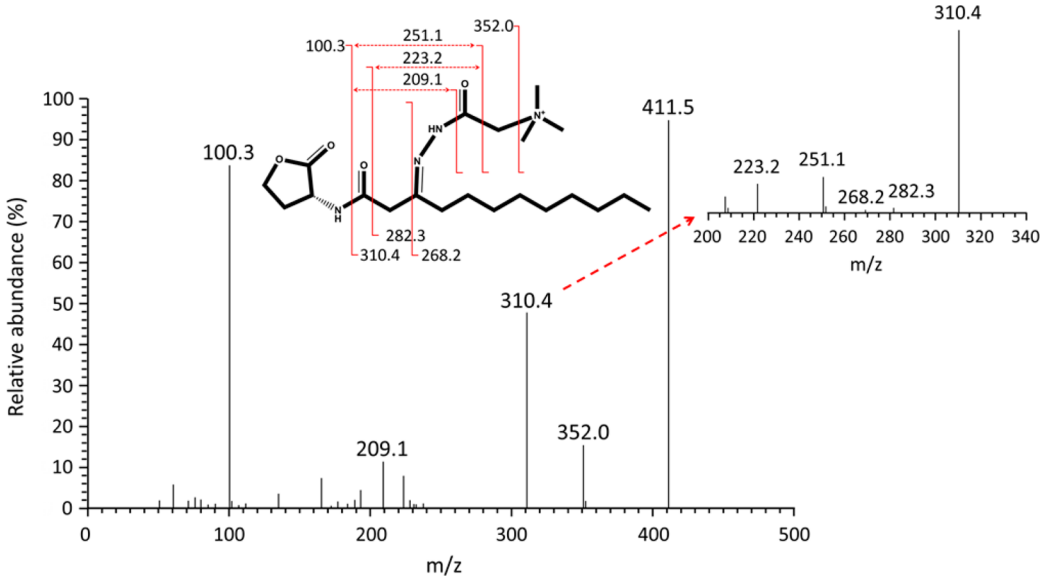

Figure 3. Tandem mass analysis of GT derivatized 3-oxo-C_{12}-HSL using QQQ LC-MS.

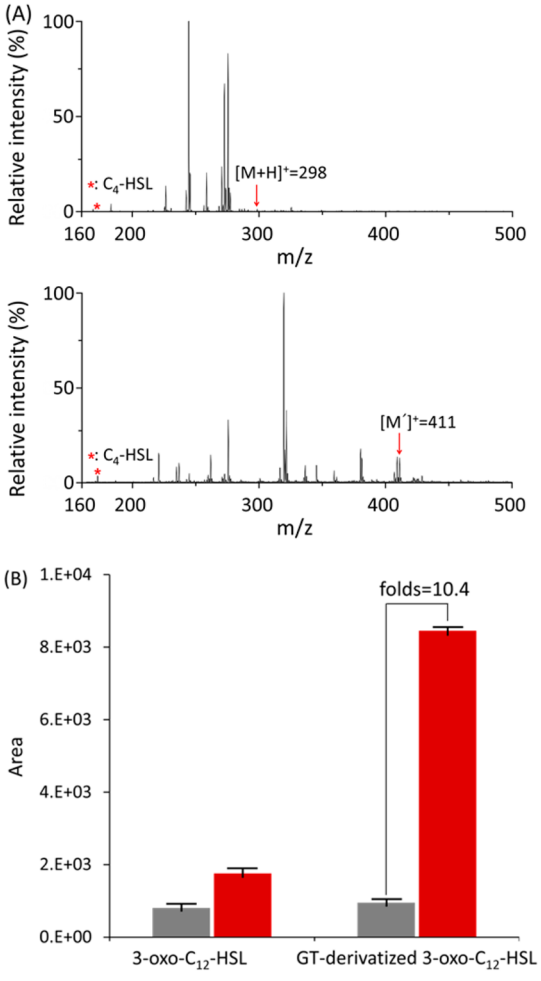

Figure 4. (A) MALDI spectra of 3-oxo-C_{12}-HSL (natural and GT-derivatized) from *Pseudomonas aureginosa* PA01 (50 mL cultured); *, C_4-HSL; M, 3-oxo-C_{12}-HSL; M′, GT derivatized 3-oxo-C_{12}-HSL; (B) quantitative analysis of 3-oxo-C_{12}-HSL from *Pseudomonas aureginosa* PA01 (gray bar: 30 mL; red bar: 300 mL cultured) using MALDI-TOF MS. The error bars represent standard deviation of triplicate samples.

CONCLUSION

In conclusion, a highly sensitive and reliable MALDI-based quantitation method for bacterial quorum sensing molecules was developed by applying a permanent singly positive charge to the AHLs. The one-step chemical derivatization, which generates a hydrazone formation between hydrazide (GT) and ketone (AHLs), enabled us to sensitively detect and reliably quantify quorum sensing molecules. In this study, we could directly quantify GT-derivatized 3-oxo-C_{12}-HSL from the more complex whole cell media without any additional chromatographic separation. The robust analytical process provides a distinct improvement in time and labor for QSMs analyses. The main drawback is that this method is only applicable to ketone-containing AHLs, but the MALDI-based quantitation method may be suitable for targeted 3-oxo AHLs high-throughput analyses. This approach will offer new insights into metabolism of quorum sensing signals in bacterial biofilm formation and regulation of pathogenic virulence factor production and antimicrobial resistance. Ongoing work is focused on investigating the role of AHLs in *P. aeruginosa* biofilm formation using the MALDI-based 3-oxo AHL quantitation method. In addition, future studies will elucidate the correlation between clinically relevant antibiotics dosages and bacterial QSMs during biofilm formation with a new high-throughput analytical format. Therefore, this analytical technology will provide a valuable approach to detecting and quantifying cellular metabolites in many areas of biological and clinical analyses.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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DOI: 10.1021/ac5039362
Anal. Chem. 2015, 87, 858−863

Author Contributions

[‖]Y.-W.K. and C.S. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) (NRF-2013R1A1A1004998) and Polar Academic Program (PAP, PD14010) from KOPRI.

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Publication Date (Web): January 8, 2015 | doi: 10.1021/ac5039362

DOI: 10.1021/ac5039362
Anal. Chem. 2015, 87, 858−863