RESEARCH ARTICLE

Identification and Quantification of *N*-Acyl Homoserine Lactones Involved in Bacterial Communication by Small-Scale Synthesis of Internal Standards and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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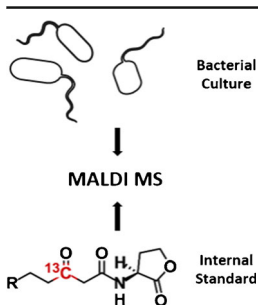
Abstract. *N*-acyl homoserine lactones (AHL) are small signal molecules involved in the quorum sensing of many gram-negative bacteria, and play an important role in biofilm formation and pathogenesis. Present analytical methods for identification and quantification of AHL require time-consuming sample preparation steps and are hampered by the lack of appropriate standards. By aiming at a fast and straightforward method for AHL analytics, we investigated the applicability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Suitable MALDI matrices, including crystalline and ionic liquid matrices, were tested and the fragmentation of different AHL in collision-induced dissociation MS/MS was studied, providing information about characteristic marker fragments ions. Employing small-scale synthesis protocols, we established a versatile and cost-efficient procedure for fast generation of isotope-labeled AHL standards, which can be used without extensive purification and yielded accurate standard curves. Quantitative analysis was possible in the low pico-molar range, with lower limits of quantification reaching from 1 to 5 pmol for different AHL. The developed methodology was successfully applied in a quantitative MALDI MS analysis of low-volume culture supernatants of *Pseudomonas aeruginosa*.

Keywords: Ionic liquid matrices, Quantification, Low molecular weight, Quorum sensing, Quantitative MALDI, Internal standard, Isotope labeling

Introduction

Quorum sensing (QS) is a mechanism of intra- and interspecies bacterial communication based on the production, release, and detection of small signaling molecules [1, 2]. First discovered in the marine bacterium *Vibrio fischeri* [3], QS is by now known as a bacterium's ability to receive a 'quorum' by the surrounding bacterial cells for the expression of a certain phenotype.

N-acyl homoserine lactones (AHL) are a widely distributed class of quorum sensing molecules in gram-negative bacteria, which have been linked to bacterial biofilm formation, adhesion, motility, and virulence [4]. AHL consist of a homoserine lactone moiety (HSL) bound to a saturated *N*-acyl chain of variable composition via a carboxamide link. Acyl chain lengths from 4 to 18 carbon atoms and substitutions at the third carbon position were observed. Accordingly, AHL can generally be grouped in unsubstituted AHL, 3-oxo-AHL, or 3-hydroxy-AHL, containing either a carbonyl or hydroxyl moiety at C3. Notably, a few cases of AHL with unsaturated acyl chains have been reported to occur in nature as well [5].

A precise and reliable measurement of AHL concentrations in biological samples is of great interest [6]. Various methods

have been developed for the extraction of AHL from biological samples, their purification, and qualitative as well as quantitative analysis. Quantitative approaches include thin-layer chromatography (TLC) combined with a bacterial biosensor strain, stand-alone high-performance liquid chromatography (HPLC), and HPLC coupled to mass spectrometry (MS) [7]. However, achievable resolution of AHL that have very similar structures is low with TLC, bearing the possibility of false identifications [7, 8]. As AHL absorb UV light at low wavelengths near vacuum UV, HPLC methods employing UV detection encounter limitations in terms of background absorbance by the mobile phases of acetonitrile/water or of methanol/water that have been used in the majority of studies [6, 7, 9]. Therefore, liquid chromatography (LC) analysis is often coupled to MS detection and structural identification by tandem MS experiments (MS/MS). MS techniques in general allow identification and structural characterization based on m/z value ratios and specific fragmentation patterns [10–12].

Matrix-assisted laser desorption/ionization (MALDI) MS is a powerful tool for the analysis of low molecular mass (LMM) biological compounds [13, 14]. The main advantage of this method is the possibility of acquiring spectral data from semi-purified samples in a high-throughput manner [15]. The use of isotope-labeled analyte analogues as internal standards allows obtaining quantitative results [16]. To our knowledge, MALDI-TOF MS has been barely applied to the analysis of AHL. Recently, derivatization of 3-oxo-AHL by Girard's reagent T, introducing a permanent cationic charge, was reported for MALDI-TOF MS analysis [8]. This led to background suppression and increased sensitivity, but this technique remains restricted to the analysis of 3-oxo-AHL.

MALDI-TOF MS of LMM-compounds faces limitations to be considered [13, 17]. Most importantly, AHL lie within the mass range of commonly used MALDI matrices. Accordingly, matrix and analyte ion signals can potentially interfere, rendering the identification of unknown analytes difficult. Furthermore, a detector saturation in the low mass range of LMM compounds can be observed frequently [13]. One approach to overcome the drawbacks of classic MALDI matrices is the use of ionic liquid matrices (ILM), which are generated from a conventional acidic matrix supplied with an equimolar amount of an organic counter base [18, 19].

Accurate MS-based quantification of AHL can be facilitated by the use of internal standards (IS). Previous studies utilized only a single internal standard to assess quantities of structurally different AHL [10, 12]. However, to account for different behavior of AHL derivatives during extraction, purification, and ionization, internal standards for each individual compound are required. Syntheses of AHL using preparative chemistry and a solid-phase procedure for C3-unsubstituted AHL were described previously [12, 20–22]; more recently the synthesis of deuterated building blocks for the generation of AHL standards was reported [23].

In this work, we present protocols for a fast and straightforward synthesis of all three structural subtypes of AHL in a small-scale setup requiring only standard laboratory equipment and readily available chemicals. The protocols can be easily implemented in analytical laboratories and yield AHL standards sufficient for MS-based analysis and quantification. MS and MS/MS analysis of AHL variants in MALDI MS were studied employing both crystalline and ionic liquid matrices. Parameters for the performance of quantitative measurements were elucidated, highlighting the potential of MALDI MS in the analysis of natural quorum sensing mediated by AHL.

Materials and Methods

Chemicals

Methylene chloride (CH_2Cl_2) was purchased from Carl Roth (Karlsruhe, Germany). All other organic solvents, polymer-bound N-benzyl-N-cyclohexylcarbodiimide (P-BCD), N,N'-dicyclohexylcarbodiimide (DCC), unlabeled carboxylic acids, ($1-^{13}C$)-decanoic acid, ($1-^{13}C$)-octanoic acid, Meldrum's acid, N,N-dimethylpyridin-4-amine (DMAP), (S)-(−)-α-amino-γ-butyrolactone hydrobromide (L-HSL), sodium cyanoborohydride, triethylamine (TEA), 2-aminopentane (AP), trifluoroacetic acid (TFA), and α-cyano-4-hydroxycinnamic acid (CHCA) were from Sigma-Aldrich (Munich, Germany). Sep-Pak Vac RC Silica cartridges were from Waters Corporation (Eschborn, Germany), 10 K centrifugal filters were from VWR International (Darmstadt, Germany). Glass vessels rather than plastic materials were used throughout this study as far as possible.

Small-Scale Synthesis of N-Acyl-L-homoserine Lactone Standards

Previously described procedures [12, 20] were modified to allow small-scale synthesis of unsubstituted, 3-oxo- and 3-hydroxy-AHLs by application of both in-solution and solid-phase synthesis routes. Detailed information and synthesis protocols are provided in the Electronic Supplementary Material.

Cultivation of Bacteria and Extraction of AHL

AHL were extracted from bacterial culture supernatants as described previously [8]. Briefly, 500 mL of liquid broth (BD Difco antibiotic medium 3; BD GmbH, Heidelberg, Germany) was inoculated with an aliquot of an overnight culture of *Pseudomonas aeruginosa* PAO1 and incubated at 37 °C. When reaching an optical density of 1.5 at 600 nm (OD_{600}), the bacterial culture was fractionated and centrifuged (3500 × g for 30 min, RT) and different volumes of the supernatant were collected for extraction and MALDI MS analysis. Prior to extraction, supernatants were spiked with ($3-^{13}C$)-3O-C_{12}-HSL [derived from DCC-based small-scale synthesis using ($1-^{13}C$)-decanoic acid as educt] as an IS. In particular, 116 nmol of IS was added to 5 mL of *P. aeruginosa* supernatant. Spiked supernatants were extracted two times with acidified ethyl acetate (0.5% acetic acid); the ethyl acetate phases were combined and

evaporated to dryness. Extracts were redissolved in 500 µL of MeOH, and SPE on a silica cartridge was carried out as described in the Electronic Supplementary Material, but with adjusted solvent volumes: cartridge activation with 3 mL of (A), 5 mL of (B), 5 mL of (A); samples were then loaded onto the cartridge with 3 mL of (A), followed by a washing step with 5 mL of (A) and elution with 5 mL of (B). The dried SPE extracts were redissolved in 70% acetonitrile for storage.

Preparation of Ionic Liquid Matrices and AHL Samples for MALDI Mass Spectrometry

Ionic liquid matrices were prepared by mixing the respective MALDI matrix solution with either one or two molar equivalents of counter base in an equivalent volume. All matrix and base stock solutions were made with 55% acetonitrile and supplemented with TFA as indicated. The ILM *mono*-AP-CHCA was prepared by mixing equal volumes of a 32 mM (6 mg/mL) CHCA matrix solution and a 32 mM solution of 2-aminopentane (AP). The *mono*-AP-CHCA preparation was supplemented with 0.1% of TFA. The ILM *di*-AP-CHCA was obtained by mixing equal volumes of a 32 mM (6 mg/mL) CHCA matrix solution and a 64 mM solution of 2-aminopentane (AP); the TFA concentration was 0.05%.

All AHL samples (syntheses, standards, or extracts of bacterial supernatant) were evaporated to dryness and directly redissolved in either CHCA matrix solution (3 mg/mL CHCA, 55% acetonitrile, 0.1% TFA) diluted 1:2 with 70% acetonitrile or ILM solution diluted 1:2 with 70% acetonitrile. One µL of sample preparation per spot was applied to a stainless steel MALDI target plate and solvent was allowed to evaporate under ambient conditions. All samples were spotted and analyzed six times.

Standard samples for quantification with internal ^{13}C-labeled AHL standards were made as follows: a dilution series of a C_{10}-AHL reference standard (RS) mixture containing C_{10}-HSL, 3O-C_{10}-HSL and 3OH-C_{10}-HSL at equal concentrations was prepared in glass micro inserts, ranging from 50 to 1500 pmol/µL of each AHL. An equal volume of the ^{13}C-labeled C_{10}-AHL IS to be tested was added to each standard sample at a concentration of 500 pmol/µL. Concentration of the IS was determined from preceding MALDI MS measurements by a 1:1 ratio of the signal-to-noise of the isotopic ion peaks of the RS compared with the IS and calculated as described previously [15]. The spiked standard samples were evaporated to dryness and the remaining solid was redissolved in CHCA matrix solution using a volume equal to the volume of the RS added beforehand. Standard samples were diluted with matrix solution to produce further sets of dilution series. For quantification of 3O-C_{12}-HSL in *P. aeruginosa* PAO1 extracts, standard curves were prepared from dilution series of a 3O-C_{12}-HSL RS at different concentrations spiked with a (3-^{13}C)-3O-C_{12}-HSL IS at a concentration of 500 pmol/µL (IS concentration estimated by quantitative MALDI MS as described above).

MALDI-TOF/TOF MS

All measurements were performed on an AB SCIEX MALDI TOF/TOF 5800 mass spectrometer (SCIEX, Darmstadt, Germany) equipped with a Nd:YAG laser (355 nm) and operating at a pulse rate of 400 Hz. Spectra were acquired in positive ion mode accumulating 2000 shots over an *m/z* range from *m/z* 50 to 500. The instrument was calibrated using peaks of AHL reference compounds and matrix peaks.

For MS/MS analysis, protonated AHL molecules were selected as precursors. MS/MS spectra were acquired by accumulation of 2500 shots; collision energy was set to 1 kV. Fragmentation was performed with air gas at a medium collision-induced dissociation-(CID) pressure of 1.25×10^{-6} Torr. Obtained spectra were analyzed in respect of diagnostic marker ions corresponding to the AHL analyte of interest.

Throughout this study, all quantitative calculations were based on peak signal-to-noise (S/N) instead of peak area [24]. S/N values were obtained by spectra processing with the SCIEX vendor software (TOF/TOF Series Explorer software v.4.0.0), with the local noise window width (LNWW) set to 250 (*m/z*).

Standard curves were calculated by plotting the average S/N ratio of the isotopic ion peaks (RS to IS) obtained from measurement of six sample spots of each standard and the respective standard amount on-target. The S/N values of the IS were corrected for the contribution of natural isotopes in the analyte molecule. For each analyte S/N value, the S/N of the *m+1* peak fraction was calculated based on the carbon isotope distribution and subtracted from the S/N value of the IS. The theoretical isotope peak distribution was evaluated experimentally by analysis of the AHL reference standards. The contribution of the enriched IS to the *m* peak fraction was neglected. Standard deviation (SD) for each data point was calculated as SD of the isotopic ion ratios of the six replicates. Lower and upper range limits of standard curves were determined depending on the outcome of the linearity coefficient (R^2) obtained. Also, data points of the lowest or highest standards were rejected, if a relative SD above 50% was calculated. No data points within the curve were removed. For determination of analyte concentrations in bacterial supernatant extracts, the standard curve slope and intercept parameters from the equation $y = a \cdot x + b$ were applied to experimental data. The analyte concentration was calculated as $C_{Analyte} = \left(\frac{S/N_{Analyte}}{S/N_{IS}} - b \right) / a \cdot C_{IS}$

As a control, concentration was also determined by using a formula based on analyte and IS sensitivities as described before [15].

Results and Discussion

Small-Scale Synthesis of N-Acyl-Homoserine Lactones

For the identification of *N*-acyl homoserine lactones from biological samples, the knowledge of their MS and MS/MS behavior is a major prerequisite; the necessary parameters can

be studied best using standard compounds. Moreover, the availability of isotope-labeled standards is mandatory for quantification by MALDI MS. As only a limited number of AHL standards are commercially available, we established easy to perform small-scale synthesis protocols for a wide range of AHL (Figure 1, Supplementary Table S1). Synthetic procedures were developed from preparative organic chemistry procedures published earlier [20], but allowed for the needs of non-specialized analytical laboratories, and were downscaled to reduce the costs, e.g., those for isotope-labeled reactants. Utilizing considerable small reaction volumes and prevention of time-consuming washing and purification steps commonly applied in preparative chemistry make this small-scale approach feasible for parallel performance of multiple syntheses.

Details about the synthesis and information on yields and purification are given in the Electronic Supplementary Material. Briefly, the synthesis of unsubstituted AHL is based on carbodiimide activation of a fatty acid, catalyzed by DMAP, to form a carboxamide bond with the amino group of α-amino-γ-butyrolactone (Figure 1, route a; Supplementary Figure S1) [12, 25]. The reaction was performed using a polymer-bound carbodiimide allowing straightforward removal of the resin and of bound excessive reactants after completion of the synthesis reaction. Syntheses were performed with C_4-, C_6-, C_8-, C_{10}-, and C_{12}-fatty acids to produce the respective unsubstituted AHL (Supplementary Table S1). By the use of commercially available (1-^{13}C)-decanoic acid, an isotope-labeled C_{10}-HSL standard was generated successfully (Figure 2a, Supplementary Figure S4A).

For synthesis of 3-oxo-AHL in preparative scale, an in-solution route was described before [20]. It is based on the reaction of a carbodiimide activated fatty acid with Meldrum's acid forming a dimethyl dioxane dione intermediate, which is then converted with L-homoserine lactone to the final product. We modified this procedure to implement a simple small-scale synthesis achievable with both solid- and solution-phase

(DCC-based) routes. Main changes were the application of polymer-bound carbodiimide, simplification of reaction clean-up, and drastically increased incubation times to induce rearrangement of the intermediate dimethyl dioxane dione to a reactive ketene (Figure 1, route b and Supplementary Figure S2). This protocol allowed the formation of the ketene at lower temperatures without the use of harsher conditions demanding reflux heating as in the existing macroscale protocol. Optimization of the protocols allowed the generation of 3O-C_6-HSL, 3O-C_8-HSL, 3O-C_{10}-HSL, 3O-C_{12}-HSL, and 3O-C_{14}-HSL from the respective C_{n-2}-fatty acids (Supplementary Table S1). (1-^{13}C)-octanoic acid was used for the generation of a ^{13}C-labeled 3O-C_{10}-HSL standard. To quantify the major *P. aeruginosa* AHL, namely 3O-C_{12}-HSL, an isotope-labeled standard was synthesized employing (1-^{13}C)-decanoic acid, yielding (3-^{13}C)-3O-C_{12}-HSL.

Synthesis of 3-hydroxy-AHL was accomplished by reduction of the 3-keto function of a 3-oxo-AHL using sodium cyanoborohydride (Figure 1, route c and Supplementary Figure S3) [20, 26].

MALDI MS of N-Acyl-Homoserine Lactones

MALDI MS was employed as a tool for quality control and fast structural characterization of AHL synthesis products. Since the basic reactions underlying our modified synthesis protocols were thoroughly studied before [20], no spectroscopic analysis additionally to MALDI MS-based structure elucidation was carried out. First steps to assess AHL ionization and fragmentation behavior in MALDI MS were taken by analyzing commercial standards for identification of key fragments, which allowed an easy assignment of spectra of newly synthesized AHL variants.

All investigated AHL (Supplementary Table S1) delivered strong signals of protonated or sodiated molecules using CHCA as matrix. Utilizing 2,5-DHB or 9-aminoacridine, two
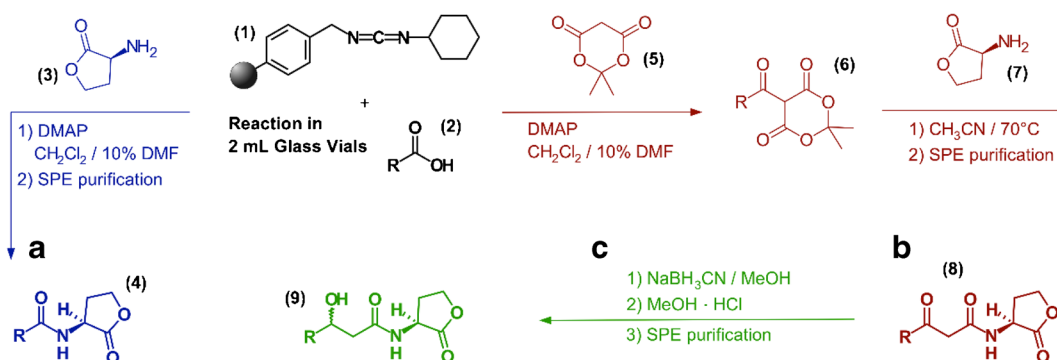
Figure 1. Small-scale synthesis approach for the generation of AHL. All reactions were performed in 2 mL glass vials. Detailed synthesis schemes are shown in Supplementary Figures S1–S3. **(a)** Synthesis of C3-unmodified AHL standards employing solid-phase chemistry. A one-pot reaction was carried out by reaction of the carboxylic acid (2) with L-HSL (3) activated by polymer-bound *N*-benzyl-*N*-cyclohexylcarbodiimide (1) and catalyzed by DMAP. The respective AHL (4) was obtained by SPE using silica cartridges. **(b)** Synthesis of 3-oxo-AHL standards using solid-phase chemistry, followed by a centrifugal filtration step and purification by SPE. **(c)** To obtain 3-hydroxy-AHL, 3-oxo-AHL (8) generated in small-scale synthesis were reduced with sodium cyanoborohydride while the pH was kept at 3–4 by addition of methanolic hydrochloric acid. The respective product (9) is extracted with ethyl acetate and concentrated by SPE
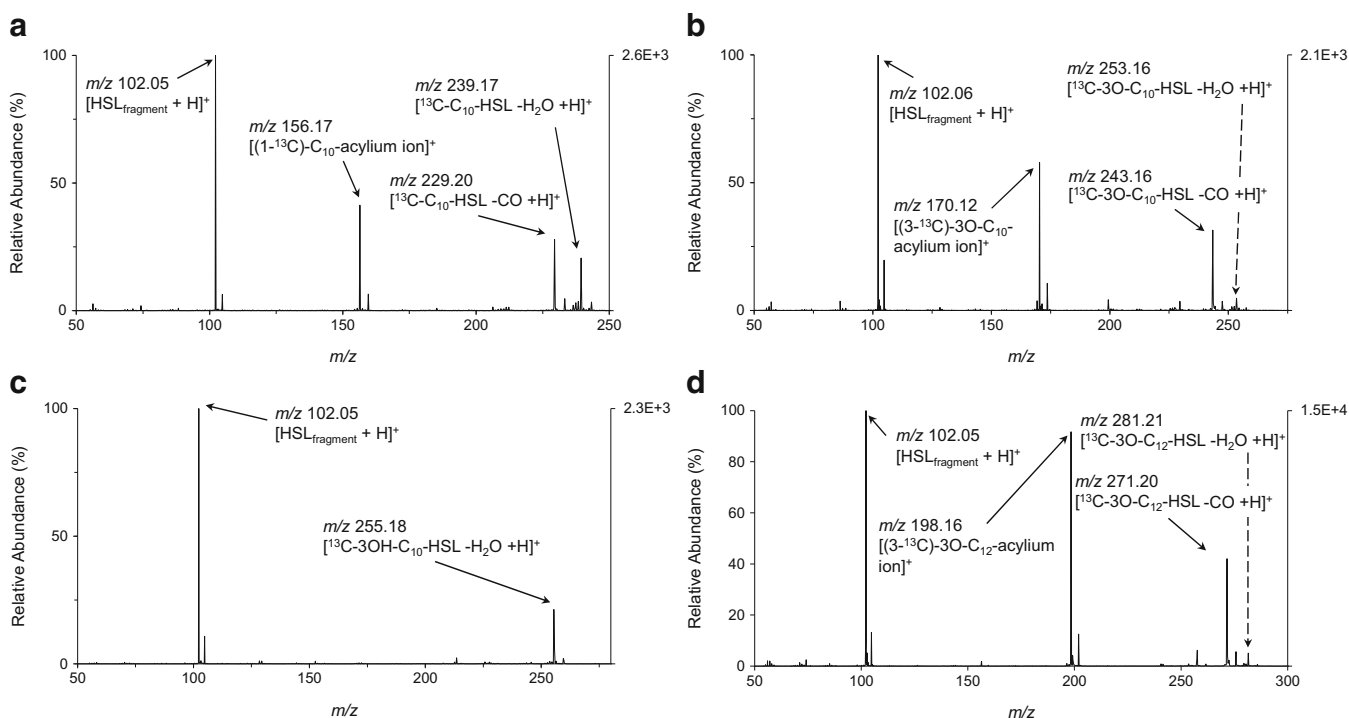
Figure 2. MALDI MS/MS spectra of isotope-labeled AHL standards generated by small-scale synthesis. Matrix: CHCA. MS/MS spectra of **(a)** $(1-^{13}C)-C_{10}-HSL$, **(b)** $(3-^{13}C)-3O-C_{10}-HSL$, **(c)** $(3-^{13}C)-3OH-C_{10}-HSL$, and **(d)** $(3-^{13}C)-3O-C_{12}-HSL$ acquired by CID are shown. Fragment ion peaks are labeled

matrices usually well suited for MALDI MS of LMM analytes, did not yield interpretable spectra. PNA (*para*-nitroaniline) delivered satisfying spectra in terms of signal intensities. However, it was excluded from further studies because of its volatility leading to sample loss by evaporation in the ion source

Reflecting one of the most common problems in MALDI MS analysis of LMM compounds, the mass region of interest in AHL spectra is widely populated by matrix background signals (Supplementary Figure S4, Figure 3a, b). Consideration of blank matrix spectra prior to AHL analysis facilitates identification of analytes in MALDI MS spectra and the selection of precursors for MS/MS evaluation. Despite the presence of matrix background ions, AHL proton and alkali adduct ions were unambiguously assigned. One potential approach to deal with the strong spectral background is the use of alternative matrices, e.g., ionic liquid matrices, as described below. The lower limit of detection (LLOD) of AHL reference standards measured in classic CHCA matrix was reached between 1 and 2.5 pmol for different AHL (Table 1).

In MS/MS experiments, all unsubstituted as well as 3-oxo- and 3-hydroxy-AHL derivatives generated a common fragment at *m/z* 102.05 upon collision-induced dissociation (see Figure 2). This characteristic fragment was the most intense in all MS/MS spectra of AHL investigated in this study and can serve as a general marker ion for AHL identification. Its structure has been annotated earlier as the protonated α-amino-γ-butyrolactone that arises from fragmentation of the carboxamide bond between the homoserine amino group and the acyl chain [10, 12].

Besides this diagnostic fragment, C3-unsubstitued AHL produced three major fragments observable in CID MS/MS spectra (Figure 2a): (1) an acylium ion originating from carboxamide cleavage, (2) a signal corresponding to the loss of water from the protonated molecule ion, and (3) a signal deriving from loss of carbon monoxide [10].

Similarly, MS/MS spectra of 3-oxo-AHL were characterized by the occurrence of a strong acylium fragment and a loss of CO from the homoserine lactone ring. In addition, both ^{13}C-$3O-C_{10}-HSL$ and ^{13}C-$3O-C_{12}-HSL$ showed fragments originating from water loss in CID experiments (Figure 2b and d).

MALDI MS spectra of 3-hydroxy-AHL showed a specific ion composition surrounded by matrix background signals. Besides the protonated molecule and the sodium adduct, all 3-hydroxy-AHL produced dominant ion signals deriving from water loss from the acyl chain hydroxy group (Supplementary Figure S4C). Notably, CID of the protonated $(3-^{13}C)-3OH$-$C_{10}-HSL$ molecule generated an analogue fragment ion (*m/z* 255.18) and the characteristic HSL fragment, but no acylium ion (Figure 2c). To examine the synthesis product further, the species at *m/z* 255.18 was isolated as MS/MS precursor, yielding additional structural information (Supplementary Figure S6A).

MALDI MS of N-Acyl-Homoserine Lactones Using Ionic Liquid Matrices

In a comprehensive screening of a variety of ionic liquid matrices (ILM) built up by different combinations of bases
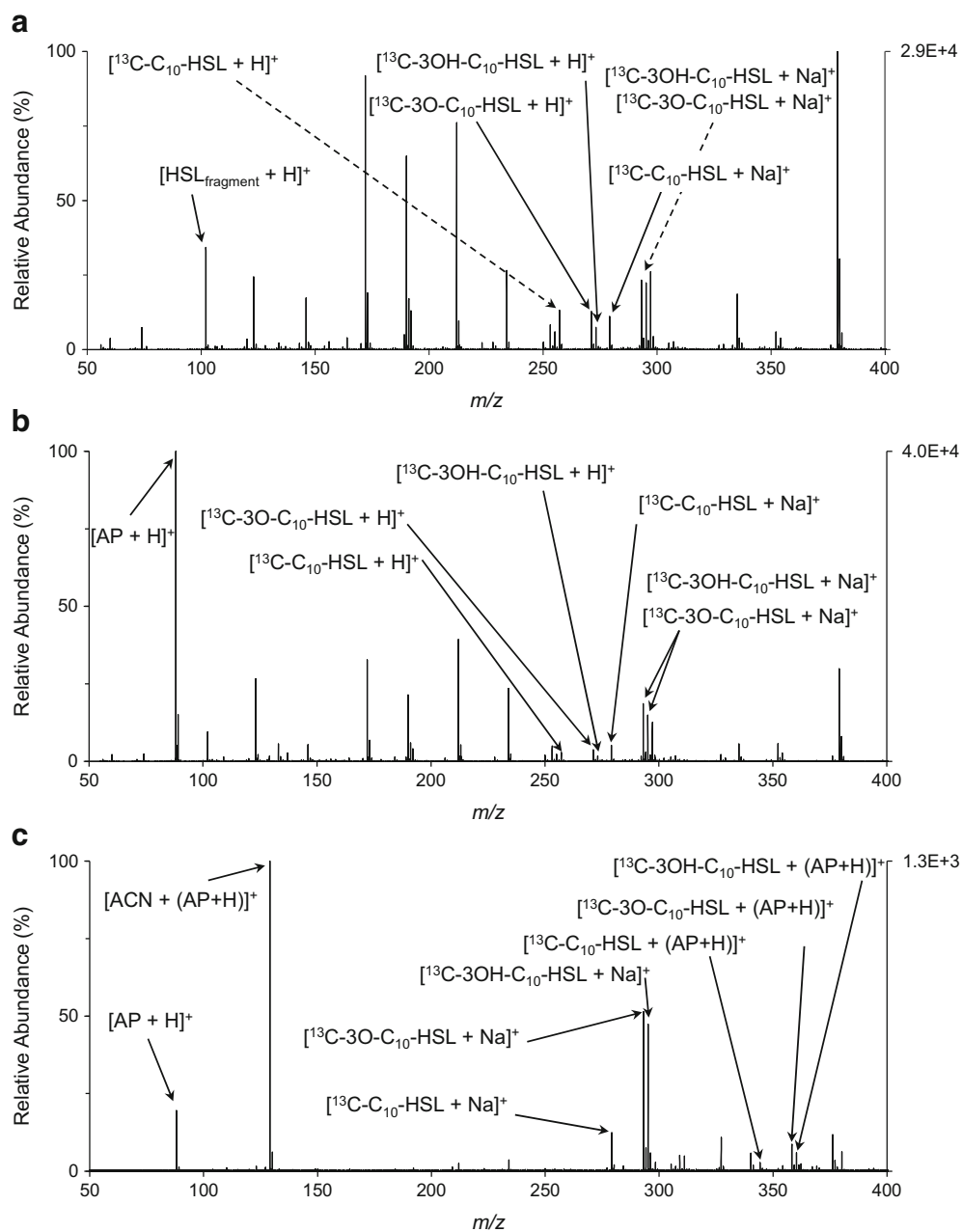
Figure 3. MALDI MS spectra of ^{13}C-labeled C_{10}-AHL standards synthesized in small-scale. **(a)** AHL standards measured in crystalline CHCA matrix. Accessible mass accuracy was between 1 and 10 ppm for all protonated and sodiated AHL molecules, if instrument calibration and internal calibration based on CHCA matrix ion signals was performed. **(b)** AHL standards measured in the ionic liquid matrix *mono*-AP-CHCA. **(c)** AHL standards measured in the ionic liquid matrix *di*-AP-CHCA. ACN = acetonitrile, AP = 2-aminopentane

Table 1. Lower Limit of Detection for Protonated and Sodiated Species of AHL Standards Measured in CHCA and *mono*-AP-CHCA. The Lowest Standard Amount On-Target with an S/N > 10 was Accepted as LLOD

	LLOD in CHCA Matrix		LLOD in *mono*-AP-CHCA ILM	
	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$
C_{10}-HSL	1 pmol	2.5 pmol	2.5 pmol	2.5 pmol
$3O$-C_{10}-HSL	2.5 pmol	5 pmol	5 pmol	0.625 pmol
$3OH$-C_{10}-HSL	1 pmol	1 pmol	0.625 pmol	0.25 pmol
$3OH$-C_{10}-HSL -H_2O	1 pmol	N/A	0.125 pmol	N/A
$3O$-C_{12}-HSL	1 pmol	5 pmol	0.125 pmol	0.125 pmol

and classically used MALDI matrices as acidic components, the ILM *di*-AP-CHCA containing a double molar excess of base showed best results in terms of quality of the AHL MALDI spectra. Figure 3c shows the spectrum of a mixture of C3-unsubstituted, 3-oxo-, and 3-hydroxy-C_{10}-AHL, all synthesized with a ^{13}C-label in the acyl chain. Matrix background ions were widely suppressed [27], only low intensity sodium or potassium adducts were observed in some cases; further, a strong signal at *m/z* 88.12 was observed, which we could assign as the protonated 2-aminopentane. However, *di*-AP-CHCA did not yield protonated molecules of the AHL analytes, but generated strong sodium adducts thereof. Furthermore, adducts of the analytes with protonated 2-aminopentane were observed. Although we were not able to detect fragment ions of sodium adduct species in CID experiments for structural elucidation, the high spectral quality compared with classic crystalline matrices rendered the described ILM a beneficial tool for qualitative screening of AHL-containing biological samples by MALDI-TOF MS. For example, *di*-AP-CHCA allowed detection of AHL sodium adducts in volumes of 5 mL of *P. aeruginosa* culture supernatants while maintaining strong suppression of matrix background signals (Supplementary Figure S7C). Another ILM preparation, *mono*-AP-CHCA, containing only one molar equivalent of 2-aminopentane, also enabled detection of protonated analyte molecules and was accompanied by improved spectral quality (Figure 3b).

Moreover, LLODs of AHL measured in *mono*-AP-CHCA were comparable to those obtained with classic CHCA matrix (Table 1). This novel matrix was also applied successfully for the detection of natural AHL in *P. aeruginosa* culture supernatant (Supplementary Figure S7B).

In some experiments we observed the signal of protonated 2-aminopentane (*m/z* 88.12) also in spectra acquired from spots prepared with crystalline CHCA matrix. As this signal was not observed in CHCA spectra measured if no ILM-preparations were present on the same target, we conclude that there is a gas-phase carryover of the ILM-base. This is in contrast to other ILM investigated earlier which did not show any instability under vacuum conditions [19]. However, this carryover does not interfere with the AHL analysis. Nevertheless, a source bake out after extensive use of this ILM is recommended.

Quantitative MALDI MS

The use of isotope-labeled standards enables accurate determination of analyte quantities by MALDI MS. In particular, suitable isotopologues can correct for ionization and detection differences of analytes and show the same behavior as the analytes in sample preparation. Furthermore, labeled compounds can facilitate elucidation of fragmentation mechanisms.

To establish a MALDI MS method for quantification of AHL, standard curves from dilution series spiked with an IS
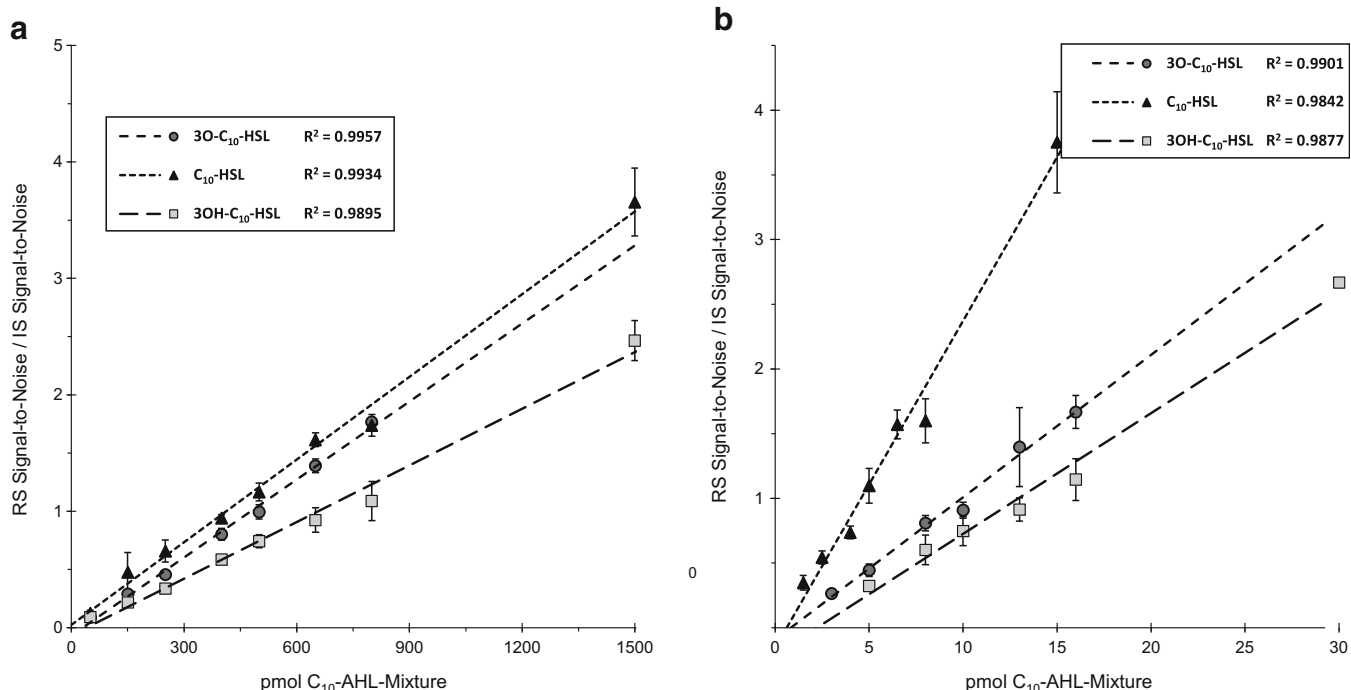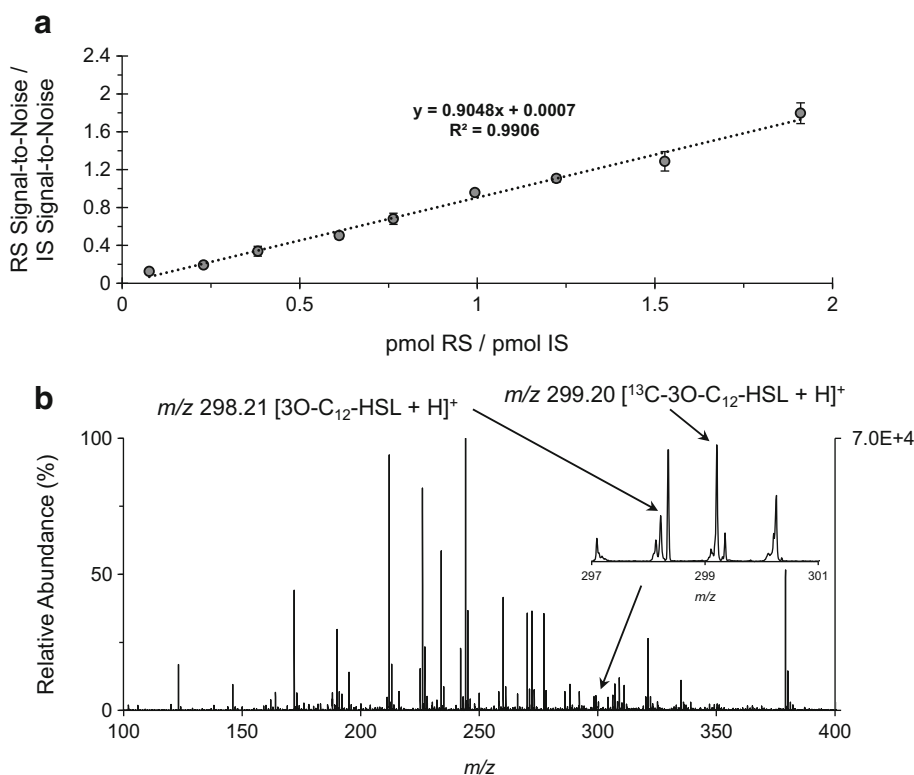

Figure 4. Standard curves for quantification of C_{10}-AHL using ^{13}C-labeled internal standards. The internal standards (1-^{13}C)-C_{10}-HSL, (3-^{13}C)-3O-C_{10}-HSL, or (3-^{13}C)-3OH-C_{10}-HSL were spiked into separate dilution series of C_{10}-AHL reference mixtures. **(a)** Standard curves for the range of 50 pmol to 1500 pmol reference standard on-target. The amount of internal standard in each sample was 500 pmol on-target. **(b)** Standard curves for definition of the lower limit of quantification (LLOQ). To produce new sets of standard curves for determination of the LLOQ, the spiked dilution series in **(a)** were further diluted 1:100 (C_{10}-HSL standard series) and 1:50 (3O-C_{10}-HSL and 3OH-C_{10}-HSL standard series). Error bars display standard deviation (SD) of isotopic ion ratios. Relative SD was below 20% for all data points, except for 150 pmol of C_{10}-HSL with a relative SD of 36%

were generated by linear regression of the isotopic ion ratios and the RS quantities applied on-target. Although both peak signal-to-noise and areas can be employed for the calculation of quantitative data, we favored signal-to-noise as a robust determinant of AHL ion signal intensities [24]. To adjust for the contribution of natural isotopes of the RS to the $m+1$ peak fraction, a correction value based on the theoretical carbon isotope distribution was used to calculate the IS signal (see Materials and Methods). For quantification of C_{10}-HSL, the lowest standard on the standard curve revealed a lower limit of quantification (LLOQ) of 1.5 pmol (Figure 4b). A dynamic range of 1.5 pmol to 15 pmol ($R^2 = 0.9842$) was found for RS samples spiked with 5 pmol (1-^{13}C)-C_{10}-HSL. Although linear range measurements without an internal standard revealed a narrower linearity for higher pico-molar amounts, an R^2 value greater than 0.99 was achieved for the respective C_{10}-HSL standard curve (Figure 4a). The LLOQ of 3O-C_{10}-HSL was found at 3 pmol of RS spiked with 10 pmol (3-^{13}C)-3O-C_{10}-HSL, whereas the dynamic range was between 3 and 16 pmol ($R^2 = 0.9901$). Similar results were obtained for the internal (3-^{13}C)-3OH-C_{10}-HSL standard (10 pmol on-target); with an LLOQ of 5 pmol and a dynamic range from 5 pmol to 30 pmol

($R^2 = 0.9897$). Alternatively, an accurate standard curve (LLOQ 2.5 pmol) based on the isotope ratios of the peaks corresponding to loss of water from the protonated 3OH-C_{10}-HSL molecule was acquired (Supplementary Figure S6B).

Analysis of AHL in Supernatants of Bacterial Cultures

After a general evaluation of our standards derived from small-scale synthesis, the (3-^{13}C)-3O-C_{12}-HSL standard was employed for the quantification of natural 3O-C_{12}-HSL from *P. aeruginosa* culture supernatants. Acquisition of standard curves spiked with 13 pmol of IS and measured in crystalline CHCA matrix revealed a dynamic range from 1 to 25 pmol with an accurate linearity ($R^2 = 0.9906$) (Figure 5a). Since CHCA generated strongest proton adduct intensities, we chose the classic matrix instead of an ILM preparation. ILM can also be used for quantification, e.g., of the sodium adducts [28]; however, the linearity of these curves was in some cases lower and the linear range more narrow than those obtained from protonated species (data not shown). To determine the concentration in microbiological samples, the standard curve equation

Figure 5.   Quantification of 3O-C_{12}-HSL from *P. aeruginosa* PAO1 supernatant using a ^{13}C-labeled internal standard. **(a)** Standard curve for 3O-C_{12}-HSL quantification. The amount of internal (3-^{13}C)-3O-C_{12}-HSL standard was 13 pmol on-target, the amount of the lowest reference standard on the curve was 1 pmol on-target. The linear regression equation was applied on experimental data in **(b)** to calculate quantitative results. Error bars display SD of the isotopic ion ratios. Relative SD was below 20% for all data points. **(b)** MALDI MS spectrum (CHCA) of the extract of 5 mL *P. aeruginosa* PAO1 supernatant diluted 5-fold. Peaks assigned to the 3O-C_{12}-HSL analyte produced by *P. aeruginosa* and the internal (3-^{13}C)-3O-C_{12}-HSL standard are labeled. A 3O-C_{12}-HSL concentration of 9.3 μM in *P. aeruginosa* supernatant was calculated by applying the standard curve equation from **(a)** on the isotopic ion ratio of the 3O-C_{12}-HSL analyte and the internal standard

was applied on MALDI MS data of the spiked extracts of *P. aeruginosa* culture supernatants. Alternatively, analyte concentration was calculated based on experimentally determined sensitivities of the analyte and the IS as described before [15]. Our in-house synthesized internal standard allowed us to determine AHL quantities in 5 mL of bacterial supernatants and calculate a 3O-C$_{12}$-HSL concentration of 9.3 µM (9.1 µM by calculation based on analyte sensitivities) derived from 500 mL of the *P. aeruginosa* culture. The signals of the 3O-C$_{12}$-HSL analyte and the isotope-labeled standard detected for quantification are displayed in Figure 5b. Overall, the performance of our standards in quantitative MALDI experiments demonstrates the effectiveness of a downscaled and simplified synthesis approach for internal standard provision. Compared with the previously described derivatization of 3O-C$_{12}$-HSL in *P. aeruginosa* supernatant with Girard's reagent T (GT) [8], our approach accounted for the analyte extraction and enabled quantification from smaller bacterial culture volumes, although the absolute LOQ determined here (1 pmol) was above that reported in the earlier study by Kim and coworkers (2.5 fmol for GT derivatized and 40 pmol for underivatized 3O-C$_{12}$-HSL).

Conclusion

We were able to show that rapid small-scale synthesis allows preparation of suitable standards for a wide range of AHL derivatives. Moreover, the synthesis of isotope-labeled AHL compounds enables accurate quantification of AHL by MS. Owing to its moderate tolerance against salts, MALDI MS is a suitable and straightforward method for fast screening of biological molecules even in complex samples, without the need for extensive sample preparation. We further showed that 2-aminopentane-based ILM are highly suitable for analysis of AHL because of efficient background ion suppression.

As all major classes of AHL could be detected with the present approach, MALDI MS has the potential for a widespread use in biotechnology and biomedical sciences to decipher processes mediating communication between bacteria. This is particularly interesting for the study of microbial interactions and biofilm organization, as well as host–microbiota interactions. Besides the application in rapid sample screening and quantification by MALDI-TOF MS, our here presented approach for provision of internal standards may be useful for sensitive and quantitative AHL detection by LC-MS/MS.

Acknowledgements

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