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Facile preparation of deuterium-labeled *N*-acylhomoserine lactones as internal standards for isotope dilution mass spectrometry

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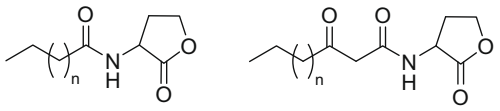
ABSTRACT

N-Acylhomoserine lactones (AHLs) are widely conserved signal molecules that mediate quorum sensing in Gram-negative bacteria. In this study, deuterium-labeled AHLs were prepared for use as internal standards for isotope dilution mass spectrometry. Their utility in the sensitive and precise quantification of AHLs in culture supernatants of bacteria by GC/MS was demonstrated.

1. Introduction

Quorum sensing (QS), the coordinated population density-dependent regulation of gene expression in individual bacterial cells, is mediated by the exchange of extra-cellular signals, and affects a diversity of behaviors in a variety of bacterial species.[1] Gram-negative bacteria utilize *N*-acylhomoserine lactones (AHLs) (Fig. 1) as their command language to coordinate behavior during the invasion and colonization of higher organisms.[2,3] AHLs are synthesized by a member of the LuxI protein family and vary with respect to the chain length (4–18 carbons) and the substituent (H, O, or OH) at the third carbon of the acyl side chain. When their concentrations reach a critical threshold, their interaction with the regulatory proteins of the LuxR family induces the expression of particular genes, operons, or regulons.

Because AHLs are mostly found in trace amounts, a sensitive method is required for their detection. First, the detection of AHLs using bacteriological monitoring systems, individually or in combination, was reported.[4–7] These methods rely typically on a phenotypic response, that is, bioluminescence, pigment production, or a reporter gene activated through an AHL-receptor protein. Separation by TLC on a C_{18} reverse phase plate coupled with detection by AHL-biosensors gives a rapid and direct visual index of the AHLs produced by the tester bacteria.[8] However, biosensors can reflect potential artifacts and not simply the ability of a bacterium to synthesize an AHL. To overcome this drawback, chemical methods



n = 3: C_6-HSL (**1**)
n = 4: C_7-HSL (**2**)
n = 5: C_8-HSL (**3**)

n = 1: 3-oxo-C_6-HSL (**4**)
n = 7: 3-oxo-C_{12}-HSL (**5**)

Figure 1. Structures of AHLs.

using GC/MS, LC/MS, or LC/MS/MS have been used for the direct and unambiguous identification of AHLs in bacterial supernatants.[9–13] In addition, determination of the levels and kinetic profiles of AHLs is required for a better understanding of the regulation and optimization of biological processes via AHL-mediated QS. To achieve accuracy and precision in a quantitative analysis, an appropriate internal standard (IS) is required. In previous studies,[10,14] *N*-heptanoylhomoserine lactone (C_7-HSL, **2**) was used to quantify AHLs in biological samples because its chemical properties are similar to those of target AHLs. However, some bacteria produce AHLs harboring odd-numbered acyl chains, including C_7-HSL (**2**).[15–17] To overcome this, isotope-labeled *N*-hexanoylhomoserine lactone (C_6-HSL, **1**) and *N*-3-oxododecanoylhomoserine lactone (3-oxo-C_{12}-HSL, **5**) were chemically or biologically prepared and used for MS-based quantification.[18,19] However, because the levels of AHLs are quite low, a relatively high level of background noise from the complex matrixes can lead to shifts in analyte/IS ratios, resulting in a decrease in accuracy and precision. Thus, a

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method of quantification which uses isotope dilution mass spectrometry is required.

In this study, we synthesized isotopically labeled AHLs as internal standards and developed a relatively simple process of purification and a GC/MS-based method for the quantification of AHLs. 3-Oxo AHLs are known to be labile during gas chromatographic runs and thus hard to detect by GC/MS. Therefore, we also exploited derivatization for the detection. These methods were used to determine the kinetics of *N*-hexanoylhomoserine lactone (C_6-HSL, **1**), *N*-octanoylhomoserine lactone (C_8-HSL, **3**), and *N*-3-oxohexanoylhomoserine lactone (3-oxo-C_6-HSL, **4**) in the culture supernatant of a plant symbiotic bacterium, *Sinorhizobium meliloti*.

2. Results and discussion

2.1. Synthesis of deuterium-labeled AHLs

Because all AHLs can be easily prepared by coupling a homoserine lactone with acyl parts, the incorporation of an isotope into the lactone ring is the best way to develop a method of quantification with GC/MS. Based on the synthesis of [3,3,4,4-$^2H_4$]-homoserine lactone hydrochloride (**9**) by Ramalingam and Woodard,[20] deuterium-labeled C_6-HSL (**1**), C_8-HSL (**3**), and 3-oxo-C_6-HSL (**4**) were prepared (Scheme 1). The hydroxy group of [1,1,2,2-$^2H_4$]-2-bromoethanol (**6**) was protected with THP ether (81%), the subsequent coupling of which with diethyl acetamidomalonate afforded **8** (65%). Hydrolysis and decarboxylation of the condensation product **8** in 6 M HCl yielded **9** (44%). The labeled lactone **9** was coupled with hexanoyl chloride or octanoyl chloride to furnish **1**-d_4 (80%) and **3**-d_4 (84%), respectively. Compound **4**-d_4 was synthesized by coupling the butanoyl derivative of Meldrum's acid with **9** (79%). In addition to the final products, the structures of the intermediates, including four deuterium atoms, were confirmed by NMR and MS. The isotopic purity of deuterated AHLs was determined to be >98% by MS.

2.2. GC/MS of deuterium-labeled AHLs

Deuterium-labeled AHLs were analyzed by GC/MS using the full-scan EI mode. The conditions for GC were set according to Cataldi et al.[10] (see Section 4). The deuterium-labeled C_6- and C_8-HSLs, **1**-d_4 and **3**-d_4, were detected at 9.4 and 12.1 min (Fig. 2A), almost identical with unlabeled AHLs. As described previously, deuterated 3-oxo-C_6-HSL (**4**-d_4) could not be observed due to thermal degradation. In the positive EI mode, alkanoyl HSLs are known to produce a characteristic fragment ion at *m/z* 143, which is due to a McLafferty rearrangement, typical of carbonyl groups having a hydrogen atom in the γ position. In deuterated compounds, the fragment ions were detected at an *m/z* position, four mass units larger than those of the corresponding unlabeled compounds (Fig. 2B–E). The deuterium label in **1**-d_4 and **3**-d_4 was stable enough not to cause any loss of deuterium during the fragmentation into the major ion at *m/z* 147. Therefore, quantitative detection was performed in the selected ion monitoring (SIM) mode using the fragmentation at *m/z* 143 for unlabeled AHLs and *m/z* 147 for labeled AHLs.

To improve stability and sensitivity, we treated 3-oxo-C_6-HSL (**4**) with *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxyamine hydrochloride (PFBHA·HCl) to obtain the PFB-oximes and analyzed them by GC/MS.[18] As shown in Figure 3A, **4**-d_4 was detected as two peaks at 16.4 and 16.8 min due to the formation of *E*/*Z* isomers of PFB-oximes. In the positive EI mode, the mass spectra of PFB derivatives of unlabeled and deuterium-labeled compounds generated a characteristic ion at *m/z* 181 due to the PFB fragment of the oximes (Fig. 3B and C). Also, the spectra displayed a major ion $[M-197]^+$, *m/z* 215 for **4**-d_4 and 211 for **4**, originating from the loss of the $C_6F_5CH_2O$ moiety. Since the latter pair of ions reflected the difference in mass between **4**-d_4 and **4**, we selected them as a marker fragment in the SIM mode. Only the deuterium-labeled compound could be detected by SIM, showing that no replacement of deuterium by hydrogen occurred during the derivatization and GC/MS processes.

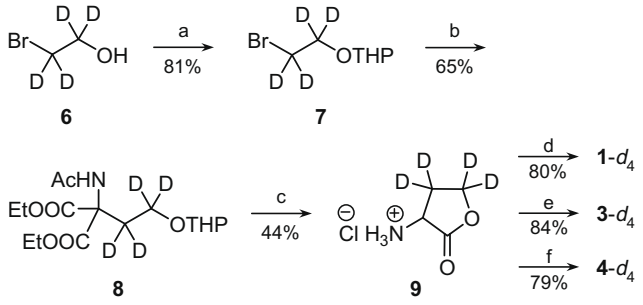
2.3. Linearity and sensitivity of GC/MS

The linearity of the response was evaluated by a GC/SIM analysis of standard mixtures containing a ratio of unlabeled to labeled AHLs of 0.1–5. Response curves were found to be linear with the correlation coefficient (R^2) from 0.992 to 0.999 (Table 1). Based on these data, standard curves for **1**, **3**, and **4** were constructed. Detection limits of **1**, **3**, and **4** were 27, 23, and 23 fmol, respectively (S/N = 3). The derivatization of 3-oxo-C_6-HSL (**4**) with PFBHA·HCl greatly improved sensitivity, comparable to alkanoyl HSLs. These values were good enough to detect trace amounts of AHLs in bacterial samples.

2.4. Quantification of AHLs in bacterial culture supernatant

S. meliloti is a Gram-negative bacterium of soil characterized by a nitrogen-fixing symbiosis with the plant *Medicago sativa* (alfalfa). *S. meliloti* Rm41 harbors at least three QS systems (Sin, Mel, and Tra) mediated by short- and long-chain AHLs.[21–23] In the present study, short-chain AHLs, C_6-HSL (**1**), C_8-HSL (**3**), and 3-oxo-C_6-HSL (**4**), were analyzed quantitatively by GC/MS. As in previous studies,[9,10] we first tried to detect AHLs in the crude extract of bacterial supernatant; however, the high background levels in SIM chromatograms affected the accuracy of the procedure. Therefore, the EtOAc extracts were fractionated by HPLC on an ODS column, and the fraction containing AHLs was subjected to GC/MS. In the case of 3-oxo-C_6-HSL (**4**), the fraction was treated with PFBHA·HCl and then subjected to GC/MS. Representative SIM chromatograms of **1**, **3**, and **4** are shown in Figure 4. All AHLs could be detected with a high S/N, indicating that contaminants were effectively removed from the analytical samples. Of course, we confirmed that their mass spectra were identical to the corresponding standards (Supplementary data, Fig. S1).

Using this protocol, we investigated the dynamics of AHLs for bacterial cultures in a period of up to 37 h (Fig. 5). In the process, appropriate amounts of isotope-labeled AHLs were added to the supernatant of bacterial cultures before the extraction step. Figure 5D shows the growth curve of *S. meliloti* Rm41 in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$. As shown in Figure 5A–C, C_8-HSL (**3**) was most abundant at all sampling times, followed by C_6-HSL (**1**) and 3-oxo-C_6-HSL (**4**). The concentrations of C_6-HSL (**1**) and C_8-HSL (**3**) continued to increase throughout the



Scheme 1. Synthesis of deuterium-labeled AHLs. Reagents and conditions: (a) dihydropyran, *p*-toluenesulfonic acid, 0 °C to rt; (b) diethyl acetamidomalonate, EtONa, EtOH, rt; (c) 20% aq HCl, reflux; (d) hexanoyl chloride, Na_2CO_3, CH_2Cl_2–H_2O, 0 °C to rt; (e) octanoyl chloride, Na_2CO_3, CH_2Cl_2–H_2O, 0 °C to rt; (f) butanoyl Meldrum's acid, Et_3N, MeCN, rt to reflux.

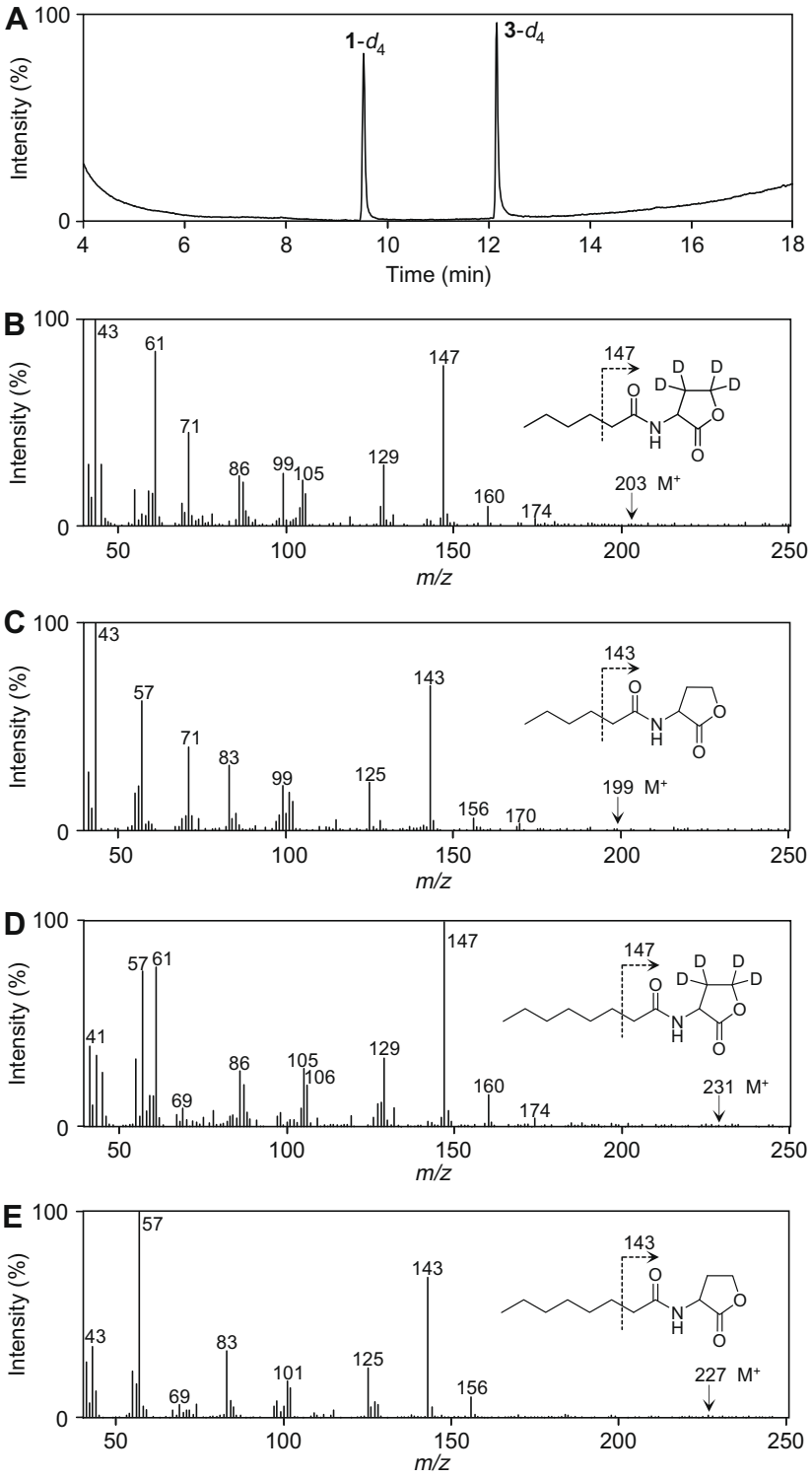


Figure 2. Analysis of labeled and unlabeled AHLs by GC/MS. Total ion chromatogram of standard mixtures of labeled AHLs (A). Mass spectra of **1**-d_4 (B), **1** (C), **3**-d_4 (D), and **3** (E).

experiment. The concentration of 3-oxo-C_6-HSL (**4**) was much lower than that of **1** or **3**, and seemed to stop increasing in the late exponential to stationary growth phase. Previously, it was reported that *S. meliloti* produces a variety of AHLs thought to have distinct biological roles. Bacteriological reporter systems easily and sensitively elucidate the AHLs produced by *S. meliloti*; however, the response of these systems to AHLs is linear only in a narrow range and depends on the chemical structure of AHLs. In this study, we quantified the short-chain AHLs in cultures of *S. meliloti* and found that C_8-HSL (**3**) is most abundant. Taken together, C_8-HSL (**3**) is suggested to play a crucial role as a chemical signal in the QS systems of *S. meliloti* Rm41.

3. Conclusions

Deuterium-labeled AHLs were easily prepared from a commercially available [1,1,2,2-$^2H_4$]-2-bromoethanol (**6**). These labeled compounds were useful as internal standards to quantify AHLs in

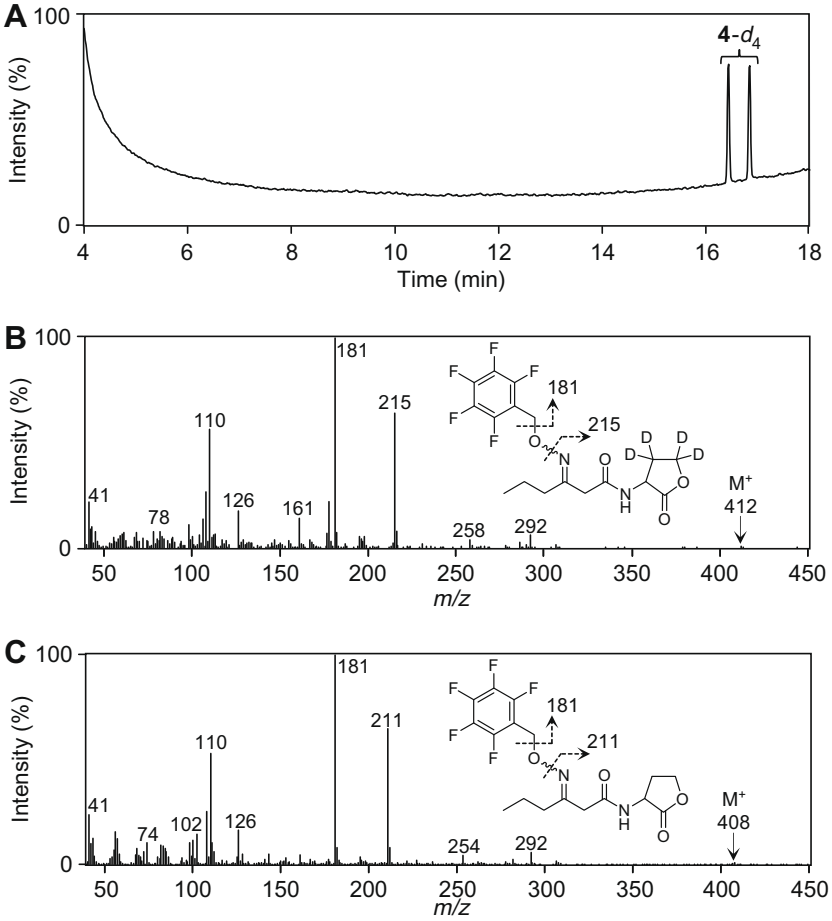


Figure 3. Analysis of the PFB-oximes of labeled and unlabeled 3-oxo-C_6-HSL by GC/MS. Total ion chromatogram of the PFB-oximes of **4**-d_4 (A). Mass spectra of the PFB-oximes of **4**-d_4 (B) and **4** (C).

Table 1
Standard curves and sensitivities of AHLs analysis by GC/MS

Analyte	Standard curve[a]	Correlation coefficient (R^2)	Linear range[b]	Detection limits[c] (fmol)
C_6-HSL (**1**)	$y = 0.7976x + 0.0334$	0.9987	0.1–5	27
C_8-HSL (**3**)	$y = 1.252x + 0.0310$	0.9990	0.1–5	23
3-Oxo-C_6-HSL (**4**)	$y = 1.008x + 0.2127$	0.9921	0.1–5	23

[a] y: response ratio (analyte/IS), x: amount ratio (analyte/IS).
[b] Amount ratio (analyte/IS).
[c] S/N = 3.

bacterial cultures by GC/MS. We also found the labeled AHLs to be applicable to quantification by LC/MS/MS (unpublished data). To date, many biological events induced by AHL-mediated QS have been elucidated in Gram-negative bacteria. However, in many cases it is not clear which AHL plays a key role in such events. Using the present methodology, we can integrate both biological and chemical data, which will provide a better understanding of the AHL-mediated QS in Gram-negative bacteria.

4. Experimental

4.1. General

^1H and ^{13}C NMR spectra were recorded on a JNM-AL 400 spectrometer (JEOL, Tokyo, Japan). High-resolution mass spectra were obtained with a JMS-T100LC AccuTOF mass spectrometer (JEOL) using PPG as an internal standard. Column chromatography was performed on Wakogel C-200 (Wako Pure Chemical, Osaka, Japan). HPLC separation was performed with a LaChrom Elite system (Hitachi, Tokyo, Japan). Solvents for HPLC were purchased from Kanto Chemical (Tokyo, Japan).

4.2. Synthesis of deuterium-labeled AHLs

4.2.1. Deuterium-labeled C_6-HSL (1-d_4)

D,L-[3,3,4,4-$^2H_4$]-Homoserine lactone hydrochloride (**9**) was prepared by a modification of the method reported by Ramalingam and Woodard.[20] [1,1,2,2-$^2H_4$]-2-Bromoethanol (**6**) (5 g, 38.8 mmol, Cambridge Isotope Laboratories, MA, USA) was added to a stirred solution of dihydropyran (5.31 mL, 58.2 mmol) and *p*-toluenesulfonic acid (30 mg, 0.175 mmol) at 0 °C. After stirring for 2 h at rt, the reaction was stopped with $NaHCO_3$ (17 mg, 0.21 mmol), and subsequently the mixture was distilled to afford **7** as a colorless liquid (6.72 g, 31.5 mmol, 81%). Diethyl acetamidomalonate

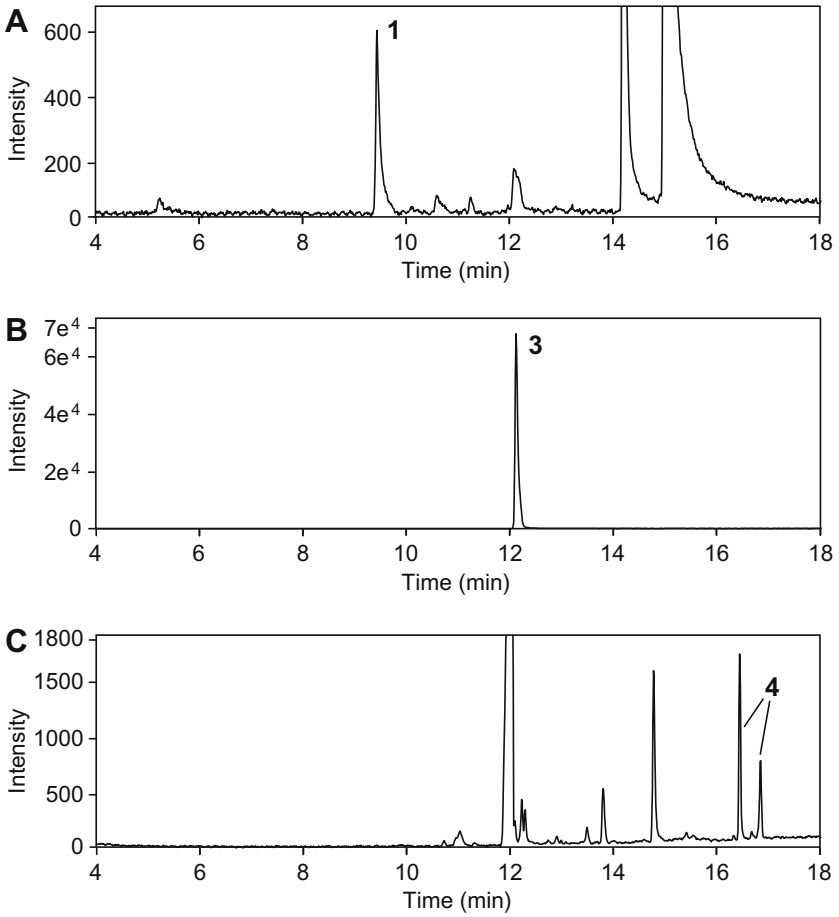


Figure 4. Representative selected ion chromatograms of C_6-HSL (**1**) (A), C_8-HSL (**3**) (B), and PFB-oximes of 3-oxo-C_6-HSL (**4**) (C) in HPLC-separated fractions from bacterial cultures of *S. meliloti* Rm41.

(10.3 g, 47.3 mmol) and EtONa (3.22 g, 47.3 mmol) were dissolved in dry EtOH (35 mL) and heated at reflux for 0.5 h under an N_2 atmosphere. After cooling to rt, **7** was added to the mixture and stirred for 3 h at rt. Furthermore, the reaction was continued for 8 h under reflux. After cooling to rt, the mixture was diluted with 100 mL of EtOAc and washed with water and brine. The organic layer was dried over Na_2SO_4, evaporated, and purified by column chromatography (hexane–EtOAc, 20% stepwise) to give **8** as a colorless oil (7.10 g, 20.3 mmol, 65%). **8** was dissolved in 20 mL of 20% aq HCl and the mixture was refluxed for 24 h. After cooling to rt, the reaction mixture was diluted with 50 mL of water and washed with EtOAc two times. The aqueous layer was concentrated and the residue was dissolved in boiling EtOH. After the undissolved sodium bromide was removed, the filtrate was kept at 4 °C overnight to give **9** as a white crystal (1.27 g, 8.97 mmol, 44%). ^{1}H NMR (400 MHz, D_2O) δ 4.30 (1H, s, H-1). MS (ESI$^+$) *m/z* 128 [M−HCl+Na]$^+$.

Next, the coupling of homoserine lactone hydrochloride with acyl chloride was performed. **9** (100 mg, 0.706 mmol) and Na_2CO_3 (225 mg, 2.12 mmol) were dissolved in CH_2Cl_2/H_2O (4 mL, 1:1) and the mixture was cooled to 0 °C. After stirring for 5 min at 0 °C, hexanoyl chloride (131 μL, 0.953 mmol) was added dropwise to the mixture. The mixture was stirred for 2 h at rt and subsequently diluted with 30 mL of EtOAc. The solution was washed with brine, dried over Na_2SO_4, and evaporated. The residue was crystallized from EtOAc to afford a white crystal of **1**-d_4 (115 mg, 0.566 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (3H, t, *J* = 7.1 Hz, H-6), 1.25–1.38 (4H, H-4,5), 1.65 (2H, m, H-3), 2.25 (2H, t, *J* = 7.3 Hz, H-2), 4.53 (1H, d, *J* = 5.6 Hz, HSL-α), 5.99 (1H, br, NH). HRMS (ESI$^+$) *m/z* 226.1355 [M+Na]$^+$ (calcd for $C_{10}H_{13}D_4NNaO_3$, 226.1357).

4.2.2. Deuterium-labeled C_8-HSL (3-d_4)

The procedure described in Section 4.2.1 was followed. White crystal (137 mg, 0.591 mmol, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* = 6.8 Hz, H-8), 1.22–1.38 (8H, H-4,5,6,7), 1.65 (2H, m, H-3), 2.25 (2H, t, *J* = 7.3 Hz, H-2), 4.53 (1H, d, *J* = 5.6 Hz, HSL-α), 6.00 (1H, br, NH). HRMS (ESI$^+$) *m/z* 254.1665 [M+Na]$^+$ (calcd for $C_{12}H_{17}D_4NNaO_3$, 254.1670).

4.2.3. Deuterium-labeled 3-oxo-C_6-HSL (4-d_4)

Butyric acid (136 μL, 1.50 mmol) was added to a solution of 4-dimethylaminopyridine (202 mg, 1.65 mmol), *N*,*N*′-dicyclohexylcarbodiimide (340 mg, 1.65 mmol), and Meldrum's acid (216 mg, 1.50 mmol) in dry CH_2Cl_2 (10 mL). After stirring at rt overnight, the urea that had formed was removed by filtration. The filtrate was evaporated, dissolved in 20 mL of EtOAc, and washed with 2 M HCl and brine. The organic layer was dried over Na_2SO_4 and evaporated dry to yield butanoyl Meldrum's acid quantitatively. Butylated Meldrum's acid, **9** (150 mg, 1.06 mmol), and Et_3N (177 μL, 1.27 mmol) were dissolved in dry MeCN (10 mL) and stirred for 2 h at rt and for 3 h under reflux. After cooling to rt, the reaction mixture was evaporated, dissolved in EtOAc (20 mL), and washed with satd $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and purified by column chromatography ($CHCl_3$/MeOH, 97:3) to afford **4**-d_4 as a white solid (177 mg, 0.826 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (3H, t,

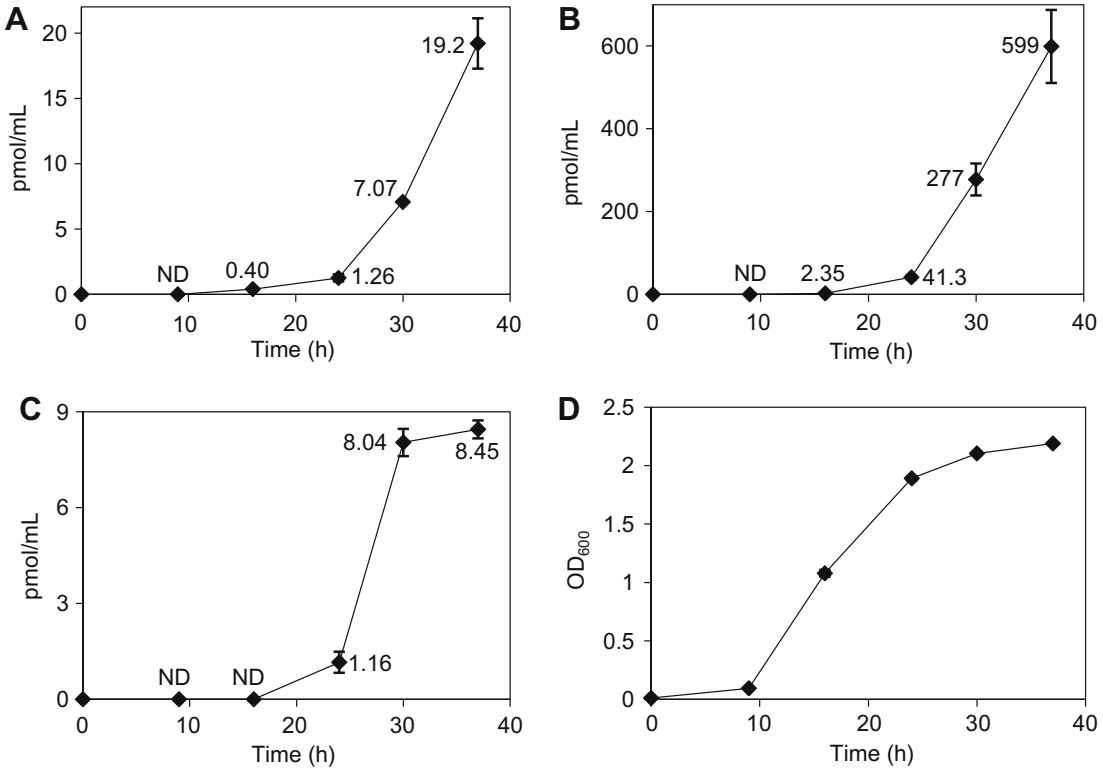


Figure 5. Time-course of the levels of AHLs in bacterial cultures of *S. meliloti* Rm41. (A) C_6-HSL (**1**), (B) C_8-HSL (**3**), (C) 3-oxo-C_6-HSL (**4**), and (D) growth curve of Rm41. The error bars indicate the standard deviation for three replicates.

J = 7.3 Hz, H-6), 1.63 (2H, m, H-5), 2.52 (2H, t, *J* = 7.6 Hz, H-4), 3.48 (2H, s, H-2), 4.58 (1H, d, *J* = 6.56 Hz, HSL-α), 7.71 (1H, br, NH). HRMS (ESI$^+$) *m/z* 240.1147 [M+Na]$^+$ (calcd for $C_{10}H_{11}D_4NNaO_4$, 240.1150).

4.3. GC/MS conditions

GC/MS data were obtained with a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) fitted with an InertCap 5MS/NP capillary column (25 m × 0.25 mm id, 0.25 mm film, GL Sciences, Tokyo, Japan). Conditions were as follows: injection, 1 μL, splitless, 60 s valve time; carrier gas, He at 0.8 mL/min; transfer line temperature, 280 °C; electron energy, 70 eV. The GC was programmed as follows: 3 min at 150 °C then to 275 °C at 15 °C/min.

4.4. Quantitative analysis

An overnight culture of *S. meliloti* Rm41 (gift from Professor J. E. González) was diluted with fresh LB medium containing 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (OD_{600} = 0.01). Fifty milliliters of this culture was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C with rotation at 120 rpm for 9, 16, 24, 30, or 37 h. Following growth, the bacterial cells were removed by centrifugation (9000×*g*, 3 min). After addition of the appropriate amount of deuterium-labeled AHLs, the culture supernatant was extracted three times with an equal volume of EtOAc. The combined EtOAc extracts were evaporated and dissolved in 200 μL of MeOH. After the insoluble material was removed by centrifugation, the supernatant was subjected to HPLC. The conditions were as follows: column, Inertsil ODS-3 250 mm × 10 mm id (GL Sciences); solvent, 30% aq MeCN for 15 min and then linear gradient from 30 to 70% aq MeCN for 15 min; flow rate, 4 mL/min, column oven, 40 °C. The factions eluted at 6–7 min for 3-oxo-C_6-HSL (**4**), 12–13 min for C_6-HSL (**1**), and 27–28 min for C_8-HSL (**3**) were collected and evaporated to dryness. The dried residues containing **1** and **3** were redissolved in 500 μL of HPLC-grade EtOAc and 1 μL of the mixture was subjected to GC/MS. The fractions containing **4** were dissolved in 2 mL of 50 mM aq PFBHA, stirred for 2.5 h at rt, and extracted twice with an equal volume of EtOAc. The dried residues were redissolved in 500 μL of HPLC-grade EtOAc and 1 μL of the mixture was injected into the GC/MS system.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.055.

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