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New Structural Variants of Homoserine Lactones in Bacteria

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N-Acylhomoserine lactones (AHLs) are used by a wide variety of bacteria for cell–cell communication in "quorum-sensing". These compounds are derived from L-homoserine lactone and a fatty acid, which varies in chain-length, degree of saturation, and the presence or absence of an oxygen atom at C-3. In this study we describe for the first time the occurrence of acyl chains carrying a methyl branch, and present a GC-MS-based method that can be used to distinguish these compounds from unbranched isomers. The bacterium *Aeromonas culicicola* produces several methyl branched AHLs. In *Jannaschia helgolandensis*—a marine bacterium of the *Roseobacter* clade—a doubly unsaturated AHL, (2*E*,9*Z*)-*N*-(2,9-hexadecadienoyl)-L-homoserine lactone, occurs. The location and configuration of the double bonds was proven by spectrometric investigation and synthesis. Finally, a method was developed to establish the absolute configuration of 3-hydroxyalkanoyl-HSLs by mild cleavage and chiral gas chromatography. The AHLs synthesized during this study were tested in sensor systems specific for certain AHL types. The results show that these compounds display varying responses to the respective sensors; this underlines the importance of determining the whole bouquet of AHLs and its function to fully understand their importance for regulatory functions in bacteria.

Introduction

Many bacteria utilize small diffusible compounds as signal molecules for cell–cell communication. In "quorum sensing" (QS) communication systems numerous important traits are regulated in a concentration- and, thus, cell-density dependent manner, including bioluminescence, virulence, the production of antibiotics and secondary metabolites, biofilm formation, and gene transfer.[1]

The predominant class of compounds used in species-specific QS in Proteobacteria is *N*-acylhomoserine lactones (AHLs). Produced by LuxI-type AHL synthases, these signal molecules directly bind to their cognate LuxR-type transcriptional regulator proteins, and thus activate the expression of target genes responsible for a specific response.[2] Many Proteobacteria produce not only one AHL but a pattern of these signal molecules that remains constant within a certain organism, but varies between different species.[3] Therefore, they might possess more than one LuxI/LuxR-like QS system synthesizing multiple AHLs. However, examples are known in which a single LuxI-type AHL synthase is responsible for the production of several structurally similar AHLs.[4]

The LuxI-type enzymes catalyze the reaction of *S*-adenosylmethionine (SAM) with acylated acyl carrier proteins derived from fatty acid biosynthesis.[5] The AHL synthase acylates SAM and generates the homoserine lactone (HSL) ring by lactonization and the concomitant release of methylthioadenosine.[6] This reaction results in the formation of specific AHLs that differ in length (4 to 18 carbon atoms), substitution at C-3 of the acyl chain (hydrogen, hydroxyl, or carbonyl group), and the degree of saturation. Most often, unbranched, saturated, or monounsaturated AHLs have been identified.[7] Long-chain AHLs with an unbranched side chain have often been identified in Alphaproteobacteria.[3,4,7] Recently, novel diunsaturated AHLs were described. *N*-(Hexadecadienoyl)homoserine lactone ($C_{16:2}$-HSL) was reported from *Jannaschia helgolandensis* and *Staleya guttiformis*, while *N*-(octadecadienoyl)homoserine lactone ($C_{18:2}$-HSL) has been found to occur in *Dinoroseobacter shibae*, an Alphaproteobacterium of the *Roseobacter* clade.[3] In both cases the location and configuration of the double bonds remained unknown. In the pink-pigmented methylotroph *Methylobacterium extorquens*, an epiphytic Alphaproteobacterium, a *N*-(tetadecadienoyl)homoserine lactone ($C_{14:2}$-HSL) was found to be produced under methylotrophic growth conditions by the *luxI*-like homologue *mlaI*.[8] One double bond could be located at C-2 in *E* configuration, but the location of the other remained unknown.

The majority of AHLs identified so far possess an even-numbered acyl side chain corresponding to fatty acids readily available in microbial cells. In some bacteria, AHLs with an odd number of carbon atoms in the chain have been detected, but these always occur in trace amounts together with large

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amounts of even-numbered AHLs. The short-chain C_7-HSL, which was first identified in *Rhizobium leguminosarum*[9] but found to be produced by different bacteria, for example, *Serratia marcescens*,[10] *Edwardsiella tarda*,[11] *Erwinia psidii*[12] and the plant pathogen *Pantoea ananatis*,[13] is the most abundant AHL with an odd-numbered side chain.

AHLs bearing a 3-hydroxyalkanoyl group are also common among the Proteobacteria. 3-Hydroxy-AHLs (3OH-HSL) with varying chain length were found in, for example, different strains of the *Roseobacter* clade,[3,14] *Pseudomonas fluorescens*,[15,16] and *Pseudomonas chlororaphis*,[17] as well as in two strains of the Rhizobiaceae.[18,19] An unsaturated analogue, (*Z*)-*N*-(3-hydroxy-7-tetradecenoyl)-L-homoserine lactone, was described from *Rhizobium leguminosarum*.[10] The substitution in the 3-position of the acyl chain introduces a new stereogenic center into the AHL, in addition to the one of the L-configured HSL. So far, the absolute configuration at this center has only been clarified in (3*R*,7*Z*)-*N*-(3-hydroxy-7-tetradecenoyl)homoserine lactone from *Rhizobium leguminosarum* after isolation by using chiral solvating agents and NMR spectroscopy.[20] Recently, a new QS compound was identified in the anoxygenic phototrophic soil bacterium *Rhodopseudomonas palustris*, which contained the HSL ring but not the common acyl group.[21] Instead, *p*-coumaric acid originating from lignin degradation products in the environment is conjugated to HSL.

In the present study we report the structure elucidation and synthesis of diunsaturated AHLs, clarify the absolute configuration of 3OH-C_{10}-HSL and 3OH-C_8-HSL by using chiral gas chromatography, and report a new type of AHL that carries a methyl branched acyl side chain from *Aeromonas culicicola* MTCC 3249^T. We also probe the biological activity of these AHLs in sensor systems specific for short-chain and long-chain AHLs.

Results

Identification of methyl branched AHLs

Aeromonas is a medically important genus of the γ-Proteobacteria, as different species are known to cause diseases in animals and are suspected to cause multiple infections in humans.[22–24] The expression of multifactorial virulence determinants in them is associated with high cell densities, and thus supposedly is under the control of quorum sensing.[25–27] Recently it has been found that the LuxRI homologues or QS system is universally present in this genus.[28] However, AHLs produced were only characterized in *A. hydrophila* and *A. salmonicida*[29,30] where they regulate serine and metalloprotease production,[29,31] biofilm development,[32] and butanediol fermentation.[33] *A. culicicola* 3249^T was isolated from the midgut of the haematophagous mosquito, *Culex quinquefasciatus*, where it is particularly abundant in blood-fed mosquitos.[34] Recently the presence of the QS system and LuxRI homologues, that is, AcuRI, were reported in this strain.[28] *A. culicicola* 3249^T also carries a conjugation related type four secretion machinery, which is involved in delivering virulence factors (proteins or protein–DNA complexes) to host target cells, as shown in several important human and plant pathogens.[35]

The GC-MS analysis of an extract of *A. culicicola* 3249^T revealed the presence of several compounds showing the typical fragmentation pattern of AHLs, *m/z* 143 and 102 (Figure 1).[3,36] The major AHL present in the extract was *N*-(3-hydroxyoctanoyl)homoserine lactone (3OH-C_8-HSL), which was unambiguously identified by its mass spectrum, gas chromatographic retention index, and comparison with synthetic reference samples (see the Supporting Information). Besides *N*-(octanoyl)homoserine lactone (C_8-HSL) several other AHLs with unknown structures were present in the extract. The mass spectra of two of these unknown AHLs, **A** and **B**, are presented in Figure 2.

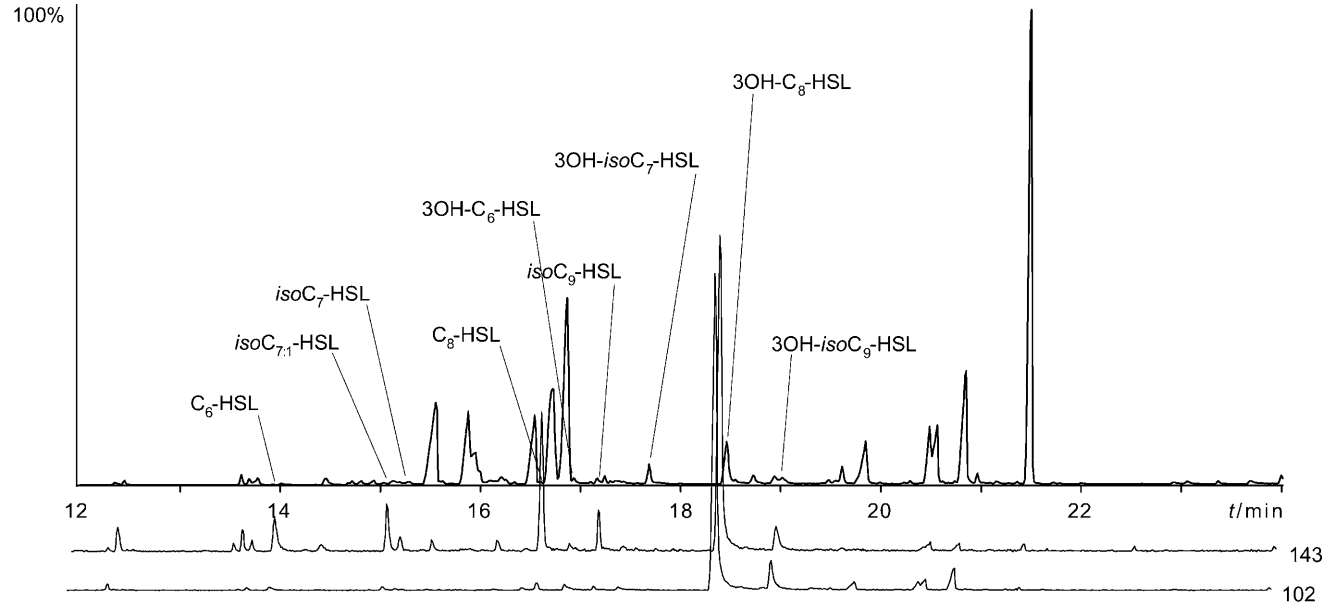

Figure 1. Total ion chromatogram of an extract of *Aeromonas culicicola* 3249^T. The characteristic ion traces *m/z* 143 and 102 (slightly offset for improved visibility) are shown and the AHLs are indicated by the labels used in Table 1.

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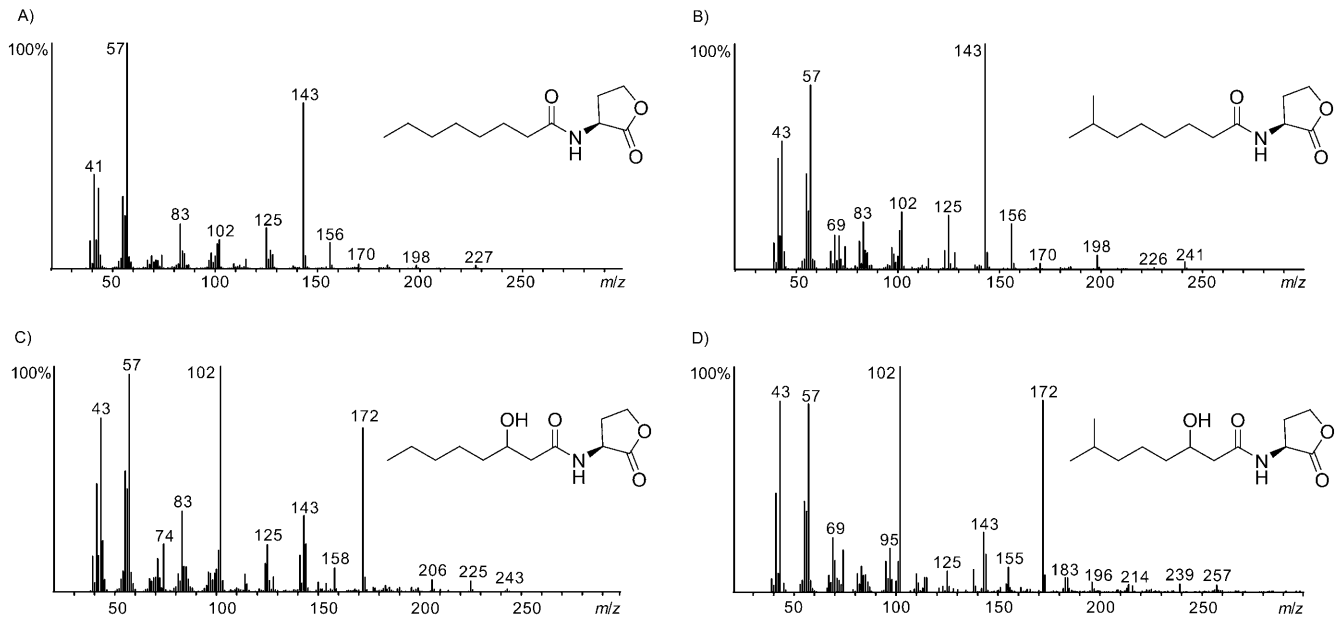

Figure 2. Mass spectra of: A) C_8-HSL, B) *iso*C_9-HSL (compound **A**), C) 3OH-C_8-HSL, D) 3OH-*iso*C_9-HSL (compound **B**).

They exhibited molecular ions at *m/z* 241 and 257, which correspond to the molecular formulas $C_{13}H_{23}NO_3$ and $C_{13}H_{23}NO_4$, respectively. These data initially suggested AHL **A** to be a higher homologue of C_8-HSL, C_9-HSL. Compound **B** was similarly assumed to be 3OH-C_9-HSL; this was supported by a prominent ion at *m/z* 172, which is specific for 3-hydroxy-AHLs.[3] A detailed analysis of the mass spectra, nevertheless, showed small but characteristic differences in the higher mass region. Compound **A** showed a $[M-43]^+$ ion, which is indicative for an acyl chain carrying a methyl at the ω-1 position. The mass spectra thus indicated that compound **A** was the *iso* compound *N*-(7-methyloctanoyl)homoserine lactone (*iso*C_9-HSL).

The presence of a methyl branch in **A** was further supported by the examination of its gas chromatographic retention index, *I*.[37] On the basis of an empirical model,[38] the retention index for long-chain methyl branched aliphatic compounds can be calculated, according to Equation (1). Here, *N* is the number of carbon atoms in the alkyl chain, FG is a functional group increment, and Me_i is an increment for each methyl group depending on the position in the chain. The values for Me_i on an apolar gas chromatographic phase have been published.[38] In general, the calculated and measured retention indices do not differ by more than 5–8 units.

$$I_c = N + \mathrm{FG} + \Sigma \mathrm{Me}_i \qquad (1)$$

The functional group increment of AHLs was determined as $FG_{AHL} = 1198$ by measuring *I* for several straight-chain AHLs with variable chain length. The calculated retention index $I_C = 2058$ for the putative *iso*C_9-HSL was obtained by adding $N = 800$ for the C_8 alkyl chain and $Me_{(\omega-1)} = 60$ for the *iso* branch to FG_{AHL}. The measured value of the unknown AHL compound ($I = 2056$) unambiguously supported our suggestion of an *iso*-methyl branched side chain. The value for the structural alternative, C_9-HSL, was calculated to be $I_C = 2098$. A similar approach revealed compound **B** to be *N*-(3-hydroxy-7-methyloctanoyl)homoserine lactone (3OH-*iso*C_9-HSL).

Then both *iso*C_9-HSL and 3OH-*iso*C_9-HSL were synthesized to confirm their putative structures. 6-Bromohexanoic acid (**1**) was transformed into the corresponding sodium salt, and alkylation with isopropylmagnesium chloride by using Li_2CuCl_4 as catalyst afforded 7-methyloctanoic acid (**2**).[39] This acid was coupled with L-homoserine lactone hydrobromide in the presence of 1-ethyl-3-(3-diaminopropyl)carbodiimide (EDC) to obtain *iso*C_9-HSL (**3**).[40] The synthesis of 3OH-*iso*C_9-HSL started from 3-bromopropanol (**4**). Alkylation with isobutylmagnesium bromide by using Li_2CuCl_4 again furnished 5-methylhexanol (**5**), which was oxidized to the corresponding aldehyde **6** by using PCC. Addition of ethyl diazoacetate under tin catalysis gave ethyl 7-methyl-3-oxooctanoate (**7**),[41] which was enantioselectively hydrogenated with a (*R*)-Ru-[Cl_2BINAP·NEt_3] catalyst[42] and simultaneously transesterified to afford methyl (*R*)-3-hydroxy-7-methyloctanoate (**8**) with an *ee* > 95%. After saponification and coupling with L-homoserine lactone hydrobromide the desired (*R*)-3OH-*iso*C_9-HSL ((*R*)-**9**) was obtained. Both synthesized compounds proved to be identical to the AHLs identified in the natural extract (Scheme 1).

A careful analysis by evaluation of the mass spectrometry data and retention times revealed the presence of additional AHLs in the *A. culicola* MTCC 3249^T extract. They are homologues of the compounds discussed so far, and contain a shorter C6 or *iso*C7 side chain (Table 1). Examination of the synthetic 3OH-AHLs revealed no signs of degradation during the analytical process. In summary, besides the major component 3OH-C_8-HSL, eight other AHLs were present in the extract.

Diunsaturated homoserine lactones

During a systematic screening of diverse bacterial strains of marine Proteobacteria for AHL activity and identification of the

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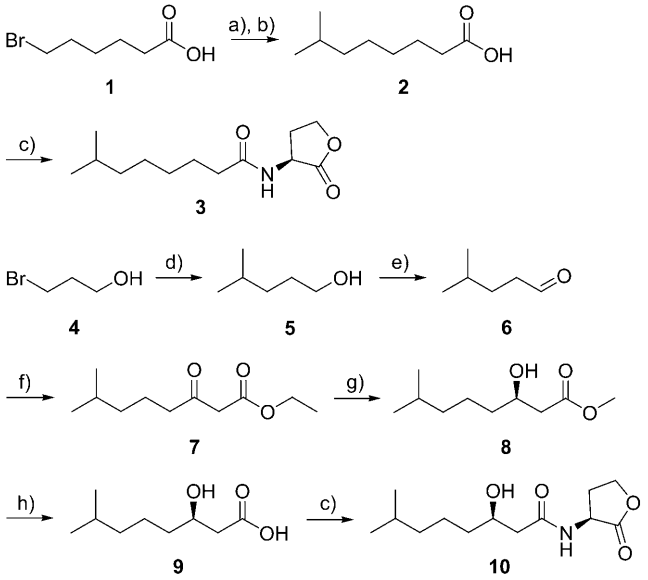

Scheme 1. Syntheses of *iso*C_9-HSL (**3**) and (*R*)-3OH-*iso*C_9-HSL (**10**): a) Na, MeOH; b) *i*PrMgCl, Li_2CuCl_4, THF; c) L-HSL, EDC, NEt_3, H_2O; d) *i*BuMgBr, Li_2CuCl_4, THF; e) PCC, CH_2Cl_2; f) $N_2CHCOOEt$, $SnCl_2$, CH_2Cl_2; g) H_2, (*R*)-Ru[Cl_2BINAP·NEt_3], MeOH; h) NaOH, EtOH.

Table 1. Acylhomoserine lactones identified in an extract of *Aeromonas culicola* 3249^T.[a]

	HSL	Identification		HSL	Identification
a	C_6-HSL	MS, *I*, syn	b	3OH-C_6-HSL	MS, *I*
c	*iso*C_7-HSL	MS, *I*, syn	d	*iso*C_7-*en*-HSL[b]	MS
e	**3OH-*iso*C_7-HSL**	MS, *I*	f	C_8-HSL	MS, *I*, syn
g	**3OH-C_8-HSL**	MS, *I*, syn	h	*iso*C_9-HSL	MS, *I*, syn
i	3OH-*iso*C_9-HSL	MS, *I*, syn			

[a] Identification based on: MS: mass spectra, *I*: gas chromatographic retention index; syn: synthetic reference compound. The major AHLs are denoted by bold typeface. Because most AHLs represent only minor or trace components of the whole extract, direct quantification in the TIC was impossible. [b] This compound was tentatively identified to be *N*-(7-methylhexenoyl)homoserine lactone with unknown location of the double bond.

individual AHLs we encountered unknown diunsaturated homoserine lactones.[3] Beside $C_{14:1}$-HSL and $C_{16:1}$-HSL, the GC-MS spectra of different isolates of *Jannaschia helgolandensis*[3] showed the presence of an unknown AHL with the expected characteristic ions at *m/z* 102 and 143, which are specific for the HSL (Figure 3). The molecular ion at *m/z* 335 ($C_{20}H_{33}NO_3$) and unspecific alkyl fragments of the series C_nH_{2n-3} dominate over the ions characteristic for AHLs; this indicates a $C_{16:2}$-HSL with two double bonds in the side chain.

An extract of *J. helgolandensis* was then derivatized with dimethyl disulfide (DMDS)[3] to localize the double bond positions in $C_{16:2}$-HSL. The mass spectrum (Figure 3) of the derivative exhibited a molecular ion at *m/z* 429, which resulted from addition of two SCH_3 groups to one double bond. The second double bond failed to react, which is typical for double bonds of α,β-unsaturated methyl esters.[43–45] Therefore, this double bond was assumed to be located at C-2. The location of the remaining double bond at C-9 could be unambiguously assigned by the fragments at *m/z* 145 $(C_7H_{14}SCH_3)^+$ and 284 (HSL-COCH=$CHC_7H_{13}SCH_3)^+$; this led to *N*-(2,9-hexadecadienoyl)homoserine lactone as the structure for this AHL.

The configuration of the double bonds was determined by comparison with a synthetic sample. While the double bond at C-2 was assumed to be *trans* configured as is usually found in similar acids in biological systems, the C-9 double bond seemed to be *cis* configured. Therefore, $C_{16:2}$-HSL was synthesized according to Scheme 2. 1,7-Heptanediol (**13**) was monoprotected with 3,4-dihydro-2*H*-pyran,[46] and oxidized with PCC to the corresponding aldehyde **14**. The following *Z*-selective Wittig reaction with heptyltriphenylphosphonium bromide furnished (*Z*)-7-tetradecen-1-ol (**15**) after deprotection in a 97:3 *Z/E* ratio.[47] Oxidation set the stage for the following Horner–Wadsworth–Emmons reaction to yield ethyl (2*E*,9*Z*)-2,9-hexadecadienoate (**17**), which was saponified to the free acid. The acid was finally coupled with L-homoserine lactone hydrobromide in the presence of EDC to furnish (2*E*,9*Z*)-*N*-(2,9-hexadecadienoyl)-L-homoserine lactone (**11**). This AHL proved to be identical to the natural compound and thus established the configuration of the two double bonds. The separation of all four geometrical isomers, which were formed as minor byproducts during the Wittig and Horner–Wadsworth–Emmons reactions, was possible with GC. Only the 2*E*,9*Z* compound **11** proved to be identical to the natural component in all aspects; this firmly established the proposed structure.

A $C_{18:2}$-HSL is present in several *Dinoroseobacter shibae* strains of the *Roseobacter* clade, but the low concentration has made double bond localization impossible.[3]

Determination of the absolute configuration of *N*-(3-hydroxyalkanoyl)homoserine lactones

Since the first identification of 3OH-C_4-HSL in the marine bacterium *Vibrio harveyi*, many other 3-hydroxy-AHLs have been reported.[1] Until now the absolute configuration of the hydroxyl group at C-3 of the side chain has only be determined for the "small bacteriocin" from *Rhizobium leguminosarum*.[21] Isolation of the pure AHL followed by NMR spectroscopy experiments with chiral solvating agents assigned this compound to be (3*R*,7*Z*)-*N*-(3-hydroxy-7-tetradecenoyl)homoserine lactone. Because this procedure is difficult and requires relatively large amounts of the target molecule, a rapid method by using chiral GC was developed to elucidate the absolute configuration of 3-hydroxy-AHLs.

Therefore, the natural extract of *A. culicicola* containing 3OH-C_8-HSL (**18**) as major AHL compound was analyzed. Direct separation of AHLs on a chiral GC phase proved to be unsuccessful. Therefore, the crude extract was treated with H_2SO_4 in methanol to cleave the AHL into the corresponding methyl ester and HSL. This procedure did not affect the stereogenic center at C-3 of the acyl side chain. Both racemic and enantiomerically pure methyl 3-hydroxyoctanoate were synthesized by using the same strategy as described above for the synthesis of **10** (see the Supporting Information). The gas chromatographic investigation of the synthetic esters and the derivatized natural extract on a chiral hydrodex-6-TBDMS phase

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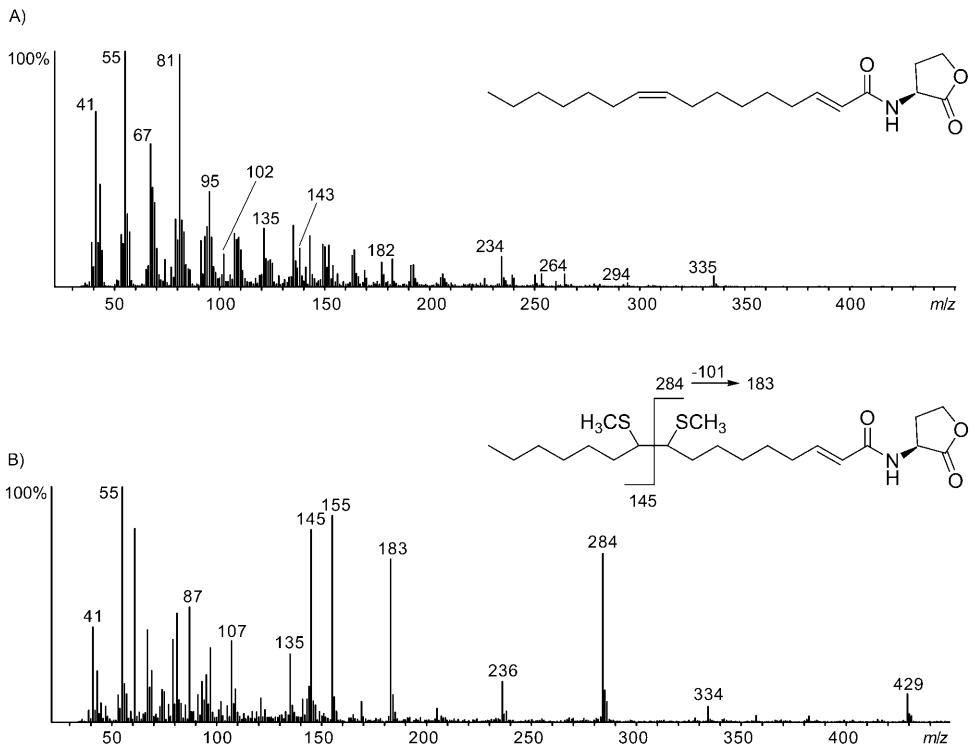
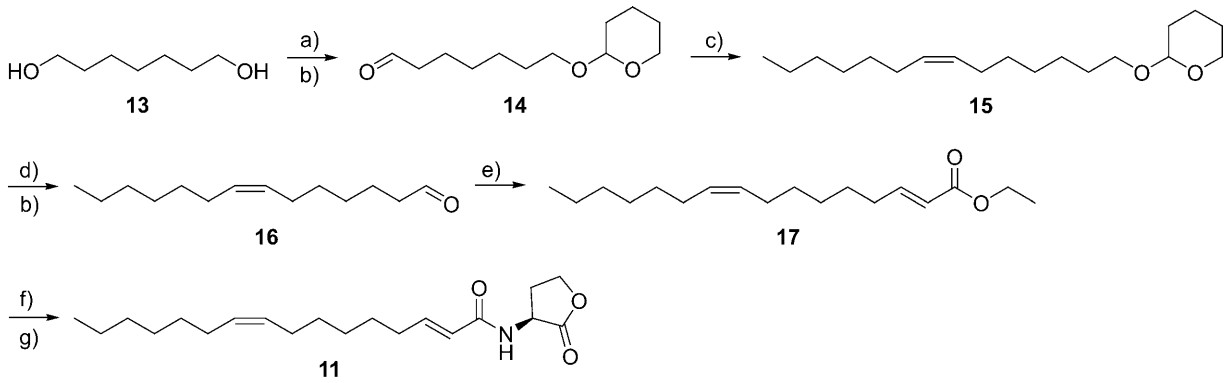


Figure 3. Mass spectra of: A) $C_{16:2}$-HSL, and B) DMDS adduct of $C_{16:2}$-HSL.

Nevertheless, the activity of a certain AHL species on sensor systems specific for another AHL type is not well evaluated. Therefore, the synthesized AHLs were analyzed for their activity in AHL-mediated QS. Two green fluorescent proteins (GFPs) equipped reporter strains[35,49] capable of sensing AHLs with a certain acyl chain length and substitution were used. *Escherichia coli* MT102 (pJBA132) is a reporter for AHLs with short acyl chains and exhibits the highest sensitivity for *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C_6-HSL)[34] while *Pseudomonas putida* F117 (pKR-C12) is a reporter that is most sensitive for 3-oxo-C_{12}-HSL.[50] A very similar activity is shown with C_{12}-HSL, which was used as a reference in this study. The maximum induction of fluorescence attained during the test period of 24 hours by different AHLs was recorded and the results are shown in Figure 5 (see also Tables S1 and S2 in the Supporting Information).



Scheme 2. Synthesis of (2*E*,9*Z*)-$C_{16:2}$-HSL (**11**): a) 3,4-dihydro-2*H*-pyran, *p*TsOH, CH_2Cl_2; b) PCC, CH_2Cl_2; c) $[Ph_3PC_7H_{13}]Br$, BuLi, THF; d) *p*TsOH, MeOH; e) $(CH_3CH_2O)_2POCH_2COOCH_2CH_3$, NaH, DME; f) NaOH, EtOH; g) HSL, EDC, NEt_3, H_2O.

showed that the naturally occurring compound is enantiomerically pure (*R*)-3OH-C_8-HSL (Figure 4). Similarly, 3OH-C_{10}-HSL found in an extract of *Phaeobacter gallaeciensis* T5, which is a strain of the *Roseobacter* clade that can produce the antibiotic tropodithietic acid[5] and can decrease the population of other bacteria under natural conditions,[48] proved to be *R* configured as well.

Activity of AHLs in quorum sensing or quorum quenching experiments with biosensor strains

We were then interested to determine how a methyl branched or a dienoic acyl chain could modify the activity of the identified AHLs on known LuxR receptors. This is of particular interest because a diverse structural array of AHLs is now known.

Activation of fluorescence of sensor plasmid pJBA132 with compounds having modified C_8 side chains was observed only with concentrations at least 100-fold higher than 3-oxo-C_6-HSL, which is highly active even at a concentration of 0.025 $\mu g\,mL^{-1}$. While *iso*C_9-HSL is inactive, an oxygen atom at C-3 of the acyl side chain increased activity, as can be seen by the higher reactivity of 3OH-C_8-HSL. The absolute configuration seems to have an effect on the reactivity. The unnatural (*S*)-3OH-C_8-HSL is more active than the natural (*R*)-3OH-C_8-HSL. Most surprising is the finding that the additional methyl group in 3OH-*iso*C_9-HSL increases reactivity, despite the fact that this structure is less similar to 3-oxo-C_6-HSL than 3OH-C_8-HSL. The

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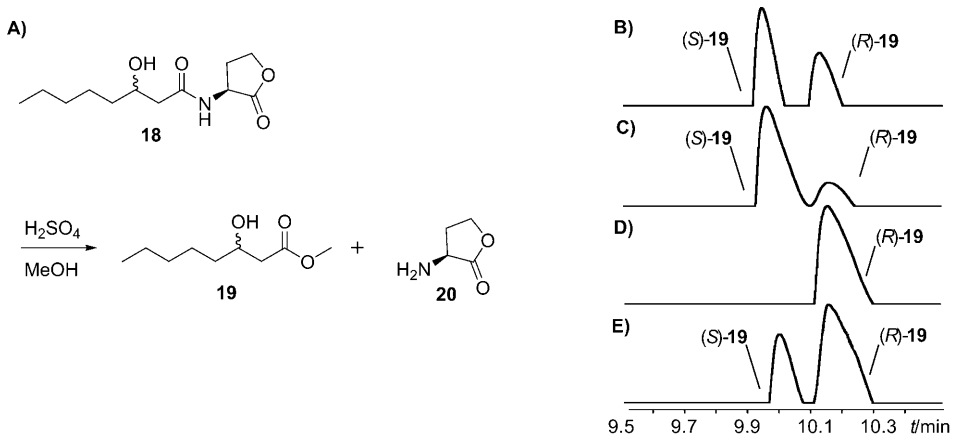

Figure 4. Enantiomer analysis of 3OH-C_8-HSL: A) derivatization procedure. Gas chromatographic analysis of: B) a racemic mixture of (*S*)-**19** and (*R*)-**19** on a hydrodex-6-TBDMS column, C) coinjection of *rac*-**19** and (*S*)-**19**, D) extract of *Aeromonas culicicola* after treatment with H_2SO_4/MeOH, E) coinjection of B) and D).

unmodified parent compound C_8-HSL has the highest reactivity. Increasing the chain length further, for example, as in 3OH-C_{10}-HSL, decreased the reactivity. Also unexpected is the relatively high activity of (2*E*,9*Z*)-$C_{16:2}$-HSL, which has a very long side chain.

The response of the sensor plasmid pKR-C12 to the investigated AHLs is at least ten-times lower than that of the best inducing compound, C_{12}-HSL. Here, the hydroxy group seems to have a negative effect on activity, because 3OH-C_8-AHLs are less active than the respective C_8-AHL. The long chain (2*E*,9*Z*)-$C_{16:2}$-HSL shows the best induction. This effect can be attributed to the chain length, which might better match this receptor than the shorter modified C_8-AHLs, except for 3OH-C_{10}-AHL. Contrary to results with pJBA132, no effect of the stereochemistry at C-3, which was investigated by the two enantiomers (*R*)- and (*S*)-3OH-C_8-AHL, was observed.

Besides investigating their functionality as QS inducers, the compounds were also evaluated for their ability to inhibit the induction of fluorescence obtained by the natural ligands of the AHL signaling system. In the presence of 3-oxo-C_6-HSL (0.25 $\mu g\,mL^{-1}$) fluorescence with pJBA132 was reduced to about 50% only with very high concentrations (250 $\mu g\,mL^{-1}$) of the hydroxy-AHLs (*R*)-3OH-C_{10}-HSL, (*S*)- and (*R*)-3OH-C_8-HSL, 3OH-*iso*C_9-HSL as well as the branched *iso*C_9-HSL. By use of pKR-C12 in the presence of C_{12}-HSL (0.25 $\mu g\,mL^{-1}$) appreciable reduction of fluorescence was found only after addition of 25 $\mu g\,mL^{-1}$ (*S*)- or (*R*)-3OH-C_8-HSL.

Discussion

The presented results show for the first time the presence of methyl-branched AHLs in the AHL bouquets of bacteria (bacterial quorum sensing systems). This finding is not surprising because bacteria are well-known for the abundant biosynthesis of methyl branched fatty acids using amino acid derived building blocks as starters in fatty acid biosynthesis.[51] The work also shows the power of GC-MS methods in the identification of unknown AHLs. Because of the similarity in EI- and most likely also in ESI-mass spectrometry the verification of compound identity by chromatographic methods by using retention times or direct comparison seems to be mandatory.

The structure of a long-chain dienoic AHL has been described here fully for the first time, and shows the usefulness of the well-known DMDS-derivatization method for localization of double bonds also in this type of fatty acid derivative. Furthermore, a simple method was established that allowed the determination of the absolute configuration of 3-hydroxyacyl-HSLs in complex mixtures. The pattern of AHLs found in *A. culicula* increases the number of AHLs known from this genus. So far, C_4-HSL, C_6-HSL, C_8-HSL, C_{12}-HSL, and C_{14}-HSL have been reported from *A. hydrophila* and *A. salmonicida*.[29,30]

Our data provide clear evidence that the reported unusual AHLs can stimulate the tested QS systems. The required concentrations are usually about tenfold higher than those of the specific AHLs. Even small structural differences, for example, an additional methyl group, can have a profound effect on the activation of the AHL receptor protein. The LuxR proteins encoded by the sensor plasmids used here are not the natural receptors for the studied unusual AHLs; thus, these effects might be even stronger for the cognate LuxR proteins of the investigated bacteria. In such a way, differences in the composition of the AHLs produced by a bacterial strain might modulate the response of the quorum sensing regulon.

The now evident enhanced structural diversity can be reflected in different responses of bacteria towards exogenous AHLs. The genetic response to AHLs can vary widely between Gram-negative bacteria.[52] Previous studies on the structural diversity of AHL-related compounds did not address the type of structural variation presented in this report,[53] but also showed large differences in the activity between structural analogues of AHLs.

Experimental Section

Culture conditions and extraction of AHLs: The various *Roseobacter* strains were cultivated and extracted as described before.[3] Homogeneity of the *A. culicicola* 3249^T culture was determined by sequencing of the nearly complete 16S rDNA gene (1525 bases) and its subsequent homology search in NCBI by using the BLAST search. The putative AHLs were extracted and purified in larger quantities from *A. culicicola* 3249^T culture supernatants in order to deduce the chemical structures as described earlier.[54] The supernatant from a stationary-phase culture (15 L) of *A. culicicola* 3249^T grown in M9 medium (Sigma) containing acid hydrolyzed casein (0.2%; Oxoid) at 30 °C and 225 rpm, were extracted three times with dichloromethane (supernatant/dichloromethane 700:300). The solvent was removed by a rotary evapoarator at 30 °C and a 2 g extract was obtained. The extract was dissolved in a minimum

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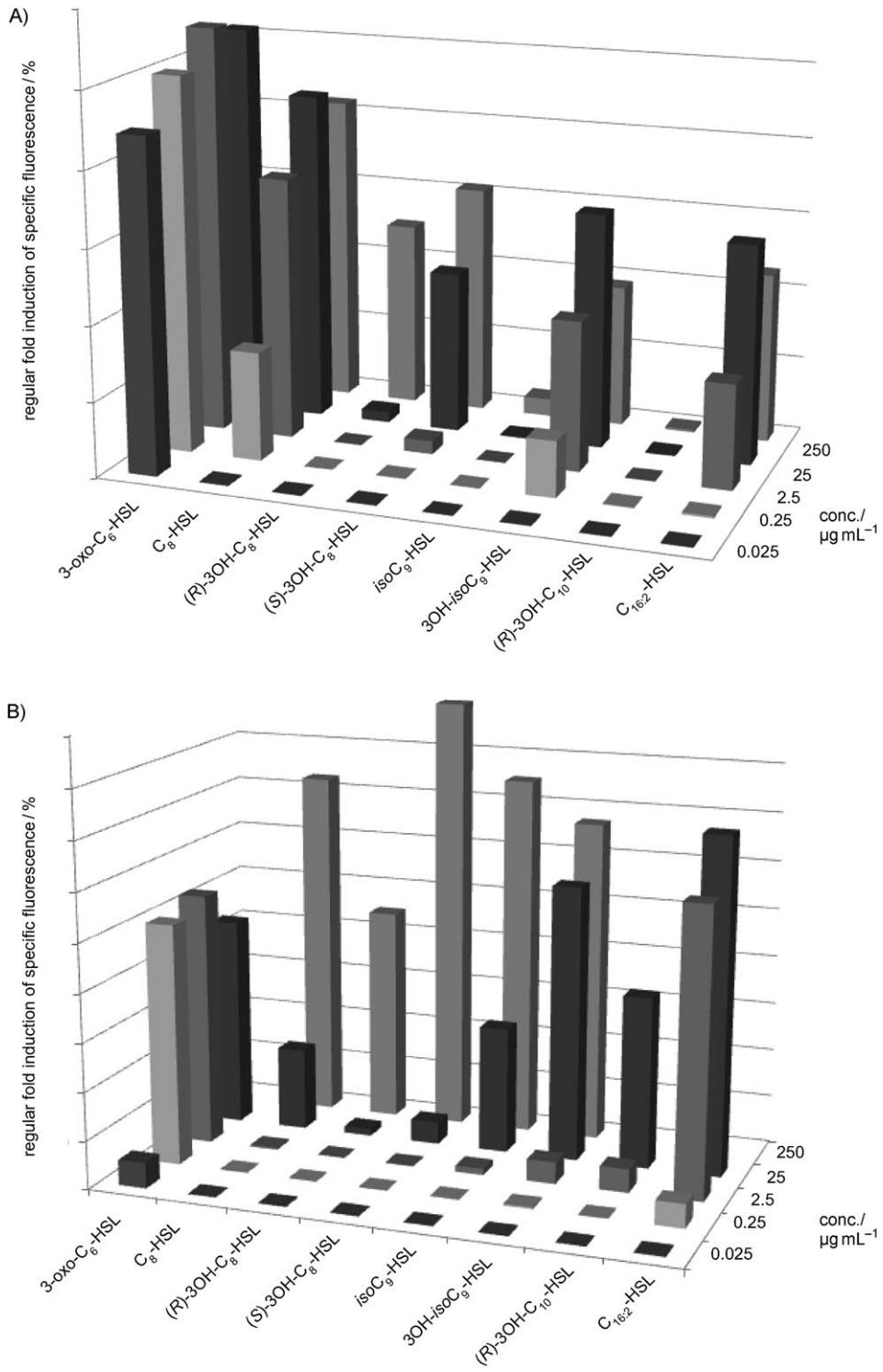

Figure 5. Relative fold induction of sensors: A) pJBA132, which is most sensitive for *N*-(3-oxohexanoyl)homoserine lactone (3-oxo-C_6-HSL), and B) pRK-C12, which is most sensitive for C_{12}-HSL. Fold induction in the presence of either 0.25 µg mL^{-1} 3-oxo-C_6-HSL or C_{12}-HSL, respectively, was arbitrarily set to 100 %.

volume of dichloromethane and filtered through a Whatman 2 V filter paper for further characterization.

GC-MS analyses: GC-MS analyses were carried out by using a HP 6890 Series GC system connected to a HP 5973 mass selective detector (Hewlett–Packard) fitted with a BPX5 fused-silica capillary column (25 m × 0.22 mm i.d., 0.25 µm film; SGE, Inc., Melbourne, Australia). Conditions were as follows: inlet pressure 77.1 kPa, He 23.3 mL min^{-1}, injection volume 2 µL, transfer line 300 °C, electron energy 70 eV. The GC was programmed as follows: 5 min at 100 °C increasing at 10 °C min^{-1} to 300 °C, and operated in splitless mode (60 s valve time). The carrier gas was He at 1 mL min^{-1}. Retention indices (*I*) were determined from a homologous series of *n*-alkanes (C_8–C_{25}).

Chiral gas chromatography: Separation of the enantiomers was carried out by using an 8000Top GC (ThermoQuest, Toronto, Canada) instrument fitted with a hydrodex-6-TBDMS column (35 m, 0.25 mm i.d.; Macherey–Nagel) and a FID. Conditions were as follows: inlet pressure 30 kPa, H_2 20 mL min^{-1}, injection volume 1 µL. The GC was programmed as follows: 1 min at 130 °C increasing at 0.5 °C min^{-1} to 180 °C. The carrier gas was H_2 at 1 mL min^{-1}.

Biological assays: The AHL biosensor strains *E. coli* MT102 (pJBA132) and *P. putida* F117 (pRK-C12) were grown in LB medium with tetracycline (25 µg mL^{-1}) at 37 °C and with gentamycin (20 µg mL^{-1}) at 30 °C, respectively. Overnight cultures (50 mL) of the strains were diluted with the same volume of corresponding fresh medium, incubated for about 1 h and adjusted to an OD_{620} of 1.0. Aliquots (100 µL) of these cultures were pipetted into wells of 96-well microtiter plates, which contained LB medium (90 µL), methanol (5 µL), and the test compounds (5 µL; 0.025 µg–0.25 mg mL^{-1}) to evaluate the compounds for QS activity. In the assays used for testing the compounds for inhibitory activity of QS, instead of methanol (5 µL) the wells contained the corresponding AHL (3-oxo-C_6-HSL for *E. coli* MT102, C_{12}-HSL for *P. putida* F117) at a concentration of 0.25 µg mL^{-1}. Samples containing only the corresponding AHL without test compound were used as positive control, and those containing solvent (10 µL) only were used as negative control. The microtiter plates were incubated at 30 °C while being shaken. After 0, 4, 8, and 24 h growth at an OD_{620}, fluorescence (excitation wavelength 485 nm, detection wavelength of 535 nm) was determined by using a Victor 1420

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Multilabel counter (Perkin–Elmer). Fold induction of fluorescence in the test samples, which were prepared at least in triplicate, was calculated by dividing their specific fluorescence (GFP_{535}/OD_{620}) by the specific fluorescence of the negative control. Relative activity was calculated by comparing the fold induction of positive control samples (only AHL) to samples containing only test compounds, and samples with test compounds and the corresponding AHL, respectively.

Synthesis procedures: See the Supporting Information.

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Keywords: *Aeromonas* · cell recognition · pheromones · quorum sensing · *Roseobacter*

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