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Discovery of Complex Mixtures of Novel Long-Chain Quorum Sensing Signals in Free-Living and Host-Associated Marine Alphaproteobacteria

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More than 100 bacterial isolates from various marine habitats were screened for AHL production by using gfp *reporter constructs based on the* lasR *system of* Pseudomonas aeruginosa *and the* luxR *system of* Vibrio fischeri. *Of the 67 Alphaproteobacteria tested, most of which belonged into the so-called* Roseobacter *clade, 39 induced fluorescence in either one or both sensor strains up to 103-fold compared to controls. Acylated homoserine lactones were identified by GC-MS analysis and shown to have chain lengths of C_8, C_{10}, C_{13}–C_{16}, and C_{18}. One or two double bonds were often present, while a keto or hydroxyl group occurred only rarely in the side chain. Most strains produced several different AHLs. C_{18}-en-HSL and C_{18}-dien-HSL were produced by* Dinoroseobacter shibae, *an aerobic anoxygenic phototrophic bacterium isolated from dinoflagellates, and are among the longest AHLs found to date. Z7-C_{14}-en-HSL, which has previously been detected in* Rhodobacter *sphaeroides, was produced by* Roseovarius tolerans *and* Jannaschia helgolandensis. *This signal molecule was synthesised and shown to induce a similar response to the culture supernatant in the respective sensor strain. The widespread occurrence of quorum-sensing compounds in marine Alphaproteobacteria, both free-living strains and those associated to eukaryotic algae, points to a great importance of this signalling mechanism for the adaptation of the organisms to their widely different ecological niches.*

Introduction

Bacteria secrete small signal molecules called autoinducers into the medium to regulate gene expression in a concentration- and thus population-density-dependent way. This type of cell-to-cell communication, known as quorum sensing, was discovered as the regulatory mechanism responsible for luminescence in the marine symbiont *Vibrio fischeri*, but is now known to control widely different traits, including the expression of virulence factors, production of antibiotics and the formation of biofilms.[1,2]

In Gram-negative proteobacteria, *N*-acyl homoserine lactones (AHLs) represent a group of well-studied autoinducers. AHLs directly bind to their cognate LuxR-type transcriptional regulators with high specificity, which is determined by the length and substitution of the acyl side chain. The pattern of AHL molecules produced by a certain organism is stable, but different species produce different patterns of AHLs. Thus, AHLs have been thought to mediate species-specific signalling. Evidence is now accumulating that they can also mediate interspecies communication.[3,4]

AHLs are present in culture media at nanomolar concentrations or less, and thus their identification by chemical methods requires large quantities of culture supernatant, solvent extraction and concentration. Therefore, for screening purposes, biosensor strains are usually used that do not produce AHLs on their own but are capable of sensing their presence through the expression of a LuxR-controlled promoter fused to a gene coding for an easily detectable output signal, for example, bioluminescence (*lux* genes), fluorescence (*gfp*) or β-galactosidase production. Although the sensitivity of these constructs matches the physiological concentrations of AHLs, their chemical identification remains a problem.

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The marine environment is also the source *V. cholerae*, *V. harveyi* and several other well-studied *Vibrio* strains, which heavily rely on autoinducer-mediated transcriptional control. However, apart from these examples, only very little information on AHL production in marine bacteria is available. Interestingly, however, in the free-living marine bacterium *Rhodobacter sphaeroides*, a quorum-sensing system was identified that utilises an unusual AHL molecule with a C_{14} side chain and a double bond at C-7—(*Z*)-*N*-(tetradec-7-enoyl)homoserine lactone.[5] By knocking out the AHL synthase *cerI*, a mutant was obtained that was defective in AHL production and showed increased exopolysaccharide production. Thus, AHL production in *R. sphaeroides* is required to prevent aggregation during growth. Recently, C_{16}-homoserinelactone (HSL) was detected in culture supernatants of the related marine species *R. capsulatus* by aid of a radiotracer method. This quorum-sensing system was shown to be required for synthesis of a phage-like gene-transfer agent.[6] A screening of 43 strains isolated from marine snow based on the *Agrobacterium tumefaciens* reporter system identified four strains from the *Roseobacter* group that produced AHLs.[7] AHL production was also found in marine sponges, and a screening of 11 bacterial isolates detected one *Roseobacter*-group-related strain that stimulated the *Chromobacterium violaceum* and *Agrobacterium tumefaciens* reporter systems.[8]

Here we report a systematic screening of a large number of ecologically diverse and phylogenetically well-characterised isolates from different marine habitats for AHL activity using reporter strains, followed by nontarget GC-MS analysis, identification of the AHLs and chemical synthesis. The reporter strains used here carry sensor plasmids with *luxR-gfp* transcriptional fusions[3,12]. The luxR receptor protein initiates transcription of green fluorescent protein (GFP) and thus fluorescence of the cell only after binding of a sufficient amount of its cognate AHL signal molecule (Figure 1). The luxR receptor protein is highly specific for a certain chain lengths and side-chain substitution of AHLs, and thus determines the specificity of the reporter strain, that is, the detection window. We used a long-chain reporter (*P. putida* F117 (pRK-C12) and a short-chain reporter (*E. coli* MT102 (pJBA132). Other known quorum-sensing molecules, for example, autoinducing peptides, autoinducer-2, bradyoxetin and *Pseudomonas* quinolone signal (PQS), do not elicit a response in these reporter strains. We used a new method to extract AHLs from culture supernatants that is based on their adsorption to resins during growth and has previously been used to extract secondary metabolites from cultures of myxobacteria.[9] Surprisingly, we found long-chain AHLs, which are usually difficult to detect in culture fluids because they tend to be retained within the cells, in more than half of the Alphaproteobacteria investigated (59%). AHLs were produced by strains isolated from such diverse habitats as dinoflagellates, marine snow, picoplankton and sediments. Overlapping patterns of AHLs were present in distantly related genera. The analytical constraints on the detection of quorum-sensing compounds might be responsible for a currently biased view of this mechanism, which might be ubiquitous and not necessarily restricted to symbiotic (and pathogenic) interactions.

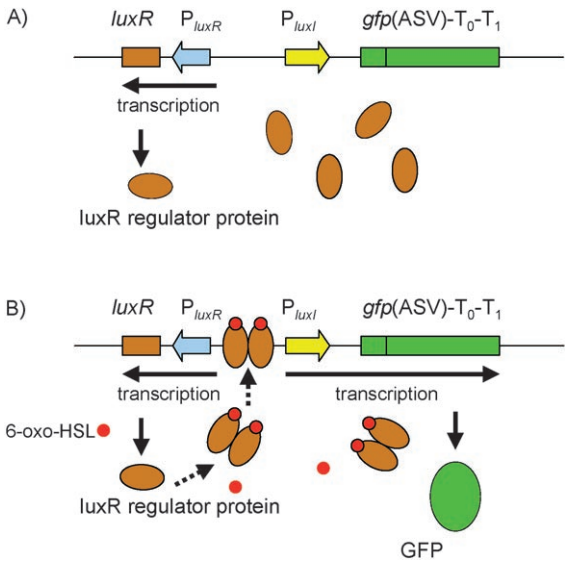


Figure 1. Schematic view of the functioning of the AHL sensor plasmids used in this investigation. A) In the absence of AHLs, the luxR regulatory protein is transcribed from its promoter P_{luxR}, but cannot fold into its active form. Thus, transcription of the *gfp* gene from the AHL-controlled promoter P_{luxI} does not take place, and green fluorescent protein (GFP) is not synthesised. B) In the presence of (exogeneously added) AHLs, which match the specificity of the luxR regulator protein, the luxR protein binds the AHL tightly and forms an active dimer, which initiates transcription from the quorum-sensing-controlled promoter P_{luxI}. Thus, the GFP is synthesised and fluorescence starts. In plasmid pKR-C12, the regulator lasR from *Pseudomonas aeruginosa* is used, which optimally binds 3-oxo-C12-HSL, while plasmid pJBA132 carries the luxR gene from *Vibrio fischeri*, which is specific for 6-oxo-C6-HSL.

Results and Discussion

Isolation and identification of marine strains

The 102 marine strains investigated here were isolated from diverse habitats of the North Sea, including dinoflagellate cultures (strain designation DFL), picoplancton (PIC), a water column sample (HEL), laminaria surfaces (LM) and diatoms (DT). Details of the isolation procedure can be found in Allgaier et al.[10] In addition, several isolates from the German Wadden Sea (e.g. strain T5), several strains of *Roseovarius tolerans* from the hypersaline Ekho Lake, Antarctica (strain designation EL)[11] and some strains from the *Roseobacter* group were tested. Strains were identified by sequencing the 16S rRNA gene as described.[10] Of these strains, 14 were Gammaproteobacteria, 65 Alphaproteobacteria, 18 belonged into the bacteroidetes phylum, three were actinobacteria and two were firmicutes. A complete list of all the tested strains including all the negative ones, their phylogenetic affiliation and the results of the bioassays can be found in Table S1 of the Supporting Information.

Detection window of the biosensor strains

The response of the biosensor strains to synthetic AHLs showed a saturation curve. Above the optimum concentration, inhibition of fluorescence induction was observed. Figure 2

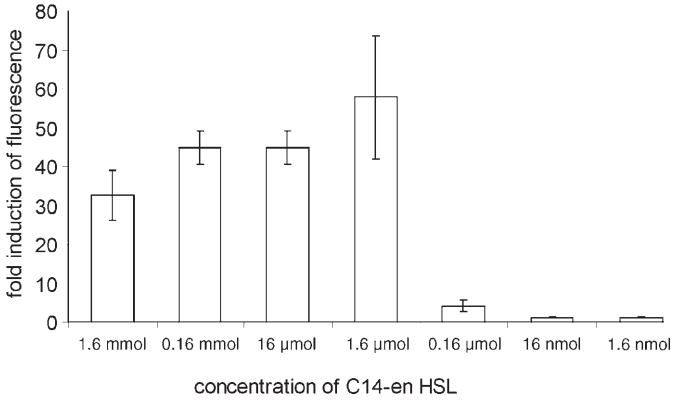


Figure 2. Effect of AHL concentration on maximum induced fluorescence (fold induction, see Experimental Section for details of calculation) in the sensor strain *P. putida* F117 (pRK-C12). The AHL tested was synthetic (*Z*)-*N*-(7-tetradecenoyl)homoserine lactone (C_{14}-en-HSL). Error bars indicate standard deviation between triplicate samples of the same extract.

shows the maximum response of the long-chain biosensor strain *P. putida* F117 (pRK-C12) to synthetic Z7-C_{14}-en-HSL. Saturation occurred at 1.6 µmol.

The detection limit of the biosensor strains was determined by testing synthetic AHLs with acyl chains between C_4 and C_{14} in length at concentrations from 0.5 mg mL^{-1} down to 0.5 ng mL^{-1} in steps of tenfold dilution. Table 1 shows that the long-chain biosensor strain has the highest sensitivity for C_{12}-HSL and is able to detect a concentration of 17.6 nmol. This corresponds to an amount of 3.5 pmol that needs to be present in the assay. The short-chain biosensor strain has its highest sensitivity for 3-oxo-C_6-HSL, detecting a concentration of 23.5 nmol, which corresponds to an amount of 4.7 pmol of AHL per assay.

Sensitivity drops dramatically, if the chain length or substitution is modified. For example, *P. putida* F117 (pRK-C12) requires tenfold higher concentrations to detect 3-oxo-C_6-HSL or Z7-C_{14}-en-HSL than its optimal substrate and is insensitive to C_4-HSL. Similarly, *E. coli* MT102 pJBA132) requires tenfold higher concentrations for C_8-HSL and 100-fold higher concentrations for C_{12}-HSL than its optimal substrate. It is also practically insensitive to C_4-HSL.

It has been shown previously that these biosensor strains are much more sensitive for the 3-oxo-substituted than for the unsubstituted HSL.[4,12] Interestingly, the only oxo-substituted HSL found here was 3-oxo-C_{14}-HSL present in two strains of *R. tolerans* and accompanied by unsubstituted C_{14}-HSL as well as unsaturated analogues. No 3-oxo-C_6-HSL was found despite the high sensitivity of the short-chain biosensor strain for this HSL.

Table 1. Detection limit of the sensor strains *P. putida* F117 (pRK-C12) and *E. coli* MT102 (pJBA132) for synthetic AHLs with chain lengths from C_8 to C_{14}.[a]

AHL	*P. putida* (pRK-C12)	*E. coli* (pJBA132)
C_4	> 2.9 mmol	2.9 mmol
3-oxo-C_6	0.2 mmol	23.5 nmol
C_8	2.2 mmol	220 nmol
C_{12}	17.6 nmol	1.76 µmol
Z7-C_{14}-en	161 nmol	160 nmol

[a] The lowest concentration that elicited a response greater than twofold induction of fluorescence is indicated. Tested AHL concentrations ranged from 0.5 mg mL^{-1} to 0.5 ng mL^{-1}.

Initially, pure aqueous culture supernatants were tested and resulted in detection of activity for only one isolate (data not shown). Subsequently, Amberlite XAD-16 was added to the culture medium during cultivation. Extraction of the resin with methanol resulted in 50-fold concentrated extracts, which were used for bioassays and chemical analyses. Figure 3 shows

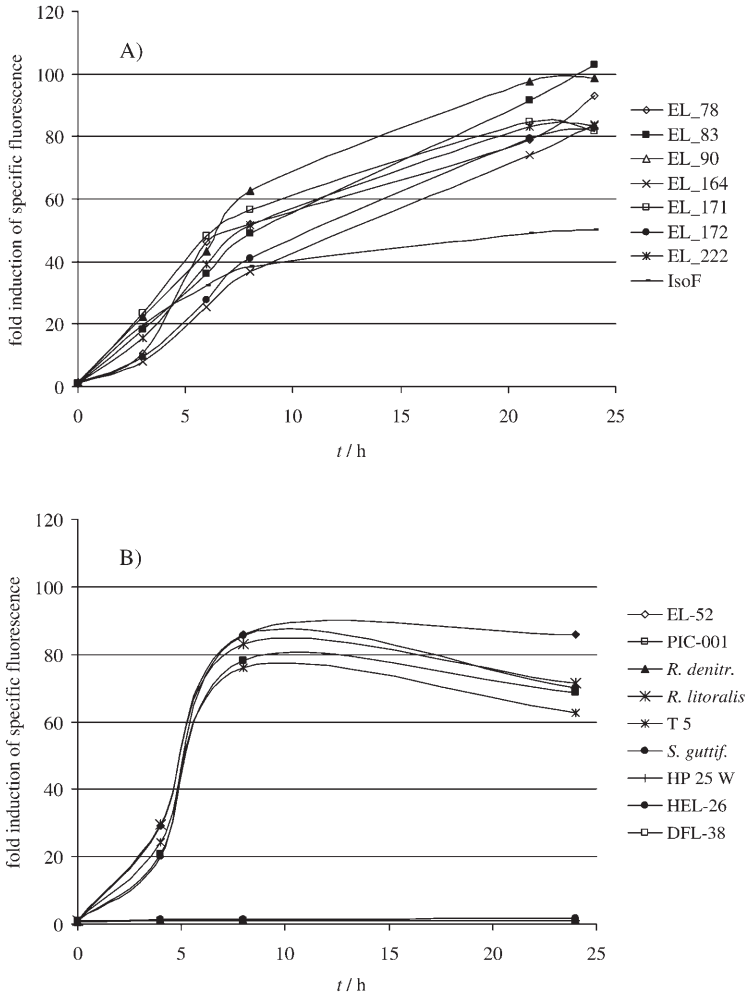


Figure 3. Example for induction of fluorescence by culture supernatants of marine isolates (see Experimental Section for details of extract preparation) with the sensor strain *P. putida* F117 (pRK-C12). Data points show the average fold induction calculated from triplicate samples of the same extract. See Tables 2 and S1 for strain designations.

Screening of marine isolates with biosensor strains

Culture supernatants from all strains were tested for induction of fluorescence with both biosensor strains.

examples of the biological screening results. In Figure 3 A, induced fluorescence in the long-chain sensor strain *P. putida* F117 (pRK-C12) is shown for AHL extracts from seven strains of *Roseovarius tolerans* as well as *Pseudomonas putida* IsoF. All strains of *R. tolerans* had similar activity, which was up to twofold higher than for AHL extracts of *P. putida* Iso F. In this organism, Steidle et al.[4] found production of small amounts of 3-oxo-C_6-HSL and large amounts of 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL. None of these compounds was detected in any of the *Roseovarius* strains by chemical analysis (see below). In Figure 3 B, the results of the analysis of several other *Roseobacter* clade isolates and strains are given. The maximum fold induction for strain EL-52 was in the same range as for the other *Roseovarius tolerans* isolates. In addition, strong activity was detected in the extracts of *Roseobacter litoralis*, strains T5, HEL-26 and DFL-38, but not in *Roseobacter denitrificans*, *Staleya guttiformis*, strains PIC-001 and HP 25 W.

Table 2 shows the complete list of strains that showed more than twofold induction of fluorescence in the bioassays, their phylogenetic affiliation based on 16S rRNA gene sequencing and the maximum fold induction observed. Of the 102 tested strains, 41 showed clear induction of fluorescence in one or both biosensor strains. In particular, 38 of the 65 Alphaproteobacteria investigated, that is, 59%, showed positive sensor sig-

Table 2. Results of bioassays, phylogenetic affiliation and homoserine lactones identified in marine bacteria. Species identification was based on BLAST searches of almost complete 16S rRNA gene sequences and is unambiguous (100%) only for strains of a type species. In all other cases, the isolated strains were schematically assigned to a described species if similarity values $\geq$97% were obtained. For similarity values $\leq$97% the most similar genus is indicated.

	Species	strain	% identity	max. fold ind. (pRK-C12)	max. fold ind. (pJBA132)	Acyl side chains of homoserine lactones
Alphaproteobacteria						
1	*Dinoroseobacter shibae*	DFL 12^T	100	48.7	$<$2.0	n.d.[b]
2	*Dinoroseobacter shibae*	DFL 16	100	55.2	$<$2.0	C_8, C_{18}-dien
3	*Dinoroseobacter shibae*	DFL 27	100	37.7	$<$2.0	C_{18}-en, C_{18}-dien
4	*Dinoroseobacter shibae*	DFL 30	100	33.3	$<$2.0	C_{18}-en, C_{18}-dien
5	*Dinoroseobacter shibae*	DFL 31	100	24.8	$<$2.0	C_{18}-en, C_{18}-dien
6	*Dinoroseobacter shibae*	DFL 36	100	70.7	$<$2.0	C_{18}-dien
7	*Dinoroseobacter shibae*	DFL 38	100	78.1	$<$2.0	n.d.[b]
8	*Hoeflea phototrophica*	DFL 33	100	17.2	$<$2.0	n.d.[b]
9	*Hoeflea phototrophica*	DFL 13	100	59.2	$<$2.0	n.d.[b]
10	*Hoeflea phototrophica*	DFL 42	100	7.5	$<$2.0	n.d.[b]
11	*Hoeflea phototrophica*	DFL 43	100	4.3	$<$2.0	n.d.[b]
12	*Hoeflea phototrophica*	DFL 44	100	6.4	$<$2.0	n.d.[b]
13	*Roseovarius mucosus*	DFL 35	100	39.7	4.8	C_{18}-en
14	*Roseovarius mucosus*	DFL 24	100	33.4	5.3	C_{14}-en, C_{18}-en
15	*Roseovarius tolerans*	EL 52	100	61.8	2.5	C_{14}, C_{14}-en
16	*Roseovarius tolerans*	EL 78	100	93.0	8.0	C_{14}, C_{14}-en, 3-oxo-C_{14}-en
17	*Roseovarius tolerans*	EL 83	100	103.1	8.8	C_{14}, C_{14}-en
18	*Roseovarius tolerans*	EL 90	100	98.7	7.7	C_{14}, C_{14}-en, 3-oxo-C_{14}-en
19	*Roseovarius tolerans*	EL 164	100	84.0	11.0	C_{14}, C_{14}-en, C_{16}, C_{16}-en
20	*Roseovarius tolerans*	EL 171	100	81.7	8.9	C_{14}, C_{14}-en, C_{16}
21	*Roseovarius tolerans*	EL 172^T	100	82.7	9.4	C_{14}, C_{14}-en
22	*Roseovarius tolerans*	EL 222	100	83.6	10.1	C_{14}, C_{14}-en
23	*Roseobacter litoralis*	DSM 7001^T	100	83.0	$<$2.0	C_8
24	*Staleya guttiformis*	LM 09	97	84.3	$<$2.0	C_{16}, C_{16}-en, C_{16}-dien
25	*Jannaschia helgolandensis*	HEL 10^T	100	65.5	5.2	C_{14}-en, C_{16}-en, C_{16}-dien
26	*Jannaschia helgolandensis*	HEL 26	100	85.4	6.3	C_{14}-en, C_{16}-en, C_{16}-dien
27	*Jannaschia helgolandensis*	HEL 43	100	11.6	4.9	C_{14}-en, C_{16}-dien
28	*Oceanibulbus indolifex*	HEL 76	99	25.3	12.1	C_{16}-en
29	*Sulfitobacter* sp.	PIC 069	96	29.3	5.2	n.d.[b]
30	*Sulfitobacter* sp.	PIC 070	96	12.3	3.1	n.d.[b]
31	*Sulfitobacter* sp.	PIC 072	96	26.7	7.0	n.d.[b]
32	*Sulfitobacter* sp.	PIC 074	96	27.0	2.5	n.d.[b]
33	*Sulfitobacter* sp.	PIC 076	96	29.5	8.8	n.d.[b]
34	*Sulfitobacter* sp.	PIC 082	96	22.8	6.9	n.d.[b]
35	*Sulfitobacter* sp.	DFL 23	96	27.3	6.0	n.d.[b]
36	*Sulfitobacter* sp.	DFL 41	96	37.8	5.4	n.d.[b]
37	*Roseobacter gallaeciencis*	T5	98	75.9	$<$2.0	3-HO-C_{10}, C_{18}-en
38	*Thalassospira lucentensis*	PIC 088	97	70.0	$<$2.0	n.d.[b]
Gammaproteobacteria						
39	*Glaciecola polaris*	PIC 002	98	$<$2.0	7.7	n.d.[b]
40	*Pseudoalteromonas atlantica*	PIC 075	99	24.8	7.7	n.d.[b]
Bacteroidetes						
41	*Flavobacterium* sp.	PIC 073	91	$<$2.0	7.0	n.d.[b]

[a] Strains DFL-13, DFL-42, DFL-43 and DFL-44 are currently described as *Hoeflea phototrophica*; strains DFL-24 and DFL-35 are currently described as *Roseovarius mucosus*. [b] not detected. If the assay was repeated, the highest fold induction to be observed is indicated here.

nals for AHLs. Only two Gammaproteobacteria were positive, namely *Pseudoalteromonas atlantica* PIC-075 (long- and short-chain activity) and *Glaciecola polaris* PIC-002 (short-chain activity), both were isolated from picoplankton enrichments. Activity in the short-chain biosensor was also observed in *Flavobacterium* sp. strain PIC-073.

Within the Alphaproteobacteria, some species showed the same result for all strains tested. All eight strains of *Roseovarius tolerans* showed very high activity, the maximum being 103-fold induction of fluorescence in the long-chain biosensor strain. Moreover, all strains of the newly described genera *Dinoroseobacter shibae,*[13] *Jannaschia helgolandensis*[14] and *Roseovarius mucosus*[15] had high activity. Within the genus sulfitobacter, which includes many diverse species, some showed AHL activity and others did not. The same was true for *Oceanibulbus indolifex,*[16] for which the type strain was inactive, while a closely related marine isolate (HEL-76) showed high activity in the short-chain biosensor assay. Interestingly, *Roseobacter litoralis* showed high activity, while *R. denitrificans* and *Staleya guttiformis* did not. Strain T5, which is closely related to *R. gallaeciensis*, also induced fluorescence.

GC-MS screening of active culture supernatants

In order to screen a large number of extracts for the presence of AHLs, a rapid method was needed that would allow identification of unknown AHLs possibly present in the extracts. For this nontarget screening approach, we choose GC-MS because of its good separation power, sensitivity and better structural information obtained in the mass spectra compared to the more often used HPLC-MS or HPLC-MS/MS methods. Other approaches with CE,[17] GC-MS,[18] or HPLC-MS[19] use target-oriented analyses to identify known AHLs or are restricted to certain classes of AHLs.[20] Initially the methanol from the resin extracts of active cultures was removed by evaporation, and the solid material was resuspended with ethyl acetate to remove unsoluble matrix material in the sample so as to facilitate GC-MS analysis. Surprisingly, these extracts gave poorer peak shapes of AHLs than direct injection of the crude methanol extract, which led to better detection of trace AHLs in the methanol extracts. The major drawback of the direct-injection method was the behaviour of the large amount of diketopiperazines (cyclic dipeptides) found in all samples. The diketopiperazines, also present in ethyl acetate extracts, were mainly derived from proline and showed up as double peaks, thus indicating the presence of diastereomeric mixtures containing D- and L-proline. Interestingly, the diketopiperazines cyclo(pro-tyr), cyclo(leu-pro), cyclo(ile-pro), cyclo(ala-ile), cyclo(phe-pro) and cyclo(pro-val) were previously reported to influence quorum-sensing activity.[21,22] However, the concentrations of diketopiperazines needed to elicit a response were in the millimolar range, and thus 1000-fold higher than those of the AHLs. We found these cyclic peptides as well as occasionally cyclo(met-pro) and cyclo(pro-trp) in every sample investigated; this points to artificial formation, possibly during autoclavation of the amino acid-containing medium.[23] Formation in the injection port of the gas chromatograph can be excluded because silylation of samples prior to GC analysis furnished silylated diketopiperazines, thus showing their existence in the original extracts. Although these compounds can be formed during autoclavation, some bacterial contribution to their formation might also be possible, because not all strains yielded identical proportions of cyclic peptides. Nevertheless, their widespread occurrence and the high concentrations that have previously been reported to be necessary for eliciting a biological effect[21] make it unlikely that these compounds play an important role in the communication of bacteria under natural conditions. The diketopiperazines tended to remain to some extent in the injection port of the gas chromatograph and were carried over to the next analysis. This behaviour was not observed for the AHLs because injections of pure methanol after an AHL-containing extract produced diektopiperazine peaks, but no AHL peaks. The major drawback in the direct-methanol-injection method is that the injector liner has to be cleaned after every 15 to 20 injections to remove superfluous matrix material.

Structural identification of AHLs in active culture supernatants

GC-MS analysis of AHL extracts was successful for the most-active active extracts (see Table 2). AHLs were detected in 22 of 41 active extracts. Extracts without activity in the biosensor strains used here were not analysed by GC-MS, except for two reference samples. The AHLs were identified by interpretation of their mass spectra,[24] their gas-chromatographic retention indices and comparison with synthetic samples. The mass spectra of saturated AHLs exhibit a base peak at *m/z* 143, together with a characteristic ion at *m/z* 102 and a small $[M^+]$. In monounsaturated AHLs, the ion at *m/z* 102 becomes more intense, while in the diunsaturated compound, the intensities of both characteristic ions decreases compared to unspecific diunsaturated alkyl fragments of the series C_nH_{2n-3}. A characteristic intense ion for 3-hydroxy-AHLs is *m/z* 172, which arises from α-cleavage next to the hydroxyl group. Similarly, 3-oxo-AHLs are identified by an ion at *m/z* 170, accompanied by the McLafferty-type rearrangement ion at *m/z* 185. Of further importance is the $[M^+]$ ion, which is small but significant in all AHL types. Furthermore, unsaturated AHLs show a characteristic loss of the homoserine moiety $[M^+-101]$. These data even allow identification of AHLs that do not have good mass spectra because of their low abundance in the extracts. Representative spectra are shown in the Supporting Information. Unfortunately, the location of double bonds in unsaturated compounds is not possible by analysis of the mass spectra, because no abundant characteristic fragmentation induced by the double bond occurs. Therefore we used dimethyl disulfide (DMDS) addition for the location of double bonds,[25,26] a method that has so far not been applied to AHLs. The DMDS addition performed with crude extracts gave mass spectra that allowed easy location of the double bond, exemplified for 7-C_{14}en-HSL (Figure 4). Preferential cleavage between the thiomethyl groups furnished two cleavage products, the ions at *m/z* 145 and 258. The latter contains the HSL ring, which is

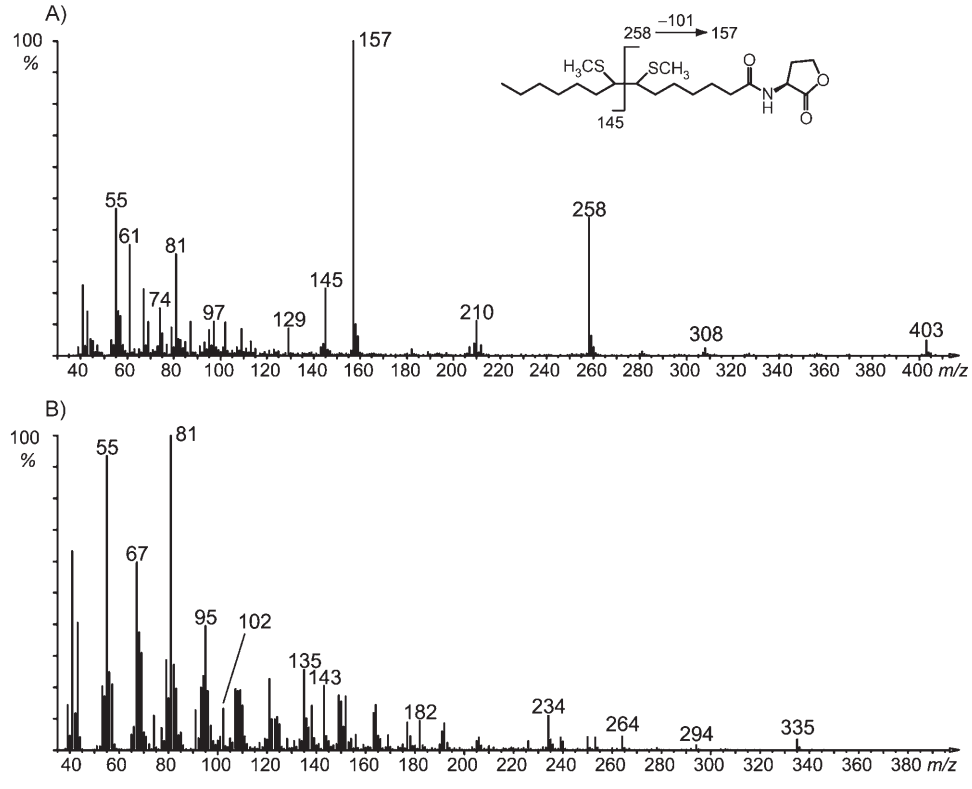


Figure 4. Mass spectrum and fragmentation pattern of the DMDS adduct of A) 7-C_{14}-en-HSL smd and B) C_{16}dien-HSL with unknown position of double bonds.

easily lost to form the base peak at *m/z* 157. These three ions indicate the position of the double bond to be at C-7.

The detection limit for the AHLs by GC-MS was about 10 ng per injection (around 40 pmol). The reporter strains required 4–5 pmol of the AHL for a clear positive response towards their most optimal AHL, thus their sensitivity was tenfold higher than that of the chemical analysis, similar to previous reports.[8] However, their sensitivity dropped by orders of magnitude outside of the detection window. Spiking experiments with 5 μg of C_6- or C_{12}-AHL added to 200 mL solution revealed that these amounts were easily detectable by GC-MS after XAD extraction. One possibility for the lack of detection in some of the positive strains is that AHLs were present, but below the limit of detection of GC-MS analysis; theoretically, unknown compounds could also have stimulated the sensors. In addition, GC-MS analysis was hindered by the frequent presence of large peaks of diketopiperazines that masked the AHLs. In view of the complexity of the AHLs produced by any single organism investigated here, an analysis of their biological effects must be based on fractionated extracts or synthesised pure compounds, rather than on bulk culture supernatants.

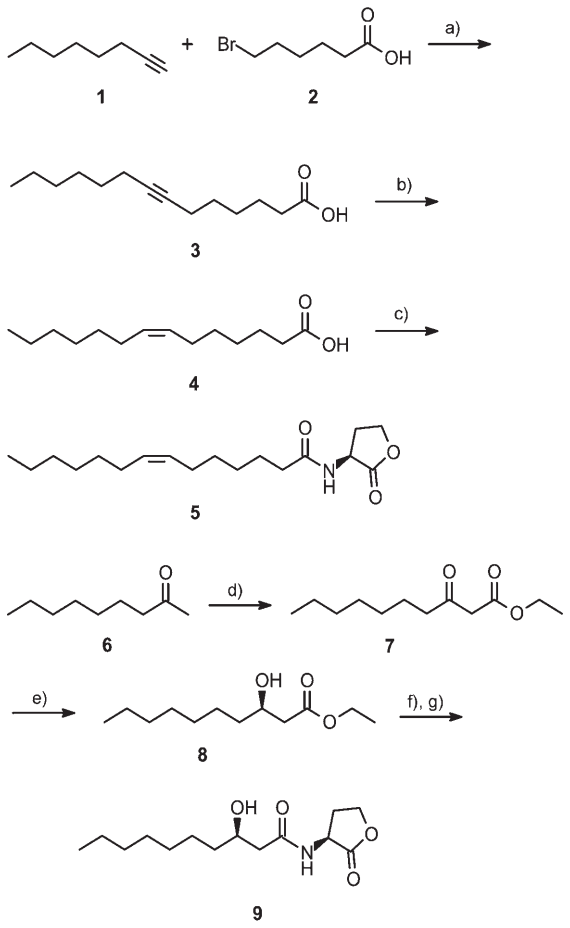
Long-chain AHLs are generally difficult to detect because only a small fraction is present in the culture supernatant, while the bulk amount is thought to be membrane bound. We therefore conclude that the amounts of long-chain AHLs produced by the investigated marine bacteria must have been very high. The adsorption of AHLs to XAD resins during cultivation might have been another important reason for the ability to detect them in such a large number of marine organisms. However, those compounds that dominated GC-MS analysis were not necessarily those that were responsible for the high activities seen in the biosensor strains.

Synthesis of reference AHLs

Several AHLs were synthesised to confirm identifications and establish gas-chromatographic retention times (Scheme 1). Saturated C_8-, C_{12}- and C_{15}-HSLs were prepared by treatment of acyl chlorides with L-homoserine lactone according to Angle and Henry[27] with shorter reaction times to give increased yields. The unsaturated Z7-C_{14}en-HSL was obtained as shown in Scheme 1. Oct-1-yne (**1**) was deprotonated with $LiNH_2$ in liquid ammonia and treated with 6-bromohexanoic acid (**2**).[28] The resulting 7-tetradecynoic acid (**3**) was then hydrogenated with Lindlar's catalyst to yield a 98:2 *Z*/*E* mixture of 7-tetradecenoic acid (**4**). This acid was finally coupled with L-homoserine lactone in the presence of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)[29] to furnish Z7-C_{14}en-HSL (**5**). Comparison of the retention times with the synthetic sample confirmed that only the *Z* diastereomer occurs naturally. The hydroxy compound 3-HO-C_{10}-AHL was synthesised from octanal (**6**). Addition of ethyl diazoacetate furnished ethyl 3-oxodecanoate (**7**). This ester was enantioselectively hydrogenated with a (*S*)-Ru-BINAP catalyst[30] with concomitant transesterifcation to furnish methyl (*S*)-3-hydroxydecanoate (**8**) with an *ee* > 95 %. After saponification, the acid was coupled with L-homoserine lactone, yielding the desired (*S*)-*N*-(3-hydroxydecanoyl)homoserine lactone (**9**). Similarly, the *R* diastereomer was obtained by using the (*R*)-Ru-BINAP catalyst in the hydrogenation step. Both diastereomers of **9** showed identical gas-chromatographic retention times, so it remains unclear whether the 3-HO group is *R* or *S* configured.

Spectrum of AHLs found in marine Alphaproteobacteria

Long-chain AHLs with chain lengths of C_{14}, C_{16} and C_{18} clearly dominated among the identified compounds. The shortest AHL found was C_8-HSL in *Dinoroseobacter shibae* DFL-16 and *Roseobacter litoralis*. These organisms appear to be physiologically very similar, but are phylogenetically relatively distant. *D. shibae* was isolated from dinoflagellates,[13] while *R. litoralis* is a free-living sediment organism.[31] Both are able to perform aerobic anoxygenic photosynthesis. The C_8-HSL has previously been reported from several bacteria.[2] The only hydroxy AHL



Scheme 1. Synthesis of AHL. a) $LiNH_2$, NH_3; b) H_2, Pd/C; c) HSL, EDC, benzene; d) $N_2CHCOOEt$, $SnCl_2$; e) (*R*)-Ru[Cl_2BINAP·NEt_3]; f) NaOH, MeOH; g) HSL, EDC, benzene;

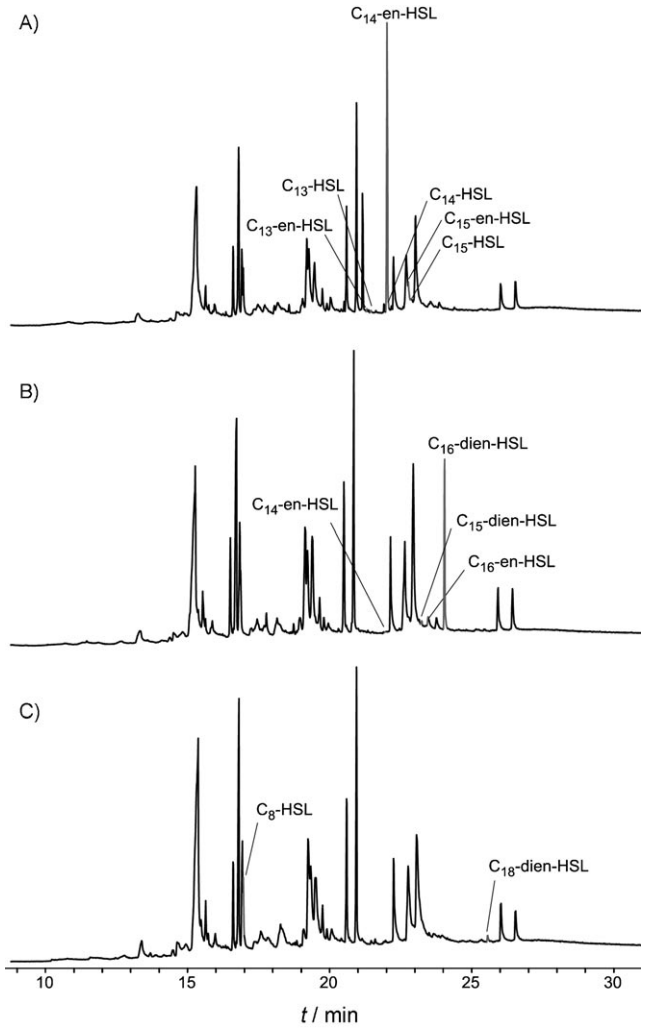


Figure 5. Total ion chromatograms of representative strains A) EL-83, B) Hel-26, C) DFL-16. AHL peaks are marked in grey. The major peaks in each analysis represent diketopiperazines, mostly derived from proline.

identified was 3-HO-C_{10}-HSL (**9**) present in strain T5. It has previously been identified in *Sinorhizobium meliloti*,[32] *Pseudomonas fluorescens*[33] and *Burkholderia pseudomallei*,[34] but the absolute configuration of the side chain is not known. Since the sensitivity of both biosensor strains was very low for C_8- and C_{10}-HSLs, either both AHLs must have been present in high concentrations or they were accompanied by small amounts of AHLs that were able to stimulate the reporter strains strongly but were not detectable by GC-MS.

Z7-C_{14}en-HSL (**5**) was identified in two species that also belong to the *Roseobacter* clade but are not strongly related, *Roseovarius tolerans* and *Jannaschia helgolandensis* (Figure 5). Both are free-living marine organisms, just like the facultative anaerobic phototroph *Rhodobacter sphaeroides*, where **5** has been previously detected.[5] By using the synthetic Z7-C_{14}en-HSL, the detection limit for this compound was determined, which, even with the long-chain biosensor strain, was relatively high (161 nmol). The weak activity seen with the short-chain biosensor strain for *R. tolerans* and *J. helgolandensis* extracts was probably caused by its response to the high concentrations of C_{14}en-HSL, since no short-chain AHLs were detected by GC-MS. While **5** is a major component of *R. tolerans*, C_{14}en-HSL occurs only in minor amounts in *J. helgolandensis*. Whether it also contains the double bond at C-7 remains unknown, because the low amounts of material did not allow determination by the DMDS method. The major AHL of *J. helgolandensis* is C_{16}dien-HSL, the first AHL reported with a diene acyl group. Determination of the double-bond positions was not performed because of the low amounts available. This AHL was identified by its mass spectrum, which showed characteristic ions at *m/z* 102, 143, 156, 234 [M^+-101], and 335 [M^+] (Figure 4). In addition, C_{14}-HSL, 3-oxo-C_{14}-HSL and C_{16}en-HSL, compounds that have been identified previously, were present in some strains of this genus.[18,20,32,35] *Oceanibulbus indolifex* contained enough C_{16}en-HSL to determine the location of the double bond by the DMDS method, it proved to be located at C-9. This compound has previously been described as being produced by *S. meliloti*,[32,35] but the position of the double bond had not been unambiguously determined. *Staleya guttiformis* contained C_{14}-AHL as well as all three C16-AHLs. C_{18}-en-HSL and C_{18}-dien-HSL were detected in the three phylogenetically unrelated species *Dinoroseobacter shibae*, *Roseovarius mucosus* and strain T5. Again the position of the double bonds

remains unknown. To the best of our knowledge, these AHLs have not so far been identified in nature.

Unexpectedly, AHLs with an uneven number of carbon atoms were also detected, namely C_{13}-HSL, C_{15}-HSL, C_{15}en-HSL and C_{15}dien-HSL. They always occurred together with large amounts of even-numbered AHLs. With the exception of C_7-AHL, detected in *Serratia marcescens,*[36] such AHLs have not been identified before. Because bacteria often contain iso- and/or anteiso-methyl-branched fatty acids, we assumed that these compounds might be methyl branched. Nevertheless, comparison of their retention times with those of authentic C_{15}-HSL confirmed the presence of odd-numbered unbranched acyl chains in these compounds. C_{15}-HSL was synthesised and tested with the long-chain biosensor strain. It showed about 10% of the activity of C_{14}-HSL.

Phylogenetic distribution of AHLs in Alphaproteobacteria

Figure 6 shows the most recent phylogenetic tree of the Rhodobacterales, into which all the Alphaproteobacteria investigated in this study belong. *Dinoroseobacter shibae* is a newly described genus of phototrophic Alphaproteobacteria that has been isolated from single cells of cultivated toxic dinoflagellates;[13] the various strains tested here were isolated from *Prorocentrum lima* (DFL-12) and *Alexandrium ostenfeldii* (DFL-16, DFL-27, DFL-30, DFL-31). *Hoeflea marina* represents a newly reclassified genus[37] in which the cluster of phototrophic strains from dinoflagellates analysed here represents a new species, described as *Hoeflea phototrophica* by Biebl et al.[38] DFL-33,DFL-13, and DFL-44 were isolated from *Alexandrium lusitanicum*, while the source of DFL-42 and DFL-43 was *Prorocentrum lima. Roseovarius mucosus* (isolated from *Alexandrium ostenfeldii*) is also a newly described species.[15] The strains of *Roseovarius tolerans*[11] investigated here are free-living, phototrophic aerobes that have been isolated from different depths of the hypersaline Antarctic Ekho Lake. This lake is also the source of *Staleya guttiformis.*[39] *Roseobacter litoralis* was isolated from marine sediment by Shiba.[31] Interestingly, the closely related species *Roseobacter denitrificans* did not show any quorum-sensing activity. While all of the 24 strains described so far were able to perform aerobic anoxygenic photosynthesis or were at least shown to contain the *pufLM* genes of the photosynthesis gene cluster, this is not the case for the remaining 17 biosensor-positive strains. *Jannaschia helgolandensis*[14] was isolated from the highest dilution of a North Sea water sample, just like *Oceanibulbus indolifex*, which produces large amounts of indol derivatives.[16] Various isolates from the genus *Sulfitobacter*, isolated from picoplankton and dinoflagellates, showed moderate activity in the AHL bioassays, but no AHL molecule could be identified by GC-MS. No activity was found in many other *Sulfitobacter* strains, but this might be due to the fact that they were outside of the detection window of the biosensor strains. Strain T5 was isolated from the German Wadden Sea and is closely related to *Roseobacter gallaeciensis*[40] derived from larval cultures of the scallop *Pecten maximus*. Finally, the closest relative of strain PIC-106 is *Thalassospira lucentensis*,[41] which was isolated from chemostat cultures at 13 °C after three months of cultivation at very low nutrient concentrations.

The AHLs found in the marine Alphaproteobacteria were very similar to those found in rhizobia, a group of Alphaproteobacteria that are able to live freely in the soil, but are also capable of triggering a complex quorum-sensing-controlled symbiotic relationship with leguminous plants that culminates in the joint formation of root nodules for the fixation of nitrogen. C_{18}-HSL was first reported in *Sinorhizobium meliloti*,[35] a fully sequenced organism that produces ten different AHLs.[32] A single AHL-synthase is responsible for the synthesis of AHLs with chain lengths from C_{12} to C_{18}, including 3-oxo-C_{14}-HSL and 3-oxo-C_{16}-HSL. However, in the marine strains, C_{18}-HSL was never found, only analogues with one or two double bonds; this indicates that these might be a specific adaptation to the marine environment. Similarly, several strains tested produced C_{14}-HSL and C_{16}-HSL in the unsubstituted form, just as in rhizobia, but also as analogues with one or two double bonds or a 3-oxo-group. It was shown by Teplitski et al.[32] that the pattern of AHLs produced by a single strain strongly depends on the medium. Here we show that marine Alphaproteobacteria produce patterns of long-chain AHLs very similar to those found in terrestrial rhizobia. This suggests that this ability is characteristic of Alphaprotebacteria as a phylum rather than being related to a specific ecological niche.

The fact that only three AHL-positive extracts outside of the Alphaproteobacteria were found in our screen might simply reflect the chain-length specificity of the biosensor strains used.

Possible physiological roles of AHLs in marine Alphaproteobacteria

In rhizobia, AHL signalling is two-sided, targeting both the bacterium and the host plant during the development of root nodules. It is therefore tempting to speculate that the AHLs produced by marine strains that were isolated from dinoflagellates (*Dinoroseobacter shibae*, *Hoeflea phototrophica*, *Roseovarius mucosus*) might be involved in regulating (potential) symbiotic interactions with their eukaryotic hosts. Interestingly, there are also genetic similarities between rhizobia and the marine *Roseobacter* clade organisms. Rhizobia have huge megaplasmids or second chromosomes, and a complex plasmid pattern with large linear and circular plasmids was discovered in the aerobic anoxygenic phototrophs from the *Roseobacter* clade.[42] However, the same compounds were also synthesised by apparently free-living strains that were isolated from sediments or marine bacterioplankton or after prolonged enrichment without any known association to higher organisms. Z7-C_{14}en-HSL has previously been detected in the free-living *Rhodobacter sphaeroides*.[5] Whole-genome sequencing revealed two copies of the quorum-sensing genes *luxR* and *LuxI* on the chromosome of *Silicibacter pomeroyi,* which has been isolated from coastal bacterioplankton. Three AHL-type signal molecules were identified in culture supernatants by using thin-layer chromatography and the *Agrobacterium tumefaciens* reporter strain.[43] One possible explanation would be that these organisms use quorum sensing to switch from the planktonic to the

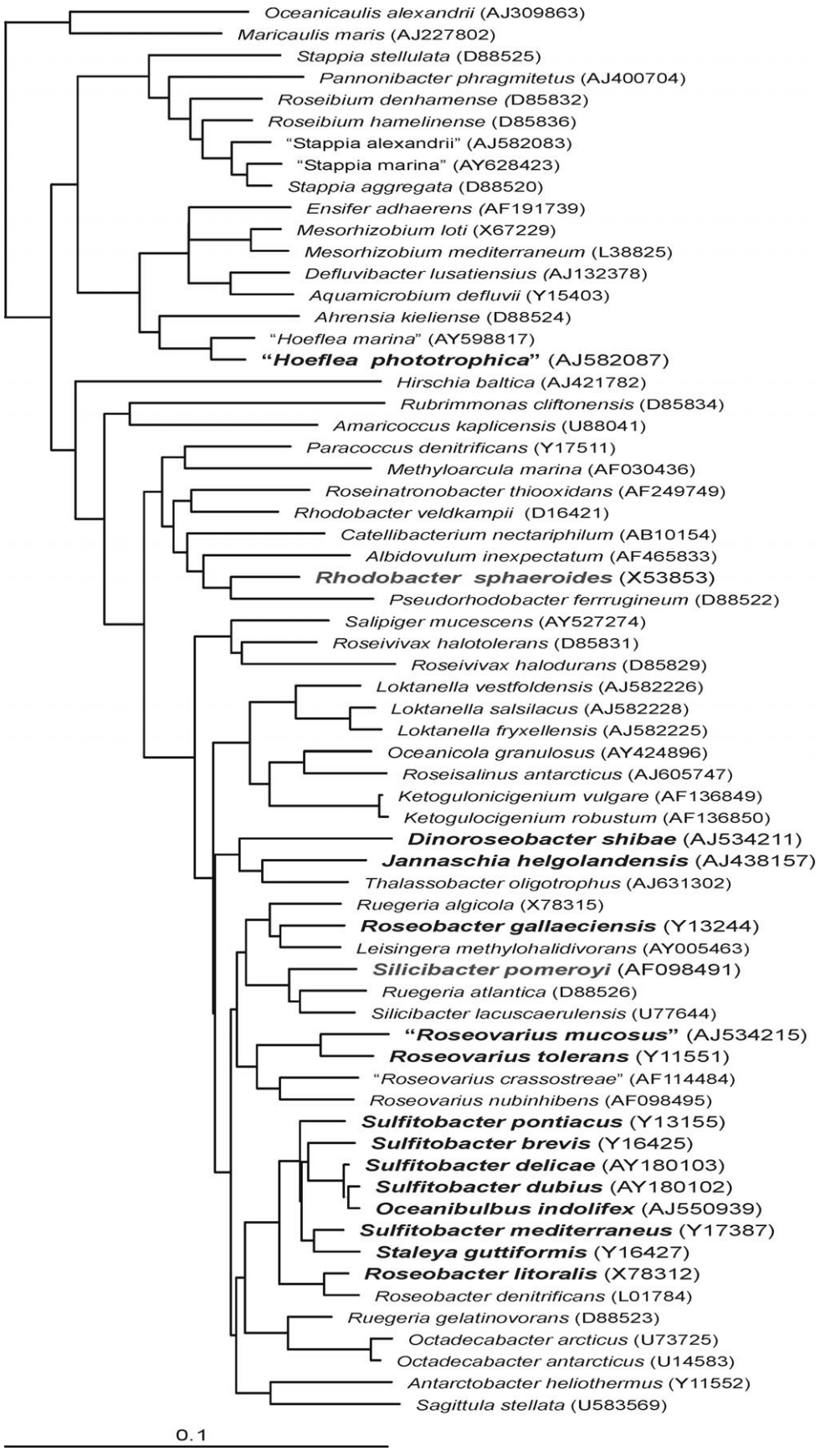


Figure 6. Phylogenetic tree of the marine Rhodobacterales within the Alphaproteobacteria based on almost complete 16S rRNA gene sequences. Results for species in inverted commas are currently submitted or in press. The tree was calculated by using the neighbour-joining algorithm and rooted with *Escherichia coli* as out group. Quorum-sensing activity has been found in species marked in bold (this work) or grey (previous publications).

biofilm mode of growth, which is required in order to form marine snow and to colonise the surfaces of marine plants and animals. Interestingly, zoospores of the seaweed *Ulva* have a variety of mechanisms to allow them to decide which surface is a good substrate for settlement, one of them being the presence of AHLs from bacterial microcolonies; *Ulva* has a slight preference for long-chain AHLs, but does not discriminate between different side-chain substitutions.[44,45] AHLs can additionally perform functions totally unrelated to quorum sensing. Recently a detailed chemical analysis of the fate of *P. aeruginosa* AHLs in aqueous environments showed that they undergo complex degradation processes that result in tetramic acids that have antibacterial activity and act as siderophores.[46]

Conclusion

The study presented here shows that AHL signal molecules are produced by more than half of the marine Alphaproteobacteria tested and was able to identify complex mixtures of long-chain AHLs. However, given the narrow windows of sensitivity of the two biosensor strains used for screening and the relatively high detection level of the GC-MS analysis, we think that we are still seeing only the tip of the iceberg. Production of AHLs might actually be the rule rather than the exception, at least in marine Alphaproteobacteria and possibly beyond. The physiological roles of these AHLs for the bacteria in their widely different ecological niches remain to be elucidated.

Experimental Section

Biosensor strains. *P. putida* F117 (pRK-C12)[3] was used for the detection of long-chain AHLs. *P. putida* F117 (Kmr; *ppuI::npt*) is a transposon mutant of the AHL producing wild-type *P. putida* IsoF. Strain F117 carries a kanamycin-resistance gene in the *ppuI* gene responsible for the synthesis of AHLs and therefore does not produce AHLs.[4] The sensor plasmid pRK-C12 (Gm^R; pBBRIMC-5–P_{lasB}-*gfp*(ASV)P_{lac}-*lasR*) is a derivative of the broad host range plasmid pBBR1MCS-5 carrying a gentamycin-resistance gene and a fusion between the *lasB* gene from *P. aeruginosa* and an unstable *gfp* variant as well as *lasR* from *P. aeruginosa* placed under the control of P_{lac}.

E. coli MT102 (pJBA132) was used for the detection of short-chain AHLs.[12] *E. coli* MT102 is a restriction-negative, streptomycin-resistant derivative of MC1000.[3] The sensor plasmid pJBA132 (Tc^R; pME6031-*luxR*-P_{luxI}-RBSII-*gfp*(ASV)-T_o-T_1) is derived from the high-copy number ColE1-based plasmid pJBA88. It carries a tetracycline-resistance gene, a transcriptional fusion between the *luxI* promoter of *Vibrio fischeri* and an unstable *gfp* variant, and the *luxR* gene from *V. fischeri*.**Preparation of AHL extracts.** Marine bacteria were grown in marine broth 2216 (Difco) or LBSS (26 g Luria Bertani broth (Sigma), 17.08 g sea salts (Sigma) per 1000 mL of distilled water). Medium (15 mL) was inoculated from fresh plates and incubated at room temperature with agitation in 50 mL polypropylen tubes for 1–7 days until an OD_{620} of 1.0–1.7 had been reached. The preculture was added to fresh medium (100 mL) in a 500 mL Erlenmeyer flask. The neutral adsorber resin Amberlite XAD-16 (2 mL, Rohm & Haas) was added, and the culture was incubated for 3 days at room temperature with shaking until an OD_{620} above 1.0 had been reached. The culture supernatant was discarded. Methanol (50 mL) was added to the resin, and the mixture was left without shaking overnight. The resin was removed by filtering through a paper filter. The methanol extract was concentrated to several mL in an evaporator and stored at $-20\,^{\circ}C$.

Phylogenetic affiliation of strains. The 16S rRNA gene sequence of the strains tested was determined as described.[10] Almost complete sequences were used for a first identification by using the BLAST program. For the construction of a phylogenetic tree, the sequences were manually aligned and compared with published sequences from the DSMZ 16S rDNA database, including sequences available from the Ribosomal Data Project[47] and EMBL. An alignment was constructed with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and used for calculating the distance matrix corrected by the Kimura′2- parameter method[48] and CLUSTALX.[49] A phlyogenetic dendrogram was inferred by using the neighbor-joining method.[50]

Bioassay. Sensor strains and positive control were grown on LB medium and incubated at 37 °C (*E. coli* MT102) or 30 °C (*P. putida* F117 and *P. putida* IsoF) on a shaking platform for 12–20 h until an OD_{620} of 1.0 had been reached. The respective strains were inoculated from plates into preculture (3 mL), which was then transferred to fresh medium (100 mL). LB medium (90 μL) and the methanol extract (10 μL) were pipetted into 96-well microtitre plates, and the sensor strain (100 μL) was added. Each methanol extract was measured in triplicate. Microtitre plates were incubated at 30 °C and 37 °C, respectively, without shaking. After 0, 2, 4, 8 and 24 h, fluorescence was determined in a Victor1420 Multilabel Counter (Perkin–Elmer) at an excitation wavelength of $\lambda = 485$ nm and a detection wavelength of $\lambda = 535$ nm. OD_{620} was also measured. Methanol (10 μL) was used as a negative control, and extracts from the wild-type strain *P. putida* IsoF or synthetic homoserine lactones were used as a positive control. Fold induction of fluorescence was calculated by dividing the specific fluorescence (gfp_{535}/OD_{620}) of the test sample by the specific fluorescence of the negative control. Assays were repeated if positive-control values were below previously determined values.

GC-MS analyses. The extracts prepared as described above were separated into two parts, which were concentrated at 50 °C under a stream of nitrogen to a volume of about 30 μL. This solution (1 μL) was injected into the mass spectrometer. It has to be mentioned that the often large amounts of diketopiperazines limit the concentration of the samples, because otherwise the GC-MS spectrometer would be flooded with material during analysis. GC-MS analyses were carried out on a HP 6890 Series GC System connected to a HP 5973 Mass Selective Detector (Hewlett–Packard, Wilmington, USA) 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: 1 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}). Identification of compounds was performed by comparison of mass spectra and retention times with synthetic compounds.

Synthesis of acylated homoserine lactones. Chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland) or Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. NMR spectra were obtained on a Bruker AMX400 (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz) spectrometer with TMS as

an internal standard. Optical rotations were determined on a Dr. Kernchen Propol Digital Automatic Polarimeter. Column chromatography was carried out with Merck Kieselgel 60. Thin-layer chromatography was carried out on 0.2 mm precoated plastic sheets Polygram Sil G/UV_{254} (Marcherey-Nagel, Düren, Germany). Solvents were purified by distillation and dried according to standard methods.

Synthesis of *N*-acyl-L-homoserine lactones: General method. According to the method of Angle and Henry,[27] a solution of L-homoserine lactone hydrobromide(0.36 g, 2.0 mmol) in H_2O (4 mL) and CH_2Cl_2 (4 mL) was cooled to 0 °C. K_2CO_3 (0.83 g, 6.0 mmol) was added, and the solution was stirred for 5 min. The acid chloride (2.35 mmol) was added dropwise, and the reaction mixture was allowed to warm to room temperature, then stirred for further 5 h. The mixture was then diluted with ethyl acetate (25 mL), and, after separation, the aqueous layer was extracted with ethyl acetate (3×). The combined organic phases were dried and concentrated to give the crude product. Flash column chromatography afforded the respective *N*-acyl-L-homoserine lactones as white solids.

***N*-Butyryl-L-homoserine lactone**: 85%; $R_F = 0.43$ (pentane/diethyl ether 2:1); $[\alpha]_D^{21}$: −26.2 ($c = 2.78$ in H_2O); m.p. 118 °C (from EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 6.06$ (s, 1H; NH), 4.56 (ddd, $^3J = 11.6$, 8.6, 5.8 Hz, 1H; NCH), 4.47 (td, $^2J = 9.1$, $^3J = 1.1$ Hz, 1H; OCH_2), 4.29 (ddd, $^2J = 11.3$, $^3J = 9.3$, 5.9 Hz, 1H; OCH_2), 2.86 (dddd, $^2J = 12.6$, $^3J = 8.5$, 5.8, 1.2 Hz, 1H; NCHCH_2), 2.24 (td, $^2J = 7.5$, $^3J = 1.4$ Hz, 2H; CH_2CO), 2.19–2.08 (m, 1H; NCHCH_2), 1.73–1.64 (m, 2H; CH_2CH_3), 0.97 ppm (t, $^3J = 7.4$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 175.5$ (NCO), 173.6 (CO_2), 66.1 (CH_2O), 49.3 (CHN), 38.0 (CH_2CON), 30.7 (CH_2CH_2O), 18.9 (CH_2CH_3), 13.7 ppm (CH_3); EI-MS (70 eV): *m/z* (%) = 171 (3) $[M]^+$, 156 (3), 143 (50), 125 (5), 113 (6), 101 (8), 85 (9), 71 (49), 57 (80), 43 (100).

***N*-Octanoyl-L-homoserine lactone**: 94%; $R_F = 0.41$ (pentane/diethyl ether 2:1); $[\alpha]_D^{21}$: −24.0 ($c = 2.10$ in MeOH); m.p. 128 °C (from EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 6.25$ (s, 1H; NH), 4.59 (ddd, $^3J = 11.6$, 8.5, 6.2 Hz, 1H; NCH), 4.47 (t, $^3J = 9.0$ Hz, 1H; OCH_2), 4.29 (ddd, $^2J = 11.2$, $^3J = 9.3$, 5.9 Hz, 1H; OCH_2), 2.87–2.80 (m, 1H; NCHCH_2), 2.27–2.23 (m, 2H; CH_2CO), 2.19–2.09 (m, 1H; NCHCH_2), 1.64 (quint, $^3J = 7.1$ Hz, 2H; CH_2CH_2CO), 1.30–1.28 (m, 8H; 4 × CH_2), 0.88 ppm (t, $^3J = 6.7$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$ 25 °C, TMS): $\delta = 175.7$ (NCO), 173.8 (CO_2), 66.1 (CH_2O), 49.2 (NCH), 36.1 (CH_2CON), 31.6 (CH_2), 30.5 (CH_2), 29.1 (CH_2), 28.9 (CH_2), 25.4 (CH_2), 22.6 (CH_2), 14.0 ppm (CH_3); EI-MS (70 eV): *m/z* (%) = 227 (2) $[M]^+$, 156 (12), 143 (75), 125 (18), 115 (4), 102 (12), 101 (15), 85 (6), 83 (20), 57 (100), 43 (35), 41 (42).

***N*-Dodecanoyl-L-homoserine lactone**: 91%; $R_F = 0.37$ (pentane/diethyl ether 2:1); $[\alpha]_D^{21}$: −24.5 ($c = 1.70$ in MeOH); m.p. 128 °C (from EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 6.13$ (s, 1H; NH), 4.56 (ddd, $^3J = 11.6$, 8.6, 5.9 Hz, 1H; NCH), 4.47 (td, $^2J = 9.0$, $^3J = 0.8$ Hz, 1H; OCH_2), 4.29 (ddd, $^2J = 11.3$, $^3J = 9.3$, 5.9 Hz, 1H; OCH_2), 2.88–2.83 (m, 1H; NCHCH_2), 2.25 (t, $^3J = 8.3$ Hz, 2H; CH_2CON), 2.19–2.08 (m, 1H; NCHCH_2), 1.64 (quint, $^3J = 7.4$ Hz, 2H; CH_2CH_2CON), 1.33–1.29 (m, 2H; CH_2CH_3), 1.26 (s, 14H; 7CH_2), 0.88 ppm (t, $^3J = 6.9$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 175.6$ (CON), 173.8 (CO_2), 66.1 (CH_2O), 49.2 (NCH), 36.2 (CH_2CON), 31.9 (CH_2), 30.6 (CH_2), 29.6 (2CH_2), 29.4 (CH_2), 29.3 (2CH_2), 29.2 (CH_2), 25.4 (CH_2), 22.7 (CH_2), 14.1 ppm (CH_3); EI-MS (70 eV): *m/z* (%) = 283 (5) $[M]^+$, 156 (20), 143 (100), 125 (18), 102 (20), 85 (8), 83 (23), 69 (10), 57 (59), 43 (40).

7-Tetradecynoic acid: In a similar manner to the procedure described by Ames and Covell,[27] 1-octyne (1.48 mL, 10.0 mmol) was added to a stirred solution of $NaNH_2$ (0.39 g, 10.0 mmol) in liquid ammonia (15 mL) at −60 °C, and the mixture was stirred under reflux for 1 h. 6-Bromohexanoic acid (0.39 g, 2.0 mmol) in dry THF (5 mL) was added, the reaction mixture was stirred under reflux for further 8 h, and then the ammonia was allowed to evaporate. The residue was dissolved in diluted HCl, and the mixture was extracted with diethyl ether (3×). The organic layers were collected and dried (Na_2SO_4), and the solvent was removed under reduced pressure to give a yellow oil. Purification by flash column chromatography yielded the product (0.34 g, 1.52 mmol, 76%) as a white solid. $R_F = 0.32$ (pentane/diethyl ether 2:1); m.p. 32 °C (from pentane/diethyl ether 1:1); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 10.79$ (br s, 1H; CO_2H), 2.36 (t, $^3J = 7.5$ Hz, 2H; CH_2CO_2), 2.18–2.11 (m, 4H; 2CH_2C), 1.65 (quint, $^3J = 7.5$ Hz, 2H; C$H_2CH_2CO_2$), 1.54–1.23 (m, 12H; 6CH_2), 0.89 ppm (t, $^3J = 7.0$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 180.2$ (CO_2), 80.5 (CCH_2), 79.7 (CCH_2), 34.0 (CH_2CO_2), 31.3 (CH_2), 29.1 (CH_2), 28.7 (CH_2), 28.5 (CH_2), 28.2 (CH_2), 24.2 (CH_2), 22.5 (CH_2), 18.7 (CH_2C), 18.5 (CH_2C), 14.0 ppm (CH_3); EI-MS (70 eV, MSTFA-derivative): *m/z* (%) = 296 (1), 281 (49), 226 (5), 206 (11), 178 (5), 164 (38), 150 (11), 129 (23), 117 (48), 94 (39), 75 (92), 73 (100), 55 (27), 41 (27).

(*Z*)-7-Tetradecenoic acid. 7-Tetradecynoic acid (0.23 g, 1.0 mmol) was dissolved in ethanol (5 mL), and Lindlar catalyst (0.02 g) was added to the solution. The mixture was stirred for 2 h under H_2 at room temperature. The reaction mixture was filtered through a short plug of silica To remove the catalyst and the solvent was evaporated to afford a yellow oil. The crude product was purified by flash chromatography to give the acid (0.20 g, 0.9 mmol, 90%) in a mixture of stereoisomers (*Z*/*E* 98:2, as determined by GC/MS) as a colourless oil. $R_F = 0.37$ (pentane/diethyl ether 2:1); m.p. 32 °C (from pentane/diethyl ether); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 10.20$ (br s, 1H; COOH), 5.40–5.29 (m, 2H; 2CH), 2.35 (t, $^3J = 7.5$ Hz, 2H; CH_2CO_2), 2.06–1.99 (m, 4H; 2CH_2CH), 1.64 (tt, $^3J = 7.3$, 3.7 Hz, 2H; C$H_2CH_2CO_2$), 1.38–1.27 (m, 12H; 6CH_2), 0.88 ppm (t, $^3J = 6.9$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 180.3$ (CO_2), 130.3 (CH), 129.4 (CH), 34.1 (CH_2CO_2), 31.8 (CH_2), 29.7 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 28.7 (CH_2), 27.2 (CH_2), 27.0 (CH_2), 24.6 (CH_2), 22.6 (CH_2CH_3), 14.1 ppm (CH_3); EI-MS (70 eV, MSTFA-derivative): *m/z* (%) = 298 (7), 283 (86), 208 (9), 199 (11), 185 (8) 166 (20), 155 (8), 145 (25), 129 (71), 117 (100), 96 (20), 84 (18), 75 (92), 73 (91), 55 (39), 41 (28).

(*Z*)-*N*-(Tetradec-7-enoyl)-L-homoserine lactone (5). As described by Chhabra et al.,[29] L-homoserine lactone hydrobromide (0.07 g, 0.4 mmol) was dissolved in H_2O. Triethylamine (0.05 mL, 0.4 mmol) was added to the stirred solution, followed either by the addition of acid (0.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.07 g, 0.4 mmol). The reaction mixture was stirred for 3 h at room temperature, and then the solvent was evaporated in vacuo. The residue was extracted with EtOAc (5×). The combined organic extracts were successively washed with H_2O, saturated $NaHCO_3$ solution and brine, dried ($MgSO_4$) and concentrated. The crude product was purified by flash column chromatography to obtain **5** (0.06 g, 0.2 mmol, 56%) in a mixture of stereoisomers (*Z*/*E* 98:2, as determined by GC/MS) as a white solid. $R_F = 0.10$ (pentane/EtOAC 2:1); $[\alpha]_D^{22}$: −17.7 ($c = 1.03$ in MeOH); m.p. 135 °C (from EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 6.49$ (br s, 1H; NH), 5.39–5.29 (m, 2H; CH=CH), 4.61 (ddd, $^3J = 11.5$, 8.6, 6.4 Hz, 1H; NCH), 4.46 (t, $^3J = 9.0$ Hz, 1H; OCH_2), 4.28 (ddd, $^2J = 11.1$, $^3J = 9.3$, 6.0 Hz, 1H; OCH_2), 2.86–2.77 (m, 1H; NCHCH_2), 2.31–2.24 (m, 2H; CH_2CON), 2.22–2.11 (m, 1H; NCHCH_2), 2.04–1.98 (m, 4H; 2CH_2CH), 1.69–1.59 (m, 2H; CH_2CH_2CON), 1.36–1.24 (m, 12H; 6CH_2), 0.88 ppm (t, $^3J = 6.7$ Hz, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 175.7$ (CON), 173.8 (CO_2), 130.1 (CH), 129.3 (CH),

66.0 (CH_2O), 49.0 (CHN), 36.0 (CH_2CON), 31.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 28.9 (CH_2), 28.8 (CH_2), 27.2 (CH_2), 27.0 (CH_2), 25.2 (CH_2), 25.0 (CH_2), 22.6 (CH_2CH_3), 14.0 ppm (CH_3); EI-MS (70 eV): m/z (%) = 309 (17) $[M]^+$, 208 (22), 196 (4), 179 (5), 156 (30), 143 (75), 125 (23), 102 (55), 83 (33), 67 (52), 55 (100), 41 (88).

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