N-Acylated Alanine Methyl Esters (NAMEs) from *Roseovarius tolerans*, Structural Analogs of Quorum-Sensing Autoinducers, *N*-Acylhomoserine Lactones

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The *Roseobacter* clade is one of the most important bacteria group living in the ocean. Liquid cultures of *Roseovarius tolerans* EL 164 were investigated for the production of autoinducers such as *N*-acylhomoserine lactones (AHLs) and other secondary metabolites. The XAD extracts were analyzed by GC/MS. Two AHLs, Z7-C14:1-homoserine lactone (HSL) and C15:1-HSL, were identified. Additionally, the extract contained five compounds with molecular-ion peaks at *m*/*z* 104, 145, and 158, thus exhibiting mass spectra similar to those of AHLs with corresponding peaks at *m*/*z* 102, 143, and 156. Isolation of the main compound by column chromatography, NMR analysis, dimethyl disulfide derivatization for the determination of the location of the C=C bond and finally synthesis of the compound with the proposed structure confirmed the compound to be (*Z*)-*N*-(hexadec-9-enoyl)alanine methyl ester. Four additional minor compounds were identified as C14:0-, C15:0-, C16:0-, and C17:1-*N*-acylated alanine methyl esters (NAMEs). All NAMEs have not been described from natural sources before. A BLASTp search showed the presence of AHL-producing *luxI* genes, but no homologous genes potentially responsible for the structurally closely related NAMEs were found. The involvement of the NAMEs in chemical communication processes of the bacteria is discussed.

Introduction. – Autoinducers are small diffusible signal molecules secreted by bacteria in a cell-to-cell communication process called *quorum sensing* that is used to regulate their gene expression in a cell density-dependent manner [1–3]. Several widely different traits such as cell differentiation, expression of virulence factors, flagella or biofilm formation are known to be quorum sensing regulated [4–7]. Bacteria of the *Roseobacter* clade constitute some of the most important bacteria in the ocean [8][9]. In this clade quorum sensing regulates, *e.g.*, antibiotic production in *Phaeobacter* [10], flagella formation, type-IV secretion, and the mode of cell division in *Dinoroseobacter* [11], inhibits biofilm formation and activates motility in *Ruegeria* [12], and influences motility in *Silicibacter* [13]. The dominant class of autoinducers used in *Gram*-negative bacteria, to which the *Roseobacter* clade belongs, are *N*-acylhomoserine lactones (AHLs) [14]. Quorum sensing mediated by AHLs is one of the best-understood bacterial systems at the molecular level [15]. Usually, the quorum-sensing circuit consists of a LuxI/LuxR-type system, with LuxI being the synthase of the autoinducer and LuxR its co-evolved receptor. After the synthesis of the autoinducer

by LuxI, it freely diffuses in and out of the cell. Productive binding to the receptor LuxR only occurs at a certain threshold concentration for which a high intracellular ligand concentration and thus a high cell density is needed. The LuxR–autoinducer complex then acts as a transcriptional regulator, binding to DNA and inducing expression of downstream genes. In many but not all bacteria, the activated LuxR complex at the same time also enhances the production of the autoinducer itself, thus resulting in a positive feedback loop [16].

AHL Autoinducers share several structural characteristics. They are comprised of a homoserine ring in L-configuration which is unsubstituted in the β- and γ-position, and *N*-acylated with an acyl chain derived from fatty-acid biosynthesis [5]. The chain length varies from 4 to 18 C-atoms with C_{14}, C_{16}, and C_{18} dominating in bacteria of the *Roseobacter* clade [17]. The acyl residue is most often unbranched and saturated, mono- or diunsaturated. The monounsaturated chain normally carries a C=C bond in (*Z*)-configuration. Diunsaturated AHLs, *e.g. N*-[(2*E*,9*Z*)-hexadeca-2,9-dienoyl]homoserine lactone, have been identified in *Jannaschia helgolandensis*, *Staleya guttiformis*, and *Dinoroseobacter shibae* [17]. AHLs with a rare methyl branching in iso position have been identified in *Aeromonas culicicola* 3249^T [18]. Additionally, modifications at C(3) of the acyl chain are possible comprising a OH or C=O function. For example, *N*-(3-hydroxydecanoyl)homoserine lactone (3-OH-C_{10}-AHL) has been identified in *Roseobacter gallaeciensis* [17], *Sinorhizobium meliloti* [19], *Pseudomonas fluorescens* [20], and *Burkholderia pseudomallei* [21]. The keto compound 3-oxo-C_{14}-AHL was identified in *R. tolerans* EL-78 and EL-90 [17], as well as in *S. meliloti* besides other 3-oxo-AHLs [22]. In addition, AHLs carrying a *p*-coumaroyl residue derived from environmental *p*-coumaric acid were identified in *Rhodopseudomonas palustris*, *Bradyrhizobium* sp., and *Silicibacter pomeroyi* [23]. Usually, several AHLs are produced by one strain in nanomolar concentrations or less. The pattern produced is stable for a certain organism under identical cultivation conditions but different species produce different patterns.

During our in-depth study on the variation of AHLs in various members of the *Roseobacter* clade, we encountered unknown related metabolites. We report here the identification of five *N*-acylated alanine methyl esters (NAMEs), the structures of which show striking similarities to AHLs produced by *R. tolerans*. A XAD enrichment method was used to extract these secondary metabolites from *Roseovarius* cultures [24]. The compounds were identified by GC/MS analysis, isolation of the major compound, and synthesis. A preliminary bioassay was performed to evaluate the function of the compounds.

Results and Discussion. – *Extraction*. The secondary metabolites released by liquid cultures of *Roseovarius tolerans* EL-164 were collected by extraction *via Amberlite XAD-16*. The resin was added to freshly inoculated liquid cultures of *R. tolerans*. This procedure removes bacterial metabolites from the aqueous phase of the culture, thus likely enhancing their production if a concentration-dependent feedback loop exists for the target compounds. The concentrated extracts were investigated by GC/MS. Two AHLs, *N*-[(*Z*)-tetradec-9-enoyl]homoserine lactone (**2**, C14 : 1-AHL) and *N*-[(*Z*)-pentadec-9-enoyl]homoserine lactone (**7**), were readily identified (*Fig. 1*).

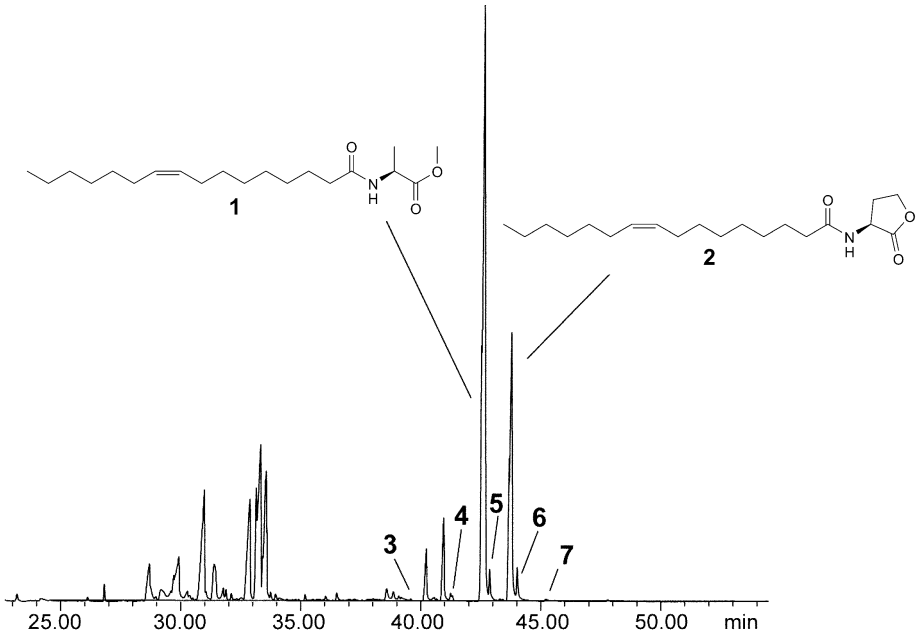


Fig. 1. *Expansion of the total ion chromatogram* (24–55 min) *of the XAD extract of* R. tolerans *showing the AHLs* **2** *and* **7**, *as well as the NAMEs* **1** *and* **3**–**6**

Compound **2** has previously been detected in *R. tolerans* in addition to the corresponding C14 : 0-, C16 : 0-, and C16 : 1-AHLs [17].

Structure Elucidation. However, five compounds, **1** and **3**–**6**, showed mass spectra very similar to those of the AHLs. These mass spectra are characterized by fragment-ion peaks at *m*/*z* 104, 145, and 158, compared to those at *m*/*z* 102, 143, and 156, in case of AHLs (*Fig. 2*). These fragment ions can be explained by cleavage of the amide-bond, and β- and γ-cleavage as indicated in the figure. The amount of the major compound **1** was sufficient for isolation by column chromatography and investigation by NMR spectroscopy. The NMR spectra showed an ester-bound Me group with a chemical shift of δ 3.75 (*s*), a CH H-atom next to a CO group with a signal at δ 4.61 (*quint.*, $J = 7.2$) and a N-atom in a N–CH–Me spin system, suggesting an alanine methyl ester structure. Moreover, the typical NMR signals of an acyl chain established that the amino group was acylated by a long-chain fatty acid with a C=C bond in (*Z*)-configuration.

By addition of dimethyl disulfide (DMDS) to the crude extract and subsequent investigation by GC/MS, the location of the C=C bond was elucidated [18][25][26]. The mass spectrum of the addition product is shown in *Fig. 3*. Cleavage preferentially takes place between the methylsulfanyl groups and results in two characteristic ion peaks at *m*/*z* 145 $[C_7H_{14}SMe]^+$ and 288 $[MeOCOCHCH_3NH–COC_8H_{15}SMe]^+$, indicating the C=C bond to be located at C(9). The latter fragment ion contains the alanine methyl ester moiety which is lost to give rise to the fragment-ion peak at *m*/*z* 185. The structure proposal was supported by HR-MS data (*Table*), indicating the molecular formula $C_{20}H_{37}NO_3$.

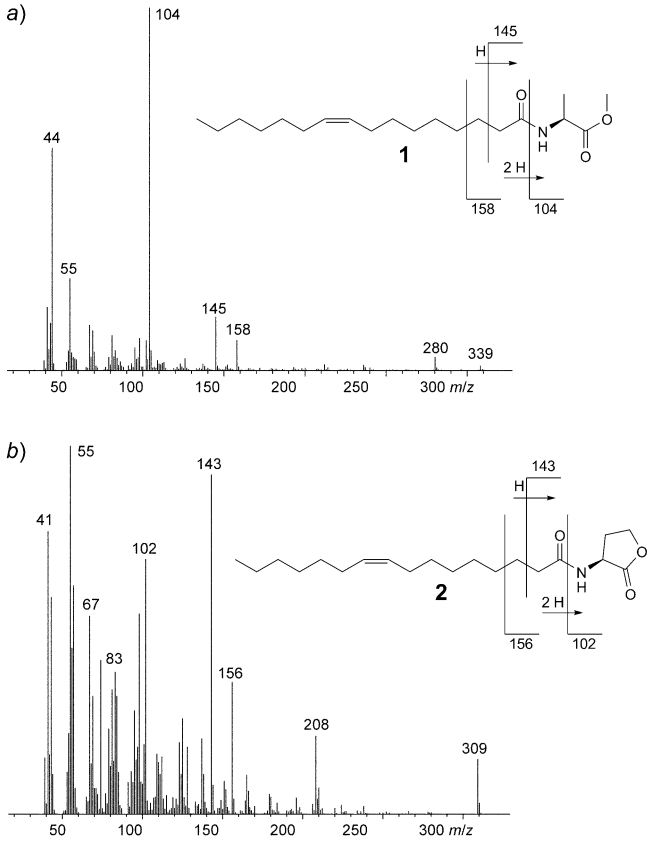


Fig. 2. *Mass spectra of* a) *(*S*)*-N-*[(*Z*)-hexadec-9-enoyl]alanine methyl ester* (**1**)*, and* b) N-*[(*Z*)-tetradec-9-enoyl]homoserine lactone* (**2**)

To establish the structure, *N*-[(*Z*)-hexadec-9-enoyl]alanine methyl ester (**1**) was synthesized by coupling palmitoleic acid with L-alanine methyl ester (*Scheme 1*). Comparison of the spectral data of the natural product with those of the synthetic sample confirmed the proposed structure. In addition, the GC retention indices (*RI*s) of the natural and synthetic compound match ($RI_{nat} = 2479$, $RI_{syn} = 2477$).

The structures of the remaining four *N*-acylated alanine methyl esters (NAMEs), **3**–**6**, were suggested based on the analysis of their mass spectra (*Fig. 4*). Compound **6** (C17:1-NAME) shows a mass spectrum almost identical to that of **1**. Since the molecular-ion M^+, peak at *m*/*z* 353 is 14 u higher compared to **1**, indicating an additional CH_2 group, **6** was proposed to be the higher homolog *N*-[(*Z*)-heptadec-9-enoyl]alanine methyl ester. The DMDS derivative evidenced the location of the double bond at C(9). Unsaturated AHLs show a characteristic loss of the homoserine moiety ($[M-101]^+$) [17]. The unsaturated C16:1- and C17:1-NAMEs exhibit peaks of corresponding fragment ions, $[M-103]^+$, resulting from the loss of the alanine methyl ester moieties at *m*/*z* 236 and 250, but in low intensities. The mass spectra of compounds

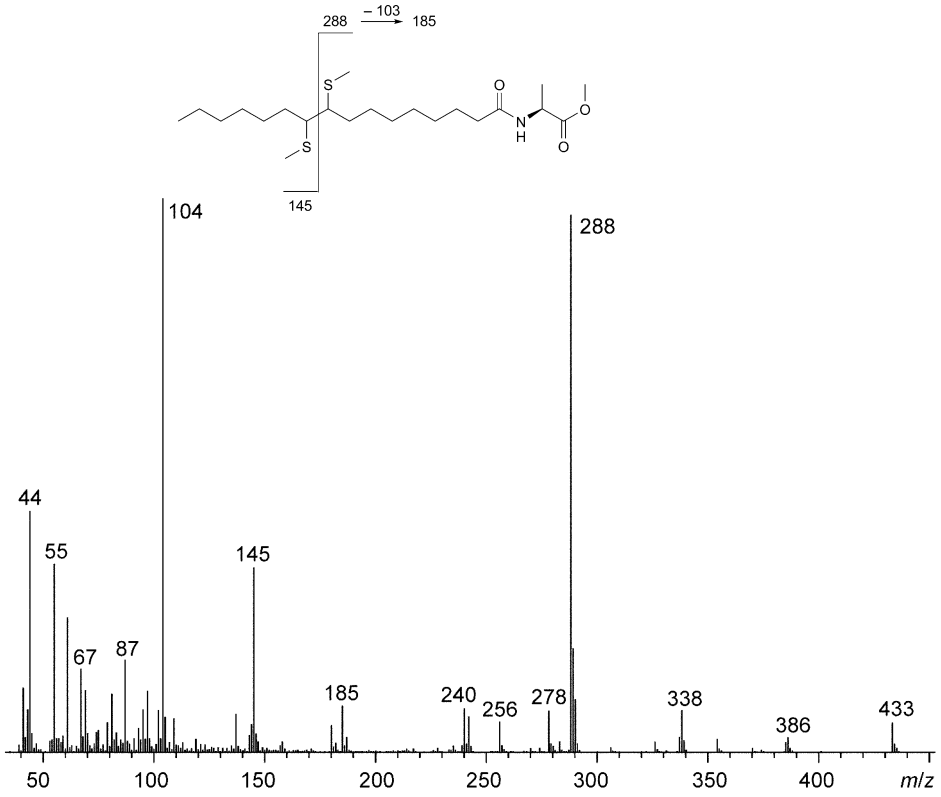


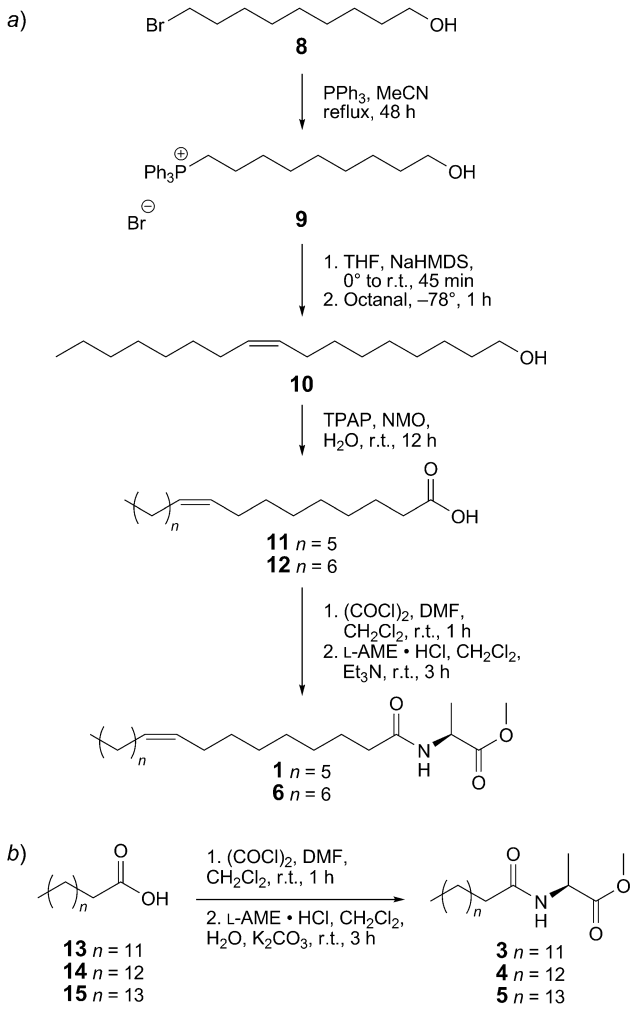
Fig. 3. *Mass spectrum of the DMDS derivative of* **1**. The ion peaks at *m*/*z* 145 and 288 indicate the C=C bond at C(9).

Table. *HR-MS Data of Natural* N-*Acylalanine Methyl Esters*

	Molecular ion	$[M+H]^+$ or $[M+Na]^+$ [*m*/*z*]		Acyl side chain
		calc.	found	
3	$C_{18}H_{35}NNaO_3^+$	336.2509	336.2509	$C_{14}H_{27}O$
4	$C_{19}H_{37}NNaO_3^+$	350.2671	350.2666	$C_{15}H_{29}O$
5	$C_{20}H_{40}NO_3^+$	342.3008	342.3003	$C_{16}H_{31}O$
1	$C_{20}H_{38}NO_3^+$	340.2852	340.2845	$C_{16}H_{29}O$
6	$C_{21}H_{40}NO_3^+$	354.3008	354.3003	$C_{17}H_{31}O$

3–**5** also exhibit the fragment-ion peaks at *m*/*z* 104, 145, and 158, typical for NAMEs, albeit with different ion intensities compared to the unsaturated NAMEs. For the latter, the base peak is at *m*/*z* 104, whereas it is at *m*/*z* 145 for compounds **3**–**5**. Similarly, mass spectra of AHLs show an increased intensity of the fragment-ion peak at *m*/*z* 102 for unsaturated AHLs and at *m*/*z* 143 for saturated AHLs [17][27]. Taking into account the molecular-ions peak at *m*/*z* 313, 327 and 341, which are small but significant,

Scheme 1. *Synthesis of* a) *Unsaturated NAMEs* **1** *and* **6**, *and* b) *Saturated NAMEs* **3**, **4**, *and* **5**. NaHMDS, Sodium hexamenthyldisilazide (=sodium bis(trimethylsilyl)amide; TPAP, tetrapropylammonium perruthenate; NMO, *N*-methylmorpholine *N*-oxide.



compounds **3**–**5** were proposed to be *N*-(tetradecanoyl)-, *N*-(pentadecanoyl)-, and *N*-(hexadecanoyl)alanine methyl esters, respectively.

Since acyl side chains with an uneven number of C-atoms are less abundant in bacteria, a Me-branched side chain was also considered for these compounds. We have previously developed a *RI* calculation model for AHLs and related compounds, allowing determination of the branch position in long-chain aliphatic compounds [18][28]. For an additional Me group in the chain, *RI* are by 30–75 units higher, while an addition within the chain leads to an increment of 100 units. The *RI* of **3**–**5**, 2292, 2395, and 2500, indicate a uniform, unbranched side chain, established also by

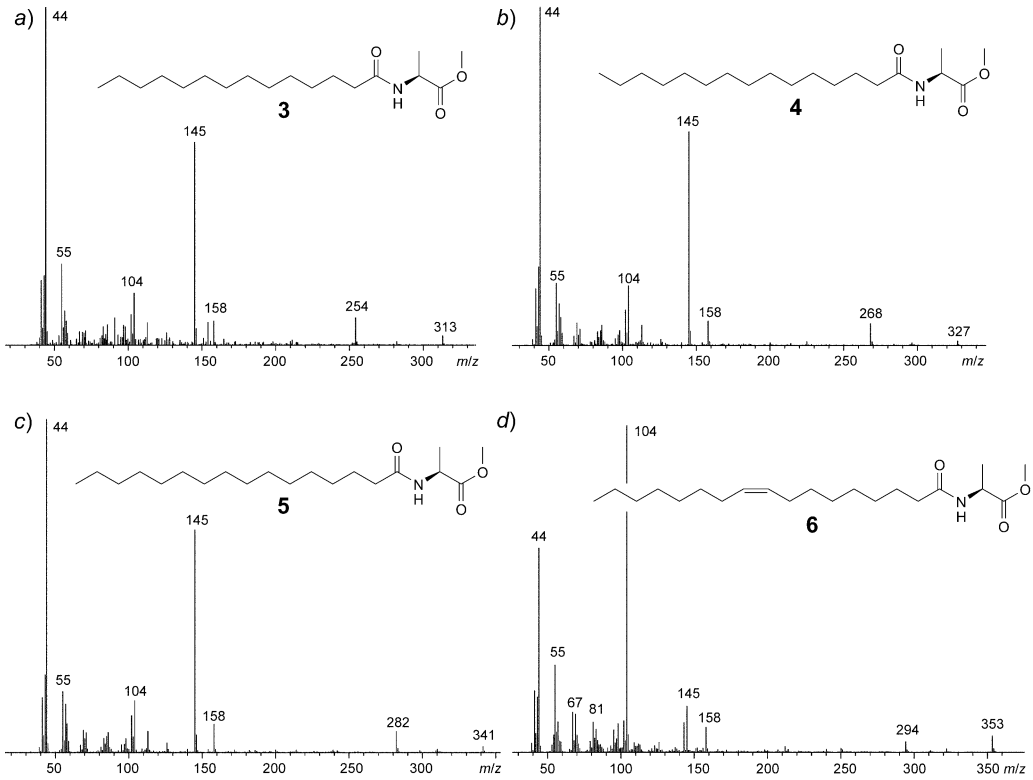


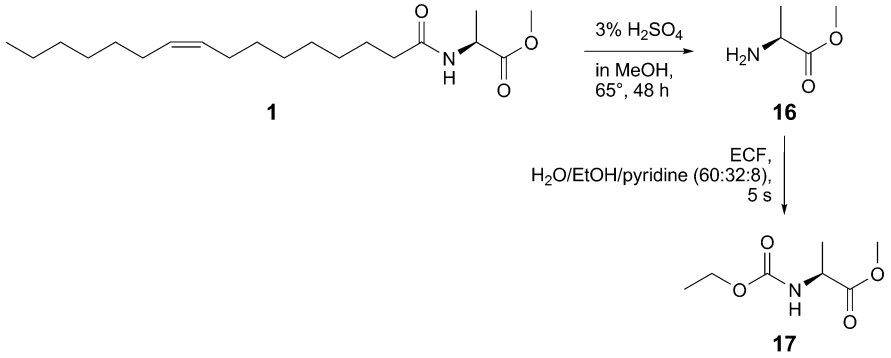
Fig. 4. *Mass spectra of the NAMEs* a) **3**, b) **4**, c) **5**, *and* d) **6**

comparison with the synthetic material (see below). All structural proposals were supported by HR-ESI-MS data (*Table*).

Synthesis. For final verification, compounds **3**–**6** were synthesized as outlined in *Scheme 1*. The synthesis of **6** started by conversion of 9-bromononan-1-ol (**8**) to the corresponding *Wittig* salt (9-hydroxynonyl)triphenylphosphonium bromide (**9**) and subsequent *Wittig* olefination with heptanal to give (*Z*)-heptadec-9-en-1-ol (**10**). Oxidation with tetrapropylammonium perruthenate (TPAP) yielded (*Z*)-heptadec-9-enoic acid (**12**). Conversion to the corresponding acid chloride with oxalyl chloride and subsequent coupling with L-alanine methyl ester hydrochloride gave target compound **6** (C17:1-NAME). In analogy to the last reaction step, conversion of tetradecanoic, pentadecanoic, and hexadecanoic acid into the corresponding acid chlorides and coupling with L-alanine methyl ester hydrochloride furnished the NAMEs **3**–**5**, respectively. All synthetic material was identical to the corresponding natural compounds, and the retention indices were in good agreement.

Assignment of Absolute Configuration. The absolute configuration of the alanine methyl ester part was assigned by cleavage of the amide bond of the NAMEs in the natural sample as well as in the synthetic **1**, followed by derivatization of the amine function with ethyl chloroformate (ClCOOEt; ECF) (*Scheme 2*). These derivatives

Scheme 2. *Cleavage of the Amide Bond and ECF Derivatization for NAME* **1**. ECF, ClCOOEt.



were analyzed by GC on chiral phases (*Fig. 5*). Comparison by coinjection experiments with a racemate of alanine methyl ester established the absolute configuration of the natural NAMEs as exclusively L.

AHL Synthethase. A search with the Pfam domain for autoinducer synthetases (PF00765) as well as BLASTp searches with the amino acid sequences of known AHL synthetases of members of the *Roseobacter* clade resulted in one AHL synthetase (ROT_01220). Examination of the surrounding neighborhood revealed the presence of a LuxR-type regulator (ROT_01219), completing the two-component system. The gene organization showed the highest synteny as well as high sequence homologies of the AHL synthase to the closely related strains *Roseovarius* sp. 217 and *Roseovarius* sp. TM1035 (*Fig. 6*). This AHL synthase seems to be responsible for the production of both identified AHLs, **2** and **7**. With **2** being the main and **7** the minor product, the normal substrate for the AHL synthase seems to be the C14 : 1 acyl chain, though C15 : 1 is also converted. There are known examples in which a single LuxI-type AHL synthase is responsible for the production of several structurally similar AHLs [22].

N-*Acylated Amino Acid Synthases* (NASs). Other *N*-acylated amino acids are structurally similar to the AHLs and NAMEs mentioned. In screenings for antibiotic compounds in cosmid libraries of environmental DNA expressed in *Escherichia coli* by *Van Wagoner* and *Clardy*, the most frequently encountered small molecules were *N*-acylated amino acids [29]. So far, *N*-acylated amino acids of tyrosine [30], tryptophan, arginine [31], and phenylalanine [32] have been described, each comprising both saturated and monounsaturated fatty-acid residues. The chain length of the fatty-acid residues of long-chain *N*-acyltyrosines ranges from C_8 to C_{18}, with C_{14} as the most frequent residue. Fatty-acid residues observed with tryptophan have a chain length of C_{16} and C_{18}, whereas both arginine and phenylalanine residues vary from C_{14} to C_{16}. These *N*-acylated amino acids are produced by a family of *N*-acyl amino acid synthases (NASs). NASs like FeeM share structural similarities with GCN5-related *N*-acyl transferases (GNATs) including acyl homoserine lactone synthases [29]. Like AHL synthases, NASs form an amide bond between an amino group and a fatty acid. In addition, the fatty acid is acyl carrier protein-bound as used by AHL synthases rather than coenzyme A-bound like used by most other GNAT like proteins. Based on these similarities in structure and substrate usage between AHL synthases and GNATs, the

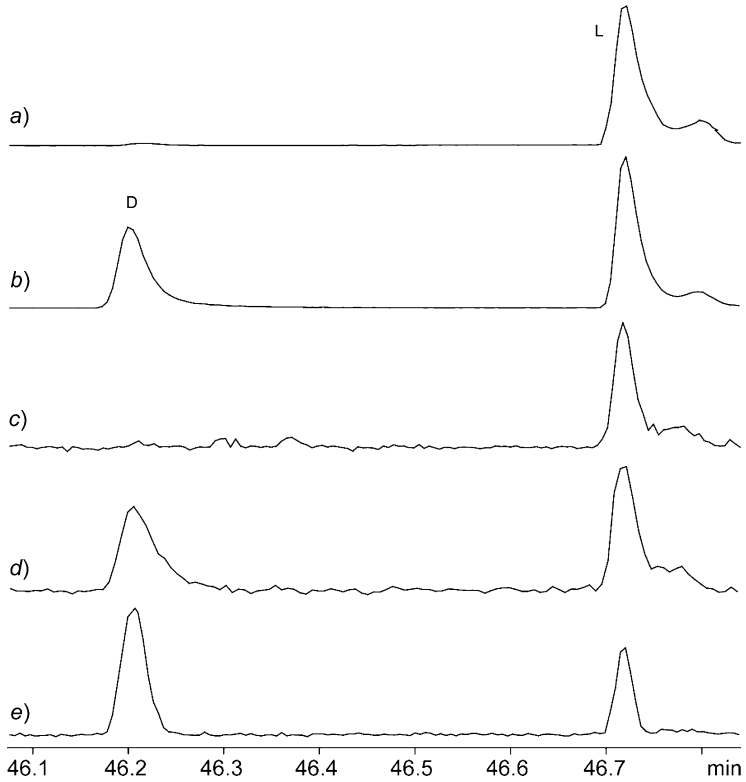


Fig. 5. *Determination of the configuration of the alanine part of NAMEs of* R. tolerans *by chiral GC on a β-DEX 225 phase. a*) NAME **1**. *b*) Co-injection NAME **1** and racemate. *c*) Natural sample. *d*) Co-injection natural sample and racemate. *e*) Racemate.

authors proposed also a functional link between the enzyme products with *N*-acyl amino acids functioning as bacterial-signaling agents [29].

The search for a *N*-acylalanine synthase proved difficult. So far, the family of *N*-acyl amino acid synthases (NASs) comprises synthases for the amino acids tyrosine, tryptophan, and arginine, as well as phenylalanine [30–32]. The known NASs show neither on the gene nor on the protein level any significant sequence similarity. Even in a group of ten sequenced *N*-acyltyrosine synthases, no absolutely conserved residues in the protein sequence were detected [33]. Thus, it is not surprising that a BLASTp search against known NASs sequences gave no results.

Bioassays. Due to the striking similarity of NAMEs and AHLs, the autoinducing activity of the NAMEs was tested in bioassays specific for long-chain AHLs. The quorum-sensing as well as the quorum-quenching activity was tested using the reporter strain *P. putida* pKR–C12 under standard conditions [34]. Even at a relatively high concentration of 5 μM, no induction of the reporter strain was observed nor did the NAMEs inhibit the induction of the reporter in the presence of structurally similar AHLs. Since neither quorum-sensing nor quorum-quenching activity was observed in these tests, NAMEs might have a different function for the bacterium. One could even

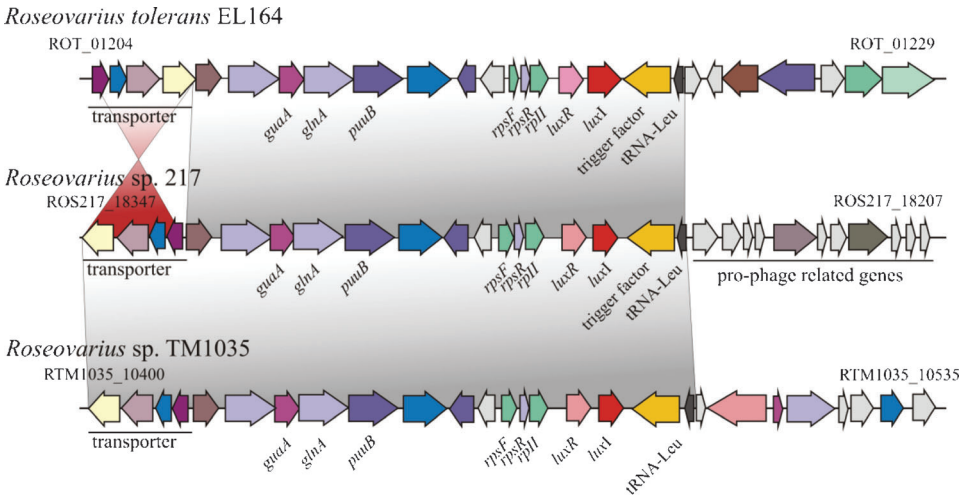


Fig. 6. *Gene organization in the neighborhood of the LuxI like synthetase* (red arrow) *in* R. tolerans *and its homology to* Roseovarius sp. 217 *and* Roseovarius sp. TM1035

speculate that, for the so far unknown function of NAMEs, it might be important that there is no interference whatsoever with the AHL quorum-sensing system of the bacterium. The inactivity in the AHL assay may be a prerequisite for proper function of NAMEs, because the actual function of the NAMEs in the physiology of *R. tolerans* is unknown.

Conclusions. – In summary, we have shown that *Roseovarious tolerans* EL-164 secretes new natural compounds, *N*-acylated alanine methyl esters during cultivation alongside with the well-known signaling compounds of the *Roseobacter* clade, AHLs. This distinguishes this bacterium from many other bacteria of this clade, which very often can secrete AHLs. Their structural analogy suggests a role in bacterial communication, but a specific function of associated genes has not been found yet. The synthetic route developed allows investigation of this bacterium and its communication system in more detail.

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Experimental Part

General. Chemicals were purchased from *Sigma–Aldrich Chemie GmbH* (DE-Steinheim) or from *Acros Organics* (Geel, Belgium), and used without further purification. Solvents were purified by distillation and dried according to standard procedures. Moisture- and/or oxygen-sensitive reactions were carried out under N_2 in vacuum-heated flasks with dried solvents. TLC: 0.20 mm *Macherey-Nagel* silica gel plates (*Polygram SIL G/UV254*). Column chromatography (CC): *Merck* silica gel *60* (0.040–0.063 mm) using standard flash chromatographic methods. GC/MS: *HP6890* GC system connected to a *HP5973 Mass Selective Detector* fitted with a *BPX-5* fused silica cap. column (25 m × 0.22 mm i.d., 0.25 μm film, *SGE Inc.*, Melbourne, Australia); conditions: inlet pressure; 97.0 kPa; He, 45.5 ml min^{-1};

injection volume; 1 µl; injector; 250°; transfer line; 300°; electron energy; 70 eV. The GC was programmed as follows: 50° (5 min isothermic), increasing at 10° min^{-1} to 320°, and operated in split mode (35 : 1); carrier gas (He); 1.2 ml min^{-1}. GC/MS Analyses of XAD extracts and of the synthesized NAMEs: *Agilent GC 7890A* system connected to a *5975C* mass-selective detector (*Agilent*) fitted with a *HP-5* MS fused silica cap. column (30 m × 0.25 mm i.d., 0.22 µm film; *Hewlett-Packard*, Wilmington, USA), conditions: inlet pressure; 67.5 kPa; He, 24.2 ml min^{-1}; injection volume; 1 µl; injector; 250°; transfer line; 300°; electron energy; 70 eV. The GC was programmed as follows: 50° (5 min isothermic), increasing at 5° min^{-1} to 320°, and operated in splitless mode; carrier gas (He): 1.2 ml min^{-1}. Chiral GC/MS analyses: the same system but fitted with a *β–DEX 225* column (30 m × 0.32 mm i.d.; 0.25 µm film, *SGE Analytical Science*, Victoria, Australia), conditions: inlet pressure; 23.1 kPa; He, 25.0 ml min^{-1}; injection volume; 1 µl; injector; 210°; transfer line; 300°; electron energy; 70 eV. The GC was programmed as follows: 30° (5 min isothermic), increasing at 10° min^{-1} to 110° and then with 25° min^{-1} to 210°, and operated in splitless mode; carrier gas (He): 2.0 ml min^{-1}. Retention indices (*RI*s) were determined from a homologous series of *n*-alkanes ($C_8 - C_{33}$). UV Spectra: *Varian Cary 100 Bio* spectrometer. IR Spectra: *Bruker Tensor 27 ATR* spectrometer. NMR Spectra: *Bruker DRX-400* (400 MHz), *AV III-400* (400 MHz), or *AV II-600* (600 MHz) spectrometers; referenced to TMS (δ 0.00 ppm) for ^{1}H-NMR and $CHCl_3$ (δ 77.01 ppm) for ^{13}C-NMR, chemical shifts are in ppm, coupling constants *J* in Hz.

Strains, Culture Conditions, and Extraction. *Roseovarius tolerans* EL-164 was obtained from the *Deutsche Sammlung für Mikroorganismen und Zellkulturen* (DSMZ). Precultures were routinely grown on marine broth medium (MB, Carl Roth, (DE-Karlsruhe)) in *Erlenmeyer* flasks at 28° on a rotary shaker at 150 rpm. *Erlenmeyer* flasks (500 ml) containing 100 ml of MB were inoculated with 2% preculture, and 2% of *Amberlite XAD-16* (*Sigma–Aldrich*, Germany) was added. After 14 d of growth, the resin was filtered off and extracted with 3 × 50 ml of CH_2Cl_2/H_2O 3 : 1 (*v*/*v*). The two phases were separated, the org. phase was dried ($MgSO_4$), and the solvent was evaporated under reduced pressure. The extract was concentrated at 60° under N_2 to a volume of *ca.* 500 µl. For HPLC analysis, 400 µl of an extract was evaporated to dryness and redissolved in 300 µl of MeCN.

Sequencing and Phylogenetic Analysis. The genome of *R. tolerans* EL164 was sequenced using the *Illumina* sequencing technology. The draft genome consists of 273 scaffolds with 3.15 Mbp. Open reading frames (ORFs) were identified and annotated using the automatic pipeline from the *Integrated Microbial Genomes* (*IMG*) Database [35]. In total, 3.243 genes could be identified of which 3.200 encode proteins.

Bioassays. The NAMEs **1** and **3**–**5** were tested according to the following procedure. The sensor strain *P. putida* pKR-C12 [34] was inoculated from plates into preculture which was grown on LB medium (20 ml with 20 µg/ml gentamycin) at 30° overnight. The next day fresh medium was added, and the culture was grown on a shaking platform for 1–2 h until an OD_{620} value of 1.0 was reached. For the test, LB medium (99 µl) and the NAME (1 µl of a 1 µM stock soln. in DMSO) were pipetted into 96-well microtitre plates, and the sensor strain (100 µl) was added (end concentration of NAMEs 5 µM). Each of the NAMEs was measured in triplicate. Microtitre plates were incubated at 30° and shaken. After 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 h, fluorescence was determined in a *Victor 1420 Multilabel Counter* (*Perkin–Elmer*) at an excitation wavelength λ of 485 nm and a detection wavelength λ of 535 nm. OD_{620} was also measured. DMSO was used as negative control, and synthetic acyl homoserine lactones (3-oxo-C12 : 0-, C14 : 0-, C15 : 0-, C16 : 0-, and C16 : 1-AHLs) were used as positive controls. 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 those previously determined. To test the quorum-quenching activity, each of the NAME **1**, **3**, and **5** (5 µM) was incubated with its structural analog AHL C14 : 0, C15 : 0, and C16 : 1, resp. (5 µM).

Derivatizations and Cleavage. To 50 µl of the sample, 50 µl of DMDS and 5 µl of I_2 soln. (5% in Et_2O) were added. The mixture was heated to 60° in a closed vial for 12 h. The soln. was hydrolyzed with sat. $Na_2S_2O_3$ soln. and extracted with AcOEt (2 × 100 µl). The combined org. layers were dried ($MgSO_4$), and the sample was concentrated at 60° under N_2 to a volume of *ca.* 30 µl [25][26].

Cleavage of the Amide Bond. For the cleavage, 100 µl of an XAD extract were evaporated to dryness and dissolved in 200 µl of MeOH containing 3% H_2SO_4. The mixture was heated to 65° in a closed vial for

48 h. Then, a spatula of $NaHCO_3$ was added for neutralization. The solvent was evaporated at 60° under N_2, and the residue was dissolved in 100 µl of CH_2Cl_2.

Ethyl Chloroformate (ClCOOEt) *Derivatization*. For the derivatization, 50 µg of sample were diluted in 100 µl of H_2O/EtOH/pyridine 60 : 32 : 8. Upon addition of 5 µl of ClCOOEt, the closed reaction tube was shaken for 5 s. Then, 100 µl of CH_2Cl_2 containing 1% ClCOOEt were added. The org. phase was removed with a syringe and dried ($MgSO_4$) [36].

Syntheses. *(*S*)-*N*-(Tetradecanoyl)alanine Methyl Ester* (= *Methyl* N-*Tetradecanoyl*-L-*alaninate*; **3**). *Representative Procedure for Amide Coupling*. Under N_2, tetradecanoic acid (0.86 ml, 2.5 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (12 ml) and a drop of DMF was added [37][38]. Upon slow addition of $(COCl)_2$ (1.48 ml, 3.75 mmol, 1.5 equiv.) at 0°, the mixture was stirred until no gas formation was observed anymore. The solvent was removed under reduced pressure. K_2CO_3 (1.04 g, 7.50 mmol, 3 equiv.) was added to a soln. of L-alanine methyl ester hydrochloride (0.35 g, 2.5 mmol, 1.0 equiv.) in H_2O/CH_2Cl_2 1 : 1 (*v*/*v*) at 0°, and the mixture was stirred for 5 min. The acid chloride was added dropwise in CH_2Cl_2 (4 ml). After stirring for 3 h, the phases were separated, and the H_2O phase was extracted with CH_2Cl_2 (3 × 5 ml). The combined org. phases were dried with $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (FC; SiO_2; pentane/AcOEt 2 : 1) to afford pure **3** (0.45 g, 57%). White solid. R_f (pentane/AcOEt 2 : 1): 0.40. *RI:* 2293. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.09 (*d*, *J* = 7.0, NH); 4.61 (*quint.*, *J* = 7.2, CH); 3.75 (*s*, MeO); 2.21 (*t*, *J* = 7.6, $COCH_2$); 1.63 (*quint.*, *J* = 7.4, $COCH_2CH_2$); 1.40 (*d*, *J* = 7.2, *Me*CH); 1.32–1.25 (*m*, 10 CH_2); 0.88 (*t*, *J* = 6.9, *Me*CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.7 (NHC=O); 172.6 (C=O); 52.4 (MeO); 47.8 (CH); 36.5 (CH_2C=O); 31.9 (CH_2); 29.6 (4 CH_2); 29.4 (CH_2); 29.3 (2 CH_2); 29.2 (CH_2); 25.2 (CH_2); 22.6 (MeCH_2); 18.5 (*Me*CH); 14.1 (*Me*CH_2). EI-MS (70 eV): 313 (< 1, M^+), 254 (6), 158 (6), 145 (54), 104 (15), 102 (8), 86 (5), 69 (5), 57 (10), 55 (14), 44 (100), 43 (16).

Compounds **4** and **5** were synthesized analogously with the respective acids.

*(*S*)-*N*-(Pentadecanoyl)alanine Methyl Ester* (= *Methyl* N-*Pentadecanoyl*-L-*alaninate*; **4**). Yield: 67%. White solid. R_f (pentane/Et_2O 2 : 1): 0.11. *RI:* 2395. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.06 (*d*, *J* = 6.8, NH); 4.62 (*quint.*, *J* = 7.2, CH); 3.75 (*s*, MeO); 2.21 (*t*, *J* = 7.7, $COCH_2$); 1.63 (*quint.*, *J* = 7.4, $COCH_2CH_2$); 1.40 (*d*, *J* = 7.2, *Me*CH); 1.30–1.25 (*m*, 11 CH_2); 0.88 (*t*, *J* = 6.9, *Me*CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.7 (NHC=O); 172.7 (C=O); 52.4 (MeO); 47.8 (CH); 36.5 (CH_2C=O); 31.9 (CH_2); 29.7(CH_2); 29.6 (4 CH_2); 29.4 (CH_2); 29.3 (2 × CH_2); 29.2 (CH_2); 25.6 (CH_2); 22.7 (MeCH_2); 18.6 (*Me*CH); 14.1 (*Me*CH_2). EI-MS (70 eV): 327 (2, M^+), 268 (7), 158 (8), 145 (64), 104 (17), 102 (8), 86 (5), 69 (6), 57 (11), 55 (15), 44 (100), 43 (17).

*(*S*)-*N*-(Hexadecanoyl)alanine Methyl Ester* (= *Methyl* N-*Hexadecanoyl*-L-*alaninate*; **5**). Yield: 65%. White solid. R_f (pentane/Et_2O 2 : 1): 0.12. *RI:* 2500. ^{1}H-NMR (400 MHz, $CDCl_3$): 5.98 (*d*, *J* = 6.8, NH); 4.61 (*quint.*, *J* = 7.2, CH); 3.75 (*s*, MeO); 2.20 (*t*, *J* = 7.7, $COCH_2$); 1.63 (*quint.*, *J* = 7.4, $COCH_2CH_2$); 1.40 (*d*, *J* = 7.2, *Me*CH); 1.30–1.25 (*m*, 12 CH_2); 0.88 (*t*, *J* = 6.8, *Me*CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.7 (NHC=O); 172.6 (C=O); 52.4 (MeO); 47.9 (CH); 36.6 (CH_2C=O); 31.9 (CH_2); 29.7(3 CH_2); 29.6 (3 CH_2); 29.5 (CH_2); 29.3 (2 CH_2); 29.2 (CH_2); 25.6 (CH_2); 22.7 (MeCH_2); 18.6 (*Me*CH); 14.1 (*Me*CH_2). EI-MS (70 eV): 341 (2, M^+), 282 (5), 158 (8), 145 (63), 104 (18), 102 (8), 86 (5), 69 (6), 57 (12), 55 (15), 44 (100), 43 (18).

*(*S*)-*N*-[(*Z*)-Hexadec-9-enoyl]alanine Methyl Ester* (= *Methyl* N-*[(9*Z*)-Hexadec-9-enoyl]*-L-*alaninate*; **1**). Under N_2, the acid **11** (0.10 g, 0.39 mmol, 1.0 equiv.) was dissolved in dry CH_2Cl_2 (5 ml), and a drop of DMF was added. Upon slow addition of $(COCl)_2$ (0.05 ml, 0.59 mmol, 1.5 equiv.) at 0°, the mixture was stirred until no gas formation was observed anymore. The solvent was removed under reduced pressure. Et_3N (0.16 ml, 1.18 mmol, 3 equiv.) was added to a soln. of L-alanine methyl ester hydrochloride (0.08 g, 0.59 mmol, 1.5 equiv.) in CH_2Cl_2 (5 ml), and the mixture was stirred for 5 min. The acid chloride was added dropwise in CH_2Cl_2 (5 ml) [37]. After stirring for 3 h, AcOEt (25 ml) was added, and the org. phase was washed with sat. $NaHCO_3$ soln. (2 × 10 ml) and H_2O (2 × 10 ml). The phases were separated, and the org. phase was dried ($MgSO_4$). The removal of the solvent under reduced pressure was followed by purification of the crude extract by FC (*RP-18*; MeCN/H_2O 6 : 1) to give **1** (64 mg, 46%). Yellow oil. R_f (MeOH/H_2O 2 : 1): 0.32. *RI:* 2477. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.04 (*d*, *J* = 6.8, NH); 5.84–5.30 (*m*, *HC*=C*H*); 4.61 (*quint.*, *J* = 7.2, CH); 3.75 (*s*, MeO); 2.20 (*t*, *J* = 7.7, $COCH_2$); 2.04–1.09 (*m*, CH_2CH=$CHCH_2$); 1.63 (*quint.*, *J* = 7.3, $COCH_2CH_2$); 1.40 (*d*, *J* = 7.2, *Me*CH); 1.35–1.24 (*m*, 8 CH_2);

0.88 (*t*, *J* = 6.9, *Me*CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.7 (NHC=O); 172.6 (C=O); 129.9 (H*C*=CH); 129.7 (HC=*C*H); 52.4 (MeO); 47.8 (CH); 36.5 (*C*H_2C=O); 31.7 (CH_2); 29.7(2 CH_2); 29.2 (2 CH_2); 29.1 (CH_2); 28.9 (CH_2); 27.2 (H_2*C*HC=CHCH_2); 27.1 (H_2CHC=C*H*CH_2); 25.5 (CH_2); 22.6 (Me*C*H_2); 18.6 (*Me*CH); 14.1 (*Me*CH_2). EI-MS (70 eV): 339 (5, M^+), 282 (3), 158 (8), 145 (13), 104 (100), 81 (8), 69 (10), 67 (12), 55 (23), 44 (62), 41 (15).

Synthesis of (9-Hydroxynonyl)(triphenyl)phosphonium Bromide (**9**). Under N_2, a soln. of 9-bromononan-1-ol (1.00 g, 4.48 mmol, 1.0 equiv.) and Ph_3P (1.29 g, 4.93 mmol, 1.1 equiv.) in MeCN was heated to reflux for 48 h [39]. The crude product was purified by FC by gradient elution (CH_2Cl_2, CH_2Cl_2/MeOH 15:1, CH_2Cl_2/MeOH 10:1) to afford **9** in quantitative yield. Viscous yellow oil. R_f (CH_2Cl_2/MeOH 15:1): 0.16. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.85–7.80 (*m*, 9 arom. H); 7.75–7.71 (*m*, 6 arom. H); 3.68–3.62 (*m*, CH_2); 3.57 (*t*, *J* = 6.6, C*H*$_2$OH); 2.73 (br. *s*, OH); 1.67–1.59 (*m*, 2 CH_2); 1.49 (*quint.*, *J* = 6.9, CH_2); 1.28–1.21 (*m*, 4 CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 134.9 (*d*, *J* = 2.9, 3 Ar); 133.3 (*d*, *J* = 10.0, 6 Ar); 103.3 (*d*, *J* = 12.5, 6 Ar); 118.0 (*d*, *J* = 85.9, 3 Ar); 62.1 (CH_2OH); 32.3 (*C*H_2CH$_2$OH); 30.0 (*d*, *J* = 15.6, CH_2); 28.7 (*d*, *J* = 10.2, CH_2); 28.5 (CH_2); 25.3 (CH_2); 22.7 (CH_2); 22.3 (CH_2), 22.2 (CH_2). ^{31}P-NMR (162 MHz, $CDCl_3$): 24.66 (*s*, PPh_3).

Synthesis of (9Z)-Heptadec-9-en-1-ol (**10**). In a round-bottom flask flushed with N_2, the *Wittig* salt **9** was dissolved in dry THF (20 ml). NaHMDS was added dropwise at 0°, and the mixture was stirred for 45 min at r.t. [40]. The bright orange soln. was cooled to −78°, and octanal was added slowly. After stirring for 1 h at −78°, the mixture was allowed to warm to r.t. It was poured on ice-cooled pentane (100 ml) and Ph_3PO was filtered off. After removing two thirds of the solvent, the mixture was precipitated on SiO_2 and purified by flash chromatography (SiO_2; pentane/AcOEt 10:1) to give **10** (0.22 g, 63%). Colorless liquid. R_f (pentane/AcOEt 10:1): 0.26. *RI:* 1975. ^{1}H-NMR (400 MHz, $CDCl_3$): 5.39–5.31 (*m*, *HC*=C*H*); 3.64 (*t*, *J* = 6.6, C*H*$_2$OH); 2.04–1.99 (*m*, *H*$_2$CHC=CHC*H*$_2$); 1.60–1.53 (*m*, C*H*$_2$CH$_2$OH); 1.37–1.22 (*m*, 10 CH_2); 0.88 (*t*, *J* = 6.9, Me). ^{13}C-NMR (100 MHz, $CDCl_3$): 130.0 (H*C*=CH); 129.8 (HC=*C*H); 63.1 (CH_2OH); 32.8 (*C*H_2CH$_2$OH); 31.9 (CH_2); 29.8 (CH_2); 29.7 (CH_2); 29.5 (CH_2); 29.4 (CH_2); 29.3 (CH_2); 29.2 (2 CH_2); 27.2 (H_2*C*HC=CH*C*H_2); 25.7 (CH_2); 22.7 (Me*C*H_2); 14.1 (*Me*CH_2). EI-MS (70 eV): 254 (< 1, M^+), 236 (14), 152 (5), 138 (10), 123 (20), 109 (36), 96 (70), 82 (83), 67 (80), 55 (100), 43 (55), 41 (91).

Synthesis of (9Z)-Heptadec-9-enoic Acid (**12**). To a soln. of **10** (0.18 g, 0.71 mmol, 1.0 equiv.), *N*-methylmorpholine *N*-oxide (NMO; 0.83 g, 7.07 mmol, 10.0 equiv.), and H_2O (0.12 ml, 7.07 mmol, 10 equiv.) in MeCN (25 ml), tetrapropylammonium perruthenate (TPAP; 0.03 g, 0.07 mmol, 0.1 equiv.) was added, and the mixture was stirred over night [41]. After removal of the solvent under reduced pressure, the crude mixture was purified by FC (SiO_2; pentane/AcOEt 10:1 with 1% AcOH) to give **12** (0.11 g, 0.24 mmol, 60%). Colorless oil. R_f (pentane/AcOEt 10:1): 0.16. ^{1}H-NMR (300 MHz, $CDCl_3$): 5.40–5.29 (*m*, *HC*=C*H*); 2.35 (*t*, *J* = 7.5, C*H*$_2$OH); 2.04–1.98 (*m*, *H*$_2$CHC=CHC*H*$_2$); 1.68–1.59 (*m*, C*H*$_2$CH$_2$OH); 1.40–1.22 (*m*, 9 CH_2); 0.88 (*t*, *J* = 6.8, CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$): 179.6 (COOH); 130.0 (H*C*=CH);129.7 (HC=*C*H); 33.9 (*C*H_2COOH); 31.9 (CH_2); 29.8 (CH_2); 29.7 (CH_2); 29.5 (CH_2); 29.3 (CH_2); 29.2 (CH_2); 29.1 (2 CH_2); 29.0 (CH_2); 27.2 (H_2*C*HC=CHCH_2); 27.1 (H_2CHC=CH*C*H_2); 24.7 (CH_2); 22.7 (*C*H_2Me); 14.1 (Me).

Compound **6** was synthesized as described for **1**.

Synthesis of *(*S*)-*N*-[(Z)-Heptadec-9-enoyl)alanine Methyl Ester* (= *Methyl* N-*[(9Z)-heptadec-9-enoyl]*-L-*alaninate*; **6**). Yield: 67%. Yellow oil. R_f (MeCN/H_2O 6:1): 0.48. *RI*: 2573. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.01 (*d*, *J* = 6.8, NH); 5.38–5.30 (*m*, *HC*=C*H*); 4.61 (*quint.*, *J* = 7.2, CH); 3.75 (*s*, MeO); 2.20 (*t*, *J* = 7.6, $COCH_2$); 2.03–1.08 (*m*, C*H*$_2$CH=CHC*H*$_2$); 1.67–1.60 (*m*, $COCH_2$C*H*$_2$); 1.40 (*d*, *J* = 7.2, *Me*CH); 1.34–1.25 (*m*, 9 CH_2); 0.88 (*t*, *J* = 6.9, *Me*CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): 173.7 (NHC=O); 172.6 (C=O); 130.0 (H*C*=CH); 129.7 (HC=*C*H); 52.4 (MeO); 47.9 (CH); 36.6 (*C*H_2C=O); 31.9 (CH_2); 29.8 (CH_2); 29.7 (CH_2); 29.3 (CH_2); 29.2 (3 CH_2); 29.1 (CH_2); 27.2 (H_2*C*HC=CH*C*H_2);25.5 (CH_2); 22.7 (Me*C*H_2); 18.6 (*Me*CH); 14.1 (*Me*CH_2). EI-MS (70 eV): 353 (5, M^+), 294 (3), 158 (8), 145 (14), 104 (100), 81 (8), 69 (9), 67 (11), 55 (22), 44 (60), 41 (14).

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