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(*Z*)-*N*-(4-Decenoyl)homoserine lactone, a new quorum-sensing molecule, produced by endobacteria associated with *Mortierella alpina* A-178

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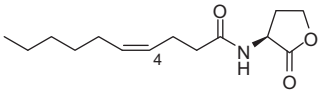
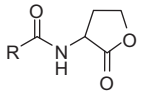
ABSTRACT

N-Acylhomoserine lactones (AHLs) are the conserved quorum-sensing signal molecules in Gram-negative bacteria. (*Z*)-*N*-(4-Decenoyl)homoserine lactone (**1**), a new AHL, was isolated from the culture broth of the fungus *Mortierella alpina* A-178 harboring bacterial endosymbionts, called endobacteria. The structure and absolute configuration of **1** were elucidated by EI-MS, chemical synthesis, and chiral GC analysis. The compound induced the expression of a QS-regulated reporter gene in *Agrobacterium tumefaciens* NTL4, although its activity was lower than that of *N*-decanoylhomoserine lactone (**6**).

Bacteria communicate with each other by producing and detecting diffusible signaling molecules. This mechanism, known as quorum-sensing (QS), allows bacteria to coordinate their activities in response to their populations.[1] The conserved QS signaling molecules of Gram-negative bacteria are *N*-acylhomoserine lactones (AHLs), which differ in the length and substitution of their respective acyl side chains, conferring upon them signal specificity.[2] Many important bacterial behaviors are regulated by AHL-dependent QS, including exoenzyme production, exopolysaccharide production, antibiotic production, motility, and biofilm formation.[1] Such coordinated bacterial actions need to succeed in developing and maintaining pathogenic and symbiotic interactions with eukaryotic hosts. Previously we discovered the production of C_6- to C_{10}-homoserine lactones (HSLs) (**2**–**6**) (Fig. 1) by bacterial endosymbionts, called endobacteria, associated with the zygomycete fungus *Mortierella alpina* A-178.[3] Molecular biological techniques revealed that an endobacterium in the fungus has the same 16S rRNA gene sequence as the β-proteobacterium *Castellaniella defragrans*. These findings suggested that AHL-mediated QS in endobacteria plays important roles in this unique symbiosis. In that study, we detected a possible new AHL in the culture extract of *M. alpina* A-178 by GC/MS. In this Letter, we describe the structure elucidation of (*Z*)-*N*-(4-decenoyl)homoserine lactone (**1**) and the activity for a QS-dependent reporter response in *Agrobacterium tumefaciens* NTL4.



L-**1**



R = $(CH_2)_4CH_3$: C_6-HSL (**2**)
R = $(CH_2)_5CH_3$: C_7-HSL (**3**)
R = $(CH_2)_6CH_3$: C_8-HSL (**4**)
R = $(CH_2)_7CH_3$: C_9-HSL (**5**)
R = $(CH_2)_8CH_3$: C_{10}-HSL (**6**)

Figure 1. Structures of AHLs produced by the endobacteria associated with *M. alpina* A-178.

The extract of the culture broth of *M. alpina* A-178 was separated by silica gel column chromatography using a gradient of *n*-hexane–EtOAc.[4] The AHL activity was evaluated with *A. tumefaciens* NTL4 harboring a plasmid with *lacZ* fused to the gene *traG* whose expression was induced by active AHLs supplied.[5] Compounds acting on *A. tumefaciens* NTL4 were eluted in 80% and 100% EtOAc fractions. The fractions were combined and subjected to separation by HPLC with an ODS column using MeCN/H_2O (10:90–70:30 over 30 min and then 70:30 for 10 min) as a mobile phase. The active eluate collected at retention time (t_R) 27–30 min, which was found to contain compound **1** by GC/MS, was further

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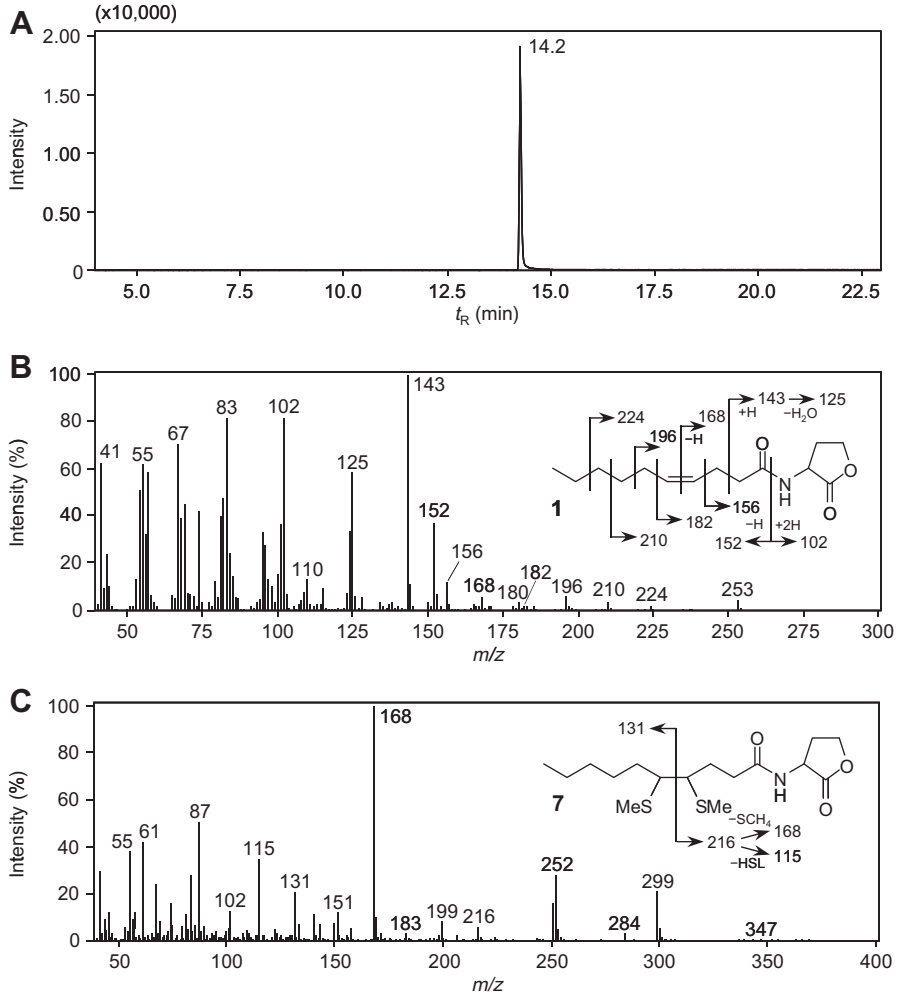
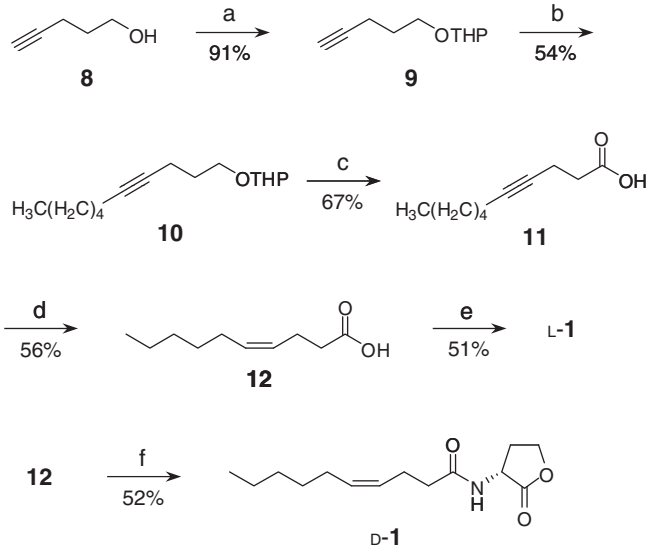


Figure 2. GC/MS analysis of compound **1** in the active HPLC eluate. (A) Selected ion monitoring chromatogram for *m*/*z* 143. (B, C) Mass spectra of **1** and its DMDS derivative (**7**). The proposed fragmentations are also described in the spectra.

purified by HPLC with a C_8 column using MeCN/H_2O (40:60) as a mobile phase to afford compound **1** (ca. 3 μg, estimated by standard curve).

The GC/MS analysis of compound **1** gave a peak at t_R 14.2 min, whose EI-MS exhibited a molecular ion peak (M^+) at *m*/*z* 253, and characteristic fragment ions for AHLs such as *m*/*z* 156, 143, 125, and 102 (Fig. 2A and B).[6] The molecular weight was consistent with that of unsaturated derivatives of C_{10}-HSL (**6**). The weak fragment ions at *m*/*z* 224, 210, 196, 182, and 168 generated by neutral loss of the aliphatic moiety of the acyl chain suggested that the unsaturation position would be C-4 in the acyl chain. To support this, compound **1** was derivatized with dimethyl disulfide (DMDS) and the product was analyzed by GC/MS.[7,8] DMDS derivative **7** provided a spectrum with a molecular ion peak (M^+) at *m*/*z* 347 and fragment ions at *m*/*z* 216 and 131, resulting from characteristic cleavage between C-4 and C-5, and also at *m*/*z* 168 and 115 originating from the fragment at *m*/*z* 216 undergoing methylsulfide and homoserine lactone losses (Fig. 2C). It was thus again suggested that compound **1** has a double bond at C-4 in the acyl chain. The double-bond geometry of **1** was expected to be *Z* because the double bond in the acyl chain of AHLs was usually *Z*.[9–11] Therefore, we proposed the structure of **1** to be (*Z*)-*N*-(4-decenoyl)homoserine lactone.

To confirm the structure of **1**, (*Z*)-*N*-(4-decenoyl)-L-homoserine lactone was synthesized according to Scheme 1.[12] The hydroxy group of 4-pentyn-1-ol (**8**) was protected as a THP ether, giving compound **9**. Treatment of **9** with bromopentane and *n*-BuLi in



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) dihydropyran, *p*-TsOH, 0 °C to rt; (b) 1-bromopentane, *n*-BuLi, HMPA, THF, −78 °C to rt; (c) CrO_3, H_2SO_4, H_2O, acetone, 0 °C to rt; (d) H_2, Lindlar catalyst, EtOH, rt; (e) L-homoserine lactone hydrochloride, EDC·HCl, Et_3N, CH_2Cl_2, rt; (f) D-homoserine lactone hydrochloride, EDC·HCl, Et_3N, CH_2Cl_2, rt.

the presence of hexamethylphosphoramide (HMPA) afforded a coupled product, **10**. Accordingly, compound **10** was treated with Jone's reagent to achieve deprotection of the THP group and subsequent oxidation, yielding carboxylic acid **11**. Hydrogenation of **11** over a Lindlar catalyst gave *Z*-olefin **12**. Finally, the acid was coupled with L-homoserine lactone hydrochloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) to afford L-**1**.[13] Analysis of synthetic L-**1** by GC/MS gave the same retention time and EI-MS data as for natural **1** (Supplementary data, Fig. S1). Thus, the planar structure of natural **1** was revealed to be (*Z*)-*N*-(4-decenoyl)homoserine lactone.

Next, to elucidate the absolute configuration of **1**, a chiral GC analysis of the natural and synthetic compounds was performed. D-**1** was synthesized from **12** and D-homoserine lactone hydrochloride (Scheme 1).[14] The GC/MS of natural **1** gave two peaks at t_R 18.7 min (minor) and 20.6 min (major) (Fig. 3).[15] These retention times were matched with those of synthetic D-**1** and L-**1**, respectively (Fig. 3), indicating that the L-form is in excess in natural **1**. The identity of the peaks was also confirmed by co-injection of synthetic standards and natural **1**. The ratio of D/L in natural **1** was calculated to be 1/9 based on standard curves.[16]

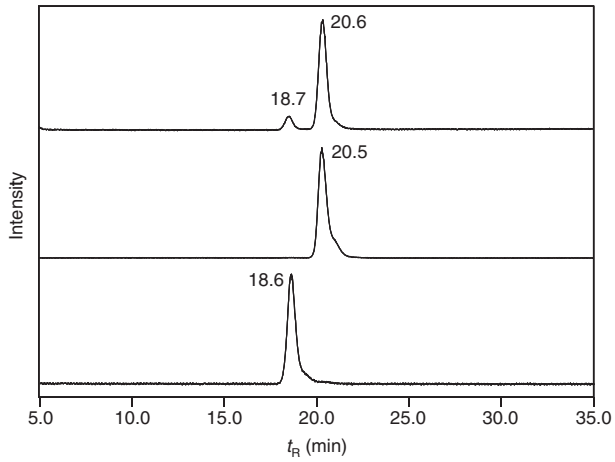


Figure 3. Chiral GC analysis of natural and synthetic **1**. Selected ion monitoring chromatograms (*m*/*z* 143) of natural **1** (top), synthetic L-**1** (middle), and synthetic D-**1** (bottom).

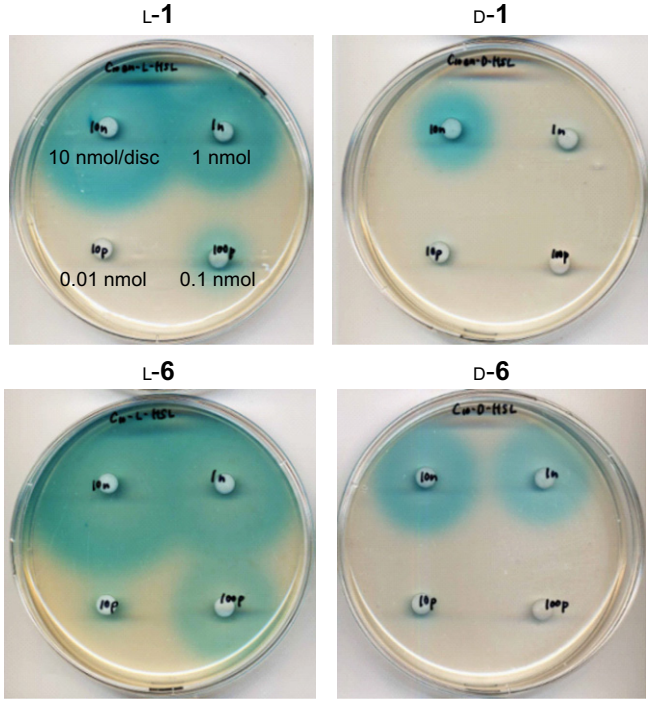


Figure 4. Activities of compounds **1** and **6** on AHL-responsive reporter gene expression in *A. tumefaciens* NTL4.

Finally, the ability of synthetic **1** to induce the reporter response in *A. tumefaciens* NTL4 was evaluated by using paper disc diffusion assay.[3] We compared the activity of (*Z*)-*N*-(4-decenoyl)homoserine lactone (**1**) with that of its saturated form, C_{10}-HSL (**6**). In both AHLs, L-forms are more active than D-forms (Fig. 4). L-**1** was about 10 times less active than L-**6**. Such difference was also observed in the activities between D-**1** and D-**6**. Thus, it was revealed that the double bond at C-4 in acyl chain weakens the activity of compound **1** on QS in *A. tumefaciens* NTL4.

In conclusion, we identified (*Z*)-*N*-(4-decenoyl)homoserine lactone (**1**) based on EI-MS and chemical synthesis, which was previously detected in the culture extract of *M. alpina* A-178. This is the first report of a novel AHL produced by endobacteria associated with fungi. Although a quantitative analysis of **1** was not conducted in this study, the production level is apparently lower than those of C_6- to C_{10}-HSLs (**2**–**6**), which were found in the culture media and mycelia of *M. alpina* A-178.[3] Furthermore, the activity of **1** for QS in *A. tumefaciens* NTL4 was weaker than that of C_{10}-HSL (**6**) or any other AHL produced by the endobacteria.[3,5] These results seem to indicate that the contribution of **1** to QS in the endobacteria would be small. However, there remains the possibility that compound **1** plays an important role in the development of the *Mortierella*-endobacterium symbiosis. For example, recently *Bradyrhizobium* spp., plant endosymbionts, were reported to use new and minor AHLs as their QS signaling molecules.[17,18] The production levels of these AHLs were low, but sufficient to invoke QS responses in these bacteria due to their high activities. Therefore, to evaluate the true contribution of **1** as a QS signaling molecule, a bioassay to assess the biological activity of **1** in endobacteria needs to be established.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.07.133.

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4. Twenty flasks, each containing 200 mL of potato dextrose broth (Difco), were inoculated with *M. alpina* A-178 and incubated for 1 week at 30 °C. The combined culture broth was extracted with equal volumes of EtOAc three times. The EtOAc layer was dried over Na_2SO_4 and evaporated, yielding a crude extract (611 mg).
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6. GC/MS data were obtained with a GCMS-QP2010 Plus (Shimadzu) fitted with an InertCap 5MS/NP capillary column (25 m × 0.25 mm i.d., 0.25 mm film, GL Sciences). The conditions were as follows: injection, 1 μL, splitless, 60 s valve time; injector temperature, 230 °C; carrier gas, He at 0.8 mL/min; transfer line temperature, 280 °C; and electron energy, 70 eV. The temperature of the column oven was programmed as follows: 150 °C for 3 min and then an increase to 275 °C at 8.33 °C/min and kept at 275 °C for 5 min.
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12. *^{1}H NMR data (400 MHz, $CDCl_3$) for synthetic intermediates. Compound* **9**: δ 1.50–1.61 (4H), 1.69–1.75 (2H, m), 1.82 (2H, quint, J = 7.1 Hz), 1.95 (1H, t, J = 2.6 Hz), 2.32 (2H, dt, J = 7.1, 2.6 Hz), 3.46–3.56 (2H, m), 3.80–3.90 (2H, m), 4.60 (1H, t, J = 3.4 Hz). Compound **10**: δ 0.90 (3H, t, J = 7.1 Hz), 1.26–1.39 (6H), 1.44–1.61 (6H), 1.78 (2H, quint, J = 6.8 Hz), 2.13 (2H, tt, J = 7.0, 2.3 Hz), 2.27 (2H, tt, J = 6.8, 2.3 Hz), 3.35–3.53 (2H, m), 3.80–3.91 (2H, m,), 4.60 (1H, m). Compound **11**: δ 0.89 (3H, t, J = 7.1 Hz), 1.24–1.37 (4H), 1.47 (2H, quint, J = 7.1 Hz), 2.12 (2H, tt, J = 7.1, 2.3 Hz), 2.44–2.64 (4H). Compound **12**: δ 0.89 (3H, t, J = 6.9 Hz), 1.24–1.38 (6H), 1.96–2.07 (2H, m), 2.33–2.44 (4H), 5.34 (1H, m), 5.44 (1H, m).
13. *Spectroscopic data for synthetic* L-**1**: $[\alpha]_D^{28}$ + 13.2 ° (c 0.450, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 7.0 Hz), 1.24–1.38 (6H, m), 1.94–2.18 (2H, m), 2.24–2.42 (4H, m), 2.83–2.90 (4H, m), 4.28 (1H, ddd, J = 11.6, 9.2, 5.9 Hz), 4.47 (1H, dt, J = 9.2, 1.0 Hz), 4.55 (1H, ddd, J = 11.7, 8.6, 5.6 Hz), 5.33 (1H, m), 5.44 (1H, m), 6.07 (1H, br). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.6, 23.1, 27.2, 29.3, 30.7, 31.5, 36.1, 49.3, 66.1, 127.2, 132.0, 173.2, 175.5. Spectra of NMR and EI-MS, see Supplementary data.
14. *Spectroscopic data for synthetic* D-**1**: $[\alpha]_D^{28}$ −11.9 ° (c 0.350, $CHCl_3$). ^{1}H and ^{13}C NMR and EI-MS data were completely identical to those of L-**1**.
15. Chiral GC was performed with an InertCap CHIRAMIX capillary column (10 m × 0.25 mm i.d., 0.25 mm film, GL Sciences). The conditions were as follows: injection, 1 μL, splitless, 60 s valve time; injector temperature, 200 °C; carrier gas, He at 2.0 mL/min; transfer line temperature, 200 °C; electron energy, 70 eV; and column oven temperature, 180 °C.
16. Other AHLs isolated or detected from the culture extract of *M. alpina* A-178 were also mixture of L- and D-forms.[3] The possibility of racemization of AHLs during the isolation processes was ruled out by the model experiments with synthetic enantiopure AHLs. We have no idea whether enantioimpure AHLs are usual in Gram-negative bacteria, because, in most previous studies, the absolute configurations of natural AHLs were concluded to be L without any experimental supports.
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