Long-Chain Acyl-Homoserine Lactones from *Methylobacterium mesophilicum:* Synthesis and Absolute Configuration

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The acyl-homoserine lactones (acyl-HSLs) produced by *Methylobacterium mesophilicum* isolated from orange trees infected with the citrus variegated chlorosis (CVC) disease have been studied, revealing the occurrence of six long-chain acyl-HSLs, i.e., the saturated homologues (*S*)-*N*-dodecanoyl (**1**) and (*S*)-*N*-tetradecanoyl-HSL (**5**), the uncommon odd-chain *N*-tridecanoyl-HSL (**3**), the new natural product (*S*)-*N*-(2*E*)-dodecenoyl-HSL (**2**), and the rare unsaturated homologues (*S*)-*N*-(7*Z*)-tetradecenoyl (**4**) and (*S*)-*N*-(2*E*,7*Z*)-tetradecadienyl-HSL (**6**). The absolute configurations of all HSLs were determined as 3*S*. Compounds **2** and **6** were synthesized for the first time. Antimicrobial assays with synthetic acyl-HSLs against Gram-positive bacterial endophytes co-isolated with *M. mesophilicum* from CVC-infected trees revealed low or no antibacterial activity.

Brazil has the largest orange groves in the world and holds an important slice of the international concentrated orange juice market. São Paulo State (Southeast Brazil) represents almost 90% of the national production.[1] However, the orange groves suffer from several plagues and diseases. Citrus variegated chlorosis (CVC) is one of the most important problems affecting sweet orange (*Citrus sinensis* Osbek) cultivation and is caused by the fastidious bacterium *Xylella fastidiosa*, which is normally found in the xylem of infected trees.[2,3]

Plants affected by CVC display remarkable reduction in fruit size and early maturation.[4] The groves may be contaminated from infected shoots and also by xylem sapsucker insects belonging to the families Cercopidae and Cicadellidae.[5,6]

Up to now there are no effective control technologies for CVC, even though *X. fastidiosa* was the first phytopathogen whose genome was successfully sequenced.[7] However, there is significant evidence that other endophytic orange bacteria may be associated with CVC, and some remarkable differences were found in endophytic flora between diseased, resistant, and healthy citrus individuals.[2,3]

The main endophytes found in orange trees from the Brazilian states of São Paulo and Minas Gerais are bacteria belonging to the genera *Bacillus*, *Curtobacterium*, *Enterobacter*, *Nocardia*, *Pantoea*, *Streptomyces*, *Xanthomonas*, and several *Methylobacterium* species (*M. extorquens, M. mesophilicum, M. fujisawaense, M. radiotolerans*, and *M. zatmanii*).[1] A seasonal study demonstrated the large prevalence of *Methylobacterium* species in symptomatic plants infected by *X. fastidiosa* in practically all seasons.[1] The presence of these bacteria also has a positive correlation with the severity of the CVC disease.

Bacteria belonging to the *Methylobacterium* genus may fix atmospheric nitrogen and can survive using MeOH and other compounds with one carbon (C1) as the sole carbon source. They are found in different environments, e.g., soil, rhizosphere, and H_2O; however, they are frequently found in symbiotic relationships with plants, contributing to them by producing many phytohormones such as indolacetic acid, kitocynins, and vitamin B12 and receiving from the plants MeOH as a carbon source.[7,8]

Recently, Poonguzhali and co-workers characterized the production of acyl-homoserine lactones (acyl-HSLs) by *Methylobacterium* spp. from different sources such as potable water, soil, and rice endophytes with the aid of bioreporters.[9] The acyl-HSLs are important signaling compounds in Gram-negative bacteria, employed in chemical communication mechanisms.[10] This phenomenon is known as quorum-sensing and plays an important role in several phenotypic factor expressions such as enzymes and multicellular behavior in many bacteria.[10] From 18 studied species, 12 promoted the induction of phenotypic factors on bioreporter assays, suggesting the production of acyl-HSLs.[9]

The strain *M. extorquens* AM1 is the sole species of this genus thoroughly studied for acyl-HSL production.[6] Interestingly, this strain produces unsaturated long-chain acyl-HSLs under methanotrophic growth conditions, while short-chain ones are produced under non-methanotrophic growth. Later work demonstrated that these signaling compounds are involved in exopolysaccharide production control.[11]

It was also reported that acyl-HSLs can exhibit antimicrobial activities against Gram-positive bacteria.[12−14] Continuing our efforts in this field, we hypothesized that acyl-HSLs produced by endophytic *Methylobacterium* could be an additional factor contributing to its large prevalence over other bacteria in CVC-diseased orange trees. Therefore, the aim of this work was to investigate if the Gram-negative orange-isolated strain *M. mesophilicum* SR 1.6/6 produces acyl-HSLs and also to evaluate the antimicrobial activity of the ensuing compounds against Gram-positive orange endophytes, such as *Bacillus* and *Curtobacterium*.

Results and Discussion

The bacterium *M. mesophilicum* SR 1.6/6 can grow in a specific cultivation medium (CHOI3) with MeOH as the sole carbon source.[8] It is an interesting characteristic offering advantages during secondary metabolite isolation and purification.

Initially, *M. mesophilicum* (5 L) was extracted with EtOAc (16.6 mg) and subjected to silica gel filtration to remove cellular and macromolecular impurities, enriching the sample in secondary metabolites.[17] This semipurified extract (8.0 mg) was subjected to a β-galactosidase assay with reporter *Agrobacterium tumefaciens* NTL4(pZLR4), which can detect several exogenous acyl-homoserine lactones.[16] In positive assays, a blue coloration is observed coming from X-Gal reagent degradation by β-galactosidase enzymes, which are expressed when the exogenous acyl-HSLs bind to the transcriptional regulator protein TraR, which controls the transcription of an *operon* containing the *lacZ* gene cloned from *Escherichia coli*.[16] The semipurified extract from *M. mesophilicum* provided positive biological activity.[17−19]

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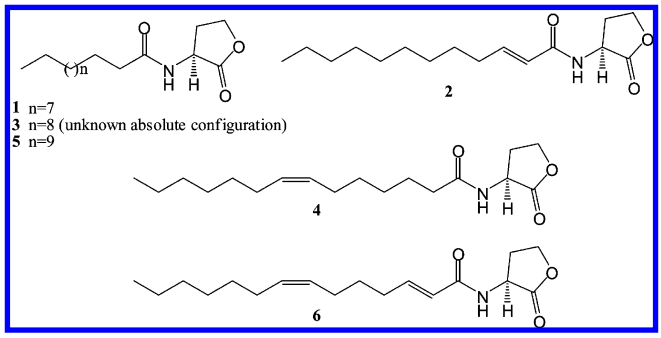


Figure 1. Acyl-HSLs produced by the orange endophyte *M. mesophilicum.*

GC-MS analysis of *M. mesophilicum* semipurified extract revealed the presence of at least six different acyl-HSLs. A cleaner fraction, more suited for spectroscopic investigations, was prepared from the EtOAc extract from 8 L of *M. mesophilicum* cultivation medium and extensive silica gel column chromatography. F22, eluted with CH_2Cl_2/EtOAc (7:3), contained 1.1 mg of the mixture of six acyl-HSLs. They were identified as (*S*)-*N*-dodecanoyl- (**1**), (*S*)-*N*-(2E)-dodecenoyl- (**2**), *N*-tridecanoyl- (**3**), (*S*)-*N*-(7*Z*)-tetradecenoyl- (**4**), (*S*)-*N*-tetradecanoyl- (**5**), and (*S*)-*N*-(2*E*,7*Z*)-tetradecadienyl-homoserine lactone (**6**) (Figure 1).

Unsaturation Positions and Geometries. The minute amounts of the natural products prevented isolation and an extensive NMR structural study. To overcome this problem, the dimethyl disulfide (DMDS) microderivatization protocol was applied with the semipurified EtOAc extract from the CHOI3 medium followed by GC-MS analysis of the DMDS derivatives, aiming to determine the unsaturation positions.[21,22]

The DMDS derivative of compound **4** provided fragments at *m/z* 145 and 258, resulting from characteristic derivative cleavage, and also at *m/z* 157 originating from the fragment at *m/z* 218 undergoing homoserine lactone loss. These results were in agreement with the literature, permitting establishment of the double-bond position at C-7 of the acyl side chain.[23]

The DMDS derivative of compound **6** provided a mass spectrum with the molecular ion at *m/z* 401, suggesting that only one of the two double bonds was derivatized, producing a characteristic fragment at *m/z* 256, two mass units smaller than that observed for the DMDS derivative of **4**. Therefore, one of the double bonds was present at C-7, while the other was located between the carbonyl group and the unsaturation at C-7. It is known that carbon−carbon double bonds conjugated to carbonyl groups are electron-deficient and do not react with DMDS under the usual conditions.[24] This, together with a less abundant *m/z* 143 fragment in the MS of the parent compound, is evidence that the second unsaturation of compound **6** was conjugated to the carbonyl group of the acyl side chain.

Interestingly, compound **2** did not react with DMDS, as observed for the energetically less favorable carbonyl-conjugated unsaturation in **6**. This and the smaller abundance of the *m/z* 143 fragment reinforced the presence of a conjugated amide moiety in the molecule.

The double-bond geometries were determined by the ^{1}H NMR spectrum of the mixture of all natural acyl-HSLs (1.1 mg). Clearly the double bond conjugated to the carbonyl group of compound **6** possesses *trans* geometry (5.83 ppm, H-2′, doublet; 6.92 ppm, H-3′, doublet of doublets; *J* 15.3 Hz). Both compounds **6** and **4** had double bonds at C-7 and shared overlapping signals in the ^{1}H NMR spectrum. A relatively pure **4** (2.3 mg total mass; 70% compound **4**) obtained from *M. mesophilicum* grown in TSB cultivation medium showed a *cis* configuration at C-7, determined by comparing the ^{13}C NMR chemical shifts of the allylic carbons (26.9 and 27.2 ppm assigned to C-6′ and C-9′) with those of *cis* (27.0 and 27.3 ppm) and *trans* (32.2 and 32.6 ppm) 7-tetradecenoic acids.[26]

Absolute Configuration Determination. The absolute configuration at the lactone moiety of the natural acyl-HSLs produced by *M. mesophilicum* was first assessed via GC-FID equipped with a Chrompack Chirasil chiral column and synthetic standards.[17−19] However analysis of the six natural acyl-HSLs mixture showed peak overlapping of the unsaturated homologues. To overcome this problem, a microscale catalytic hydrogenation reaction using Pd/C transformed the mixture of HSL into *N*-tetradecanoyl-HSL and *N*-dodecanoyl-HSL. The reaction efficiency was attested by GC-MS, clearly showing a complete conversion of all unsaturated homologues into the saturated ones (see Supporting Information), allowing retention time comparisons and co-injection with racemic and chiral HSL standards.

All natural acyl-HSLs displayed *S* absolute configuration without any traces of natural *R* enantiomers (Table 1). The absolute configuration of these compounds had not been characterized before. Consequently this simple and elegant procedure allowed the absolute configuration determination of five compounds in just one experiment. Up to now, only a few papers have described the absolute configuration of acyl-HSLs and consistently reported the occurrence of only *S* enantiomers at the homoserine lactone moiety.[19]

Synthesis of Acyl-Homoserine Lactones. The final unequivocal structural characterization relied on the synthesis of compounds **1**, **2**, **3**, **5**, and **6** for GC-MS mass spectra comparisons and co-injection with natural products. Compounds **1**, **3**, and **5** were synthesized by coupling α-amino-γ-butyrolactone salts with dodecanoic, tridecanoic, or tetradecanoic acids, as previously described.[17−19,30]

Compound **2** was synthesized in four steps starting from a Hell−Volhard−Zelinsky bromination of dodecanoic acid followed by nucleophilic substitution with iodide and thermal elimination, furnishing the target (2*E*)-dodecenoic acid.[31,32] The last step was analogous to that employed with the saturated homologues, i.e., carbodiimide-mediated coupling with the homoserine lactone moiety. The MS of synthetic **2** displayed a less abundant fragment at *m/z* 143 (21%) than other HSLs similar to that of the natural product.

Compound **6** was synthesized in five steps in 67% overall yield (Scheme 1) using Meldrum reaction with commercially available (5*Z*)-dodecenoic acid and 2,2-dimethyl-1,3-dioxane-4,6-dione.[34] The corresponding (5*Z*)-dodecenoyl derivative of Meldrum acid was subjected to nucleophilic attack and a thermal decarboxylation reaction with (*S*)-α-amino-γ-butyrolactone hydrobromide, furnishing the 3-oxo intermediate **7**. The ketone group was reduced using $NaBH_4$ to the corresponding secondary alcohol, which was mesylated and treated with DBU to afford compound **6** (76% yield). This is the first synthesis of compound **6**.

Perfect peak overlaps were observed under GC-MS co-injection with all synthetic compounds and natural products, as well as identical mass spectra.

All synthetic and natural products were also assayed with reporter *A. tumefaciens* NTL4(pZLR4), and a blue coloration was observed, indicating that these acyl-HSLs were detected by the biosensor. Consequently these acyl-HSLs were responsible for the positive test observed with EtOAc extracts from *M. mesophilicum.*

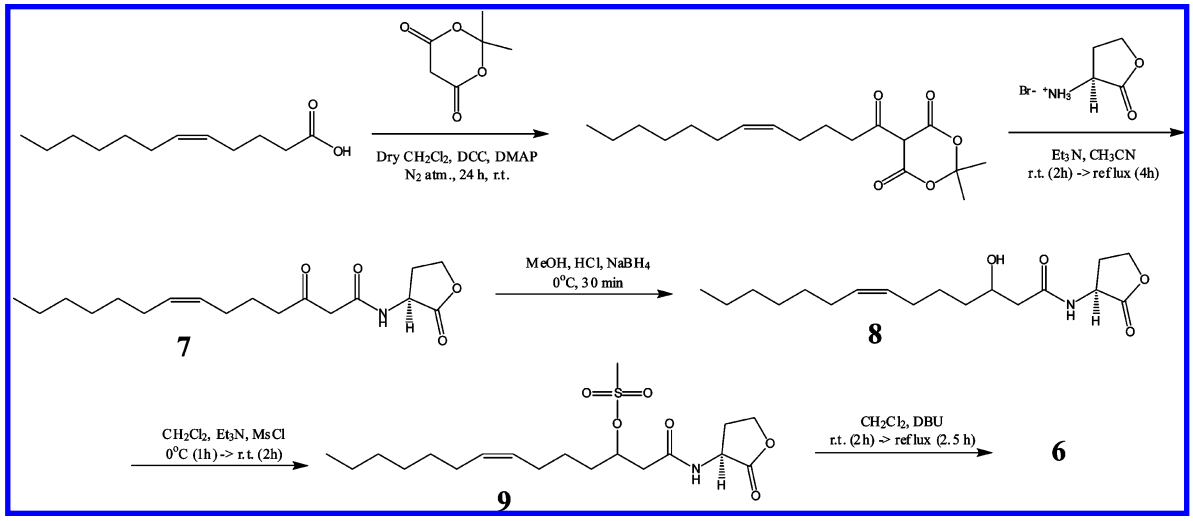
Influence of Cultivation Media on Acyl-HSL Production. The influence of the cultivation media on the production of acyl-HSLs by *M. mesophilicum* was also investigated. Two non-methanotrophic conditions were assayed: (1) CHOI3 cultivation medium with glucose instead of MeOH as carbon source and (2) complex trypticase soy broth cultivation medium (TSB). The estimated yields of acyl-HSLs were 137.5 μg/L in CHOI3 medium, 50 μg/L in CHOI3/glucose, and 287.5 μg/L in the TSB medium, which was the highest acyl-HSL production. Natural products from non-methanotrophic growth were identified by retention index comparison with previously studied acyl-HSLs from CHOI3 medium.[34] The microorganisms produced only long-chain acyl-

Table 1. Absolute Configuration of Acyl-HSLs (dodecanoyl and tetradecanoyl homologues) Produced by *M. mesophilicum* via GC-FID (Chrompack Chirasil column)

sample	retention time (min) and % (*R*)	retention time (min) and % (*S*)	ee (major)
	N-Tetradecanoyl-HSL (**5**)		
(±)-*N*-tetradecanoyl-HSL synt.	103.8 (50%)	105.4 (50%)	1:1 peaks
(*S*)-*N*-tetradecanoyl-HSL synt.		105.4 (97%)	94% (*S*)
F22 natural product		105.4 (>99%)	>99% (*S*)
co-injection (±)-*N*-tetradecanoyl-HSL and hydrogenated natural product[a]	103.6 (29%)	105.3 (71%)	13% (*S*)
	N-Dodecanoyl-HSL (**1**)		
(±)-*N*-dodecanoyl-HSL synt.	46.9 (50%)	47.7 (50%)	1:1 peaks
(*S*)-*N*-dodecanoyl-HSL synt.		47.7 (97%)	94% (*S*)
F22 natural product		47.7 (>99%)	>99% (*S*)
co-injection (±)-*N*-dodecanoyl-HSL and hydrogenated natural product[b]	46.8 (32%)	47.5 (68%)	4% (*S*)

[a] Co-injection: 1:1 mixture of (±)-*N*-tetradecanoyl-HSL at 1 mg/mL and fraction F22 at 1 mg/mL. [b] Co-injection: 1:1 mixture of (±)-*N*-dodecanoyl-HSL at 0.1 mg/mL and fraction F22 at 1 mg/mL.

Scheme 1. Synthesis of Compound **6**



HSLs in the three different media. However, some changes were observed, such as compound **2** was produced in smaller amounts in the TSB medium, and compound **3** was not detected in both non-methanotrophic conditions. Compound **6** was produced in smaller proportions under non-methanotrophic growth. Compound **4** was almost 70% pure in TSB cultivation media, allowing a more extensive NMR structural study.

Antimicrobial Assays against Gram-Positive Orange Endophytes. The large prevalence of *Methylobacterium* over other endophytic bacteria in orange tissues stimulated the investigation on whether the acyl-HSLs produced could constitute an evolutionary and colonization weapon, by Gram-positive bacteria suppression caused by acyl-HSL antimicrobial activity.[12−14] To test this hypothesis, the microorganisms *Bacillus* sp. CL15, *Bacillus* sp. CL16, *Curtobacterium flaccumfaciens* ER 1/5, and *Nocardiopsis* sp., all isolated from CVC-affected orange tissues, together with *M. mesophilicum*, were assayed against the compounds (*S*)-**1**, (*S*)-**2**, (*S*)-**5**, and (*S*)-**6**. The assays were conducted according to a previously adapted colorimetric protocol.[14] Results are shown in Table 2.

In general, all compounds showed negligible antibacterial activity if one takes into consideration that *M. mesophilicum* produces only 0.1375 ppm of acyl-HSLs in CHOI3 medium (1.1 mg for 8 L of cultivation media), while the most active compound was active at about 500 ppm.

Therefore, it is improbable that compounds produced by *M. mesophilicum* have an effective role in suppressing Gram-positive orange endophytes. The absence of antimicrobial activity can be explained in several ways. First, the Gram-positive bacteria may be resistant to the acyl-HSLs produced by *M. mesophilicum*, or the produced concentration may be too small for effective activity. However, it is also known that 3-oxo-acyl-HSLs are much more active than those without the 3-keto group.[12−14] In this case, the acyl-HSLs produced by *M. mesophilicum* do not have this oxygenation level.

Table 2. MIC (ppm) of Synthetic Acyl-HSLs against Orange Endophytic Bacteria

	(*S*)-**1**	(*S*)-**2**	(*S*)-**5**	(*S*)-**6**
Bacillus sp. CL 15	−[a]	−	−	1000
Bacillus sp. CL 16	−	−	−	1000
C. flaccumfaciens ER 1/5	−	−	−	500
Nocardiopsis sp.	1000	1000	1000	500

[a] − Inactive until the highest concentration assayed (1000 ppm).

This is the first study of metabolites produced by *M. mesophilicum*, a strain isolated from citrus variegated chlorosis disease. Six rare acyl-HSLs were detected and synthesized; compound **2** being a new natural product. In addition, the first syntheses for compounds **6** and **2** and the first absolute configuration characterization of five acyl-HSLs in just one chromatographic analysis, based upon an elegant hydrogenation protocol, are reported. The antimicrobial assays showed that the metabolites produced by *M. mesophilicum* do not seem to be associated with antimicrobial competition against orange endophytic Gram-positive bacteria. Currently, our group is making efforts to understand the effects of the synthetic acyl-HSLs on the *Xylella fastidiosa* gene expression profile.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter or Jasco J-720 spectropolarimeter using MeOH (HPLC grade) as solvent. The IR spectra were obtained with Bomem Michelson MB equipment using KBr pellets as sample supports for solids and NaCl crystals for oils (films). NMR spectra were obtained with Varian Inova-500, Bruker Gemini-300, Bruker-250, or Bruker-300 equipment, using $CDCl_3$ as solvent and TMS as internal reference (δ 0.0). The chemical shifts are provided in ppm and the coupling constants in hertz (Hz). GC-MS analyses were carried out on an Agilent 6890 gas chromatograph equipped with an HP 5973 mass selective detector operating in electron impact mode at 70 eV. Separations were carried out on HP-5 or MDN-5S columns (30 m × 0.25 mm × 0.25 μm). Highly pure He was employed as carrier gas (1 mL/min flow rate). The injector and interface temperatures were 250 and 280 °C, respectively. Chromatographic separations were carried out with Acros 0.035−0.070 mm silica gel columns. TLC analyses were performed on Merck 60 F254 aluminum sheets and revealed under exposure to UV light or chemically with acidic anisaldehyde solution. All solvents were of high analytical grade, distilled before use. Reagents for acyl-HSL synthesis and X-GAL were ordered from Sigma-Aldrich (S.Paulo, Brazil).

Cultivation Media. Nutrient broth (NB, 20 g/L in distilled H_2O) and Müller-Hinton media were from Oxoid. Luria-Bertani medium was prepared with 1% peptone (Oxoid), 0.5% NaCl, and 0.5% yeast extract (Oxoid). For solid media, 2% agar was added (Sigma). Trypticase soy broth (TSB, 20 g/L in distilled H_2O) medium was from Oxoid. CHOI3 medium was prepared as previously reported.[15] The adapted CHOI3/glucose medium was prepared with glucose (10 g/L, separately autoclaved) instead of MeOH as carbon source.

Bacterial Strains. The bacterium *M. mesophilicum* SR 1.6/6 was isolated as an endophyte in CVC-diseased citrus plants and conserved under refrigeration (5 °C) in slants with selective solid CHOI3 medium for routine use or under ultrafreezer refrigeration (−80 °C) for storage. The bioreporter *Agrobacterium tumefaciens* NTL4(pZLR4) was prepared by Cha and co-workers[16] and was maintained in solid LB medium supplemented with gentamicine (50 μg/mL). *Bacillus* sp. CL15, *Bacillus* sp. CL16, *Curtobacterium flaccumfaciens* ER 1/5, and *Nocardiopsis* sp. were isolated as endophytes from infected CVC orange trees and were maintained under refrigeration in slants containing nutrient broth medium (NB, Oxoid).

General Procedures for Extract Preparation. The inoculums of *M. mesophilicum* were prepared in test tubes containing 10 mL of liquid CHOI3, CHOI3/glucose, or TSB medium, incubated at 30 °C without shaking. After four days, the inoculums were transferred to Erlenmeyer flasks (2 L) containing 1 L of cultivation media, incubated at 29 °C for five days under shaking (110 rpm). The media were filtered with conventional paper filters (the cells were densely aggregated), and the aqueous phases (1 L) were extracted with EtOAc (3 × 500 mL). The organic layers were washed with distilled H_2O (1 × 500 mL) and evaporated under reduced pressure at 40−45 °C. For the biological assays with reporter *A. tumefaciens* NTL4(pZLR4) the EtOAc extracts from 1 L of cultivation media (CHOI3, CHOI3/glucose, or TSB) were filtered with silica gel columns (3 g, 1 cm diameter column) with *n*-hexane (50 mL), EtOAc (100 mL), and MeOH (50 mL). However, for more accurate purifications aiming at spectral studies, the combined extracts from 8 L of cultivation media were purified by silica gel chromatography using *n*-hexane, CH_2Cl_2, and EtOAc in mixtures of increasing polarity. Fractions were analyzed by GC-MS.

(*S*)-*N*-Dodecanoyl-HSL (1) (GC-MS, EI, 70 eV): *m/z* 283 ($M^{+\bullet}$, 4), 170 (2), 156 (19), 143 (100), 125 (17), 102 (20), 83 (15), 57 (46), 43 (24).

(*S*)-*N*-(2*E*)-Dodecenoyl-HSL (2) (GC-MS, EI, 70 eV): *m/z* 281 ($M^{+\bullet}$, 11), 180 (13), 156 (11), 143 (25), 125 (46), 102 (31), 101 (30), 83 (27), 67 (23), 57 (34), 41 (30).

***N*-Tridecanoyl-HSL (3) (GC-MS, EI, 70 eV, SIM mode):** *m/z* 297 ($M^{+\bullet}$, 7), 201 (23), 156 (20), 143 (100), 125 (17), 102 (21), 83 (22), 57 (54), 43 (35).

(*S*)-*N*-(7*Z*)-Tetradecenoyl-HSL (4) (GC-MS, EI, 70 eV): *m/z* 309 ($M^{+\bullet}$, 19), 228 (12), 208 (23), 156 (40), 143 (100), 125 (28), 102 (77), 55 (76), 43 (39), 41 (52).

(*S*)-*N*-Tetradecanoyl-HSL (5) (GC-MS, EI, 70 eV): *m/z* 311 ($M^{+\bullet}$, 5), 156 (20), 143 (100), 125 (14), 102 (20), 83 (14), 57 (38), 43 (23).

(*S*)-*N*-(2*E*,7*Z*)-Tetradecadienyl-HSL (6) (GC-MS, EI, 70 eV): *m/z* 307 ($M^{+\bullet}$, 2), 236 (14), 206 (20), 169 (30), 143 (55), 125 (17), 121 (38), 102 (20), 81 (100), 68 (48), 55 (59), 43 (28), 41 (45).

Biological Assays with Reporter *Agrobacterium tumefaciens* NTL4(pZLR4). The bioreporter assays were performed essentially as previously reported[17−19] with semipurified EtOAc extracts from different cultivation media, fractions, and synthetic products. Ethanol was employed as blank and synthetic (*S*)-*N*-(3-oxo-octanoyl)-HSL as positive control. The stock solutions were prepared in EtOH at 1−4 mg/mL.

Natural Acyl-HSL Dimethyl Disulfide (DMDS) Derivatization. To a round-bottom flask (25 mL) were added 0.5 mL of CH_2Cl_2 and 0.5 mg of semipurified EtOAc fraction from *M. mesophilicum* cultivated in CHOI3 medium. To this mixture were added 0.5 mL of DMDS and 40 μL of an I_2 solution (60 mg/mL in Et_2O). The mixture was stirred at 40 °C for 22 h. Then, CH_2Cl_2 (3 mL) and 5% sodium thiosulfate (3 mL) were added. The mixture was vigorously stirred for 5 min, and the organic layer was dried over magnesium sulfate, filtered, and evaporated under a N_2 flow, yielding a crude oil, which was subsequently analyzed by GC-MS.

Syntheses and Spectroscopic Characterization of (*S*)-*N*-Dodecanoyl-, (±)-*N*-Tridecanoyl-, (*S*)-*N*-Tetradecanoyl-, (*S*)-*N*-Dodecenoyl-, and (*S*)-*N*-(2*E*,7*Z*)-Tetradecadienyl-HSL. Complete synthetic procedures and spectroscopic data are provided in the Supporting Information.

Absolute Configuration Determination of Natural Acyl-HSLs Produced by *M. mesophilicum*. Catalytic Hydrogenation Microderivatization Reaction. To a round-bottom flask (5 mL) were added 0.3 mg of fraction F22 from the *M. mesophilicum* CHOI3 cultivation (containing the mixture of acyl-HSLs), EtOAc (400 μL), and a catalytic amount of Pd/C (10%, Merck). The flask was sealed with a rubber septum, connected to a hydrogen reservoir (1 atm), and magnetically stirred for 6 h. The reaction was filtered through a silica gel column (2 g, 1 cm diameter) and eluted with EtOAc (150 mL). Approximately 0.3 mg of reduction product was obtained, which was submitted to GC-MS and GC-FID (chiral column) analyses.

GC-FID (chiral column). Chiral discrimination analyses were carried out with an Agilent 6850 gas chromatograph equipped with a Chrompack Chirasil CB chiral column (25 m × 0.25 mm × 0.25 μm). Highly pure H_2 was employed as carrier gas (1 mL/min flow rate). The injector temperatures were 240 °C for *N*-tetradecanoyl-HSL and 220 °C for *N*-dodecanoyl-HSL. Analytes (hydrogenated natural product and synthetic standards) were dissolved in EtOAc (1 mg/mL) and injected in a 1/100 split mode. The oven was maintained isotermically at 180 °C for 150 min. The detector temperatures were 240 °C for *N*-tetradecanoyl-HSL and 280 °C for *N*-dodecanoyl-HSL. Results are summarized in Table 1.

Antimicrobial Assays. The antimicrobial assays were performed with synthetic acyl-HSLs and the Gram-positive bacteria *Curtobacterium flaccumfaciens* ER 1/5, *Bacillus* sp. CL16, *Nocardiopsis* sp., and *Bacillus* sp. CL15.

Inoculums of *Curtobacterium flaccumfaciens* ER 1/5 and *Bacillus* sp. CL16 were prepared in slants with solid nutrient broth medium, grown at 30 °C for 24 h in BOD. Inoculum of *Bacillus* sp. CL15 was prepared in the same way, but using 48 h of incubation. After growth, the cells were collected with flamed wire loops and suspended in sterile distilled H_2O (10 mL), until a 3×10^8 cells/mL solution was obtained, according to the McFarland scale. For *Nocardiopsis* sp., the inoculum was prepared in an Erlenmeyer containing liquid Müller-Hinton medium (50 mL) incubated under shaking for 72 h at 30 °C (100 rpm). The cells were decanted, washed with sterile distilled H_2O (2×), suspended in distilled H_2O (10 mL), vortexed, and diluted until the desired concentration. The antimicrobial assays were performed as previously adapted, using sterile distilled water and DMSO (20%) as blank and chloramphenicol as positive control.[14] The compounds (*S*)-**1**, (*S*)-**2**, (*S*)-**5**, and (*S*)-**6** were assayed, using stock solutions at 2 mg/mL. The highest concentration assayed was 1000 ppm.

For *Bacillus* sp. CL16 and *Curtobacterium flaccumfaciens* ER 1/5 addition of coloring agent was performed after 24 h of microplate incubation at 30 °C, while *Nocardiopsis* sp. and *Bacillus* sp. CL15 were incubated for 48 h.[14]

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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