Absolute Configuration and Antimicrobial Activity of Acylhomoserine Lactones

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(*S*)-*N*-Heptanoylhomoserine lactone is an uncommon acyl odd-chain natural product employed by many Gram-negative bacteria as a signaling substance in chemical communication mechanisms known as quorum sensing. The absolute configuration determination of the metabolite produced by the phytopathogen *Pantoea ananatis* Serrano is reported herein. As with all other substances of this class, the lactone moiety possesses *S* configuration, corroborating the hypothesis that it shares the same biosynthetic pathway as the (*S*)-*N*-hexanoylhomoserine lactone and also that some LuxI homologues can accept both hexanoyl- and heptanoyl-ACP as precursors. Evaluation of the antimicrobial activity of enantiomeric acylhomoserine lactones against three Gram-positive bacteria (*Bacillus cereus, B. subtilis*, and *Staphylococcus aureus*) revealed important features between absolute configuration and antimicrobial activity. The *N*-heptanoylhomoserine lactone was considerably less active than the 3-oxo derivatives. Surprisingly, non-natural (*R*)-*N*-(3-oxo-octanoyl)homoserine lactone was as active as the *S* enantiomer against *B. cereus*, while the synthetic racemic product was less active than either enantiomer.

Chemical communication is a phenomenon explored by many different organisms and nowadays is one of the most prominent research areas at the chemistry−microbiology interface. For almost 20 years, science has recognized the ability of different bacteria to coordinate phenotype expression using signaling substances, a crucial process for successful environment colonization in plants, human beings, and other animal hosts. This multicellular-like behavior is known as quorum sensing.[1]

Different compound classes are employed by bacteria as signaling substances. Among Gram-positive bacteria, γ-butyrolactones play a major role in antibiotic production and sporulation of the *Streptomyces* species.[2,3] Low molar mass peptides are employed, for example, by *Staphylococcus aureus* to control virulence factor expression.[4] The Gram-negative bacterium *Pseudomonas aeruginosa* employs quinolones in a complex cascade mechanism to regulate elastase-encoding genes expression.[5] However, the most studied compounds in Gram-negative bacteria signaling mechanisms are the acylhomoserine lactones (acyl-HSLs).[1]

There is a predominance of even-numbered acyl-HSLs assigned to the major production of even-numbered fatty acids in microbial cells. However, the number of odd-chain acyl-HSLs-producing microorganisms is increasing, e.g., *Serratia marcescens*,[6] *Sinorhizobium leguminosarum,*[7] *Edwardisiella tarda,*[8] *Erwinia psidii*,[9] and the phytopathogen *Pantoea ananatis*,[10] which produces *N*-heptanoyl-HSL (**1**) together with large amounts of *N*-hexanoyl-HSL. Long-chain acyl-HSLs such as *N*-tridecanoyl- and *N*-pentadecanoyl-HSL have been found in marine bacteria (Alphaproteobacteria).[11]

It is worth pointing out that odd-chain derivatives always occur in trace amounts together with large amounts of even-numbered acyl-HSL-homologues. In *S. leguminosarum*, it was demonstrated that a lower expression of the *cinI* gene results in smaller production of three short-chain acyl-HSLs, namely, *N*-hexanoyl-, *N*-heptanoyl-, and *N*-octanoyl-HSL, suggesting that these substances are produced by the same acyl-HSL synthesizing protein (RhiI).[7] This is very strong evidence that both even- and odd-acyl-HSLs of similar acyl length can share the same biosynthetic enzyme (LuxI homologue), most probably due to the minor structural differences between substrates (hexanoyl- and heptanoyl-ACP, for example). However, the absolute configuration of an odd-numbered acyl-HSL at the lactone moiety has not been reported before. The *S* absolute configuration of even-numbered acylhomoserine lactones derives from the aminoacid portion of the *S*-adenosyl methionine precursor.[9,10,12−15] From a chemical point of view, if both odd and even acyl-HSLs share the same absolute configuration, the hypothesis of acceptance of similar acyl-ACPs by some LuxI homologues would have a firmer foundation.

In addition to the important role of acyl-HSLs in chemical communication, alternative biological activities have been found. These include interferences in eukaryotic organisms, such as induction of apoptosis in macrophages and neutrophils with important reflexes in immunological systems.[16−18] The antimicrobial activity of some acyl-HSLs against Gram-positive bacteria (even though at high concentrations) is another important characteristic, especially under Biofilm conditions, where the metabolites, cells, and enzymes are much more concentrated.[19] One of the most studied substances of this class is (*S*)-*N*-(3-oxododecanoyl)-HSL produced by *P. aeruginosa*, which can be toxic to several Gram-positive bacteria, but not for the Gram-negative ones.[19]

The production of bactericidal acyl-HSLs by *P. aeruginosa* confers a competitive advantage to this microorganism in lung colonization, in relation to *Staphylococcus aureus,* in cystic fibrosis patients. It is known that *S. aureus* is stepwise substituted by *P. aeruginosa* in advanced lung diseases. Probably, the bactericidal property of *N*-(3-oxododecanoyl)-HSL against *S. aureus* may contribute to this process, since this metabolite was found in relatively high concentrations (600 μM/L) in lung secretions of affected patients in comparison with its production by *P. aeruginosa* under planktonic conditions (5 μM/L).[20,21]

The antimicrobial activity of acyl-HSLs against *S. aureus* has been extensively studied. Generally, acyl-HSLs with no chemical substituents such as hydroxy or carbonyl groups at position 3 did not show any activity against this microorganism, while 3-oxo-acyl-HSLs with alkyl side chains ranging from 8 to 14 carbon atoms were active at micromolar concentrations.[20] The antimicrobial activity of *N*-3-oxododecanoyl-HSL was attributed to interferences in *S. aureus* quorum-sensing regulated phenotypes, including important changes in cellular membrane.[19,20] In spite of these new findings, the importance of absolute configuration for antimicrobial activity has not been mentioned previously.

Thus, we report herein the absolute configuration determination of the acyl-odd-numbered (*S*)-*N*-heptanoylhomoserine lactone produced in trace amounts by *P. ananatis* and also the importance of absolute configuration for antimicrobial activity against Gram-positive bacteria, including synthetic 3-oxoacyl-HSLs, which are

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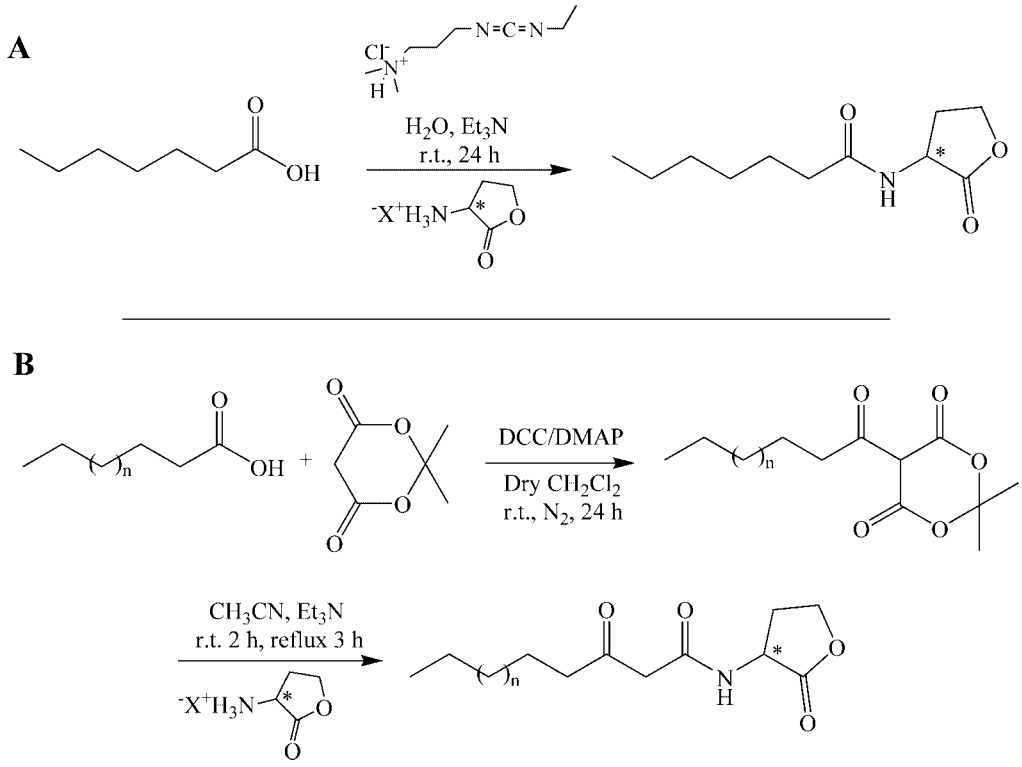
Scheme 1. (A) Synthesis of the *N*-Heptanoyl-HSL Racemic Mixture and the Enantiomers; (B) General Synthetic Procedure for *N*-3-Oxo-acyl-HSLs ($n = 1$, 5, or 7)



known to be more active than nonsubstituted acyl-HSLs.[20] The results are discussed in the face of the current theories.

Results and Discussion

In a previous paper we reported the occurrence of three acylhomoserine lactones from the phytopathogen *P. ananatis* Serrano [(*S*)-*N*-hexanoyl-, *N*-heptanoyl-, and *N*-octanoyl-HSL, in decreasing abundance].[10] This pathogen was initially described in pineapple rot disease (*Ananas comosus* L.).[22] Nowadays, it also represents an important drawback in onion (*Allium cepa* L.) production in Georgia (USA)[23] and has also been found to be a pathogen in rice (*Oryza sativa* L.),[24] eucalyptus (*Eucalyptus* hyb.),[25] and sorghumn (*Sorghum sudanense* S.).[26]

In order to determine the absolute configuration of **1** produced by *P. ananatis*, 8 L of conditioned cultivation media was extracted with ethyl acetate. Purification by column chromatography and GC-MS analyses allowed the detection of one fraction with trace amounts of **1** eluting with DCM/EtOAc (3:1), sufficiently pure for chiral column analysis. The natural product was characterized by MS comparison and co-injection with a synthetic standard.[10] Racemic and chiral synthetic standards were obtained from heptanoic acid and (±)- or (*S*)-α-amino-γ-butyrolactone using the synthetic strategy previously reported (Scheme 1) and were fully characterized by spectroscopic analyses (^{1}H NMR, ^{13}C NMR, IR, mass spectra, optical rotation, and circular dichroism).[9,12,28] Fractions and extracts from *P. ananatis* were also evaluated with bioreporter *A. tumefaciens* NTL4(pZLR4), and positive bioactivities were observed in fractions containing the acylhomoserine lactone.

The absolute configuration determination was performed using the chiral column Chirasil cyclodextrin (Chrompack). Analytical conditions were optimized using the synthetic racemic product. The *S* absolute configuration (90% ee) for the natural product was determined by co-injection and retention time comparison with racemic and chiral products. Results are shown in Table 1. Like in even-numbered acyl-HSLs the *S* absolute configuration was observed in compound **1**, reinforcing the hypothesis that some acyl-HSL synthesizing proteins (LuxI homologues) are not substrate specific and may accept similar acyl-ACPs with minor differences in their side chain length, minimizing the probability of the existence of LuxI homologues specifically devoted to the synthesis of odd-numbered acyl-HSL.

Table 1. Absolute Configuration Determination of Natural (*S*)-*N*-heptanoyl-HSL Produced by *Pantoea ananatis* CCT 6481 by GC-FID (chiral column)

N-heptanoyl-HSL	*R* retention time (min)	*S* retention time (min)	ee (major)
(±) synthetic	60.87	61.05	1:1 peaks
(*S*) synthetic		61.06	97% *S*
(*R*) synthetic	60.88		>99% *R*
(*S*) natural	60.86	61.03	90% *S*
co-injection (±)-synthetic and natural product[a]	60.85	61.03	22% *S*

[a] Co-injection: 1:1 mixture of (±)-*N*-heptanoyl-HSL (1 mg/mL) and fraction M (0.02 mg/mL). Fraction M (total mass: 4.8 mg) contains approximately 1.4% of **1**.

In spite of the importance of the acyl-HSLs bactericidal properties under environmental conditions, the relevance of the absolute configuration for the antimicrobial activity was not previously reported. Therefore, we investigated the activities of the synthetic racemic mixture and the *S* and *R* enantiomers of *N*-heptanoyl-, *N*-(3-oxo-octanoyl)-, *N*-(3-oxo-dodecanoyl)-, and *N*-(3-oxo-tetradecanoyl)-HSL against *B. subtilis* CCT 0089, *B. cereus* CCT 4060, and *S. aureus* CCT 1295, looking for the most active substance. For this purpose the desired substances were synthesized according to the route shown in Scheme 1. Each product was fully characterized by spectroscopic analyses.

In order to select the most appropriate substance/microorganism set, a preliminary antimicrobial assay was carried out applying a simple microtiter plate-based colorimetric assay dependent on the reduction of the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] to its formazan and on microorganism growth.[30]

In general, *B. subtilis* was the sole microorganism susceptible to *N*-heptanoyl-HSL, at 500 ppm. This microorganism seemed to

Table 2. Results of Preliminary Antimicrobial Assays of Some Acyl-HSLs against Gram-Positive Bacteria: Range of Enantiomers and Racemic Product Activities

solutions	concentration range for growth inhibition (μg/mL)		
	B. cereus	*B. subtilis*	*S. aureus*
water/DMSO 20%	[a]	[a]	[a]
cloranfenicol	15.62−3.91	3.91	31.25−7.81
N-heptanoyl-HSL	[a]	500	[a]
N-(3-oxo-octanoyl)-HSL	125−62.5	125−62.5	500
N-(3-oxododecanoyl)-HSL	500−125	250−125	500
N-(3-oxotetradecanoyl)-HSL	500	500−250	500

[a] Inactive even at the highest concentration assayed (μg/mL).

be the most susceptible against these substances, while *S. aureus* was the most resistant (Table 2). The 3-oxo-acyl-HSLs were more active than nonsubstituted *N*-heptanoyl-HSL, while *N*-(3-oxo-octanoyl)-HSL was the most active. All substances were considerably less active than the positive control (cloramphenicol).

Therefore for better accuracy the quantitative absorbance assay using spectrophotometric absorbance measurements (A at 650 nm)[19] was chosen together with the *B. cereus*/*N*-(3-oxo-octanoyl)-HSL set. The results are shown in Figure 2. No statistically significant activity difference (medium SD = ±0.012) between the *R* and *S* enantiomers was observed. This result, *per se,* is interesting, as the non-natural (*R*)-acyl-HSL is about as active as the natural *S* enantiomer. Another remarkable result is that the racemic product is, in general, less active than either of the pure enantiomers.

These results could fit into a model of two independent bacterial receptors for (*R*)- or (*S*)-acyl-HSL, each with a specific affinity to one enantiomer. Therefore, in the racemic product each enantiomer would be diluted by half, leading to an almost 50% activity decrease. The hypothesis of the enantiomers being each other's antagonist should also be considered; however the results are more consistent with the first hypothesis.

In summary, several findings concerning the absolute configuration of acylhomoserine lactones, including chiral characterization of a rare metabolite and also the importance of chirality for antimicrobial activity against Gram-positive bacteria, have been reported. Other cross-species correlations involving acylhomoserine lactones and important Brazilian phytopathogenic bacteria are currently under investigation.

Experimental Section

Materials and Methods. NMR analyses were carried out on a Varian Inova-500 or a Bruker Gemini-300 spectrometer, using $CDCl_3$ as solvent and TMS as internal reference. The IR spectra were recorded on a Bomen Michelson MB spectrophotometer, using KBr (Merck) pellets as sample support. A Perkin-Elmer 341 polarimeter equipped with a sodium lamp was employed for $[\alpha]^{20}_D$ analyses, using MeOH (HPLC grade) as solvent. Microplates (96-well) were analyzed with a FlashScan 530 Analytik Jena spectrophotometer. Column chromatography purifications were carried out with Si gel (Acros, 0.035−0.070 mm) and high-quality solvents. All solvents were treated before using, usually by drying with anhydrous Na_2SO_4 followed by distillation with Vigreux columns. For synthetic purposes, DCM was dried by refluxing and distilled over CaH_2. The reagents were from Aldrich or Sigma.

Microorganisms. The microorganisms *Pantoea ananatis* CCT 6481 (=ATCC 33244, type strain), *Bacillus subtilis* CCT 0089, *B. cereus* CCT 4060, and *Staphylococcus aureus* CCT 1295 were maintained in slants containing solid nutrient broth medium (OXOID, 20 g/L), under refrigeration at 5 °C. The bioreporter *Agrobacterium tumefaciens* NTL4(pZLR4) was maintained in Luria-Bertani medium (LB: 1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with gentamicine (50 μg/mL) and was provided by Dr. Welington L. Araújo (originally from Dr. Stephen K. Farrand, University of Illinois). Miller-Hinton medium was from OXOID.

Chiral GC-FID Analyses. GC-FID analyses were conducted in an Agilent 6890 chromatograph using the chiral column Chrompack Chirasil CD (25 m × 0.25 mm × 0.25 μm). Highly pure hydrogen was employed as carrier gas (1 mL/min). The injector and detector temperatures were 220 and 250 °C, respectively. Routinely, samples were dissolved in highly pure EtOAc (1 mg/mL) and injected in split mode (1/100). The temperature program for *N*-(3-oxo-octanoyl)-HSL was 50−180 °C (1 °C/min) held for 40 min at 180 °C. For *N*-heptanoyl-HSL the temperature program was 50−180 °C (2 °C/min) held for 5 min at 180 °C.

GC-MS Analyses. GC-MS analyses (70 eV) were carried out in an Agilent 6890 chromatograph coupled to a Hewlett-Packard 5973 mass detector. Routine trace analyses were carried out with a capillary Si gel column (HP5 or MDN-5S, 30 m × 0.25 mm × 0.25 μm), with the temperature program 100−290 °C (10 °C/min) held at 290 °C for 10 min, using helium (1 mL/min) as carrier gas. Samples were injected in splitless mode (1 μL) in ethyl acetate (1 mg/mL), with the injector at 250 °C.

Cultivation of *P. ananatis* and Detection of (*S*)-*N*-Heptanoylhomoserine Lactone. The inoculum was prepared in a test tube containing liquid NB medium (10 mL) incubated in BOD at 30 °C without shaking. After 24 h, the inoculum was transferred to a 2 L Erlenmeyer containing NB medium (1 L) for incubation at 28 °C for 24 h, under shaking (110 rpm). After the incubation period, the medium was centrifuged under refrigeration (18 °C, 5000 rpm, 20 min). The aqueous layer was extracted with EtOAc (3 × 500 mL), and the combined organic layers were extracted with distilled water (500 mL) and evaporated under reduced pressure at 40−45 °C. This procedure was repeated eight times, yielding 557.0 mg of crude extract. This extract was purified by silica gel column chromatography (18 g of silica; column diameter 2 cm), with *n*-hexane, DCM, EtOAc, and MeOH in increasing polarity as eluents. Fractions were grouped by TLC similarities and analyzed by GC-MS. Some fractions were also assayed with *A. tumefaciens* bioreporter. The metabolite (*S*)-*N*-heptanoyl-HSL was identified in trace amounts (<1%, by GC-MS chromatogram peak area integration) in the complex fraction M with 4.8 mg of total mass, eluting at DCM/EtOAc (3:1) polarity.

Bioassays with *Agrobacterium tumefaciens* NTL4(pZLR4). The β-galactosidase bioassay for detection of acylhomoserine lactones was carried out with extracts and fractions from *P. ananatis* cultivation media, as well as with synthetic products. It was performed as previously described.[9]

Synthesis of Acylhomoserine Lactones. General Procedure for *N*-Heptanoylhomoserine Lactone Enantiomers.[28] To a round-bottomed flask (5 mL) with a magnetic stirrer were added ultrapure Milli-Q water (2.5 mL), Et_3N (0.105 mmol), (*S*)-, (*R*)-, or (±)-α-amino-γ-butyrolactone hydrobromide (or hydrochloride) (0.105 mmol), and heptanoic acid (0.157 mmol). Then, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.157 mmol) was added. The reaction mixture was stirred at room temperature. After 24 h, it was extracted with ethyl acetate (3 × 10 mL), and the combined organic layers were extracted with 5% aqueous $NaHCO_3$ (2 × 6 mL), 1 mol/L $KHSO_4$ (1 × 6 mL), and saturated NaCl solution (1 × 6 mL). The solvent was evaporated under reduced pressure, yielding **1** as a white solid.

(±)-*N*-Heptanoylhomoserine Lactone. Yield: 64%. GC-MS (EI, 70 eV): *m/z* 213 (2), 156 (15), 143 (100), 125 (20), 113 (13), 102 (12), 101 (16), 100 (6), 85 (16), 57 (50). IR (KBr): 3315, 2955, 2858, 1776, 1646, 1545, 1384, 1173, 1013 cm^{-1}. ^1H NMR (499.88 MHz, $CDCl_3$, TMS): δ 0.88 (t, 3H, *J* 7.3 Hz, H-7′); 1.29 (m, 6H, H-4′, H-5′, H-6′); 1.64 (quintet, 2H, *J* 7.6 Hz, H-3′); 2.14 (m, 1H, H-4); 2.25 (t, 2H, *J* 8.9 Hz, H-2′); 2.84 (m, 1H, H-4); 4.28 (ddd, 1H, *J* 11.3; 9.5; 5.8 Hz, H-5a); 4.47 (t, 1H, *J* 8.9 Hz, H-3); 4.56 (ddd, 1H, *J* 11.3; 8.6; 5.8 Hz, H-5b); 6.15 (d, NH, *J* 3.7 Hz). ^{13}C NMR (125.71 MHz, $CDCl_3$, TMS): δ 14.0 (C-7′); 22.4 (C-6′); 25.3 (C-3′); 28.8 (C-5′); 30.5 (C-4); 31.4 (C-4′); 36.1 (C-2′); 49.2 (C-3); 66.1 (C-5); 173.7 (C-1′); 175.5 (C-2).

(*S*)-(−)-*N*-Heptanoylhomoserine Lactone. Chemical yield and GC-MS, IR, ^1H, and ^{13}C data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ −18 (*c* 0.42 MeOH).

(*R*)-(+)-*N*-Heptanoylhomoserine Lactone. Chemical yield and GC-MS, IR, ^1H. and ^{13}C data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ +17 (*c* 0.42 MeOH).

General Procedure for *N*-3-Oxo-acylhomoserine Lactones.[29] **Meldrum Acid Derivatives.** A round-bottomed flask (50 mL) containing dry DCM (20 mL), the fatty acid (hexanoic, decanoic, or dodecanoic acid, 2.0 mmol), 4-DMAP (2.1 mmol), DCC (2.2 mmol), and Meldrum acid (2,2-dimethyl-1,3-dioxane-4,6-dione; 2.0 mmol) was stirred under a nitrogen atmosphere for 24 h. Then, the reaction medium was filtered

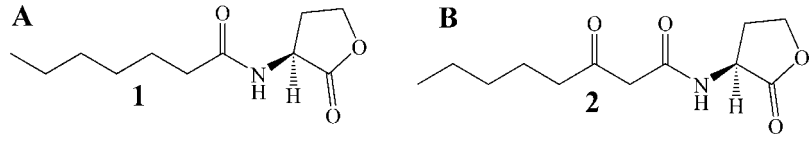


Figure 1. (A) (*S*)-*N*-Heptanoyl-HSL produced by *P. ananatis*. (B) Antimicrobial (*S*)-*N*-(3-oxo-octanoyl)-HSL.

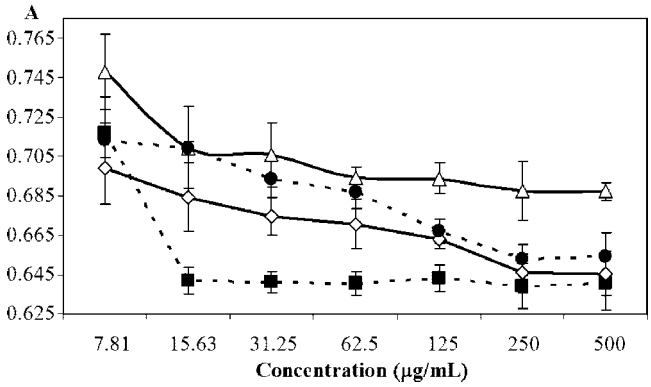


Figure 2. Antimicrobial activity of *N*-(3-oxo-octanoyl)-HSL against *B. cereus* evaluated by turbidimetry. Legend: (±): △; (*R*): ●; (*S*): ◇; cloramphenicol: ■. Blank absorbance: 0.724 ± 0.01 (water/DMSO 20%). The results represent a quadruplicate average.

through cotton wool, and the organic layer was evaporated under reduced pressure, producing a yellow oil, which was dissolved in ethyl acetate (20 mL). This was extracted with aqueous 2 mol/HCl (3 × 10 mL) and distilled H_2O (1 × 10 mL) and then dried over anhydrous $MgSO_4$. The organic layer was filtered and evaporated under reduced pressure. All Meldrum acid derivatives were used as fast as possible in the next synthetic step.

***N*-(3-Oxo-acyl)homoserine Lactones.** To a round-bottomed flask (100 mL) equipped with a refluxing condenser and magnetic stirrer were added acetonitrile (22.5 mL, HPLC grade), the Meldrum acid derivative (0.75 mmol), Et_3N (1.2 mmol), and (*S*)-, (*R*)-, or (±)-α-amino-γ-butyrolactone hydrobromide (or hydrochloride) (0.75 mmol). The reaction mixture was stirred at room temperature for 2 h and then under reflux for 3 h. Then, the reaction medium was evaporated under reduced pressure, and the white solid remaining was dissolved in ethyl acetate and methanol (20/5 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ solution (3 × 10 mL), 1 mol/L $KHSO_4$ (3 × 10 mL), and saturated NaCl (3 × 10 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated, yielding the *N*-(3-oxo-acyl)homoserine lactone as a pale yellow solid, which was further purified by Si gel column chromatography (12 g, Acros, 0.035−0.070 mm particle size; 2 cm diameter column) with hexane, DCM, and EtOAc in increasing polarity as eluents. The acyl-HSLs eluted at DCM/EtOAc (3:1) polarity.

(±)-*N*-(3-Oxo-octanoyl)homoserine Lactone, 2. Global yield: 28%. GC-MS (EI, 70 eV): *m/z* 241 (1), 224 (24), 185 (7), 143 (25), 102 (7), 99 (31), 56 (100). IR (KBr) 3258, 2934, 1777, 1716, 1646, 1546, 1173, 1019, 599 cm^{-1}. ^1H NMR (300.06 MHz, $CDCl_3$, TMS): δ 0.89 (t, 3H, *J* 7.0 Hz, H-8′); 1.30 (m, 4H, H-6′, H-7′); 1.59 (quintet, 2H, *J* 7.3 Hz, H-5′); 2.28 (m, 1H, H-4); 2.53 (t, 2H, *J* 7.3 Hz, H-4′); 2.75 (m, 1H, H-4); 3.47 (s, 2H, H-2′); 4.28 (ddd, 1H, *J* 11.0, 9.1; 6.2 Hz, H-5a); 4.47 (td, 1H, *J* 8.2; 1.4 Hz, H-3); 4.60 (ddd, 1H, *J* 11.0; 8.8; 6.2 Hz, H-5b); 7.70 (d, NH, *J* 5.1 Hz). ^{13}C NMR (75.45 MHz, $CDCl_3$, TMS): δ 13.8 (C-8′); 22.32 (C-7′); 23.0 (C-6′); 31.1 (C-5′); 29.8 (C-4); 43.8 (C-4′); 48.1 (C-2′); 49.0 (C-3); 65.8 (C-5); 166.4 (C-1′); 174.8 (C-2); 206.5 (C-3′).

(*S*)-(−)-(3-Oxo-octanoyl)homoserine Lactone. Global yield and GC-MS, IR, ^1H NMR, and ^{13}C NMR data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ −17 (*c* 0.53 MeOH), 94% ee [GC-FID(chiral column)].

(*R*)-(+)-(3-Oxo-octanoyl)homoserine Lactone. Global yield and GC-MS, IR, ^1H NMR, and ^{13}C NMR data are similar to those observed for the racemic compound. $[\alpha]^{20}_D$ +16 (*c* 0.51 MeOH), 99% ee [GC-FID(chiral column)].

(±)-*N*-(3-Oxo-dodecanoyl)homoserine lactone, 3. Global yield: 39.5%. IR (KBr): 3298, 2925, 2850, 1782, 1721, 1641, 1546, 1382, 1178, 1019, 721 cm^{-1}. ^1H NMR (300.06 MHz, $CDCl_3$, TMS): δ 0.87 (t, 3H, *J* 6.2 Hz, H-12′); 1.26 (m, 12H, H-11′, H-10′, H-9′, H-8′, H-7′, H-6′); 1.58 (quintet, 2H, *J* 7.0 Hz, H-5′); 2.25 (m, 1H, H-4); 2.53 (t, 2H, *J* 7.3 Hz, H-4′); 2.75 (m, 1H, H-4); 3.47 (s, 2H, H-2′); 4.28 (ddd, 1H, *J* 11.0; 9.5; 6.2 Hz, H-5); 4.47 (td, 1H, *J* 9.1; 1.2 Hz, H-3); 4.60 (ddd, 1H, *J* 11.4; 8.8; 6.7 Hz, H-3); 7.69 (d, NH, *J* 6.2 Hz). ^{13}C NMR (75.45 MHz, $CDCl_3$, TMS): δ 14.2 (C-12′); 22.7 (C-11′); 31.87 (C-10′); 29.0 (C-9′); 29.3 (C-8′); 29.38 (C-7′); 29.41 (C-6′); 23.4 (C-5′); 43.9 (C-4′); 48.2 (C-2′); 65.9 (C-5); 29.8 (C-4); 49.1 (C-3); 166.2 (C-1′); 174.6 (C-2); 206.3 (C-3′).

(*S*)-(−)-(3-oxo-dodecanoyl)homoserine Lactone. Global yield and IR, ^1H NMR, and ^{13}C NMR data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ −19 (*c* 0.50 MeOH).

(*R*)-(+)-(3-Oxo-dodecanoyl)homoserine Lactone. Global yield and IR, ^1H NMR, and ^{13}C NMR data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ +15 (*c* 0.50 MeOH).

(±)-*N*-(3-Oxo-tetradecanoyl)homoserine Lactone, 4. Global yield: 46.8%. IR (KBr): 3298, 2920, 2850, 1772, 1716, 1641, 1549, 1178, 1015, 721 cm^{-1}. ^1H NMR (300.06 MHz, $CDCl_3$, TMS): δ 0.88 (t, 3H, *J* 6.7 Hz, H-14′); 1.26 (m, 16H, H-13′, H-12′, H-11′, H-10′, H-9′, H-8′, H-7′, H-6′); 1.58 (quintet, 2H, *J* 7.0 Hz, H-5′); 2.25 (m, 1H, H-4); 2.53 (t, 2H, *J* 7.3 Hz, H-4′); 2.74 (m, 1H, H-4); 3.47 (s, 2H, H-2′); 4.27 (ddd, 1H, *J* 11.0; 9.2; 6.1 Hz, H-5); 4.48 (td, 1H, *J* 9.2; 1.2 Hz, H-3); 4.62 (ddd, 1H, *J* 11.0; 8.9; 7.0 Hz, H-5); 7.73 (d, NH, *J* 6.4 Hz). ^{13}C NMR (75.45 MHz, $CDCl_3$, TMS): δ 14.1 (C-14′); 22.7 (C-13′); 23.4 (C-12′); 29.0 (C-11′); 29.3 (C-9′); 29.4 (C-8′); 29.4 (C-7′); 29.6 (C6′, C5′); 29.7 (C-4); 31.87 (C-10′); 43.9 (C-4′); 48.2 (C-2′); 49.0 (C-3); 65.9 (C-5); 166.5 (C-1′); 175.0 (C-2); 206.6 (C-3′).

(*S*)-(−)-(3-Oxo-tetradecanoyl)homoserine Lactone. Global yield and GC-MS, IR, ^1H NMR, and ^{13}C NMR data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ −14 (*c* 0.50 MeOH).

(*R*)-(+)-(3-Oxo-tetradecanoyl)homoserine Lactone. Global yield and GC-MS, IR, ^1H NMR, and ^{13}C NMR data are identical to those observed for the racemic compound. $[\alpha]^{20}_D$ +11 (*c* 0.50 MeOH).

Preliminary Antimicrobial Assays: Colorimetric. The preliminary bioassays were performed against the Gram-positive bacteria *S. aureus* CCT 1295, *B. cereus* CCT 4060, and *B. subtilis* CCT 0089. The inocula were prepared in slants with solid NB medium (3 tubes each) and grown in BOD at 30 °C for 24 h. After growing, the cells were removed with a flamed wire loop and transferred to autoclaved distilled H_2O (10 mL) tubes until the suspension reached 3 × 10^8 cells/mL according to the MacFarland scale. Then 5 mL of this suspension was added to 50 mL of Miller-Hinton medium, and the mixture was pipetted (100 μL for each well) to 96-well plates (the plates were previously exposed to a UV lamp for 1 h). The solutions to be assayed (100 μL for each well) were added to the top column wells, homogenized, and pipetted in the following lines (50% dilution factor). The test was performed in duplicate. A solution of DMSO in H_2O (20%) was used as negative control (blank). Cloramphenicol (1000 μg/mL, positive control) and synthetic acylhomoserine lactones (1000 μg/mL) were prepared in DMSO (20%) in distilled H_2O. After adding the reagents, the microtiter plates were incubated at 30 °C for 24 h and analyzed by adding 100 μL of MTT solution (0.025% in H_2O). The purple color was indicative of normal microbial development, while growth inhibition was observed in the yellow wells.

Quantitative Antimicrobial Assays: Absorption. The quantitative bioassay was performed against *B. cereus* CCT 4060 with each *N*-(3-oxo-octanoyl)-HSL enantiomer and with the racemic mixture. The inoculum was prepared by adding 500 μL of a *B. cereus* 3 × 10^8 cells/mL suspension to 50 mL of Miller-Hinton medium. The bioassays were performed in gamma-sterilized, DNA-free plates. A solution of H_2O/DMSO (4:1) (100 μL) was added to each well. Then, the solutions to be assayed were added to the top wells (100 μL), homogenized, and pipetted in the following lines (50% dilution factor). Finally, the inoculum was added (100 μL). As blank, DMSO (20%) in H_2O was employed. The cloramphenicol and acylhomoserine lactones stock solutions were also prepared in DMSO (20%)/distilled H_2O (2000 μg/

mL). After adding the substances, the plates were incubated at 30 °C for 24 h and analyzed in an Analytik Jena 96-well plate reader spectrophotometer in the absorbance mode (*A* at 650 nm). The results represent an average of four measurements per well at three points in each well. Each substance was assayed in quadruplicate, while the whole test was performed in duplicate. Standard deviation calculations and graph design were carried out with Microsoft Excel.

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