The Synthesis of Optically Pure Enantiomers of *N*-Acyl-homoserine Lactone Autoinducers and Their Analogues

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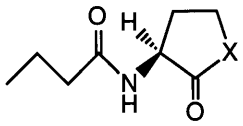
(Received December 7, 2000; CL-001103)

Optically pure enantiomers of *N*-acyl-homoserine lactones and thiolactones, whose optical purities were unambiguously determined by chiral stationary phase HPLC analyses, were firstly synthesized. Bioassays revealed that L-isomers were essential as the autoinducers in quorum sensing, while no effect was observed with D-isomers.

N-Acyl-homoserine lactones play an important role in cell density-dependent gene expression, termed quorum sensing, in many gram-negative bacteria. These diffusive signal compounds are called autoinducers.[1] For example, *N*-butyryl-L-homoserine lactone (BHL, **1**) has been found to regulate two quorum sensing systems, *las* and *rhl*, in *Pseudomonas aeruginosa*.[1] To investigate the autoinduction mechanism at a molecular level, a number of autoinducers and their analogues have been chemically synthesized.[2] However, little information is available on the absolute configuration of such synthetic compounds. Moreover, virtually nothing is known about the absolute configuration of natural autoinducers. We here report the synthesis of optically pure enantiomers of *N*-acyl-homoserine lactones (**1**~**4**) and thiolactones (**5**~**8**) whose optical purities were determined by chiral stationary phase HPLC analyses. Bioassays revealed that only L-isomers had autoinducing activity.



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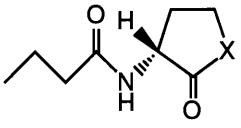

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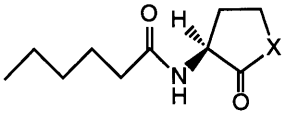

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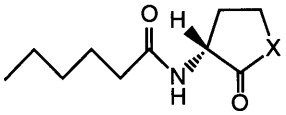

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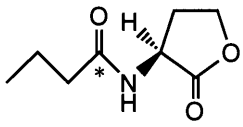
8:X=S

Homoserine lactone (HSL) derivatives (**1** and **3**) as original autoinducers, homocystein thiolactone (HCT) derivatives (**5** and **7**) as the analogues, and their optical isomers (**2**, **4**, **6** and **8**) were synthesized by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in an aqueous solution as described previously.[2] We used optically pure L- or D-HSL (ALDRICH Chemical Corporation Inc.) and L- or D-HCT (SIGMA Chemical Co.) as starting materials.[3] To confirm the optical purities of **1**~**8**, CHIRALPAK AS and its modified type columns[4] (Daicel Chemical Industries, Ltd.) were used for the HPLC system with a *n*-hexane/ethanol (80/20 v/v) eluent and a refractive index detector. The compounds **1**~**4** gave only a single peak with a different retention time, respectively.[5–7] In addition, the co-injection of L- and D-isomers gave 2 peaks corresponding to each of the isomers. These results revealed that **1**~**4** were in high states of optical purity. Since BHL extracted from the culture supernatant of *P. aeruginosa* gave the same peak as the chemically synthesized **1**, the natural autoinducer is likely a L-isomer. In contrast, the synthesized HCT derivatives gave two peaks, indicating that they were racemates. Racemization is likely to occur at the step of acylation. These racemates could be resolved into enantiomers by the HPLC system.[5,7] To further synthesize optically pure **5**~**8**, DCC coupling of the acid and HCT was accomplished in DMF with HOBt, as employed in the protection method for racemization in peptide synthesis. This allowed us to obtain optically pure HCT derivatives (**5**~**8**).[5–7] HSL derivatives **1** and **2** were stable in the solution, while **5** and **6** were readily racemized if the stock solution contained ethanol.

The natural autoinducer (BHL) added in the medium was taken up by *P. aeruginosa*.[1] Both synthetic autoinducers, L- and D-isomers are likely to be taken up by *P. aeruginosa*. To demonstrate this, ^{13}C-labelled compounds (**9** and **10**) were synthesized by using butylic acid-1-^{13}C in the same manner employed for the synthesis of **1** and **2**.[6] Cell extracts were prepared from the culture of *P. aeruginosa* strain PAO-MW1 (Δ*lasI* Δ*rhlI*), which is defective in autoinducer biosynthesis,[8] after grown in the presence of either **9** or **10**. ^{13}C NMR spectra of the cell extracts showed only one amide carbon peak at the same position (around 178 ppm) as the ^{13}C-labelled carbon of **9** or **10**.[9] These results suggest that *P. aeruginosa* cells were capable of taking up both L- and D-isomers.



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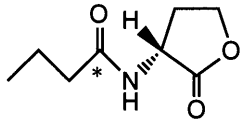

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In order to assess the autoinducing activity of optically pure compounds **1**, **2**, **5** and **6**, we monitored activation of the transcription of the *lasB*- and *rhlA-lacZ* genes in *P. aeruginosa* strain PAO-MW1 at the early stationary phase of growth. β-Galactosidase activities were determined by the Miller's standard procedure.[10] Bioassays with *P. aeruginosa* PAO-MW1 showed that the HCT derivative **5** had autoinducing activity similar to the original autoinducer **1.** On the other hand, D-iso-

mer (**2** and **6**) showed no autoinducing activity. Importantly, L-isomer activities were not inhibited by D-isomers. (Table 1) We also examined **1**–**8** for their ability to induce violacein biosynthesis by *Chromobacterium violaceum* strain CV026.[11] The violacein biosynthesis in *C. violaceum* was induced by the HCT derivatives **5** and **7** as well as the HSL derivatives **1** and **3**. *C. violaceum* did not produce violacein when D-isomer was added to the medium. Thus, the present data showed that optically pure L-HCT derivatives had autoinducing activity similar to optically pure L-HSL derivatives. Previous workers have detected relatively low autoinducing activities with HCT derivatives.[2] It was possible that their HCT derivaties were not optically pure enantiomers.

Table 1. Autoinducing activities of **1**, **2**, **5** and **6** in *P. aeruginosa*

Strain	β-Galactosidase activity[c, d]					
	control	**1**	**2**	**1+2**	**5**	**6**
PAO-MW1 (pβ01)[a]	1	15.4	1.18	15.8	13.2	1.26
PAO-MW1 (pβ02)[b]	1	14.8	0.77	14.5	15.3	0.85

[a] pβ01 : A pQF50 derivative containing the *lasB-lacZ* transcriptional fusion.
[b] pβ02 : A pQF50 derivative containing the *rhlA-lacZ* transcriptional fusion.
[c] Miller units relative to control.
[d] β-Galactosidase assays were performed at 10 μmol dm^{-3}.

Experimental evidence that optically pure D-isomers were not inhibitory for the autoinduction by L-isomers suggests that D-isomers do not bind to autoinducer binding proteins, LasR and RhlR, in *P. aeruginosa*. Since the orientation of lactone ring moiety is completely different between L- and D-isomers, the autoinducer binding proteins may recognize this difference. It is also likely that these proteins have flexibility for binding autoinducer analogues with different affinities as shown with the HCT derivatives. Little information is available on the structural requirements necessary for activation of autoinducer binding proteins. To further investigate the interaction between autoinducers and their binding proteins, we are currently purifying LasR and RhlR from *P. aeruginosa*.

This research was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

References and Notes

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3 Optical purities of commercially available L- or D-HSL and L- or D-HCT were confirmed by chiral stationary phase HPLC analyses.

4 Only a modified type of CHIRALPAK AS (Column No. T300AS00CE-JL041) provided by Daicel Chemical Industries, Ltd. was effective for separating the HSL derivatives into enantiomers. The HCT derivatives could be separated by using the original type of CHIRALPAK AS.

5 Retention times of **1**~**8** under the flow rate of 1 mL/min were:**1**, 9.0 min; **2**, 10.0 min; **3**, 7.6 min; **4**, 8.4 min; **5**, 9.4 min; **6**, 8.2 min; **7**, 8.0 min; and **8**, 7.0 min.

6 Synthetic yields of **1**~**10** were:**1**, 40 %; **2**, 35 %; **3**, 65 %; **4**, 60 %; **5**, 20 %; **6**, 15 %; **7**, 32 %; **8**, 11 %; **9**, 40 %; and **10**, 35 %.

7 We thank for the measurements of optical rotation, EI-MS, ^{1}H and ^{13}C NMR spectra using JASCO DIP-370, JEOL SX-102A and JEOL JNM-LA500 at the Instrument Center for Chemical Analysis, Hiroshima University.

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9 NMR samples were prepared from the supernatant (D_2O, 1 mL) of the homogenate of the cell (50 mg) grown in the LB medium (20 mL) containing **9** or **10** (1 mmol dm^{-3}). Both samples gave only one amide carbon peak respectively with a similar intensity.

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