Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 3897–3900

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Chemical Synthesis of *S*-Ribosyl-L-homocysteine and Activity Assay as a LuxS Substrate

Gang Zhao,[a] Wei Wan,[a] Shahrzad Mansouri,[a] Joshua F. Alfaro,[a] Bonnie L. Bassler,[b] Kenneth A. Cornell[c] and Zhaohui Sunny Zhou[a,*]

[a]*Department of Chemistry and School of Molecular Biosciences, Washington State University, Pullman, WA 99164, USA*
[b]*Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA*
[c]*Division of Vascular Surgery, L-456, Oregon Health & Sciences University, Portland, OR 97201, USA*

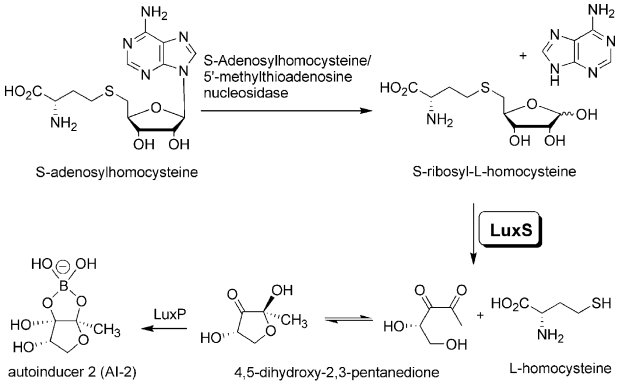
Received 15 August 2002; accepted 9 September 2003

Abstract—Bacterial quorum sensing is mediated by autoinducers, small signaling molecules generated by bacteria. It has been proposed that the LuxS enzyme converts *S*-ribosyl-L-homocysteine to 4,5-dihydroxy-2,3-pentanedione, the precursor of autoinducer 2 (AI-2). We report here a chemical synthesis of *S*-ribosyl-L-homocysteine and its analogue using Mitsunobu coupling. Chemically synthesized ribosylhomocysteine has been confirmed as a substrate for LuxS in both an enzyme assay and a whole cell quorum sensing assay. The chemical entities of products from the LuxS reaction were also established. Several ribosylhomocysteine analogues have been tested as LuxS inhibitors.

Bacteria communicate actively within their own species and across species boundaries. The process by which they sense cell density is termed quorum sensing, and is found in a broad spectrum of bacterial species.[1–3] This signaling pathway plays pivotal roles in bacterial toxin production, biofilm formation, sporulation, bioluminescence, and bacterial virulence. Quorum sensing is mediated by autoinducers (AI), small signaling molecules generated by bacteria. In general, Gram-negative bacteria produce acyl-homoserine lactone as autoinducers, while Gram-positive species generate peptides as signals.[1–3] Recently, a new type of signaling molecule composed of a furanosyl borate diester, autoinducer 2 (AI-2), has been discovered.[4] Its proposed metabolic pathway is depicted in Scheme 1.[4–7]

The first step in the pathway is the hydrolysis of *S*-adenosyl-L-homocysteine (AdoHcy) to *S*-ribosyl-L-homocysteine (SRH) and adenine by *S*-adenosyl-L-homocysteine/5′-methylthioadenosine nucleosidase (EC 3.2.2.9).[8–10] The enzyme LuxS then cleaves *S*-ribosylhomocysteine to form L-homocysteine and presumably 4,5-dihydroxy-2,3-pentanedione (DPD).[5–7,11]

LuxS is likely to be the enzyme ribosylhomocysteinase (or *S*-ribosyl-L-homocysteine hydrolase, EC 3.2.1.148, formerly EC 3.3.1.3) first described by Duerre and co-workers in the 1960s.[12] However, the chemical nature of the LuxS substrate has not been confirmed with chemically synthesized *S*-ribosyl-L-homocysteine. In previous reports, the substrate for LuxS was generated from *S*-adenosyl-L-homocysteine either by enzymatic cleavage using *S*-adenosylhomocysteine/5′-methylthioadenosine nucleosidase or by acid catalyzed hydrolysis; neither has been purified nor spectroscopically



Scheme 1.

*Corresponding author. Tel.: +1-509-335-7135; fax: +1-509-335-8867; e-mail: sunnyz@wsu.edu

0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.09.015

characterized.[5,11,13] Subsequently, 4,5-dihydroxy-2,3-pentanedione is converted to AI-2, an unusual furanosyl borate diester that has been shown by X-ray crystallographic analysis to bind to its receptor LuxP by Bassler and Hughson's groups in early 2002.[4]

AI-2 has been found in many bacteria and is thought to mediate inter-species communication, in contrast to other autoinducers that are generally specific to a given bacterial species. Sequence analysis indicates that the AI-2 pathway is widespread in a broad range of bacteria, including *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague).[2,11] Mekalanos and co-workers showed that the quorum sensing pathway regulated the expression of an array of virulence genes in *Vibrio cholerae*, a Gram-negative pathogen responsible for severe diarrheal disease. The genes implicated in their study were involved in bacterial colonization, motility, protease production and biofilm formation.[14] Thus, disruption of the AI-2 quorum sensing pathway presents an attractive strategy for developing novel antibiotics for infectious diseases; and one approach is to inhibit the LuxS enzyme. We report here a chemical synthesis of *S*-ribosyl-L-homocysteine and its analogue using Mitsunobu coupling. Subsequently, we demonstrate that chemically synthesized *S*-ribosylhomocysteine is a substrate for LuxS by both enzyme assay and whole cell quorum sensing assay. We also establish the chemical identities of the LuxS reaction products. In addition, several ribosylhomocysteine analogues have been tested as LuxS inhibitors.

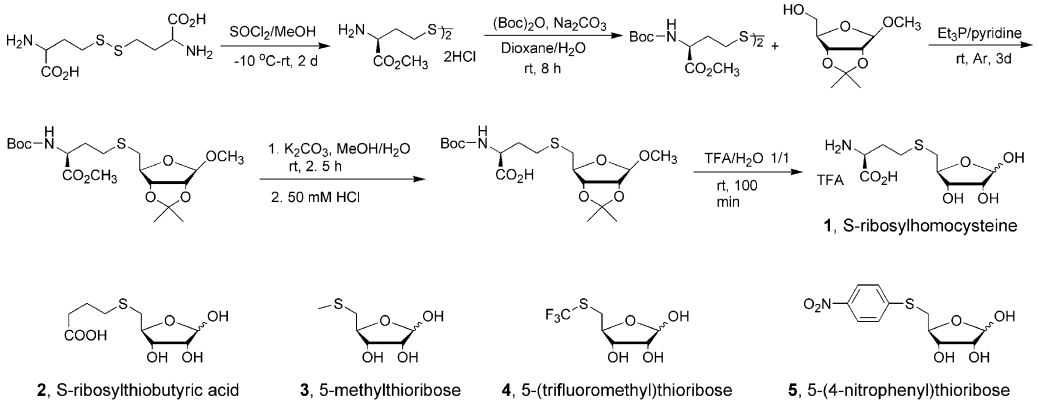
Synthesis of *S*-Ribosyl-L-homocysteine

Only one chemical synthesis of *S*-ribosyl-L-homocysteine has been reported from the Guillerm's laboratory.[15] Their key step was the formation of the S–C bond by nucleophilic substitution of C5-ribosyltosylate with the disodium salt of L-homocysteine in 22% yield. The low yield may be attributed to several factors, for example the L-homocysteine thiol group is prone to oxidation and the intermediate containing the free amino acid moiety is difficult to purify. To circumvent these problems, we prepared *S*-ribosyl-L-homocysteine following the synthetic route shown in Scheme 2. Mitsunobu coupling between the air stable disulfide of (L,L)-homocystine and the free 5-hydroxyl group of the ribose effected the S–C bond formation.[16–18]

Following standard procedures, the amino and carboxyl groups in (L,L)-homocystine (Sigma) were protected in two steps as *tert*-butyl carbamate (Boc) and methyl ester, respectively, in two steps without isolating the intermediate. The protected homocystine was obtained with an overall yield of 83%. Mitsunobu coupling of the fully protected homocystine and commercial 2,3-*O*-isopropylidene-β-D-ribofuranoside in the presence of triethylphosphine (3:1:6 molar ratio) was carried out in anhydrous pyridine at room temperature under Argon for 3 days. Fully protected ribosylhomocysteine was isolated after flash chromatography in 50–70% yields based on ribose. Standard deprotection procedures resulted in *S*-ribosyl-L-homocysteine in 90% overall yield. Our compound produced spectroscopic data that was identical to the reported values.[15] The ratio of α-form and β-form was 1 to 2, determined from NMR integration of the anomeric proton peaks (α-form at 5.19 ppm, $J = 3.9$ Hz; and β-form at 5.03 ppm, $J = 1.2$ Hz). Employing the same strategy, a ribosylhomocysteine analogue, *S*-ribosylthiobutyric acid (compound **2** in Scheme 2), was obtained in good yield from dithiodipropionic acid (Aldrich).

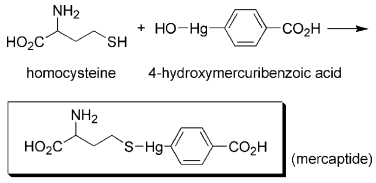
Enzyme and Whole Cell Activity Assays

Chemically synthesized *S*-ribosyl-L-homocysteine was tested as a LuxS substrate in both enzyme-based and whole cell assays. In the enzyme assay, *S*-ribosyl-L-homocysteine was incubated with recombinant *Bacillus subtilis* LuxS in 100 mM Tris–HCl, pH 8.0 at 37 °C. The LuxS expression strains were provided by Drs. Mark Hilgers and Martha Ludwig at the University of Michigan; the recombinant *B. subtilis* LuxS containing a C-terminal histidine tag was expressed and purified as previously described.[19] The L-homocysteine product was quantified by reacting a 250-μL aliquot of the assay solution with 750 μL of 133 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) in 8 M guanidinium chloride, 100 mM potassium phosphate and 1 mM EDTA, at pH 7.2 and 37 °C for 15 min, and monitoring



Scheme 2.

the absorbance changes at 412 nm.[20] With 2.3 μM LuxS, 1 mM *S*-ribosyl-L-homocysteine was completely converted into products in 35 min. Additionally, homocysteine was confirmed as a reaction product. As shown in Scheme 3, homocysteine reacts with 4-hydroxymercuribenzoic acid specifically and rapidly (reaction completes during sample mixing). The resulting mercaptide was then separated on an Apollo C-18 reverse-phase HPLC column (4.5 mm × 250 mm), eluted isocratically with a mixture of methanol (40%) and water (60%) containing 0.1% trifluoroacetic acid at 1 mL/min, and monitored at 245 nm. As shown in Figure 1 (top trace), the mercaptide formed between authentic homocysteine and 4-hydroxymercuribezoic acid co-eluted with the mercaptide generated from the LuxS reaction. Furthermore, 4,5-dihydroxy-2,3-pentanedione had been proposed as the other LuxS product. It was reported that this diketone reacted with 1,2-phenylenediamine to form a quinoxaline, as depicted in Scheme 4.[21] Indeed, when the LuxS product was incubated with 1,2-phenylenediamine, an increase of absorption around 330 nm was observed (see Fig. 2). This result was in agreement with the proposed quinoxaline formation. HPLC analysis also supported the formation of the quinoxaline shown in Scheme 4 (data not shown). After our manuscript was submitted, Pei and co-workers reported a detailed structure analysis of the quinoxaline adduct using the same method shown in Scheme 4.[22] In sum, our results confirm that LuxS cleaves *S*-ribosylhomocysteine to form L-homocysteine and 4,5-dihydroxy-2,3-pentanedione.



Scheme 3.

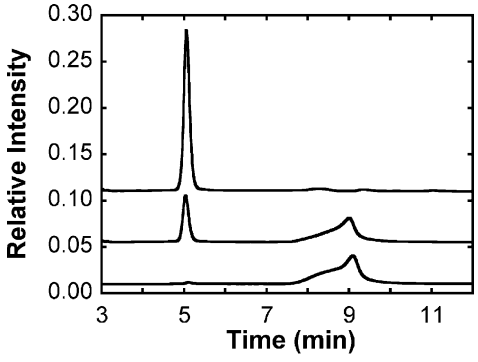
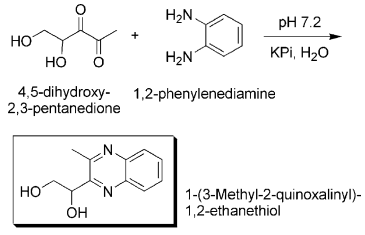


Figure 1. HPLC traces of 4-hydroxymercuribenzoic acid (200 μM, bottom trace), mixture of 4-hydroxymercuribenzoic acid and the LuxS reaction products (middle trace), and mixture of 4-hydroxymercuribenzoic acid (200 μM), the LuxS reaction products and authentic homocysteine (700 μM, top trace). The LuxS reaction products were generated by incubating 1.6 mM of *S*-ribosylhomocysteine and 20 μM of LuxS in 100 mM Hepes at pH 8 and 37 °C for 2 h; and were diluted 16-fold when mixed with 4-hydroxymercuribenzoic acid in 100 mM Hepes at pH 8.

After confirming *S*-ribosylhomocysteine as a substrate for LuxS, we also tested several *S*-ribosylhomocysteine analogues as LuxS substrates. *S*-ribosylthiobutyric acid (compound **2** in Scheme 2) did not show any activity as a LuxS substrate, despite prolonged incubation at high enzyme concentration. In addition, three other ribose analogues, compounds **3**–**5** in Scheme 2, also showed no activity when assayed as LuxS inhibitors. Compounds **3**–**5** were gifts from Drs. Michael K. Riscoe and Rolf Winter (Portland Veterans Affairs Medical Center, Portland, OR, USA). The syntheses of these compounds were previously reported.[8,23,24] These results demonstrate that the amino group in ribosylhomocysteine is important for substrate binding and catalysis, useful information for inhibitor design.

Chemically synthesized *S*-ribosylhomocysteine was further verified as a substrate for LuxS in a whole cell quorum sensing assay. *S*-Ribosylhomocysteine and *S*-ribosylthiobutyric acid were incubated in identical reaction mixtures containing purified LuxS enzyme according to the method of Schauder et al.[5] Following the incubation, LuxS protein was removed by filtration and the products of the in vitro reactions were tested for AI-2 activity in the *Vibrio harveyi* quorum sensing bioassay.[25,26] After 1 h of incubation with LuxS, ribosylhomocysteine was converted into homocysteine and 4,5-dihydroxy-2,3-pentanedione. The latter (1 μM final concentration) was capable of stimulating 500-fold light production in the *V. harveyi* AI-2 reporter strain *V. harveyi* BB170.[25,26] No increase in light production



Scheme 4.

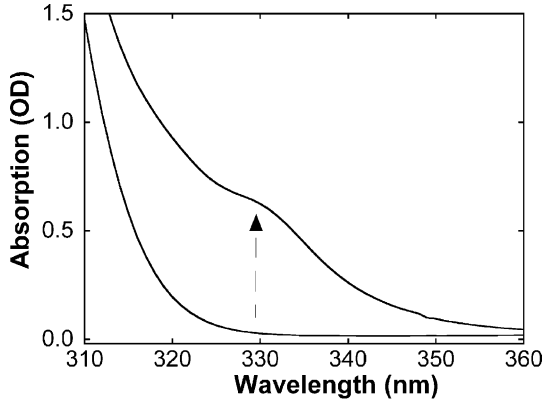


Figure 2. Spectra of 1,2-phenylenediamine (4 mM, bottom trace), and the mixture of 1,2-phenylenediamine and the LuxS reaction products (top trace). The LuxS reaction products were prepared as described in the Figure 1 legend, and were diluted 16-fold when mixed with 1,2-phenylenediamine (4 mM, final concentration) in 100 mM potassium phosphate at pH 7.2 and 37 °C. The spectral changes completed in 60 min, and the final spectrum is shown (top trace).

occurred when the identical reaction products were added to a *V. harveyi* strain lacking the AI-2 detection apparatus (*V. harveyi* BB886).[25,26] Incubation of S-ribosylthiobutyric acid with purified LuxS enzyme did not result in a product capable of stimulating bioluminescence in *V. harveyi* BB170 (4-fold stimulation). Finally, co-incubation of S-ribosylthiobutyric acid with synthetic ribosylhomocysteine (10:1, molar ratio) did not decrease the production of AI-2 activity, indicating that *S*-ribosylthiobutyric acid does not act as an inhibitor of LuxS.

Discussion and Conclusion

We report here a chemical synthesis of *S*-ribosyl-L-homocysteine and its analogue using Mitsunobu coupling. Using the chemically synthesized compound, our results confirm that *S*-ribosyl-L-homocysteine is indeed a substrate for LuxS and leads to homocysteine and 4,5-dihydroxy-2,3-pentanedione formation. Inhibitory studies of four ribose analogues suggest that the amino acid moiety in ribosylhomocysteine is crucial for binding and activity. Our synthetic route is applicable to other ribosylhomocysteine analogues. The accessibility to ribosylhomocysteine and its analogues will allow us to investigate the enzyme mechanism of LuxS and devise LuxS inhibitors as potential antimicrobial agents.

Acknowledgements

We are indebted to Professor Martha L. Ludwig and Dr. Mark T. Hilgers at the University of Michigan for providing recombinant LuxS expression strain and their support. We also thank Drs. Michael K. Riscoe and Rolf Winter at Portland Veterans Affairs Medical Center, for providing compounds **3–5** shown in Scheme 2.

References and Notes

1. Fuqua, C.; Parsek, M. R.; Greenberg, E. P. *Annu. Rev. Genet*. **2001**, *35*, 439.
2. Winans, S. C.; Bassler, B. L. *J. Bacteriol*. **2002**, *184*, 873.
3. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. *Science* **1999**, *284*, 1318.
4. Chen, X.; Schauder, S.; Potier, H.; Dorsselaer, A. V.; Pelczer, I.; Bassler, B. L.; Hughson, F. M. *Nature* **2002**, *415*, 545.
5. Schauder, S.; Shokat, K.; Surette, M. G.; Bassler, B. L. *Mol. Microbiol*. **2001**, *41*, 463.
6. Surette, M. G.; Miller, M. B.; Bassler, B. L. *Proc. Natl. Acad. Sci. U.S.A*. **1999**, *96*, 1639.
7. Taga, M. E.; Semmelhack, J. L.; Bassler, B. L. *Mol. Microbiol*. **2001**, *42*, 777.
8. Cornell, K. A.; Swarts, W. E.; Barry, R. D.; Riscoe, M. K. *Biochem. Biophys. Res. Commun*. **1996**, *228*, 724.
9. Lee, J. E.; Cornell, K. A.; Riscoe, M. K.; Howell, P. L. *Acta Crystallogr. D Biol. Crystallogr*. **2001**, *57*, 150.
10. Lee, J. E.; Cornell, K. A.; Riscoe, M. K.; Howell, P. L. *Structure* **2001**, *9*, 941.
11. Winzer, K.; Hardie, K. R.; Burgess, N.; Doherty, N.; Kirke, D.; Holden, M. T.; Linforth, R.; Cornell, K. A.; Taylor, A. J.; Hill, P. J.; Williams, P. *Microbiology* **2002**, *148*, 909.
12. Duerre, J. A.; Miller, C. H. *J. Bacteriol*. **1966**, *91*, 1210.
13. Palmer, J. L.; Abeles, R. H. *J. Biol. Chem*. **1979**, *254*, 1217.
14. Zhu, J.; Miller, M. B.; Vance, R. E.; Dziejman, M.; Bassler, B. L.; Mekalanos, J. J. *Proc. Natl. Acad. Sci. U.S.A*. **2002**, *99*, 3129.
15. Guillerm, G.; Allart, B. *J. Labelled Compd. Radiopharm*. **1992**, *31*, 717.
16. Mitsunobu, O. *Synthesis* **1981**, 1.
17. Nakagawa, I.; Hata, T. *Tetrahedron Lett*. **1975**, *16*, 1409.
18. Serafinowski, P. *Synthesis* **1985**, 926.
19. Hilgers, M. T.; Ludwig, M. L. *Proc. Natl. Acad. Sci. U.S.A*. **2001**, *98*, 11169.
20. Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol*. **1983**, *91*, 49.
21. Nedvidek, W.; Ledl, F.; Fischer, P. *Z. Lebensm.-Unters. Forsch*. **1992**, *194*, 222.
22. Zhu, J.; Dizin, E.; Hu, X.; Wavreille, A. S.; Park, J.; Pei, D. *Biochemistry* **2003**, *42*, 4717.
23. Gianotti, A. J.; Tower, P. A.; Sheley, J. H.; Conte, P. A.; Spiro, C.; Ferro, A. J.; Fitchen, J. H.; Riscoe, M. K. *J. Biol. Chem*. **1990**, *265*, 831.
24. Winter, R. W.; Cornell, K. A.; Johnson, L. L.; Riscoe, M. K. *Bioorg. Med. Chem. Lett*. **1993**, *3*, 2079.
25. Surette, M. G.; Bassler, B. L. *Proc. Natl. Acad. Sci. U.S.A*. **1998**, *95*, 7046.
26. Bassler, B. L.; Greenberg, E. P.; Stevens, A. M. *J. Bacteriol*. **1997**, *179*, 4043.