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Catalytic Mechanism of *S*-Ribosylhomocysteinase (LuxS): Direct Observation of Ketone Intermediates by ^{13}C NMR Spectroscopy

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Bacterial cell−cell communication, i.e., quorum sensing, is mediated through the production, release, and detection of small signaling molecules called autoinducers (AIs).[1] There are two types of AIs. The type 1 AIs (AI-1s) are species-specific and are acylhomoserine lactone and oligopeptide derivatives for Gram-negative and Gram-positive bacteria, respectively. The type 2 AI (AI-2) is a universal signal shared by all bacteria, presumably for interspecies communication.[2] AI-2 is biosynthesized from *S*-adenosylhomocysteine (SAH), which is hydrolyzed to adenine and *S*-ribosylhomocysteine (SRH) by the nucleosidase Pfs (Figure 1). Subsequently, SRH is converted into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DHPD) by *S*-ribosylhomocysteinase (LuxS).[3−5] DHPD spontaneously cyclizes and complexes with borate to form a furanosyl borate diester as the AI-2.[6]

The reaction catalyzed by LuxS is mechanistically intriguing; it is formally the nonredox cleavage of a stable thioether bond. LuxS contains a tetrahedrally coordinated, catalytically essential Fe^{2+} ion, which can be substituted by Co^{2+} without significant change in catalytic properties.[7] Deuterium was incorporated at the C1, C4, and C5 positions of DHPD when the LuxS reaction was carried out in D_2O.[7] On the basis of these data, we have proposed a metal-catalyzed carbonyl migration mechanism for the LuxS reaction (Figure 2).[7] SRH exists as an equilibrium between the aldehyde and hemiacetal forms. Coordination of the aldehyde carbonyl with the metal ion increases the acidity of the C2 proton, which is abstracted by a general base (possibly Cys-84 in *Bacillus subtilis* LuxS) to form an enolate. Isomerization of the enolate and protonation at C1 forms a 2-keto intermediate (**1**). Repetition of the above sequence shifts the keto group to the C3 position to give a 3-keto intermediate (**2**). Subsequent β-elimination releases homocysteine and DHPD. Here we provide direct evidence for the involvement of the ketone intermediates by taking advantage of the slow turnover rate of *B. subtilis* LuxS ($k_{cat} = 0.03\ s^{-1}$ at 23 °C) and monitoring reactions in real time by ^{13}C NMR spectroscopy. In addition, the 2-keto intermediate was chemically synthesized and demonstrated as a true intermediate on the catalytic pathway of LuxS.

To observe the 2-keto intermediate, Co^{2+}-substituted *B. subtilis* LuxS was rapidly mixed with 1 equiv of [2-^{13}C]SRH at 4 °C, and broadband-decoupled ^{13}C NMR spectra were collected every 4 min. The free substrate (no LuxS) showed two peaks at δ 76.2 and 71.6, reflecting the presence of two hemiacetal anomers (Figure 3a). Upon the addition of LuxS, three new peaks appeared immediately (∼4 min) at δ 213.3, 74.9, and 74.0. The intensity of the δ 74.9 and 74.0 peaks increased as the substrate was depleted and reached maximum when all other peaks disappeared. These two peaks were assigned as the two furanone products. The peak at δ 213.3 reached maximal intensity at 4 min, gradually decreased with time, and finally disappeared at ∼4 h when most of the substrate was depleted. Similar spectra were obtained with two LuxS mutants, E57D and E57A, although their reactions were much slower. When a ^{13}C NMR spectrum was recorded under off-resonance conditions, the

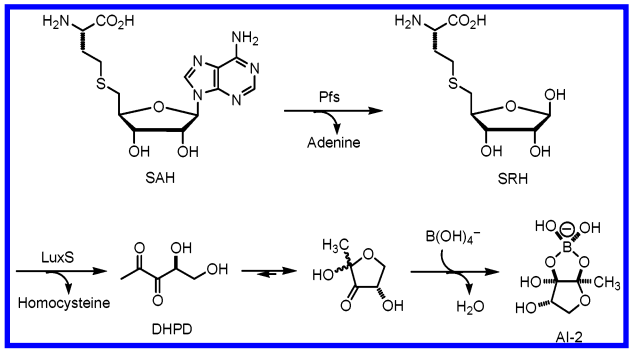


Figure 1. Biosynthetic pathway of AI-2.

δ 213.3 peak remained a singlet, while the other peaks were split into doublets. When the LuxS reaction was quenched with methanol and incubated with NH_2OH, the δ 213.3 peak disappeared, while two new peaks appeared at δ 160.9 and 161.2. These results indicate that the peak at δ 213.3 is the ^{13}C-labeled 2-keto intermediate **1**. The two peaks at δ ∼161 upon treatment with NH_2OH correspond to the cis and trans isomers of the oxime derivative. When the above experiment was repeated with a catalytically impaired C84D mutant, the slow accumulation of the δ 213.3 peak was observed (Figure 3b). Most importantly, the appearance of ketone **1** preceded the products, suggesting that the 2-keto species is a bona fide intermediate of the LuxS reaction. A control experiment conducted with an inactive mutant (C84A) resulted in no signal for either the intermediate or product.

The involvement of 3-keto intermediate **2** was investigated using [3-^{13}C]SRH as substrate. The free substrate has two peaks at δ 74.8 and 74.2 (Figure 3c). Upon incubation with E57A LuxS, a weak singlet appeared at δ 214.5 at 2 h, reached maximal intensity at ∼6 h, and decayed after 14 h. Following the appearance of the δ 214.5 peak, two new peaks emerged at δ 100.5 and 100.1 at 4−6 h and remained even after the δ 214.5 signal decayed. We assign the δ 214.5 peak as the 3-keto intermediate and the signals at δ 100.5 and 100.1 as the hydrated cyclic furanone products. Note that an intense peak appeared at δ 78.3 immediately after the reaction was initiated and remained throughout the experimental period (0−14 h). The kinetics of its formation and its chemical shift suggest that it is the 3-^{13}C-labeled ketone **1**. A very weak peak at δ 214.0 was also observed with wild-type LuxS when a spectrum was acquired over the entire 2.5-h reaction period. The 3-keto intermediate was not detected for C84D LuxS. In fact, the 3-keto intermediate was also observed as a singlet at δ 77.0 in the above experiments with [2-^{13}C]SRH as substrate and two of the less active mutants (E57D and E57A), but not with wild-type or C84D LuxS.

We noted that the peak widths for the keto intermediates (4−6 Hz) were unusually narrow for enzyme-bound species, especially when the paramagnetic Co^{2+} ion is expected to further broaden the bound ketone signals. We performed further experiments to test whether the keto intermediates were released from the LuxS

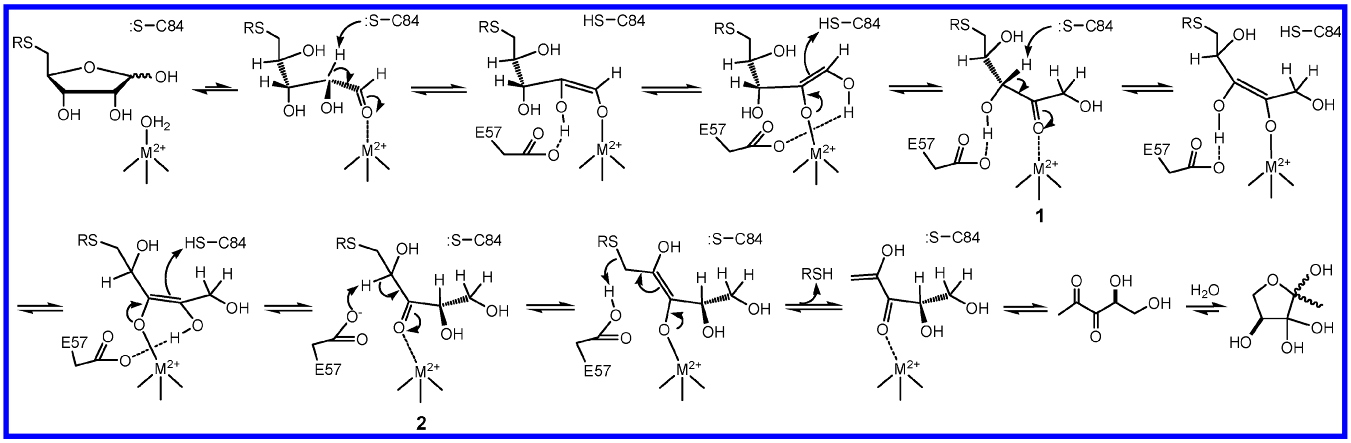


Figure 2. Proposed mechanism for LuxS-catalyzed reaction. RSH, L-homocysteine.

active site (see *Supporting Information for details)*. First, NMR experiments with different LuxS/SRH ratios (1:1−8 mol/mol) all gave the same relative intensity for ketone **1** and SRH signals (ketone **1**/SRH ∼1:6), indicating that the 2-keto intermediate and SRH are in rapid equilibrium (vide infra). Next, a pulse-chase experiment showed that addition of excess unlabeled SRH into the reaction of LuxS and [2-^{13}C]SRH can dramatically slow the decay of the ^{13}C-labeled 2-keto intermediate. Third, severe substrate inhibition was observed, especially for the less active mutants. Taken together, our data strongly argue that the keto intermediates are at least partially released into the solution during catalysis.

The intermediacy of ketone **1** was further assessed through its chemical synthesis and kinetic characterization. Ketone **1** is a 2-ketopentose linked to L-homocysteine through a thioether bond. We decided to prepare the corresponding alditol, couple it to homocysteine, and then oxidize the alditol to a ketose (Scheme 1). Synthesis of the alditol started with commercially available D-lyxose, which was converted into allyl D-lyxoside, followed by protection of the 2-, 3-, and 5-hydroxyl groups with the *p*-methoxybenzyl (PMB) group. The resulting glycoside **3** (as a mixture of α and *β* anomers) was treated with diisobutylaluminum hydride (DIBAL) in the presence of $NiCl_2$(dppp)[8] to give alditol **4**.[9] Note that oxidation of the 4-OH of alditol **4** gives a ketopentose of the same stereochemical configuration as that in intermediate **1**. Coupling of diol **4** and *N*-Boc-homocysteine *tert*-butyl ester (**5**) proved quite problematic. After numerous other attempts failed, we converted the diol **4** into cyclic sulfate diester **6** by reacting it with thionyl chloride followed by oxidation of the resulting sulfite with periodate.[10] Cyclization should make it more accessible and reactive to nucleophilic attack. Indeed, sulfate ester **6** readily reacted with homocysteine **5** to give the desired alcohol **7** in good yield and a small amount of alcohol **8**, due to nucleophilic attack at the secondary C4 position. Oxidation of the mixture of alcohols **7** and **8** by the method of Dess and Martin[11] gave ketone **9** and aldehyde **10**, respectively, which were readily separated on a silica gel column. Treatment of ketone **9** with trifluoroacetic acid removed all of the protecting groups to afford ketone **1** in 25% overall yield (10 steps).

Ketone **1** is stable for weeks in neutral aqueous solution at room temperature. The addition of LuxS resulted in rapid release of homocysteine as detected by a DTNB assay.[7] When the LuxS reaction was carried out in the presence of 1,2-phenylenediamine, a single quinoxaline derivative (compound **11** in Scheme 2) was isolated and was found to be identical to that from LuxS catalyzed cleavage of SRH, indicating that DHPD is the second reaction product. Thus, intermediate **1** is chemically competent.

To demonstrate the kinetic competence of intermediate **1**, the catalytic activities of various LuxS forms toward ketone **1** were

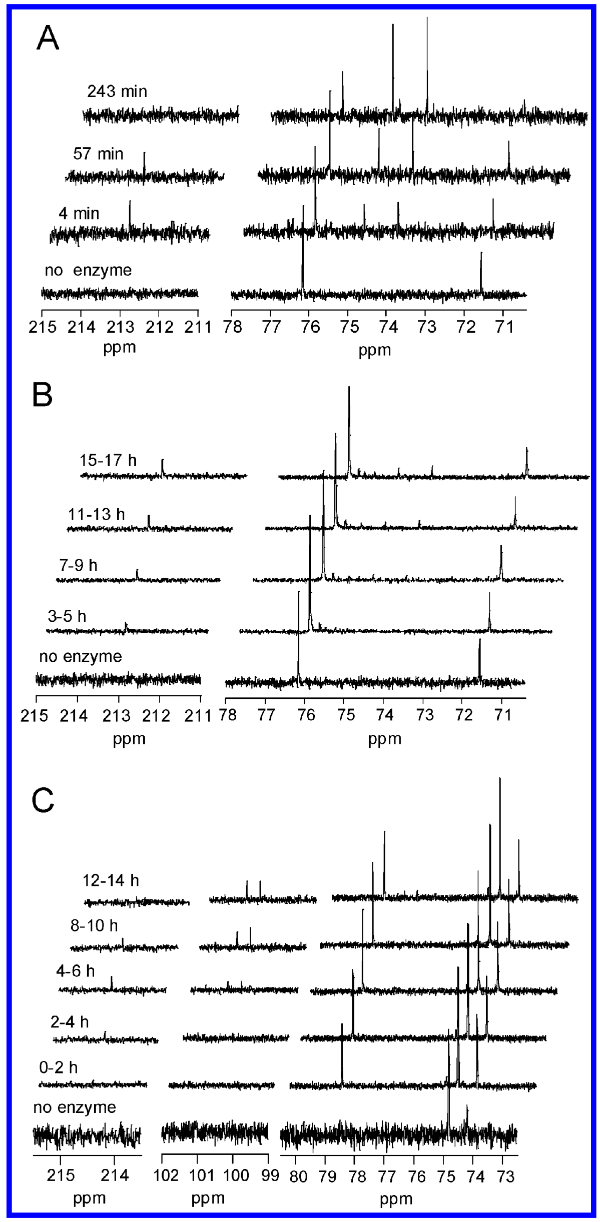


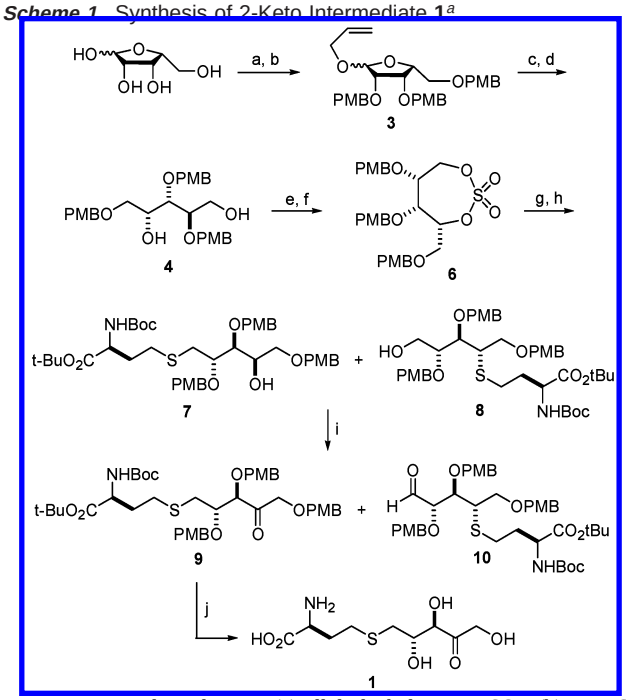
Figure 3. ^{13}C NMR spectra (150 MHz) of LuxS-catalyzed reactions. (A) [2-^{13}C]SRH (2.0 mM) and wild-type *B. subtilis* LuxS (2.0 mM) were mixed at 4 °C, and spectra were recorded every 4.1 min. (B) [2-^{13}C]SRH (2.8 mM) and C84D LuxS (2.8 mM) were mixed at 4 °C, and spectra were recorded every 2 h. (C) [3-^{13}C]SRH (1.8 mM) and E57A LuxS (1.4 mM) were mixed at 4 °C, and spectra were recorded every 2 h.

Table 1. Catalytic Properties of Co(II)-Substituted LuxS Variants toward SRH and 2-Ketone Intermediate **1**

	SRH			2-ketone intermediate **1**		
LuxS variant[a]	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
WT (*Bs*)	0.030 ± 0.005	1.4 ± 0.1	2.1×10^4	0.031 ± 0.004	1.5 ± 0.3	2.1×10^4
WT (*Vh*)	0.48 ± 0.01	39 ± 1	1.2×10^4	0.48 ± 0.02	38 ± 1	1.3×10^4
E57D (*Vh*)	ND	ND	148	0.010 ± 0.001	29 ± 2	3.4×10^2
C83D (*Vh*)	ND	ND	5	0.007 ± 0.001	50 ± 4	1.4×10^2
C83S (*Vh*)	ND	ND	18	0.030 ± 0.001	58 ± 3	5.2×10^2

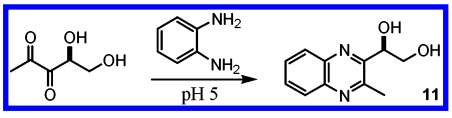
[a] *Bs*, *B. subtilis* LuxS; *Vh*, *V. harveyi* LuxS; WT, wild-type; ND, not determined.

Scheme 1. Synthesis of 2-Keto Intermediate **1**[a]



[a] Reagents and conditions: (a) allyl alcohol, cat. H_2SO_4; (b) PMBCl, Bu_4NBr, 50% aq KOH/THF (1/1), 90 °C, 76% for 2 steps; (c) DIBAL, cat $NiCl_2$(dppp), THF; (d) $NaBH_4$, EtOH, 73% for 2 steps; (e) $SOCl_2$, TEA, CH_2Cl_2, −78 °C; (f) $NaIO_4$, cat. $RuCl_3$, $CCl_4/CH_3CN/H_2O$ (4/4/6), 0 °C, 86% for 2 steps; (g) BocNH−CH(CH_2CH_2SH)CO_2-*t*-Bu (**5**), BuLi, DMF, 0 °C; (h) H_2SO_4/H_2O/THF, rt, 74% for 2 steps, **7**:**8** = 4:1; (i) Dess−Martin, CH_2Cl_2, rt, 76%; (j) TFA/anisole (5:1), rt, 93%.

Scheme 2



determined and compared with those of SRH (Table 1). Wild-type LuxS from either *B. subtilis* or *Vibrio harveyi* exhibited similar activity toward the keto intermediate and SRH. This is consistent with the notion that formation of intermediate **1** is a rapid step in WT LuxS (vide infra). However, three catalytically compromised *V. harveyi* LuxS mutants (E57D, C83D, and C83S) all had much higher activities (2−29-fold) toward the keto intermediate than SRH (Table 1). For example, the C83D LuxS has a k_{cat} value of 0.007 s^{-1} and a k_{cat}/K_M value of 140 M^{-1} s^{-1} toward the keto intermediate, whereas its activity toward SRH was barely detectable by the DTNB assay ($k_{cat}/K_M = 5$ M^{-1} s^{-1}). These results indicate that the 2-keto intermediate is kinetically competent. They also suggest that formation of the 2-keto intermediate is at least partially rate limiting in the mutants.

The above results provide additional insight into the LuxS mechanism. Accumulation of intermediate **1** indicates that its formation is fast relative to its decay. The lack of significant accumulation of intermediate **2** for WT LuxS suggests that it decays faster than its formation. Thus, the rate-limiting step is the shift of the carbonyl group from C2 to C3 (**1** to **2**). The C84D mutation slows down the formation of ketone **1** and causes the disappearance of ketone **2**. The C84A mutant is catalytically inactive and does not form the 2-keto intermediate. These results suggest that Cys-84 (Cys-83 in *V. harveyi* LuxS) is involved in the early steps of the reaction, likely responsible for the proton transfer at C1−C3 positions (Figure 2). Increased accumulation of the 3-keto intermediate upon the E57A mutation suggests that Glu-57 plays a critical role in its decay, presumably acting as the general base for the final β-elimination reaction. Mutation of Glu-57 also slowed the formation of the 2-keto intermediate (Table 1), as evidenced by its peak intensity reaching maximum at ∼4 min for WT LuxS (Figure 3a) but at 4−6 h for the E57A mutant (Figure 3c). We propose that Glu-57 is also involved in the early steps of the mechanism, by acting as a proton shuttle to facilitate the isomerization of the enolate intermediates (Figure 2). Structural studies show that both Glu-57 and Cys-84 are properly positioned in the active site to perform the proposed functions.[12]

In summary, we have obtained direct evidence for the involvement of 2- and 3-ketone intermediates in the LuxS-catalyzed reaction, validating the catalytic mechanism previously proposed. The keto intermediates are at least partially released from the enzyme active site during catalysis, providing a rare example of one active site catalyzing three distinct chemical reactions. The data also suggest Cys-84 and Glu-57 as the possible general acids/bases in the proposed catalytic mechanism.

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Supporting Information Available: Experimental details and additional NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Miller, M. B.; Bassler, B. L. *Annu. Rev. Microbiol.* **2001**, *55*, 165−199.
(2) Bassler, B. L.; Wright, M.; Showalter, R. E.; Silverman, M. R. *Mol. Microbiol.* **1993**, *9*, 773−786.
(3) Miller, C. H.; Duerre, J. A. *J. Biol. Chem.* **1968**, *243*, 92−97.
(4) Surette, M. G.; Miller, M. B.; Bassler, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1639−1644.
(5) Schauder, S.; Shokat, K.; Surette, M. G.; Bassler, B. L. *Mol. Microbiol.* **2001**, *41*, 463−476.
(6) Chen, X.; Schauder, S.; Potier, N.; Van Dorsselaer, A.; Pelczer, I.; Bassler, B. L.; Hughson, F. M. *Nature* **2002**, *415*, 545−549.
(7) Zhu, J.; Dizin, E.; Hu, X.; Wavreille, A.; Park, J.; Pei, D. *Biochemistry* **2003**, *42*, 4717−4726.
(8) Taniguchi, T.; Ogasawara, K. *Angew. Chem., Int. Ed.* **1998**, *37*, 1136−1137.
(9) Reduction by DIBAL gave alditol **3** plus a small amount of hemiacetal, which was converted into **3** by reduction with $NaBH_4$.
(10) Byun, H.-S.; He, L.; Bittman, R. *Tetrahedron* **2000**, *56*, 7051−7091.
(11) Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4155−4158.
(12) Lewis, H. A.; Furlong, E. B.; Laubert, B.; Eroshkina, G. A.; Batiyenko, Y.; Adams, J. M.; Bergseid, M. G.; Marsh, C. D.; Peat, T. S.; Sanderson, W. E.; Sauder, J. M.; Buchanan, S. G. *Structure* **2001**, *9*, 527−537. (b) Hilgers, M. T.; Ludwig, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11169−11174. (c) Ruzheinikov, S. N.; Das, S. K.; Sedelnikova, S. E.; Hartley, A.; Foster, S. J.; Horsburgh, M. J.; Cox, A. G.; McCleod, C. W.; Mekhalfia, A.; Balckburn, G. M.; Rice, D. W.; Baker, P. J. *J. Mol. Biol.* **2001**, *313*, 111−122.

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