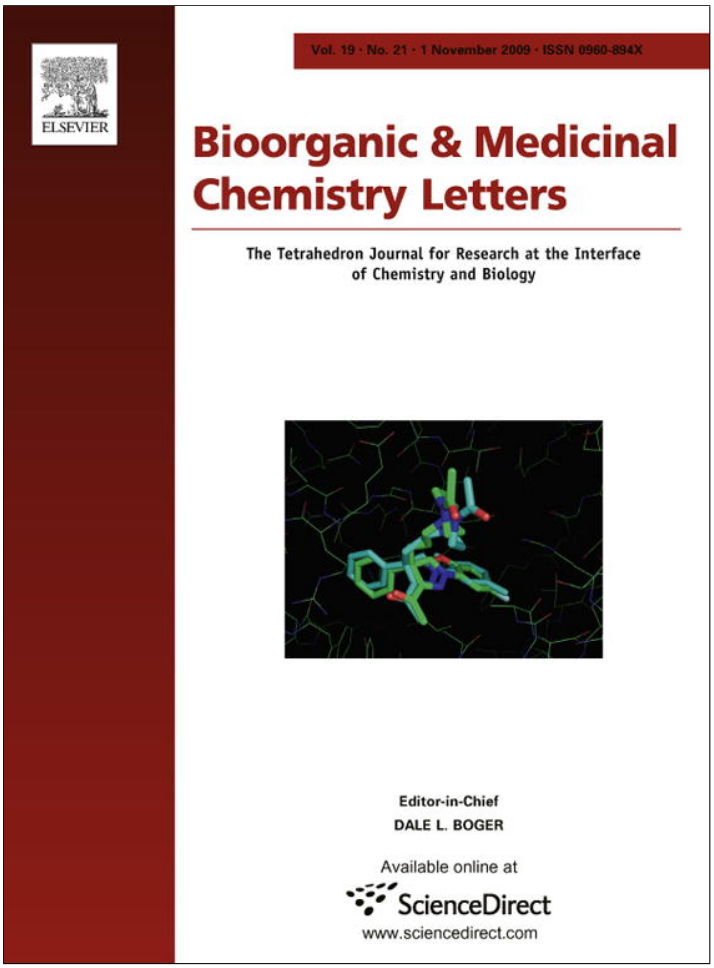

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A naturally occurring brominated furanone covalently modifies and inactivates LuxS

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ABSTRACT

Halogenated furanones, a group of natural products initially isolated from marine red algae, are known to inhibit bacterial biofilm formation, swarming, and quorum sensing. However, their molecular targets and the precise mode of action remain elusive. Herein, we show that a naturally occurring brominated furanone covalently modifies and inactivates LuxS (*S*-ribosylhomocysteine lyase, EC 4.4.1.21), the enzyme which produces autoinducer-2 (AI-2).

Antibacterial activities such as inhibition of biofilm formation and swarming have been reported for halogenated furanones, a class of natural products isolated from the marine red algae *Delisea pulchra*.[1] These compounds and their derivatives have served as lead compounds for the development of broad spectrum antibacterial agents.[2] This is particularly the case against biofilm formation, a major clinical problem that currently has no effective treatment.[3] Studies have shown that compounds **1–4** depicted in Figure 1 promote the clearance of *Pseudomonas aeruginosa* in mouse pulmonary infection models[4] and furanone **1** has been further demonstrated to inhibit *Bacillus anthracis* growth and expression of its virulence genes.[5] The effects of these compounds on several biological pathways have been investigated but the molecular targets and the precise modes of action remain elusive.[6] One attractive target is bacterial quorum sensing, the process by which bacteria sense and respond to population density, and consequently behave as a coordinated community. Quorum sensing involves the synthesis, secretion, and detection of signaling molecules, commonly referred to as autoinducers (AI's).[7] Typically, a particular autoinducer is recognized only by the species producing the molecule and hence, mediates intra-species communication. On the other hand, autoinducer-2 (AI-2) has been shown to mediate inter-species quorum sensing among many bacteria.[8] Previous work from our laboratory and others suggested that halogenated furanones disrupt the AI-2 biosynthetic pathway.[9] We present herein biochemical and chemical evidence that halogenated furanones covalently modify and inactivate the AI-2 producing enzyme LuxS.

The AI-2 biosynthetic pathway is found in roughly half of all bacterial species, both Gram-positive and Gram-negative.[10] Biosynthesis of AI-2 begins with *S*-adenosyl-homocysteine (AdoHcy), the product of *S*-adenosyl-methionine dependent transmethylation. AdoHcy is hydrolyzed to *S*-ribosyl-homocysteine (SRH) and adenine by *S*-adenosyl-homocysteine nucleosidase (EC 3.2.2.16). LuxS (*S*-ribosylhomocysteine lyase, EC 4.4.1.21) then cleaves SRH to yield L-homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) which cyclizes to form various forms of AI-2,[11] one of which is a borate diester shown in Scheme 1.[12]

Four brominated furanones were synthesized following literature procedures and tested for inhibition against LuxS.[1a,9]

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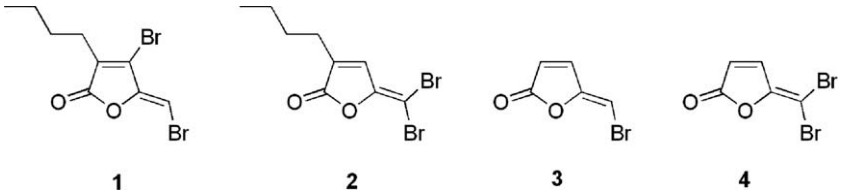
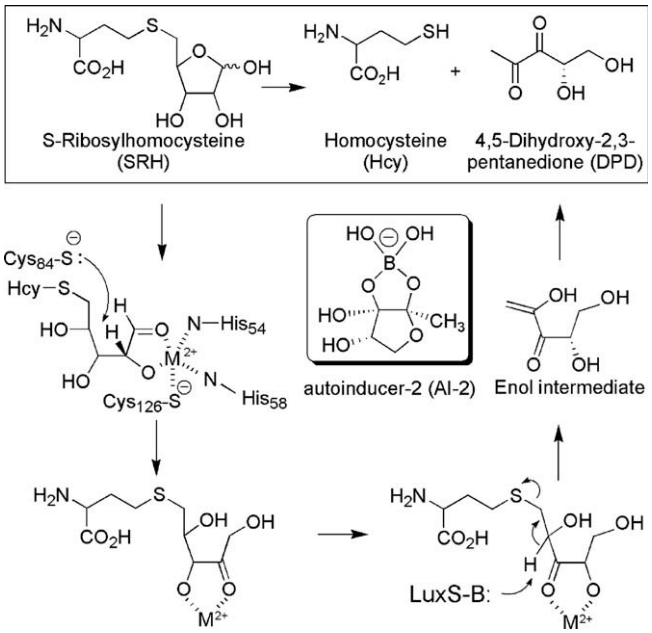


Figure 1. Structures of brominated furanones.



Scheme 1. Proposed mechanism of the LuxS enzyme and roles of Cys84 and Cys126.

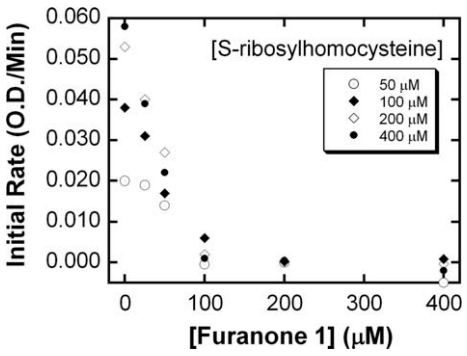


Figure 2. Effect of furanone **1** concentration on the initial rates of the LuxS reaction.

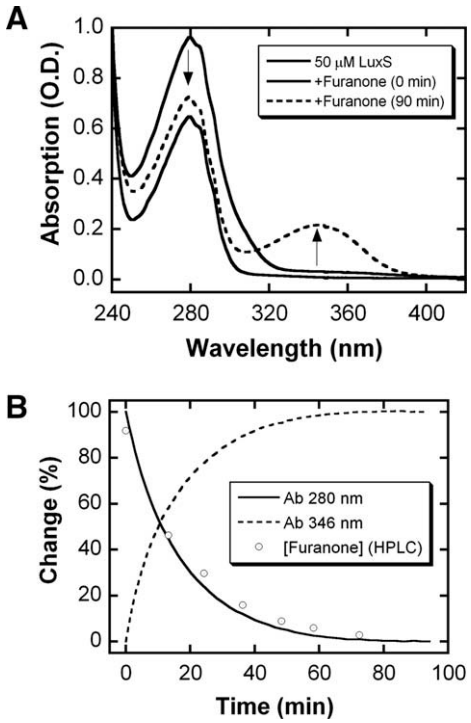
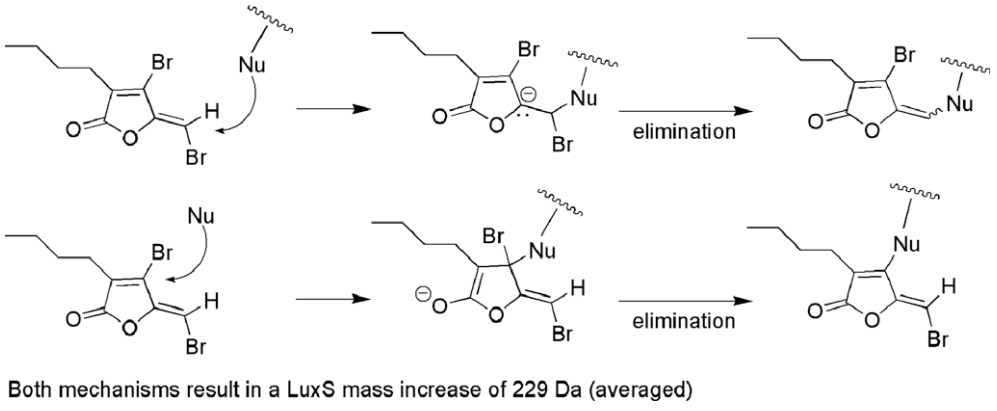


Figure 3. (A) UV/vis absorbance changes associated with the addition of furanone **1** to LuxS. (B) Time-course of absorbance changes at 346 nm (broken line) and 280 nm (solid line), and the disappearance of furanone as monitored by HPLC (circles).

Recombinant LuxS was prepared as previously described and enzyme activity was assayed after preincubation of the enzyme with furanone at a range of concentrations for 10 min (see Supplementary data for details).[11b,13] As shown in Figure 2, furanone **1** inhibits the LuxS enzyme in a concentration-dependent manner. Comparable inhibition was observed for furanone **3** (see Supplementary data). In contrast, furanone **2** and **4** displayed much weaker inhibition (see Supplementary data). The difference in inhibitory activity



Scheme 2. Proposed mechanisms for LuxS modification by brominated furanone **1**.

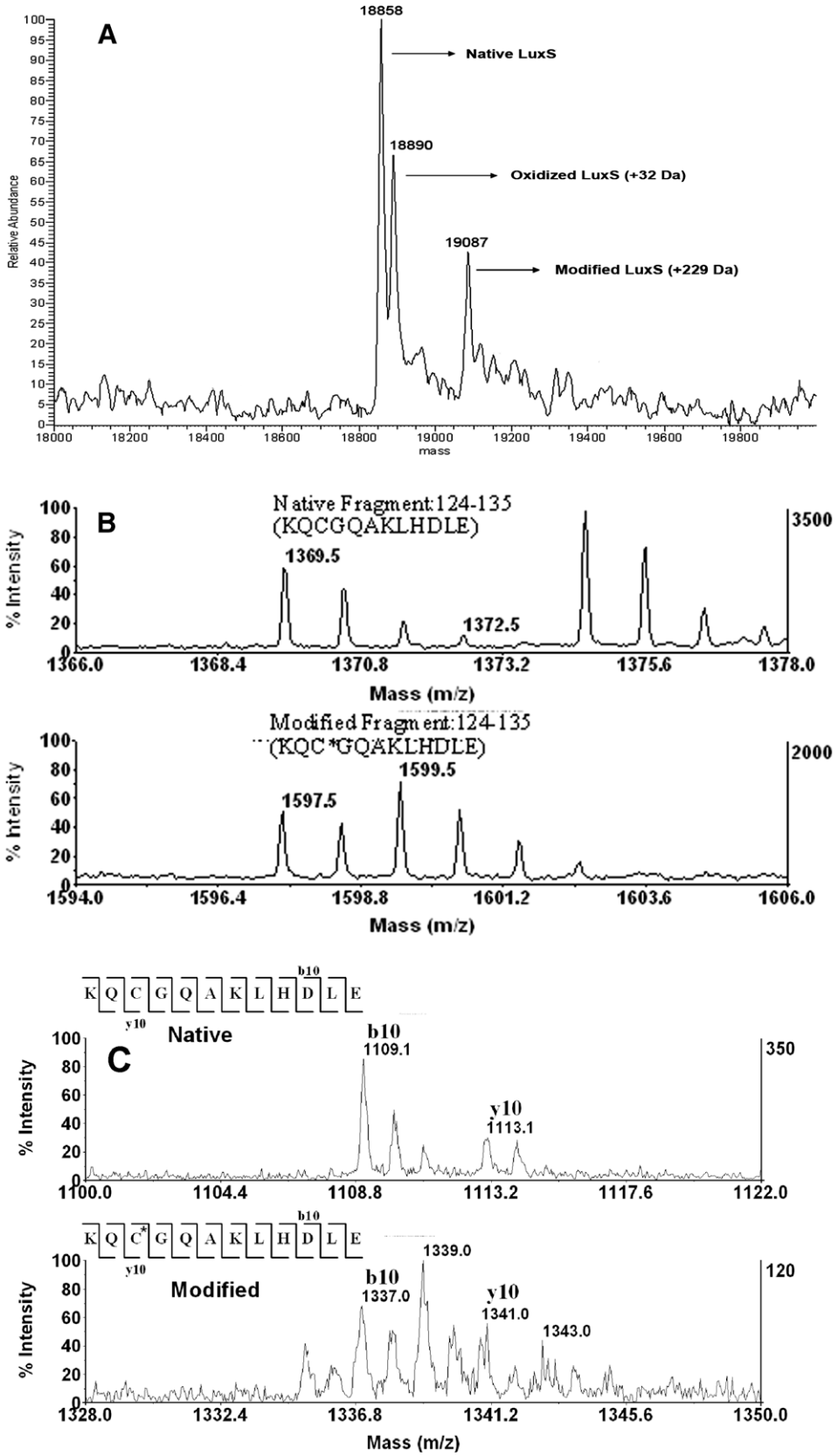


Figure 4. (A) Mass spectrum of LuxS modified by furanone **1**. (B) Mass spectra of native peptide fragment 124–135 (top) and modified fragment 124–135 (bottom) after Glu-C digestion. (C) MS/MS of native residue fragment 124–135 (top) and modified (bottom). C* indicates the site of modification by furanone **1**.

may be attributed to the difference in their structures, as both furanones **1** and **3** contain a vinyl monobromide, whereas compounds **2** and **4** are substituted with a vinyl gem-dibromide (see Fig. 1).

Detailed mechanistic studies were performed with furanone **1**, which displayed the highest specific residual activity. It appears that relative enzyme activities after preincubation of furanone

was not affected by varying SRH concentrations (data not shown), suggesting that furanone **1** covalently modifies the LuxS enzyme during preincubation. Indeed, time-dependent modification of LuxS in the presence of this furanone was observed (Fig. 3). Spectroscopic analysis of the reaction of LuxS and furanone **1** reveals the formation of a new chromophore with maximal absorbance at 346 nm (Fig. 3A). The formation of this new species correlates to the disappearance of the furanone, which has an absorbance maximum at 280 nm, as monitored by both HPLC analysis and UV-measurements (Fig. 3B). Furthermore, the absorption peak at 346 nm persisted with LuxS after the removal of excess furanone by dialysis or size-exclusion chromatography (see Supplementary data). Similar spectral changes have been observed when diethylamine is reacted with halogenated furanones to form enamines in ethereal solutions.[14] Taken together, these data suggest that LuxS undergoes covalent modification by furanone **1**.

Based on these studies, an addition–elimination mechanism for the covalent modification of LuxS by furanone **1** is proposed (Scheme 2). First, a nucleophile in LuxS adds directly to the vinyl bromide to give an intermediate which subsequently collapses to eliminate the bromide. Both the exocyclic and the ring vinyl bromine may be displaced and our data cannot rule out either of these pathways. On the other hand, the aforementioned model study also indicated that the exocyclic bromide, rather than the ring vinyl bromide, was replaced with amines, suggesting that the former is more reactive. Certainly, the LuxS enzyme may alter the intrinsic chemical reactivities of the two bromides or confer steric effects. Detailed structural characterization of the complex formed between LuxS and furanone **1** may ultimately distinguish these two pathways.

It is also worth noting that the exocyclic vinyl bromide appears to be essential for biological activities.[1,2,15] All known naturally occurring brominated furanones display either an exocyclic vinyl monobromide or dibromide moiety, but do not require the ring vinyl bromide for activity. The exocyclic vinyl bromide groups were also determined as essential structural elements for inhibition of *Escherichia coli* biofilm formation by synthetic analogs of brominated furanones.[2,15b] Nonetheless, since halogenated furanones may also target proteins other than LuxS, the above mentioned biological effects may or may not directly correlate to LuxS modification.

To lend additional support to the proposed mechanism and to identify the site or sites of modification in LuxS by furanone **1**, the protein-inhibitor complex was analyzed by mass spectrometry. Illustrated in Figure 4A, the mass spectrum of the LuxS protein treated with furanone **1** shows peaks corresponding to the native LuxS (18,858, theoretical 18,861), its oxidized form (18,890, +32 Da), and a new LuxS adduct (19,087, +229 Da). The 229 Da mass increase is consistent with the addition of one molecule of furanone **1** followed by the loss of one bromide (expected average mass 229 Da) and further supports the direct addition/elimination mechanism outlined in Scheme 2.

The modified LuxS was subsequently digested by Glu-C endoprotease and trypsin, and the peptide fragments were analyzed by MALDI-MS. The modification site was localized to Cys126 in the 124–135 peptide fragment. Conclusively, the mass shifts of 228 and 230 Da (1597.5 and 1599.5 vs 1369.5 for the unmodified peptide) and the distinct isotopic signature shown in Figure 4B and C are consistent with the addition of one molecule of furanone **1** minus one bromide. As indicated by the crystal structure of LuxS, Cys126 coordinates an active site divalent metal ion, which is thought to catalyze the aldose–ketose isomerization steps depicted in Scheme 1.[13,16] Alkylation of Cys126 may perturb proper metal binding and/or block substrate binding, resulting in the reduction of enzymatic activity. This reminisces the selective alkylation of a cysteine ligand to the catalytic zinc ion in methionine synthase (MetE) and other metalloenzymes.[17]

In addition, we find that furanone **1** modifies LuxS in a highly sequence selective manner. As shown in Figure 3A, only one adduct appeared after treatment of LuxS with furanone. In contrast, when LuxS was treated with chloroacetone, addition of four ketone moieties was observed (see Supplementary data), likely resulting from the alkylation of all four cysteine residues in LuxS. In addition, Cys84, a catalytic acid/base in the active site of LuxS, has a pK_a value around 6 and thus exists predominantly in the thiolate form;[18] and is also more solvent accessible than Cys126 (see Supplementary data). Yet, no modification of Cys84 was detected.

In summary, the biochemical and chemical studies reported herein identify a molecular target for halogenated furanones and suggest an inhibition mechanism. Since the LuxS enzyme and the AI-2 pathway are widespread in bacteria and absent in humans, our results open new avenues for the development of antibacterial agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.095.

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