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Regulation of the *Escherichia coli* AtoSC two component system by synthetic biologically active 5;7;8-trimethyl-1;4-benzoxazine analogues

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ABSTRACT

The *Escherichia coli* AtoSC two component system;upon acetoacetate induction;regulates the expression of the *atoDAEB* operon;through His → Asp phopshotransfer;thus modulating important cellular processes. In this report the effect of seven 5,7,8-trimethyl-1,4-benzoxazine derivatives on the regulation of the *E. coli* AtoSC system was studied. The new compounds were tested for their effectiveness on the expression of the *atoC* and the regulated *atoDAEB* operon. The non-substituted 5,7,8-trimethyl-1,4-benzoxazine (**4a**), was the most potent inducer on *atoC* transcription;resulting in accumulation of AtoC protein. The induction of *atoC* by **4a** was specific;since no effect was observed on the other genes of the system (*atoS* and *atoDAEB*). Moreover;compound **4a** was shown to significantly up-regulate the in vitro kinase activity of the histidine kinase AtoS without altering the protein levels in the cell. Interestingly;this compound appeared to modulate the acetoacetate-mediated induction of the *atoDAEB* promoter by the AtoSC system. These data provide the first evidence for a differential modulator role of 5,7,8-trimethyl-1,4-benzoxazine;on the AtoSC two component system mediated signaling.

1. Introduction

AtoSC is a well characterized two component system (TCS) in *Escherichia coli*, which relies on transmembrane sensor AtoS histidine kinase (HK) autophosphorylation and subsequent AtoC response regulator (RR) phosphorylation. AtoS was reported to be *in trans*-phosphorylated between the monomers of a homodimer in a conserved H-box containing the phosphorylation site His-398 residue.[1] His-398 is lying in proximity to the AtoC Asp-55 residue upon AtoSC interaction for the subsequent phosphotransfer and AtoC phosphorylation necessary for AtoSC regulatory actions,[2] the transcriptional regulation of the *atoDAEB* operon that encode for proteins involved in short-chain fatty acid metabolism.[2–5] By governing the *atoDAEB* operon;this system regulates;amongst others;the biosynthesis and the intracellular distribution of cPHB,[6,7] the flagella synthesis;[8] the chemotactic behaviour and the involvement of extracellular Ca^{2+} on cPHB synthesis regulated by the AtoSC system.[9]

We have recently reported the regulation of the AtoSC system by polyamines and histamine, as cellular components that enable pathogenic bacteria to survive and overcome host defence mechanisms.[10–12] Putrescine, the non-natural diamine 1,3-diaminopropane and their synthetic analogues elicited a pronounced *atoC* transcriptional activation, without activating neither the *atoDAEB* operon promoter nor *atoS*.[11] Histamine, a biogenic amine, elicited a polyamine-mediated inductive phenotype and induced *atoC* expression, without activating the *atoDAEB* operon promoter.[12] Furthermore, the involvement of TCSs including AtoSC, in specialized functions including the bacterial pathogenicity, virulence and host-microbe interactions[3,4] warrant the investigation of a number of synthetic amine-containing compounds, including 1,4-benzoxazines, on their possible involvement on the signaling cascade.

The 2*H*-1,4-benzoxazine-3-(4*H*)-one and 3,4-dihydro-2*H*-1,4-benzoxazine systems have been studied extensively for building natural and designed biologically active compounds, which span from herbicides, fungicides, cardiovascular agents, K_{ATP} channel openers, compounds against diabetes, neuroprotectants and agents against anxiety and depression.[13–17] Thus, the above mentioned heterocycles can be considered as privileged scaffolds for the development of potential new drugs. We recently reported, the synthesis of 5,7,8-trimethyl-1,4-benzoxazine derivatives encompassing the pharmacophore aminoamide functionality of lidocaine and their biological evaluation as novel antiarrhythmics against ischemia-reperfusion injury.[18] Furthermore, the 5,7,8-trimethyl-1,4-benzoxazine moiety can be considered as a bioisostere of the 5,7,8-trimethyl-1,4-benzopyran nucleus of the well known antioxidant vitamin E.

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As a continuation of our studies on the effect of amines on the AtoSC system and its implication in important biological pathways;we investigated the effect of a series of 5,7,8-trimethyl-1,4-benzoxazine derivatives (Fig. 1) on the regulation of the *E. coli* AtoSC system. We investigated their effect on: (i) the transcription of *atoSC* system genes, (ii) the modulation of AtoS and AtoC endogenous protein levels, (iii) the alteration of activity of AtoS HK, (iv) the dimerization/oligomerization ability of its cytoplasmic AtoS form and (v) the effect on the acetoacetate-mediated *atoDAEB* operon induction.

In order to obtain initial information on structure–activity relationships we chose to include in our study the unsubstituted 5,7,8-trimethyl-1,4-benzoxazine (**4a**) as well as 2-, 3-, and 6-substituted derivatives. More specifically, we introduced a 4-methoxyphenyl substituent at positions C2, C3 and C6, compounds **4b**, **8**, and **13**, respectively. In addition, we incorporated a halogen substituent at C6, compounds **6a, b** as well as a phenyl group at C2 and a chloro-substituent at C6, compound **4c**. Compounds **4b, c**, and **8** were obtained as racemic mixtures and were studied as such.

2. Results and discussion

2.1. Chemistry

The synthesis of the compounds of the present study **4a, b, 6a**, **b**, **8**, and **13** is depicted in Schemes 1–4. Compounds **4a**, **4c**, and **6a** were prepared as previously reported.[18]

More specifically, 2,3,5-trimethyl-6-nitro-phenol (**1**)[18] was alkylated with the appropriate α-bromoester in the presence of Cs_2CO_3 and a catalytic amount of TBAI to give ethers **2a, b**. Hydrogenation of **2a** gave the corresponding aniline which spontaneously cyclized to benzoxazinone **3a**. Reduction of the nitro group in compound **2b** was effected using CuCl/$NaBH_4$ in ethanol[19] to give benzoxazinone **3b** after spontaneous cyclization. Subsequent reduction of benzoxazinones **3a, b** with $BH_3.SMe_2$ in THF afforded the desired compounds **4a, b** (Scheme 1). Treatment of benzoxazinone **3a** with a mixture of acetic acid/hydrogen peroxide/hydrogen chloride in petroleum ether afforded the 6-chloro-derivative **5a**, while bromination with a mixture of acetic acid/hydrogen peroxide/hydrogen bromide in petroleum ether afforded the 6-bromo-benzoxazinone **5b**.[20] Reduction of compound **5a** with $BF_3.Et_2O$ and $NaBH_4$ in THF, at 0–5 °C afforded the desired 6-chloro-benzoxazine **6a**, while reduction with $BH_3.SMe_2$ in THF at rt afforded 6-bromo benzoxazine **6b** (Scheme 2). The synthesis of the 3-aryl-substituted benzoxazine derivative **8** was effected through alkylation of 2,3,5-trimethyl-6-nitrophenol (**1**) with 2-bromo-4′-methoxyacetophenone in the presence of Cs_2CO_3 and TBAI in DMF to afford compound **7**, followed by reduction of the nitro group with CuCl, $NaBH_4$ and cyclization to the corresponding imine which was reduced further in situ to afford compound **8** (Scheme 3).

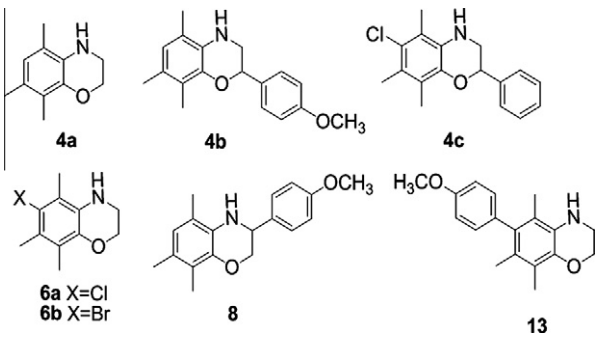


Figure 1. Structures of 5,7,8-trimethyl-1,4-benzoxazine derivatives.

Bromination of 2,3,5-trimethyl-6-nitrophenol with a mixture of acetic acid/hydrogen peroxide/hydrogen bromide in petroleum ether afforded **9**, which upon cross-coupling reaction with 4-methoxyphenyl boronic acid in the presence of $Pd(PPh_3)_4$ in DMF at 100 °C gave 6-(4-methoxyphenyl)-2,3,5-trimethylnitrophenol (**10**), which was alkylated with ethyl-2-bromoacetate in the presense of Cs_2CO_3 and a catalytic amount of TBAI to give ether **11**, which was hydrogenated to the corresponding aniline, which spontaneously cyclized to benzoxazinone **12**, which upon subsequent reduction with $BH_3.SMe_2$ in THF afforded the desired 6-aryl-benzoxazine **13** (Scheme 4). Compound **4c** was synthesized as previously reported.[18]

2.2. Effect of compounds 4a–c, 6a, b, 8, and 13 on the transcription of the atoC and atoDAEB operon genes

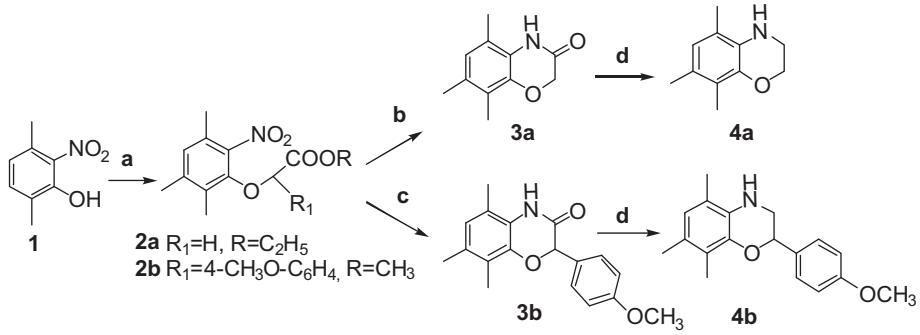
Compounds **4a–c, 6a**, **b**, **8**, and **13** were initially tested for their effect on the growth of *E. coli* strain BW25113 (*atoSC*$^+$). Following a longer lag period in the presence of the compounds, the bacteria displayed similar doubling times during the logarithmic phase irrespective of the compound used (data not shown).

Subsequently compounds **4a–c, 6a**, **b**, **8**, and **13** were tested for their effect on *atoC* gene and the *atoDAEB* operon transcription. The ability of the previously synthesized constructs[11,21] carrying *lacZ* fused to either of the promoters of the *atoSC* system (i.e., the *atoC* and the *atoDAEB* promoter) to respond to the compounds of the present study was evaluated in *E. coli* strain BW25113 (Fig. 2). The compounds were added in the growth medium at a final concentration of 1 mM. To facilitate comparisons between different sets of data, the β-galactosidase activity measured in the wild-type strain BW25113 growing in the absence of the compounds, was arbitrarily defined as 100%. The new compounds, demonstrated a differential effect on gene expression and more specifically, compounds **4a–c** and **13** activated the transcription of the *atoC,* while analogues **6a** and **8** reduced it. Compounds **6b** and **13** did not exhibit any significant effect on *atoC* or *atoDAEB,* respectively (Fig. 3a and b). Compounds **4a**, **6a**, and **6b** induced a repression of *atoDAEB*, while compounds **4b**, **4c**, and **8** stimulated this promoter.

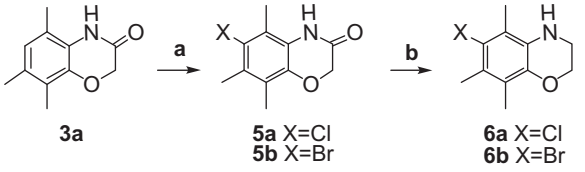
These data may provide an indication on the effect of the substitution pattern on the 5,7,8-trimethyl-1,4-benzoxazine scaffold on activity on both promoters. More specifically, the presence of a 4-methoxyphenyl substituent at position C2 or C6 (compounds **4b** and **13**, respectively) results in *atoC* activation which is also the case for the C2 and C6 disubstituted derivative **4c**. The presence of a halogen group at C6 exerts opposite effects with the chloro derivative **6a** to repress *atoC* while, the bromo analogue **6b** to induce slight activation. Finally, the presence of a 4-methoxyphenyl substituent at C3 (compound **8**) induces a repression of *atoC* similar to compound **6a**. Interestingly, the non-substituted derivative **4a** results in pronounced stimulation of *atoC*.

Concerning the *atoDAEB* promoter the effects were not as drastic. The presence of a halogen substituent at C6 (compounds **6a** and **b**) induced repression of the *atoDAEB*, while the presence of a 4-methoxyphenyl group at C2 or C3 or C6 (compounds **4b**, **8**, and **13**, respectively) resulted in stimulation. Interestingly, compounds **4b**, **4c**, **6a**, and **13** activate the transcription of both promoters, while, compounds **4a**, **6b**, and **8** activate *atoC* and repress *atoDAEB*.

Overall, the most potent transcriptional activator of the *atoC* gene was the non-substituted derivative **4a** (Fig. 3a). The specificity of this effect was demonstrated through the lack of activation (with a slight repression) of the *atoDAEB* operon promoter (Fig. 3b). In the cases where both *atoC* and *atoDAEB* were activated by the compounds (i.e., **4b** and **c**), the activation of *atoDAEB* could not be attributed to the enhanced intracellular accumulation of AtoC, the product of *atoC*, but rather to their direct effects on



Scheme 1. Reagents and conditions: (a) $R_1CH(Br)COOR$ (R = Me or Et), Cs_2CO_3, TBAI, DMF, rt; (b) H_2, Pd/C, EtOH, 70 °C; (c) CuCl, $NaBH_4$, EtOH, 85 °C; (d) $BH_3{\cdot}SMe_2$, THF, rt.



Scheme 2. Reagents and conditions: (a) CH_3COOH-H_2O_2-HCl or HBr, petroleum ether, 60 °C; (b) $BF_3{\cdot}Et_2O$, $NaBH_4$, THF, 0–5 °C or $BH_3.SMe_2$, THF, rt.

atoDAEB transcription. Accordingly, this is a possible explanation of the fact that the potent activation of the *atoC* by **4a** does not enhance but rather slightly repress the *atoDAEB* operon expression (Fig. 3).

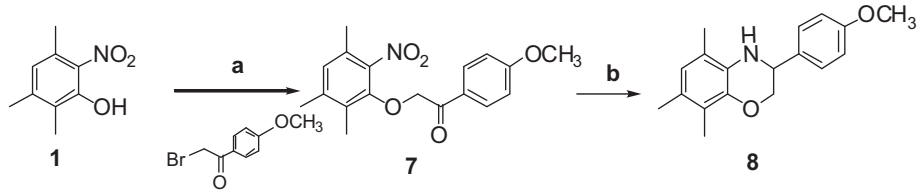
In an effort to investigate the concentration-depended activation of the *atoC* by **4a**, we repeated the same experiment in cells of *E. coli* BW25113 carrying plasmid pCPG6 (*atoC-lacZ*) in the presence of increasing concentrations of compound **4a** (Fig. 4). The results of the β-galactosidase assay confirmed that **4a** caused activation of transcription of the *atoC* gene in a concentration-dependent manner (Fig. 4).

2.3. Effect of compound 4a on atoSC two component system genes transcription

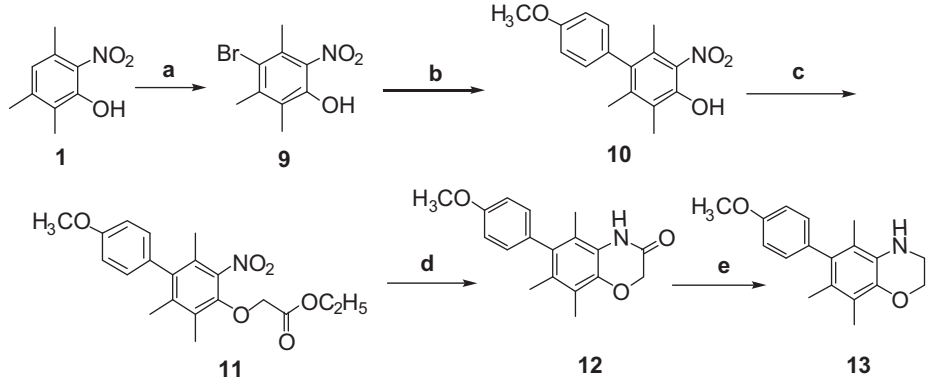
The next issue we addressed was whether compound **4a** affects the transcription of other genes in a similar manner. Thus, we investigated genes that share a topological and/or functional relevance with *atoC*, that is, the neighboring *atoS* gene, encoding the AtoS kinase and the regulated *atoDAEB* operon.

Therefore, the same experiment was performed using *E. coli* BW25113/pCPG6 (*atoC-lacZ*), BW25113/pCPG4 (*atoS-lacZ*) and BW25113/pCPG5 (*atoA-lacZ*) cells grown in the presence of compound **4a** at 1 and 2.5 mM concentration, respectively. Taking into consideration the ability of the construct pCPG6 (*atoC-lacZ*) to respond to the natural precursor, putrescine, for *atoC* activation in *E. coli* strain BW25113,[11] putrescine was used as a positive control of induction in this experiment (data not shown).

As shown in Figure 5 compound **4a** activated in a concentration-dependent manner the *atoC* gene, while neither of the other



Scheme 3. Reagents and conditions: (a) Cs_2CO_3, TBAI, DMF, rt; (b) CuCl, $NaBH_4$, EtOH, 85 °C.



Scheme 4. Reagents and conditions: (a) CH_3COOH-H_2O_2-HBr;petroleum ether, rt; (b) 4-$OCH_3C_6H_5B(OH)_2$, Cs_2CO_3, $Pd(PPh_3)_4$, DMF, 100 °C; (c) $BrCH_2COOC_2H_5$, Cs_2CO_3, TBAI, DMF, rt; (d) H_2, Pd/C, EtOH, 70 °C; (e) $BH_3{\cdot}SMe_2$, THF.

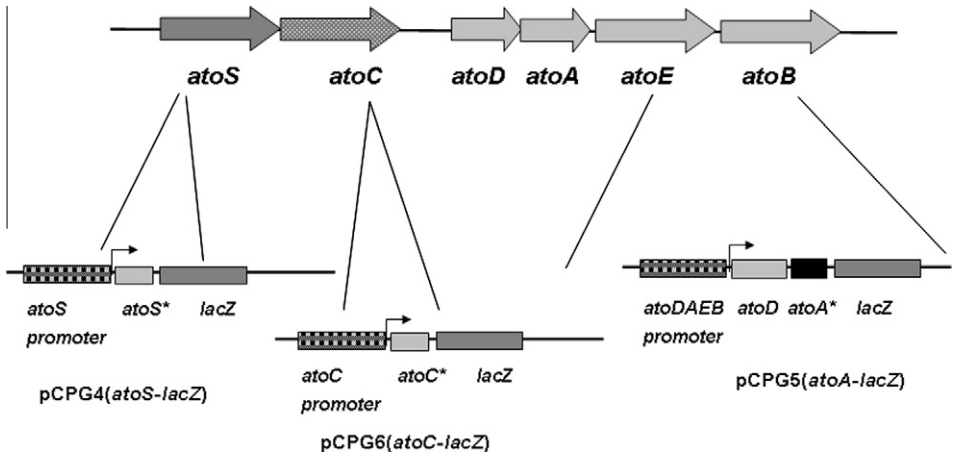


Figure 2. Structural organization of the *atoSC* and *atoDAEB* operon genes and constructs of plasmid pMLB1034 carrying various promoters fused to a promoterless *lacZ* gene. pCPG4 (*atoS-lacZ*) represents the promoter of *atoS* and a part of the *atoS* gene, pCPG5 (*atoA-lacZ*) represents the promoter of the *atoDAEB* operon extending 460 bp upstream the translational start of the *atoD* gene and pCPG6(*atoC-lacZ*) represents the promoter of *atoC* and a part of the *atoC* gene (*denotes part of the gene).

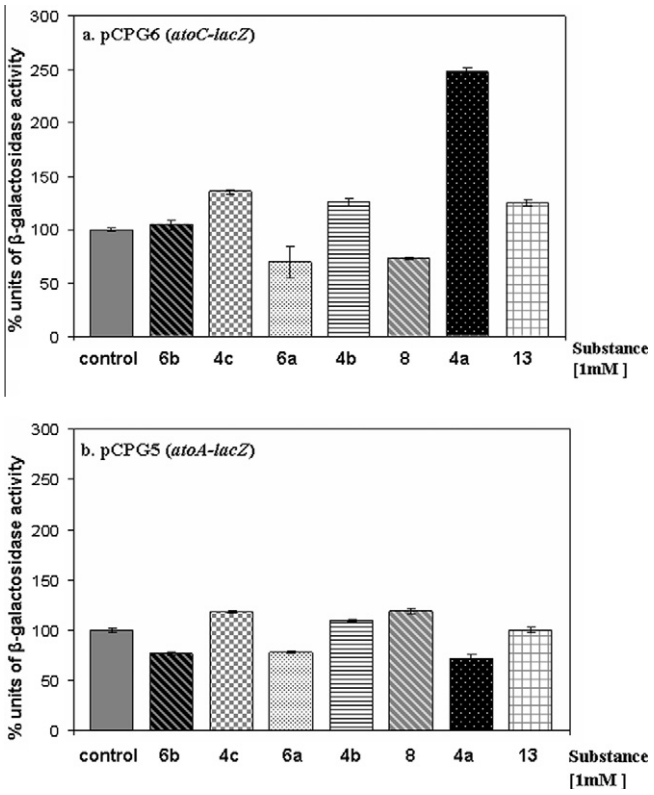


Figure 3. Effect of 5,7,8-trimethyl-1,4-benzoxazine derivatives in the transcription of *atoC* gene and the *atoDAEB* operon in E. *coli* strain BW25113. The *E. coli* K12 cells BW25113 carrying plasmids (a) pCPG6 (*atoC-lacZ*) and (b) pCPG5 (*atoA-lacZ*) were cultured in the presence of 1 mM of the indicated 5,7,8-trimethyl-1,4-benzoxazine derivatives, respectively. The transcriptional activity of the constructs was measured by assaying β-galactosidase activity.

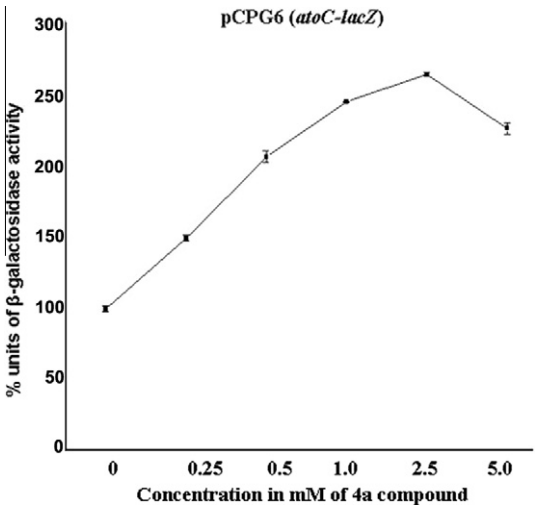


Figure 4. Induction of *atoC* mediated by compound **4a.** The transcriptional activation of the *atoC* was measured by assaying β-galactosidase expression in BW25113/ pCPG6 (*atoC-lacZ*) cells growing in the presence of increasing concentrations of **4a** (0, 0.25, 0.5, 1.0, 2.5 and 5 mM) (see Section 4). The results are presented from two independent experiments, while in each experiment two clones from each transformant were tested.

two promoters (*atoS* and *atoDAEB*) was affected in a similar manner. Specifically, the results of the β-galactosidase assay suggested that **4a** elicited a pronounced effect in the transcriptional activation of the *atoC* gene, whereas failed to induce *atoS* gene and slightly suppressed the *atoDAEB* operon.

2.4. Effect of compound 4a on the expression of AtoSC protein levels

Since compound **4a** activated the transcription of *atoC* in a concentration-dependent manner, it was investigated whether this activation leads to AtoC protein accumulation in the cell, thus affecting the translational mechanism. *E. coli* BW25113 cells transformed with the multicopy plasmid pUC-Az, overexpressing the AtoS and AtoC proteins[22] were grown and compound **4a** was added half at OD_{600} 0.2 and the other half at OD_{600} 0.4, respectively and the growth was continued to OD_{600} 0.9.

Surprisingly, under these culture conditions, cells exposed to higher concentrations of **4a** indicated a reduction on AtoC protein levels (Fig. 6a). Similar observations were obtained for AtoS protein levels, by immunoblot analysis of the same cell extracts with the purified polyclonal anti-AtoS (Fig. 6b). The protein levels of AtoS and AtoC under the specific cell culture conditions is irreversibly proportional to the concentration of **4a** added in the growth cultures (Fig. 6).

This result could be attributed to a broad effect of this compound under the specific culture conditions causing stress/ heat shock response and the subsequent protein degradation[23,24] since a concentration-dependent increase of the heat shock protein DnaK was observed whereas the protein levels of the heat shock Hsp60 remained unchanged (Fig. 6c and d). This result is also

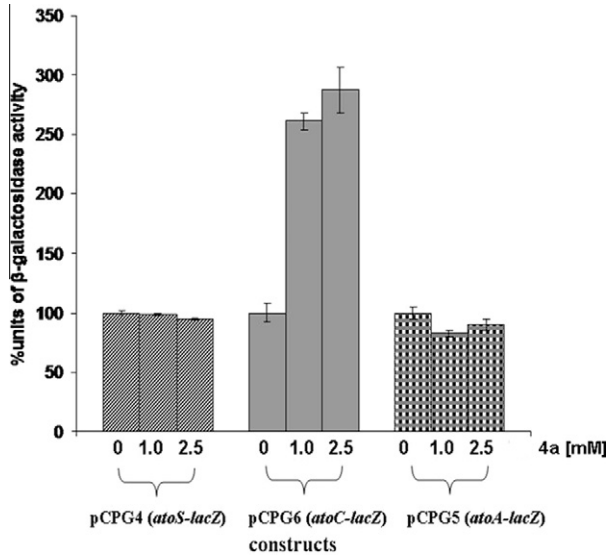


Figure 5. Effect of 5,7,8-trimethyl-1,4 benzoxazine (**4a**) on *atoSC* and the regulated *atoDAEB* operon gene expression. The *E. coli* K12 strain BW25113 was transformed with plasmids pCPG4(*atoS-lacZ*), pCPG6(*atoC-lacZ*) and pCPG5(*atoA-lacZ*). Induction mediated by compound **4a** was measured by assaying β-galactosidase expression in the presence of the indicated concentrations of the compound (1.0 and 2.5 mM, respectively).

indicative of the fact that upregulation of the Hsp proteins under stress/heat shock-induced *E. coli* cells is critical for survival and Hsp70 (DnaK) acts as a negative modulator for Hsp60 expression.[25,26]

Furthermore, the effect of **4a** on the protein levels of the AtoC was observed in cells growing in different culture conditions. *E. coli* BW25113/pUC-Az cells were exposed to final concentrations 1 and 2.5 mM of **4a** at the beginning of the growth phase and grown to an OD_{600} 0.5–0.6. The protein levels of AtoC in the cell extracts were monitored by immunoblotting using affinity-purified rabbit polyclonal anti-AtoC antibody.[2] As shown in Figure 7a, *atoC* transcriptional activation also produced increased AtoC accumulation. This indicates that compound **4a** under these specific culture conditions does not enhance AtoC degradation. On the other hand, AtoS protein levels remained constant with a decrease at the higher concentration of the tested compound (Fig. 7b).

2.5. Effect of the 5,7,8-trimethyl-1,4-benzoxazine derivatives on AtoS kinase activity

It is conceivable that the possible effect of the 5,7,8-trimethyl-1,4-benzoxazine derivatives on the biological output of the AtoS–AtoC two-component system (i.e., the activation of the *atoDAEB* operon expression) could be due to their influence on AtoS kinase activity. To test whether these compounds could indeed influence the autophosphorylation of AtoS, the in vitro autophosphorylation assay was carried out with the purified cytosolic protein, cytoAtoS (see Section 4). Equal concentration (7 μM) of cytoAtoS autophosphorylated in the absence or presence of each of the 5,7,8-trimethyl-1,4-benzoxazine derivatives of the present study at a final concentration of 1 mM.

Interestingly, of all the compounds tested, **4a**, resulted in an enhancement of the in vitro autophosphorylation activity of the truncated cytosolic protein of AtoS (cytoAtoS), whereas the other 5,7,8-trimethyl-1,4-benzoxazine derivatives **4b, c, 6a, b, 8**, and **13** had no/ or slight effect on the induction of AtoS autophosphorylation (Fig. 8a).

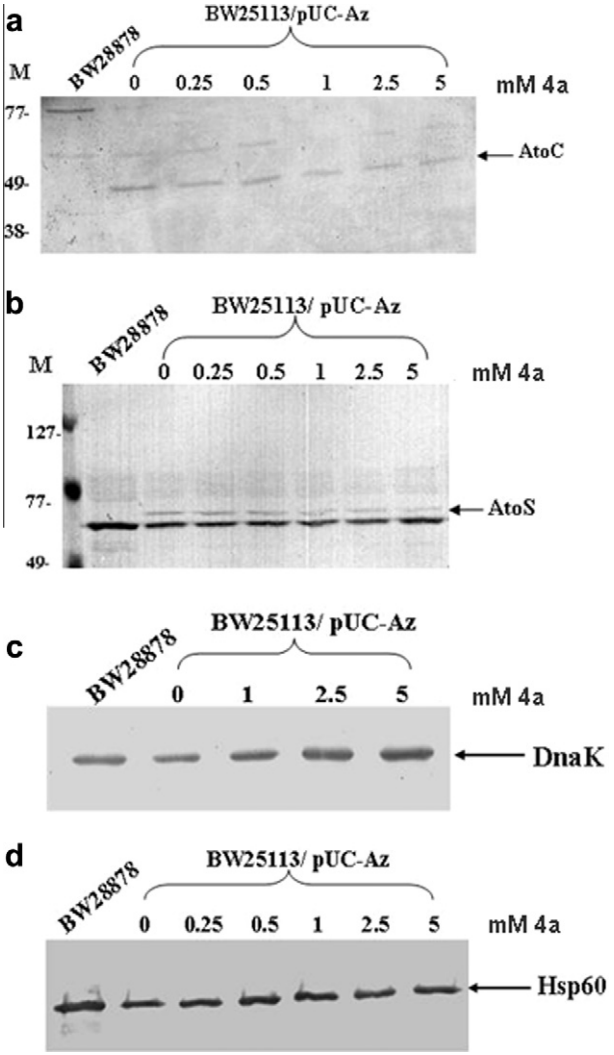


Figure 6. (a, b) Effect of compound **4a** on AtoSC protein levels. *E. coli* K12 cells BW25113/pUC-Az were grown in the presence of various concentrations of **4a** to a cell density of 0.9 (see Section 4). The total cell extracts from each culture (15 μL) were subjected to SDS–PAGE 10% w/v and immunostained with (a) anti-AtoC (1:500) and (b) anti-AtoS (1:1000). BW28878 cells, not expressing the AtoC and AtoS proteins, were used as negative control. (c, d) Effect of compound **4a** on endogenous DnaK and Hsp60 expression. *E. coli* K12 cells BW25113/ pUC-Az were grown in the presence of the indicated increasing concentrations of compound **4a** to an OD_{600} 0.9. Western blot of total cell extracts (15 μL) was performed to detect the expression of the heat shock proteins: (c) DnaK (1:2000) and (d) Hsp60 (1:1000).

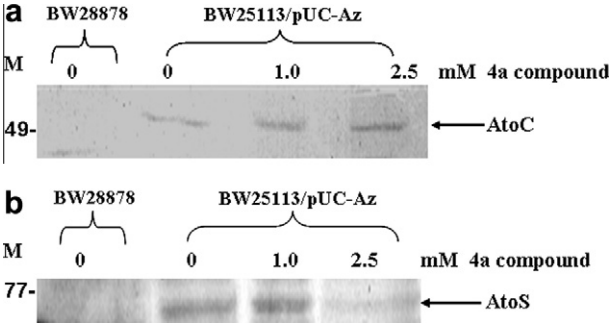


Figure 7. Effect of compound 4a on AtoSC protein expression. The *E. coli* K12 cells BW25113/pUC-Az were cultured in the absence or presence of 1.0 and 2.5 mM of compound **4a**. Total cell extracts (15 μL) of each culture grown to an OD_{600} 0.5–0.6 were analyzed. Immunoblot analysis was performed using the purified polyclonal rabbit antibody of (a) AtoC and (b) AtoS respectively. BW28878 cells, not expressing the AtoS and AtoC proteins, were used as negative control.

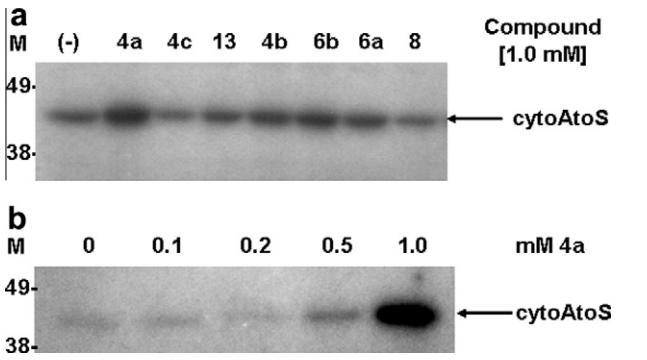


Figure 8. Effect of the 5,7,8-trimethyl-1,4-benzoxazine derivatives on AtoS autophoshorylation activity. (a) Autophosphorylation activity of AtoS kinase was measured when purified cytoAtoS (7 μM) was incubated with [γ-^{32}P]-ATP for 30 min at room temperature, in the absence or presence of the indicated 5,7,8-trimethyl-1,4-benzoxazine derivative at the final concentration of 1 mM or (b) purified cytoAtoS (4 μM) was incubated with [γ-^{32}P]-ATP for 30 min at room temperature, in the absence or presence of 0.1, 0.2, 0.5 and 1.0 mM compound **4a**, respectively. Prior the addition of [γ-^{32}P]-ATP each protein sample was preincubated for 15 min with the appropriate compound. The reactions were stopped by the addition of SDS loading buffer followed by separation on SDS–PAGE 12% w/v and autoradiography.

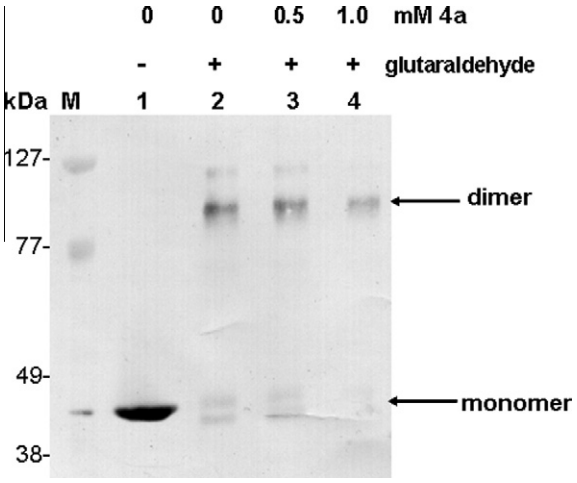


Figure 9. Chemical cross-linking of purified cytoAtoS in the presence of compound **4a**. Purified cytoAtoS (2 μg) was cross-linked with glutaraldehyde 0.1% v/v for 30 min at room temperature in the absence or presence of 0.5 and 1 mM **4a**, respectively (see Section 4). Each sample (25 μL) was withdrawn at the time indicated, subjected to SDS–PAGE 8.5% w/v and immunostained with anti-His (1:500). **Lane 1**: cytoAtoS with no glutaraldehyde added, **2**: cytoAtoS treated with glutaraldehyde, **3 and 4**: cytoAtoS treated with glutaraldehyde in the presence of 0.5 and 1 mM **4a**, respectively.

2.6. Compound 4a affects AtoS kinase activity

Since it was demonstrated that the AtoS protein levels remained constant in cells exposed to **4a** at the final concentration of 1 mM (Fig. 7b), we next examined the involvement of this compound on the activation of AtoS kinase activity.

In order to test whether **4a** could induce the autophosphorylation of AtoS sensor histidine kinase, the in vitro autophosphorylation assay was carried out with the purified cytosolic protein, cytoAtoS (4 μM), in the presence of increasing concentrations of compound **4a**. As shown in Figure 8b, the presence of increasing concentrations of the compound **4a** in the reaction mixture stimulated the enzyme autophosphorylation activity. AtoS kinase activation by compound **4a** occurs at higher concentrations of the tested compound (Fig. 8b).

2.7. Determination of the effect of compound 4a on AtoS dimerization/oligomerization

Since the mechanism of AtoS activation suggests that AtoS molecules function as dimers[1] we examined if the kinase dimerization is affected by compound **4a**.

The ability of AtoS to form dimers/oligomers in vitro in the absence or presence of **4a** was determined by glutaraldehyde cross-linking of its recombinant, purified cytosolic domain (cytoAtoS). Western blot analysis of the products of such cross-linking experiments indicated that the presence of **4a** at the final concentration of 0.5 mM in the reaction mixture does not enhance cytoAtoS dimerization. Conversely, the dimeric form of AtoS appeared to slightly decrease when **4a** was added at the concentration of 1 mM, suggesting that the AtoS oligomers (probably remaining in the stacking gel) may be stimulated by this compound (Fig. 9). The possibility that **4a** may influence AtoS ATP binding ability, thus leading to the kinase activation remains to be elucidated.

2.8. Compound 4a enhances the acetoacetate effect on atoDAEB induction

The effects of compound **4a** on *atoC* transcription and AtoS in vitro kinase activity raised the question if they could influence the biological output of the AtoSC system, that is, the acetoacetate dependent activation of the *atoDAEB* operon expression. To test this possibility the effect of acetoacetate on the activity of the *atoDAEB* promoter was measured in the presence of compound **4a**.

Thus, *E. coli* BW25113 cells carrying plasmid pCPG5 (*atoA-lacZ*) were grown in the presence of 2.5 or 10 mM acetoacetate and their effects on acetoacetate-dependent induction of *atoDAEB* promoter were compared for various concentrations of compound **4a**. Acetoacetate (10 mM) strongly induced *atoDAEB* promoter activity in cells of atoSC^{+} genetic background. In contrary, acetoacetate

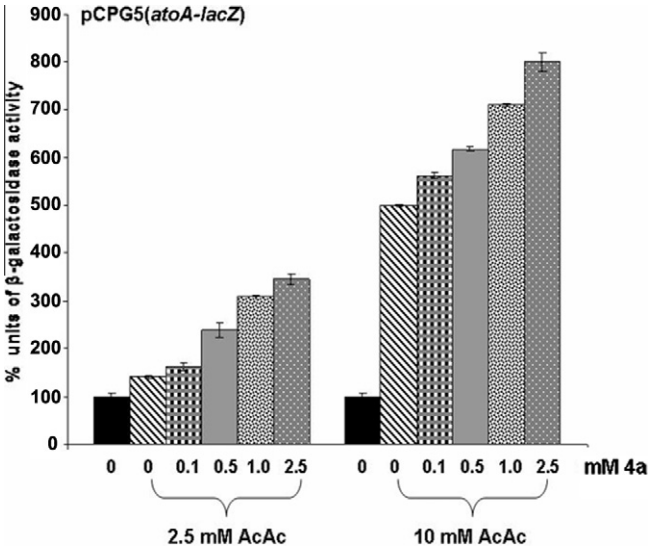


Figure 10. Effect of compound **4a** on acetoacetate-activation of the *atoDAEB* operon. Effect of the simultaneous presence of acetoacetate and **4a** on the activation of the *atoDAEB* promoter compared to the acetoacetate effect in BW25113 cells. The activity of the *atoDAEB-lacZ* construct (pCPG5) was measured in the presence of increasing concentrations of compound **4a** (0.1, 0.5, 1.0, 2.5 mM) in *E. coli* BW25113 cells. The cells were grown in a modified M9 mineral medium in the absence or presence of 2.5 or 10 mM acetoacetate respectively and the β-galactosidase activity was measured.

(2.5 mM) induced to a lesser extent the *atoDAEB* operon (Fig. 10). Compound **4a** added at increasing concentrations in the cultures produced further activation of the *atoDAEB* expression for both acetoacetate concentrations (2.5 and 10 mM, respectively).

More specifically, cells growing in the presence of both acetoacetate 10 mM and compound **4a** demonstrated the most potent effect on *atoDAEB* promoter activation (Fig. 10). This result indicates the cooperative effect of **4a** and acetoacetate-mediated TCS induction, in contrast to the competition between acetoacetate and spermidine-mediated TCS induction occurred previously.[7]

The enhancement of acetoacetate-mediated TCS induction was not due to alterations on either AtoS (Fig. 7b) or AtoC accumulation protein levels (Fig. 3b, and 7a), but rather to AtoS kinase activity enhancement, or even to an increase of the ability of the phosphorylated AtoC to bind to the *atoDAEB* operon promoter in the presence of this compound. The compound itself on the other hand does not promote the activation of *atoDAEB* transcription, indicating that neither **4a** nor one of its metabolic products in the cell may influence AtoSC in the absence of the natural inducer, acetoacetate (Fig. 3b). This effect might be due to the opposite activities of histidine kinases that can phosphorylate or dephosphorylate their response regulators.[27] Therefore, compound **4a** in the absence of the acetoacetate-binding may influence the phosphatase activity of the AtoS leading to AtoC dephosphorylation that slightly suppresses the *atoDAEB* operon expression (Fig. 3b).

In general, acetoacetate-binding further promotes AtoS autophosphorylation in the cell without affecting the AtoS dimerization/oligomerization state.[1] As stated, ligand binding alters the conformation of the histidine kinase thus leading to the 'on kinase active state'.[28] It is therefore possible the kinase domain of AtoS to be 'switched on' and **4a** binding to the kinase domain may induce conformational changes in order to facilitate ATP binding, thus activating AtoS, leading to further *atoDAEB* activation. After all, **4a** was indeed shown to be an activator of AtoS enzymatic activity in vitro with the enhancement of the autophosphorylation activity of the truncated protein of AtoS (Fig. 8) that is even in the absence of the natural inducer in the 'active kinase state', thus similarly leading to **4a** binding.

Taken together, the above data provide evidence that multiple regulatory interactions may occur between the recognized signal by the AtoS histidine kinase and its cognate AtoC response regulator phosphorylation site and the mechanisms underlying the enhancement of acetoacetate-mediated induction by compound **4a** remain to be elucidated. Overall, the prominent role of the 5,7,8-trimethyl-1,4-benzoxazine (**4a**) in the bacterial microenvironment may provide the lead for the elucidation of unresolved implications of AtoSC in pathophysiology and the development of novel therapeutic drugs possibly through cross-regulation with other systems.

3. Conclusions

A series of seven 5,7,8-trimethyl-1,4-benzoxazine derivatives have been tested for their effectiveness on the expression of the *atoC*, as well as that of the *atoDAEB* (*ato*) operon. 5,7,8-Trimethyl-1,4-benzoxazine (**4a**), activated *atoC* transcription in a specific manner since none of the other tested genes of the system (*atoS* and *atoDAEB*) showed similar effect. In addition, compound **4a** resulted in accumulation of the AtoC protein and was also shown to significantly up-regulate the in vitro kinase activity of the histidine kinase AtoS without altering the protein levels in the cell. Consequently, compound **4a** appeared to modulate the acetoacetate-mediated *atoDAEB* operon induction indicating a modulator role of 5,7,8-trimethyl-1,4-benzoxazine on AtoSC two component system-mediated signaling.

4. Experimental

4.1. Materials and methods

NMR spectra were recorded on a Bruker AC 300 and a Varian 600 MHz spectrometer operating at 300 MHz for ^{1}H and 75.43 MHz for ^{13}C, and at 600 MHz for ^{1}H, respectively. ^{1}H NMR spectra are reported in units of δ relative to the internal standard of signals of the remaining protons of deuterated chloroform, at 7.26 ppm. ^{13}C NMR shifts are expressed in units of δ relative to $CDCl_3$ at 77.0 ppm. ^{13}C NMR spectra were proton noise decoupled. All NMR spectra were recorded in $CDCl_3$. Silica gel plates Macherey-Nagel Sil G-25 UV_{254} were used for thin layer chromatography. Chromatographic purification was performed with silica gel (200–400 mesh). Mass spectra were recorded on a Varian Saturn 2000 GC–MS instrument in the EI mode. High resolution mass spectra were obtained by the Department of Chemistry and Biochemistry, University of Notre Dame, USA

4.1.1. (4-Methoxy-2,3,5-trimethyl-6-nitro-phenoxy)-phenyl-acetic acid methyl ester (2b)

To a solution of 2-nitro-3,5,6-trimethylphenol (**1**) (0.182 g, 1 mmol) in dry DMF (3.33 mL) was added cesium carbonate (0.358 g, 1.1 mmol), TBAI (catalytic amount) and methyl 2-bromo-2-(4-methoxyphenyl) acetate (0.285 g, 1.1 mmol) under nitrogen. The mixture was stirred for 5 min at room temperature and the mixture was diluted with water and was extracted with ethyl acetate. The organic phase was washed with brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash column chromatography using (cyclohexane/acetone 9:1) as eluting solvent to afford 4-methoxy-2,3,5-trimethyl-6-nitro-phenoxy)-phenyl-acetic acid methyl ester, as a viscous oil in 95% yield (341 mg). R_f 0.38 (petroleum ether 40–60 °C/acetone 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34 (d, J = 8.6 Hz, 2H), 6.84 (d, J = 8.6 Hz, 2H), 6.79 (s, 1H), 5.26 (s, 1H), 3.74 (s, 3H, –OCH_3), 3.67 (s, 3H, –OCH_3), 2.17 and 2.16 (s, 6H, ArCH_3), 1.94 (s, 3H, ArCH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 170.0, 160.7, 147.6, 144.6, 141.1, 129.7, 129.4, 128.2, 127.9, 127.5, 114.3, 84.4, 55.4, 52.9, 20.4, 17.2, 13.5.

4.1.2. 2-(4-Methoxyphenyl)-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (3b)

To a solution of 4-methoxy-2,3,5-trimethyl-6-nitro-phenoxy)-phenyl-acetic acid methyl ester (0.359 g, 1 mmol) in absolute ethanol (5 mL) at 0 °C was added CuCl (0.495 g, 5 mmol) followed by $NaBH_4$ (0.378 g, 10 mmol) over 5 min. The resulting mixture was refluxed for 20 min to 3 h and the reaction was cooled to room temperature and was diluted with CH_2Cl_2. The mixture was filtered through celite and the filtrate was evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 9:1). Yield: 252 mg (85%); white solid; mp 176–177 °C; R_f 0.31 (petroleum ether 40–60 °C/acetone 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.75 (s, 1H, –N*H*CO–), 7.34 (d, J = 8.5 Hz, 2H, Ar*H*), 6.85 (d, J = 8.5 Hz, 2H, Ar*H*), 6.60 (s, 1H, Ar*H*), 5.64 (s, 1H, C*H*), 3.77 (s, 3H, OCH_3), 2.19 (s, 3H, ArCH_3), 2.18 (s, 3H, ArCH_3), 2.15 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 166.3, 159.8, 140.8, 132.3, 128.1, 127.4, 125.0, 122.7, 122.2, 120.5, 113.9, 77.8, 55.2, 19.4, 16.1, 11.6; MS (EI), m/z 297 (M^+, 100); HRMS (ESI^+) calcd for $C_{18}H_{20}O_3N$ $[M+H]^+$ 298.1438, found 298.1438.

4.1.3. 2-(4-Methoxyphenyl)-5,7,8-trimethyl-3,4-dihydro-2*H*-1,4-benzoxazine (4b)

To a solution of 2-(4-methoxyphenyl)-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (**3b**) (0.297 g, 1 mmol) in THF (50 mL) was

added dropwise at 0 °C a solution of borane· dimethylsulfide (24 mmol, 2.28 mL). The reaction mixture was stirred at room temperature until completion. The reaction was stopped by dropwise addition of water at 0 °C until gas evolution has ceased. The resulting mixture was extracted with ethyl acetate. The organic layer was extracted with brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the residue was purified by flash column chromatography using (cyclohexane/acetone 9:1) as elution solvent to afford 2-(4-methoxyphenyl)-5,7,8-trimethyl-3,4-dihydro-2*H*-1,4-benzoxazine (**4b**) in 89% yield (251 mg) as a white solid. mp 99–100 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46 (d, J = 8.5 Hz, 2H, Ar *H*), 7.04 (d, J = 8.5 Hz, 2H, Ar*H*), 6.66 (s, 1H, Ar*H*), 5.10 (dd, J = 2.4 Hz, J = 8.9 Hz, 1H, C*H*), 3.92 (s, 3H, OCH_3), 3.61 (dd, J = 2.4 Hz, J = 11.8 Hz, 1H, CH_2), 3.38 (dd, J = 8.9 Hz, J = 11.8 Hz, 1H, CH_2), 2.31 (s, 3H, ArCH_3), 2.27 (s, 3H, ArCH_3), 2.22 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 159.5, 142.7, 132.0, 128.6, 127.5, 126.7, 123.4, 122.4, 120.4, 114.0, 75.3, 55.4, 48.2, 19.4, 16.7, 11.6; HRMS (FAB$^+$) calcd for $C_{18}H_{21}O_2N$ [M]$^+$ 283.1572, found 283.1551.

4.1.4. 6-Bromo-5,7,8-trimethyl-2H-1,4-benzoxazin-3(4*H*)-one (5b)

To a solution of 5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (**3b**) (0.191 g, 1 mmol) in petroleum ether 40–60 °C (10 mL) was added a mixture of CH_3COOH/H_2O_2 (30%) /HBr (48%) (6.5 mmol: 6.5 mmol:2.5 mmol) and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with brine, was dried over anhydrous Na_2SO_4, and the solvent was evaporated in vacuo. The residue was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 85:15) to afford compound **5b** as a white solid. Yield: 202 mg (75%); mp 234–237 °C; R_f 0.24 (petroleum ether 40–60 °C/ethyl acetate 4:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.54 (br s, 1H, NHCO), 4.56 (s, 2H, CH_2), 2.37 (s, 3H, $ArCH_3$), 2.34 (s, 3H, $ArCH_3$), 2.22 (s, 3H, $ArCH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 165.9, 141.1, 132.3, 124.0, 123.1, 121.2, 120.9, 67.0, 20.3, 17.2, 12.9; MS(EI), m/z 269 [M$^+$,91], 271 ([M +2]$^+$,100); HRMS (ESI$^+$) calcd for $C_{11}H_{13}O_2NBr$ [M+H]$^+$ 270.0124, found 270.0121.

4.1.5. 6-Bromo-3,4-dihydro-5,7,8-trimethyl-2*H*-1,4-benzoxazine (6b)

To a solution of 6-bromo-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (**5b**) (0.270 g, 1 mmol) in THF (20 mL) at 0 °C was added dropwise borane dimethylsulfide (24 mmol, 2.28 mL). The reaction mixture was stirred at room temperature until completion. The reaction was stopped by dropwise addition of water at 0 °C until gas evolution has ceased. The organic layer was extracted with saturated aqueous $NaHCO_3$ and brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the residue was purified by flash column chromatography (hexane/acetone 9:1). Yield: 250 mg (98%); white solid; mp 99–100 °C; R_f 0.24 (petroleum ether 40–60 °C/acetone 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.20 (t, J = 4.2 Hz, 2H, CH_2), 3.42 (br s, 2H, CH_2), 3.30 (br s, 1H, NH), 2.32 (s, 3H, ArCH_3), 2.24 (s, 3H, ArCH_3), 2.15 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 141.1, 129.9, 126.2, 123.4, 120.1, 119.5, 64.8, 41.3, 20.0, 17.2, 12.7; MS (EI), m/z 255 [M$^+$, 100], 257 ([M+2]$^+$, 100); HRMS (FAB$^+$) calcd for $C_{11}H_{14}ONBr$ [M]$^+$ 255.0259, found 255.0255.

4.1.6. 1-(4-Methoxyphenyl)-2-(2,3,5-trimethyl-6-nitrophenoxy) ethanone (7)

To a solution of 2-nitro-3,5,6-trimethylphenol (**1**) (0.182 g, 1 mmol) in dry DMF (3.33 mL) was added cesium carbonate (0.358 g, 1.1 mmol), TBAI (catalytic amount) and 2-bromo-4′-methoxyacetophenone (0.252 g, 1.1 mmol) under nitrogen. The mixture was stirred for 5 min at room temperature and the mixture was diluted with water and was extracted with ethyl acetate. The organic phase was washed with brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 9:1) to afford 1-(4-methoxyphenyl)-2-(2,3,5-trimethyl-6-nitrophenoxy)ethanone (**7**) in 98% yield (322 mg) as a pale yellow sticky solid. R_f 0.44 (petroleum ether 40–60 °C/ethyl acetate 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 8.9 Hz, 2H, Ar*H*), 6.84 (d, J = 8.9 Hz, 2H, Ar*H*), 6.79 (s, 1H, Ar*H*), 5.08 (s, 2H, CH_2), 3.76 (s, 3H, OCH_3), 2.17 (s, 3H, ArCH_3), 2.15 (s, 3H, ArCH_3), 2.10 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 191.5, 164.2, 148.4, 144.7, 141.4, 130.4, 129.2, 128.2, 127.9, 127.4, 114.1, 76.8, 55.7, 20.3, 17.1, 12.5; MS (EI), m/z: 283 (38, M$^+$-NO_2), 135 (100); HRMS (ESI$^+$) calcd for $C_{18}H_{20}O_5N$ [M+H]$^+$ 330.1336, found 330.1337.

4.1.7. 3-(4-Methoxyphenyl)-5,7,8-trimethyl-3,4-dihydro-2*H*-benz]oxazine (8)

To a solution of 1-(4-methoxyphenyl)-2-(2,3,5-trimethyl-6-nitrophenoxy) ethanone (**7**) (0.329 g, 1 mmol) in absolute ethanol (5 mL) at 0 °C was added CuCl (0.495 g, 5 mmol) followed by $NaBH_4$ (0.378 g, 10 mmol) over 5 min. The resulting mixture was refluxed for 20 min and the reaction was cooled to room temperature and was diluted with CH_2Cl_2. The mixture was filtered through celite and the filtrate was evaporated in vacuo. The crude product was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 4:1) to afford compound **8**. Yield: 260 mg (92%); pale yellow solid; mp 90 °C; R_f 0.41 (petroleum ether 40–60 °C/ethyl acetate 3:2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.33 (d, J = 8.5 Hz, 2H, Ar*H*), 6.88 (d, J = 8.5 Hz, 1H, Ar*H*), 6.67 (s, 1H, Ar*H*), 5.01 (dd, J = 8.7 Hz, J = 2.8 Hz, 1H, C*H*), 4.03 (dd, J = 10.4 Hz, J = 2.9 Hz, 1H, CH_2), 3.86–3.80 (m, 4H, CH_2 and OCH_3), 3.63 (br s, 1H. N*H*), 2.15 (s, 3H, ArCH_3), 2.14 (s, 6H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 159.5, 145.1, 135.6, 132.7, 127.8, 127.3, 127.2, 126.9, 121.1, 114.1, 78.1, 73.1, 55.5, 19.5, 17.2, 12.8; HRMS (FAB$^+$) calcd for $C_{18}H_{22}O_2N$ [M+H]$^+$ 284.1651, found 284.1627.

4.1.8. 4-Bromo-2,3,5-trimethyl-6-nitrophenol (9)

To a solution of 2,3,5-trimethyl-6-nitrophenol (**1**) (0.181 g, 1 mmol) in petroleum ether 40–60 °C (10 mL) was added a mixture of CH_3COOH/H_2O_2 (30%) /HBr (48%) (6.5 mmol: 6.5 mmol:2.5 mmol) and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with brine, was dried over anhydrous Na_2SO_4, and the solvent was evaporated in vacuo. The residue was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 95:5) to afford compound **9** as a yellow solid. Yield: 166 mg (64%); mp 156–157 °C; R_f 0.38 (petroleum ether 40–60 °C/ethyl acetate 95:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.64 (s, 1H, OH), 2.64 (s, 3H, $ArCH_3$), 2.50 (s, 3H, $ArCH_3$), 2.32 (s, 3H, $ArCH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 150.4, 144.7, 135.5, 131.6, 125.4, 120.6, 22.0, 21.9, 13.3; MS(EI), m/z 259 [M$^+$,74], 261 ([M +2]$^+$,68); HRMS (ESI$^-$) calcd for $C_9H_9O_3Br$ [M-1]$^-$ 257.9771, found 257.9779.

4.1.9. 4′-Methoxy-2,3,6-trimethyl-5-nitro[1.1′-biphenyl]-4-ol (10)

A mixture of tetrakis(triphenylphosphine)palladium (0.115 g, 0.1 mmol), 4-methoxyphenyl boronic acid (0.167 g, 1.1 mmol), 4-bromo-2,3,5-trimethyl-6-nitrophenol (**9**) (0.260 g, 1.0 mmol), and Cs_2CO_3 (0.489 g, 1.5 mmol) in THF (5 mL) was degassed and then was heated under reflux overnight. After cooling to room temperature, the reaction mixture was partitioned between water and diethyl ether. The organic extracts were washed with brine and

dried over anhydrous Na_2SO_4, the solvent was evaporated and the residue was purified by flash column chromatography (petroleum ether 40–60 °C/diethyl ether/dichloromethame 97:2:1) to afford the desired product **10** in 75% yield (215 mg) as a yellow sticky solid; R_f = 0.30 (petroleum ether 40–60 °C/diethyl ether 95:5); ^{1}H NMR δ 10.16 (s, 1H,OH), 6.97 (s, 4H, Ar*H*), 3.86 (s, 3H, OCH_3), 2.26 (s, 3H, ArCH_3), 2.17 (s, 3H, ArCH_3), 1.97 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 158.9, 151.4, 144.7, 135.7, 132.6, 131.1, 130.9, 124.2, 114.4, 55.5, 19.5, 19.3, 12.6; MS (EI), *m*/*z*: 287 [M^+, 100]; HRMS (ESI^-) calcd for $C_{16}H_{16}O_4N$ $[M-1]^-$ 286.1085, found 286.1085.

4.1.10. Ethyl 2-[(4′-methoxy-2,3,6-trimethyl-5-nitro[1,1′-biphenyl]-4-yl)oxy]acetate (11)

To a solution of 4′-methoxy-2,3,6-trimethyl-5-nitro[1,1′-biphenyl]-4-ol (**10**) (0.287 g, 1 mmol) in dry DMF (3.33 mL) was added cesium carbonate (0.358 g, 1.1 mmol), TBAI (catalytic amount) and ethyl bromoacetate (0.184 g, 1.1 mmol) under nitrogen. The mixture was stirred for 5 min at room temperature and the mixture was diluted with water and was extracted with ethyl acetate. The organic phase was washed with brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash column chromatography (cyclohexane/diethyl ether 9:1) to afford ethyl 2-[(4′-methoxy-2,3,6-trimethyl-5-nitro[1,1′-biphenyl]-4-yl)oxy]acetate (**11**) in 98% yield (346 mg) as a pale yellow oil; R_f = 0.52 (petroleum ether 40–60 °C/ethyl acetate 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.98 (br s, 4H, Ar*H*), 4.54 (s, 2H, CH_2), 4.31 (m, 2H, CH_2CH$_3$), 3.86 (s, 3H, OCH_3), 2.27 (s, 3H, ArCH_3), 1.97 (s, 3H, ArCH_3), 1.91 (s, 3H, ArCH_3), 1.34)m, 3H, CH_2CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 168.1, 158.8, 146.3, 144.8, 139.7, 139.2, 131.7, 130.1, 128.7, 126.5, 114.1, 71.5, 61.4, 55.2, 18.2, 15.7, 14.1, 13.0; MS (EI), *m*/*z*: 373 [M^+, 25], 327 ([$M-NO_2]^+$, 78).

4.1.11. 6-(4-Methoxyphenyl)-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (12)

To a solution of ethyl 2-[(4′-methoxy-2,3,6-trimethyl-5-nitro[1,1′-biphenyl]-4-yl)oxy]acetate (**11**) (0.373 g, 1 mmol) in dry ethanol (10 mL) was added 10% palladium on carbon (25% w/w of starting material) and the mixture was hydrogenated at 70 °C overnight. After filtration and evaporation in vacuo, the crude product was purified by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate 85:15) to afford 6-(4-methoxyphenyl)-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (**12**). Yield: 267 mg (90%); white crystals; mp 211–213 °C; R_f 0.29 (petroleum ether 40–60 °C/ethyl acetate 3:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.50 (s, 1H, -N*H*CO-), 6.98 (d, *J* = 8.8 Hz, 2H, Ar*H*), 6.95 (d, *J* = 8.8 Hz, 2H, Ar*H*), 4.61 (s, 2H, CH_2), 3.85 (s, 3H, OCH_3), 2.19 (s, 3H, ArCH_3), 1.90 (s, 3H, ArCH_3), 1.87 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 165.8, 158.3, 141.0, 136.0, 133.2, 131.3, 130.6, 122.6, 122.1, 119.9, 113.8, 67.1, 55.2, 17.3, 14.0, 12.0; HRMS (FAB^+) calcd for $C_{18}H_{19}O_3N$ $[M]^+$ 297.1365, found 297.1352; MS (EI), *m*/*z*: 297 [M^+, 100].

4.1.12. 6-(4-Methoxyphenyl)-5,7,8-trimethyl-3,4-dihydro-2*H*-1,4-benzoxazine (13)

To a solution of 6-(4-methoxyphenyl)-5,7,8-trimethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (**12**) (0.297 g, 1 mmol) in THF (50 mL) was added dropwise at 0 °C a solution of borane· dimethylsulfide (24 mmol, 2.28 mL). The reaction mixture was stirred at room temperature until completion. The reaction was stopped by dropwise addition of water at 0 °C until gas evolution has ceased. The organic layer was extracted with saturated aqueous $NaHCO_3$ and brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo and the residue was purified by flash column chromatography using (hexane/acetone 9:1) as elution solvent to afford 6-(4-methoxyphenyl)-5,7,8-trimethyl-3,4-dihydro-2*H*-1,4-benzoxazine (**13**) in 84% yield (237 mg) as a white solid**.** mp 127–129 °C; R_f 0.37 (petroleum ether 40–60 °C/ethyl acetate 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.03 (d, *J* = 8.4 Hz, 2H, Ar*H*), 6.94 (d, *J* = 8.4 Hz, 2H, Ar*H*), 4.30 (t, *J* = 4.3 Hz, 2H, CH_2), 3.85 (s, 3H, OCH_3), 3.49 (br s, 2H, CH_2), 2.16 (s, 3H, ArCH_3), 1.88 (s, 3H, ArCH_3), 1.79 (s, 3H, ArCH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 158.0, 141.1, 134.5, 134.1, 130.9, 128.7, 125.4, 121.8, 119.3, 113.5, 65.0, 55.2, 41.5, 17.1, 14.2, 11.8; MS (EI), *m*/*z*: 283 (100, M^+); HRMS (FAB^+) calcd for $C_{18}H_{21}O_2N$ $[M]^+$ 283.1572, found 283.1562.

4.2. Biological assays

4.2.1. Bacterial strains and plasmids

E. coli strain BW25113 [*lacI*^{q}*rrnB3* δ*lacZ4787 hsdR514* DE*(araBAD)567* DE*(rhaBAD)568 rph-1*] (a gift from Dr. Hirofumi Aiba, Nagoya University, Japan) were transformed with recombinant plasmids.

The plasmids were carrying various promoters of genes, fused to a promoterless *lacZ* gene on pMLB1034 vector, that is, pCPG4 (*atoS-lacZ*), pCPG5 (*atoA-lacZ*) and pCPG6 (*atoC-lacZ*), carrying the *atoS* promoter, the *atoDAEB* promoter and *atoC* promoter, respectively (Fig. 2). These constructs, carrying the promoter of the *atoDAEB* operon and the regulatory *atoS–atoC* two component system genes, can direct the synthesis of the *lacZ* gene under the control of their cognate promoters.[11,21] The ability of these constructs to respond to various substances induction, driving the expression of *lacZ* gene, reflects the transcription of the reporter genes. Plasmid pMLB1034 was used as negative control.

Plasmid pUC-Az containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon (*atoD*, *atoA*, and two-thirds of *atoE*) has been described previously.[22]

4.2.2. Culture conditions

E. coli cells carrying the appropriate plasmids (Fig. 2) were grown at 37 °C with vigorous shaking in M_9 minimal medium,[29] supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5% w/v glucose, 1.7×10^{-3} mM $FeSO_4$, 1 μg/mL thiamine and 80 μg/mL DL-proline. Ampicillin was used at the final concentration of 100 μg/mL. The strains to be tested were grown overnight, then diluted 1:15 in the growth medium containing various substances (1 mM concentration), or various concentrations of **4a** or acetoacetate (2.5 or 10 mM) in combination with **4a**. Cultures were grown to OD_{600} 0.4–0.7 and the cells were harvested by centrifugation at 8000×*g* and washed twice with M_9 before the β-galactosidase assay.

In addition, BW25113 cells carrying plasmid pUC-Az were grown in supplemented M_9 mineral medium, in the absence or presence of various concentrations of compound **4a** added at the beginning of the growth or in portions of ½ of the compound tested at an OD_{600} 0.2 and 0.4, respectively. The cells were harvested when cultures reached logarithmic phase (OD_{600} 0.5–0.6) or late logarithmic phase (OD_{600} 0.9) by centrifugation at 8000×*g* for 10 min. The cell pellets were washed twice with ice-cold buffer saline and stored at −20 °C until use.

4.2.3. β-Galactosidase assay

β-Galactosidase activity assays were performed using the *E. coli* BW25113 (*atoSC*$^+$) carrying the appropriate plasmids, as described.[29] In all experiments, BW25113 carrying plasmid pMLB1034 was used as negative control.

4.2.4. Immunoblot analysis

An aliquot of 1 mL of each culture was centrifuged and resuspended in SDS loading buffer of 1.5× (75 mM Tris–HCl pH 6.8, 3% w/v SDS, 0.15% w/v bromophenol blue, 15% v/v glycerol and

β-mercaptoethanol at a final concentration of 10% v/v), boiled for 5 min and kept at −20 °C for immunoblot analysis.

4.2.5. Purification of recombinant cytosolic AtoS protein form

The recombinant His_6-tagged protein cytoAtoS was purified as described (**5**) by immobilized metal affinity chromatography (IMAC) on Ni^{2+}-NTA agarose (Qiagen) columns and dialyzed against a storage buffer containing 50 mM Tris–HCl pH 7.6, 0.5 mM DTT and 50% v/v glycerol. Following the determination of the cytoAtoS-His_6 concentration and an analysis of its purity by SDS–polyacrylamide gel electrophoresis, the protein was stored in aliquots at −80 °C.

4.2.6. Protein concentration

Protein concentrations were determined by the Bradford method, [30] using bovine serum albumin as the reference stan dard.

4.2.7. Kinase activity assay

Purified recombinant histidine kinase cytoAtoS-His_6 was incubated at room temperature for approximately 30 min with 1.6 μM [γ-^{32}P]-ATP (4 nmoles per reaction) in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT and 10 mM NaF at a final reaction volume of 45 μL. Equal concentrations of cytoAtoS were pre-incubated at room temperature with the phosphorylation buffer in the absence or presence of each of the 5,7,8-trimethyl-1,4-benzoxazine derivatives (at the final concentration of 1 mM) or compound **4a** (in increasing molar concentrations, 0.1, 0.2, 0.5 and 1.0 mM) for 15 min, prior the addition of [γ-^{32}P]-ATP. Each phosphorylation reaction was terminated by adding 4× SDS sample buffer and analyzed by SDS–PAGE and autoradiography.

4.2.8. In vitro cross-linking of purified cytoAtoS

Purified cytoAtoS-His_6 protein (2 μg) was initially incubated with the cross-linking buffer (20 mM Hepes-KOH, pH 7.9, 0.2 mM EDTA, 0.1 M NaCl, 20% v/v glycerol and 0.5 mM DTT) and the indicated concentrations of compound **4a** (0.5 and 1 mM) for 15 min at 25 °C. CytoAtoS-His_6 was further cross-linked for 30 min at 25 °C with 0.1% v/v glutaraldehyde added in the reaction. The reaction volume was 25 μL and the cross-linking was terminated by addition of an equal volume of 2× SDS–PAGE loading buffer and boiling for 3 min. The complexes were analyzed by SDS–PAGE on 8.5% w/v gels and immunoblotting analysis was performed using the polyclonal antibody against the histidine-tag (Santa Cruz Biotechnology).

4.2.9. Electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 8.5, 10 and 12% w/v polyacrylamide gels.[31] Proteins were transferred to immobilon PVDF membranes[32] and immunostained with the rabbit polyclonal antibodies against, DnaK, Hsp60, AtoC and AtoS, respectively, prepared as described.[2,11,33]

Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra for compounds **2a, b**, **3a**, **b**, **4a–c**, **5a**, **b**, **6a**, **b**, and **7–13**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.029.

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