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Inhibitory Mechanism of Novel Inhibitors of UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase from *Haemophilus influenzae*

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Bacterial UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to uridine diphospho-*N*-acetylglucosamine (UNAG), which is the first step of bacterial cell wall synthesis. We identified thimerosal, thiram, and ebselen as effective inhibitors of *Haemophilus influenzae* MurA by screening a chemical library that consisted of a wide range of bioactive compounds. When MurA was preincubated with these inhibitors, their 50% inhibitory concentrations (IC_{50}s) were found to range from 0.1 to 0.7 μM. In particular, thimerosal suppressed the growth of several different Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* at a concentration range of 1–2 μg/ml. These inhibitors covalently modified the cysteine residue near the active site of MurA. This modification changed the open conformation of MurA to a more closed configuration, which may have prevented the necessary conformational change from occurring during the enzyme reaction.

Keywords: *Haemophilus influenzae*, MurA, cell wall synthesis, inhibitors, Cys117 loop

Bacterial cell walls consist of peptidoglycan layers that protect bacteria from destruction by osmotic pressure, and the biosynthesis pathway of the cell wall serves as a major target for the development of antibacterial agents. Peptidoglycan is a polymer that consists of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid, and the first biosynthetic reaction in the synthesis of peptidoglycan is the transfer of the enolpyruvyl group of PEP to the 3'-hydroxyl group of uridine diphospho-*N*-acetylglucosamine (UNAG), which is catalyzed by UDP-NAG enolpyruvyl transferase (MurA) [7]. MurA is an essential enzyme that is conserved in both Gram-positive and Gram-negative bacteria and has no mammalian homolog. Deletion of the MurA gene in *Escherichia coli* or *Streptococcus pneumoniae* leads to lethal mutants [6, 11]. These properties make MurA an attractive target for the development of novel antibiotics.

Various inhibitors of MurA have been developed. Among them, fosfomycin, which is a broad-spectrum antibiotic produced by *Streptomyces*, was shown to inhibit MurA [14]. Analysis of the detailed mechanism of MurA revealed that fosfomycin covalently attached to a cysteine residue at the active-site loop of MurA and irreversibly inactivated its activity [26]. However, fosfomycin resistance occurs with high frequency *via* overexpression of MurA [13], enzymatic modification of the antibiotic [5], decreased uptake of the antibiotic [1], or substitution of the cysteine near the active-site loop with aspartate [15].

A few novel inhibitors of MurA, other than fosfomycin, have been discovered by various research groups including pharmaceutical companies. A derivative of 5-sulfonoxy-anthranilic acid (T6361) binds to the catalytic loop of *E. cloacae* MurA with a K_i of 16 μM and prevents the transition from the open (unliganded) to the closed (UNAG-liganded) conformation [12]. The Bristol–Myers Squibb compound Cpd1 inhibited *E. coli* MurA with an IC_{50} value of 6 μM [2]. In addition, peptide inhibitors against *E. coli* MurA were also discovered from biopanning of phage display [19]. R. W. Johnson compounds (RWJ-3981, RWJ-140998, and RWJ-110192) showed efficient inhibition against MurA with IC_{50} values between 0.2 and 0.9 μM and minimum inhibitory concentration (MIC) values between 4 and 32 μg/ml against *Staphylococcus aureus*, and these compounds also prevented biosynthesis of nucleic acids and proteins [4]. Although several MurA inhibitors have

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been identified, more potent inhibitory compounds with distinct structures are required for the development of antibiotics that specifically target MurA.

In this study, we identified novel inhibitors by screening a drug library against *Haemophilus influenzae* MurA and examined their inhibitory mechanism. All of the identified compounds covalently modified the cysteine residues at the active-site loop and prevented MurA from undergoing the necessary conformational changes required for functional activity.

Materials and Methods

Materials

NINDS Custom Collection II and Prestwick Chemical Library were purchased from MicroSource Discovery Systems Inc. (Gaylordsville, CT, U.S.A.) and Prestwick Chemicals (Illkirch, France), respectively. Fosfomycin disodium salt, thimerosal, and tetramethylthiuram disulfide (thiram) were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphoenolpyruvate and UDP-*N*-acetylglucosamine were also obtained from Sigma. *M. tuberculosis* H37Rv (ATCC 27294), H37Ra (ATCCC 35835), K-strain, and 5 clinically isolated *M. tuberculosis* strains from tuberculosis patients were obtained from the Culture Collection of Mycobacterial Strains in the Korean Institute of Tuberculosis. K-Strains were identified using RFLP by The Korea Center for Mycobacterial DNA Data Base, Korean Institute of Tuberculosis. Other bacterial strains for MIC determination were obtained from the Culture Collection of Antimicrobial Resistant Microbes at Seoul Women's University (Seoul, Korea).

Expression and Purification of *H. influenzae* MurA

The coding region of *Haemophilus influenzae* MurA (GenBank ID: AAC 22737.1) was cloned into the NdeI and XhoI sites of pET21a to generate an expression vector for MurA, as described previously [27]. The expression vector was transformed into *E. coli* Rosetta2 (DE3), and the recombinant MurA was expressed and purified as previously described [27].

Assay

The activity of MurA was measured as previously described [17]. Briefly, 20 μl of 100 nM *H. influenzae* MurA in 50 mM Tris-HCl, pH 7.8, was mixed with 20 μl of substrate solution containing 200 μM phosphoenolpyruvate (PEP) and 400 μM UDP-*N*-acetylglucosamine (UDP-GlcNAc) and incubated for 30 min at 25°C. The reaction was terminated by adding 200 μl of malachite green reagent containing 0.045% malachite green and 4.2% ammonium molybdate in 4 N HCl, and the amount of phosphate released from the cleavage of PEP was estimated by measuring the optical density at 650 nm.

Screening of *H. influenzae* MurA Inhibitors

To screen the library for MurA inhibitors, 1 μl of dimethylsulfoxide solution containing 10 mM of the compound from a 96-well formatted chemical library was preincubated with 20 μl of 100 nM *H. influenzae* MurA for 20 min at room temperature, and then the residual activity of MurA was measured as described above.

Fluorescence Measurements

Binding of the isolated inhibitors to the lid-like loop region of *H. influenzae* MurA was examined using 8-anilino-1-naphthalene sulfonate (ANS). To measure the fluorescence of the ANS bound to MurA, 50 μM ANS was mixed with 1.5 μM MurA and incubated in 25 mM sodium-potassium phosphate, pH 6.9, for 20 min at 30°C. The effect of UNAG and inhibitors on the fluorescence of MurA-bound ANS was examined using MurA pretreated with 10 μM of each inhibitor or 1 mM UNAG for 20 min at 30°C. The ANS fluorescence was excited at 366 nm and the emission spectra were monitored between 400 and 600 nm using the FluoroSENS fluorimeter (Gilden Photonics Ltd., Glasgow, U.K.).

Determination of the Cysteine Reactivity of the Inhibitors

To determine whether the identified inhibitors were reactive to the sulfhydryl group, each inhibitor was incubated in the absence or presence of dithiothreitol (DTT) in 50 mM Tris-HCl, pH 7.8, at room temperature for 30 min. The inhibitory activities of the inhibitors were then determined as described above. The number of cysteine residues on MurA before and after incubation with the isolated inhibitors was determined by titration of the free thiols using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). MurA was incubated with 400 μM of UNAG for 30 min at room, and then incubated with 20 μM of each isolated inhibitor or 200 μM of fosfomycin for 20 min. After MurA was isolated on a desalting column, the cysteine residues of MurA were titrated with 0.9 mM DTNB in 100 mM sodium phosphate, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) for 40 min at room temperature. The number of cysteine residues was calculated from the absorbance at 412 nm and using an extinction coefficient of 13,600 $cm^{-1} M^{-1}$, as described previously [21].

Determination of Minimum Inhibitory Concentration (MIC) Values of the Inhibitors

The MIC values of the *H. influenzae* MurA inhibitors against Gram-positive and -negative bacteria were determined using the antimicrobial susceptibility test according to the Clinical Laboratory Standards Institute recommendations [10]. The MIC values of the MurA inhibitors against *Streptococcus pyogenes* were measured by a broth dilution method using Mueller Hinton II broth containing 5% lysed horse blood. The MIC values of the inhibitors against the

other bacterial strains were determined by an agar dilution method using a Mueller Hinton I agar plate containing various concentrations of the tested inhibitors. The MIC values of the inhibitors against *Mycobacterium tuberculosis* were measured according to the recommendation by the World Health Organization using Löwenstein–Jensen medium [8, 9].

RESULTS

Novel MurA Inhibitors are Identified

The purified *H. influenzae* MurA was used to screen for potential inhibitors from commercial libraries consisting of bioactive chemicals including drug compounds. In the initial screen of the library, compounds that reduced *H. influenzae* MurA activity by more than 90% at 100 μM were selected for further analysis. The IC_{50} values of these compounds were determined, and three compounds (ebselen, thiram, and thimerosal) whose IC_{50} values were less than 1 μM were identified (Fig. 1). These compounds displayed an anti-MurA activity that was above 90% at a chemical concentration below 2 μM (Fig. 2), and their IC_{50} values were determined to be 0.1 (ebselen), 0.4 (thimerosal), and 0.7 μM (thiram) (Table 1). The IC_{50} value of fosfomycin against *H. influenzae* MurA was determined to be 40 μM, which was 4–5-folds higher than the reported values against *E. coli* MurA [4]. The inhibitory activities of the newly identified compounds against *H. influenzae* MurA were 50–100-folds higher than that of fosfomycin. These compounds share no apparent structural similarity to fosfomycin or previously reported inhibitors of MurA [2, 4, 12].

Thimerosal and Thiram Effectively Prevent the Growth of Several Bacteria

The antibacterial activities of thimerosal and thiram were tested against various bacteria, and the MIC values were obtained by measuring the diameter of the disk diffusion zone on the agar after incubation in the presence of different concentrations of the inhibitors (Table 2). Thimerosal effectively inhibited the growth of Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella oxytoca*, and *Enterobacter cloacae* in the concentration range of 0.5–2 μg/ml. In Gram-positive bacteria, it effectively inhibited the growth of *Staphylococcus aureus* at a concentration of 1 μg/ml, but showed low inhibitory activity against the growth of *Streptococcus pyogenes*. Thiram showed a less potent antimicrobial activity than thimerosal, and it inhibited the growth of both Gram-negative and Gram-positive bacteria

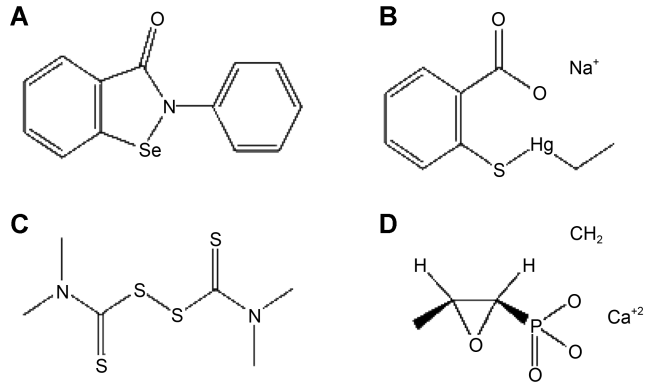

Fig. 1. Structures of MurA inhibitors: Ebselen (**A**), thimerosal (**B**), thiram (**C**), and fosfomycin (**D**).

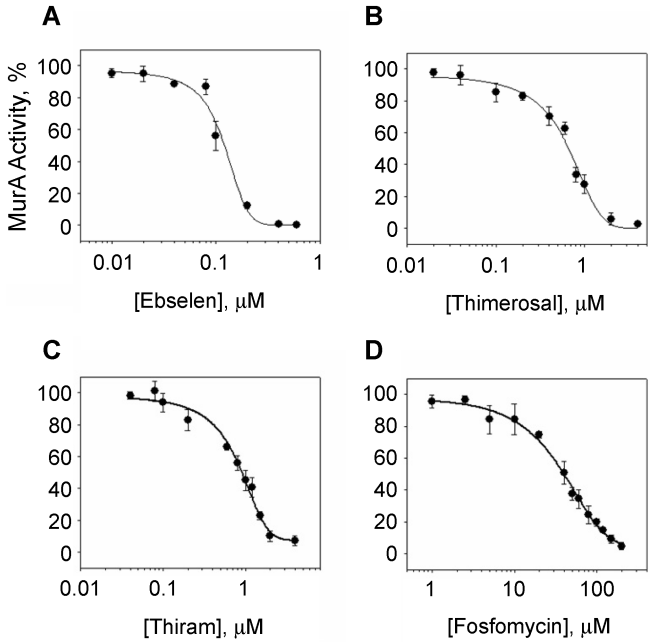

Fig. 2. Concentration dependent inhibitory activity of ebselen (**A**), thimerosal (**B**), thiram (**C**), and fosfomycin (**D**) against *H. influenzae* MurA.

Various concentrations of the compounds were simultaneously incubated with MurA for 30 min at room temperature, and then the residual activity of MurA was measured. The concentrations of *H. influenzae* MurA, PEP, and UNAG were 100 nM, 0.1 mM, and 0.2 mM, respectively. All experiments were performed in triplicate.

Table 1. The IC_{50} of the inhibitor, calculated from the inhibitory curve measured for *H. influenzae* MurA activity at various concentrations of the inhibitor.

Compound	IC_{50} (μM)
Ebselen	0.1
Thimerosal	0.4
Thiram	0.7
Fosfomycin	40

The concentrations of *H. influenzae* MurA, PEP, and UNAG were 100 nM, 0.1 mM, and 0.2 mM in 50 mM Tris-HCl, pH 7.8, respectively.

Table 2. MIC values, obtained by measuring the diameter of the disk diffusion zone on the agar after incubation with a variety of bacteria in the presence of different concentrations of the inhibitors.

No.	Strain Name	MIC (μg/ml))	
		Thimerosal	Thiram
1	*Streptococcus pyogenes* CCARM0206	>128	>128
2	*Streptococcus pyogenes*CCARM0208	>128	>128
3	*Streptococcus pyogenes* CCARM0207	>128	>128
4	*Staphylococcus aureus* CCARM0201	1	1
5	*Staphylococcus aureus* CCARM0204	1	8
6	*Staphylococcus aureus* CCARM0205	1	4
7	*Escherichia coli* CCARM0230	1	32
8	*Escherichia coli* CCARM0237	2	64
9	*Escherichia coli* CCARM0238	1	32
10	*Escherichia coli* CCARM0235	2	64
11	*Escherichia coli* CCARM0236	1	64
12	*Pseudomonas aeruginosa* CCARM0219	2	>128
13	*Pseudomonas aeruginosa* CCARM0223	2	>128
14	*Pseudomonas aeruginosa* CCARM0224	2	>128
15	*Pseudomonas aeruginosa* CCARM0225	0.5	>128
16	*Salmonella typhimurium* CCARM0240	1	64
17	*Klebsiella oxytoca* CCARM0248	1	64
18	*Klebsiella oxytoca* CCARM0249	1	64
19	*Enterobacter cloacae* CCARM0252	2	64
20	*Enterobacter cloacae* CCARM0253	1	64
21	*Mycobacterium. tuberculosis* H37Rv (ATCC 27294)	>40	>40
22	*Mycobacterium. tuberculosis* H37Ra (ATCCC 35835),	>40	>40
23	*Mycobacterium. tuberculosis* K-strain,	>40	>40
24	Five clinically isolated *M. tuberculosis* strains from tuberculosis patients	>40	>40

in the concentration range of 1–64 μg/ml, with the exception of *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. These results indicate that thimerosal has a more potent antibacterial activity than thiram, although the IC_{50} values of the two compounds were within the same range. Ebselen was reported to have antibacterial activity against various strains and was found to efficiently block the growth of *Staphylococcus aureus* or *Bacillus subtilis* with MIC values of 0.2–0.4 μg/ml [20].

The Isolated Inhibitors are Reactive to Sulfhydryl Group

Ebselen, thimerosal, and thiram contain heavy metal element or disulfide bonds (Fig. 1), implying that they could react with sulfhydryl groups. To test whether these compounds can react with the sulfhydryl group, the residual inhibitory activities of these compounds after incubation with 10 mM dithiothreitol (DTT) were examined. When these compounds were incubated for 30 min with DTT, their inhibitory activities against *H. influenzae* MurA were almost completely eliminated (Fig. 3). These results indicate that the identified inhibitors have a strong reactivity toward the sulfhydryl group, and they most likely inhibit MurA through a covalent linkage to surface-exposed cysteine residues.

The Identified Inhibitors Covalent Modify Cysteine Residues of MurA

Fosfomycin, a previously known MurA inhibitor, covalently modified Cys115 of *E. coli* MurA at the active site loop region [16], which is equivalent to Cys117 of the *H. influenzae* enzyme. The inactivation of the identified MurA inhibitors by treatment with DTT suggests that these compounds may covalently modify the cysteine residue at the active-site loop of MurA, as was reported for fosfomycin. To test whether ebselen or thiram could modify cysteine residues, the number of free cysteine residues on MurA was determined by DTNB titration before and after treatment with these compounds. The number of DTNB-accessible -SH groups of MurA was measured as 2.3. This value is reasonable, since the crystal structure of *H. influenzae* MurA showed that only 2 out of 6 cysteines are exposed to the surface, and Cys117 is partially exposed when UNAG binds to the active site of MurA [28]. When MurA was incubated with 200 μM of fosfomycin, the number of free cysteines decreased to 1.8 (Table 3), indicating that the C117 residue, which is exposed to the surface in the closed conformation, is partially modified by fosfomycin. In comparison, the number of free cysteines of

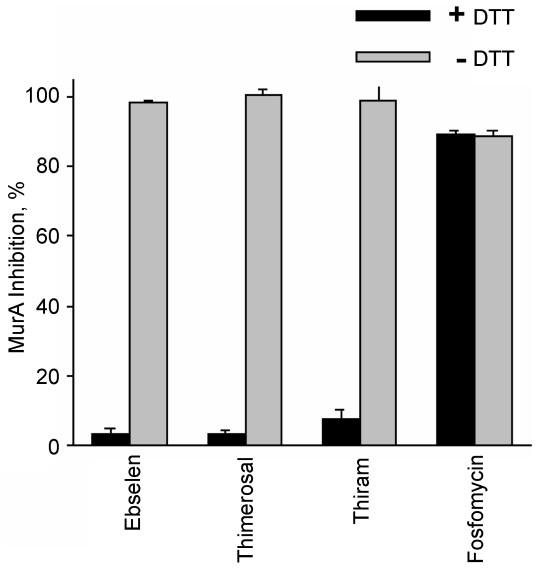

Fig. 3. The reactivities of ebselen, thimerosal, thiram, and fosfomycin toward the sulfhydryl group.
Ebselen, thimerosal, thiram, or fosfomycin were incubated in the presence or absence of 500 μM DTT, and their inhibitory activities against MurA were determined as described in Material and Methods. The concentrations of ebselen, thimerosal, thiram, and fosfomycin in the reaction mixture were 2, 5, 5, and 500 μM respectively. All experiments were performed in triplicate.

MurA treated with 20 μM of ebselen or thiram decreased to 1.8 and 0.9, respectively (Table 3). The effect of thimerosal was not investigated because it interferes with DTNB titration (data not shown). The reduction of DTNB-accessible cysteines indicates that ebselen and thiram could covalently modify C117 of MurA. In particular, thiram showed a higher reactivity to surface-exposed free cysteines than ebselen or fosfomycin.

The Conformation of MurA is Changed by the Identified MurA Inhibitors

The fluorescence enhancement of ANS upon specific binding to MurA was exploited to monitor the conformational change after treatment with the inhibitors. ANS specifically binds to the open conformation of *Enterobacter cloacae* MurA, and the fluorescence of MurA-bound ANS is significantly enhanced as compared with the fluorescence of free ANS, whereas occupation of the active site by UNAG induces a closed conformation of *E. cloacae* MurA, which has a low affinity to ANS [23, 24]. To examine whether the identified compounds affect the conformation of MurA, the fluorescence spectrum of ANS bound to inhibitor-treated MurA was examined. The intensity of the fluorescence spectrum of ANS when bound to MurA was increased by more than 5-fold and there was a shift in the peak maximum from 520 nm to 470 nm compared with the spectrum of free ANS (Fig. 4A, filled circle and inverted triangle). When ANS was mixed with UNAG-treated MurA, the fluorescence intensity of ANS was reduced by 80% (Fig. 4A, open circle), indicating that the binding affinity of ANS to the UNAG–MurA complex was slightly lower compared with the affinity with MurA in the open conformation. It is worth noting that the reduction in fluorescence intensity was less than the intensity change observed for UNAG–*E. cloacae* MurA [28]. The smaller reduction in the ANS intensity is most likely due to the less compact conformation of the UNAG–*H. influenzae* MurA complex compared with UNAG–*E. cloacae* MurA [23]. The fluorescence spectra of ANS in the presence of MurA treated with thimerosal, ebselen, or thiram showed lower intensity than the ANS spectrum with MurA in the open conformation (Fig. 4B, 4C, and 4D), indicating that the modification of MurA by these inhibitors induced a conformational change. In particular, the fluorescence intensity of ANS with thimerosal- or ebselen-treated MurA was lower than that of UNAG–MurA (Fig. 4B, 4C), suggesting that thimerosal or ebselen modifies MurA to a more compact structure than the structure of UNAG–MurA.

DISCUSSION

We have identified three novel inhibitors of *H. influenzae* MurA by screening libraries that consisted of bioactive compounds. These inhibitors have distinct structures from fosfomycin or other MurA inhibitors. Ebselen (Fig. 1A) has a benzoisoselenazolone scaffold, and is known as a selenium-based mimic of glutathione peroxidase and peroxynitrite scavenger [18, 22]. Thimerosal (Fig. 1B) is an ethyl (2-mercaptobenzoato-S) mercury sodium salt that has been used as an antiseptic and antifungal agent in childhood vaccines [3]. Thiram (Fig. 1C) is a 1-(dimethylthiocarbamoyldisulfanyl)-*N*,*N*-dimethyl-methanethioamide, in which two diethyl, dithiocarbamato moieties are connected through a disulfide bond. It had been used as a fungicide, seed disinfectant, and bactericide [25]. Even though these compounds have

Table 3. The number of free cysteines of *H. influenzae* MurA before and after treatment of inhibitors in the presence of UNAG was measured by DTNB titration. All experiments were conducted in triplicate.

			+UNAG		
			Ebselen	Thiram	Fosfomycin
# of Free Cys (±S.D.)	2.3 (±0.1)	2.3 (±0.1)	1.8 (±0.2)	0.9 (±0.1)	1.8 (±0.0)

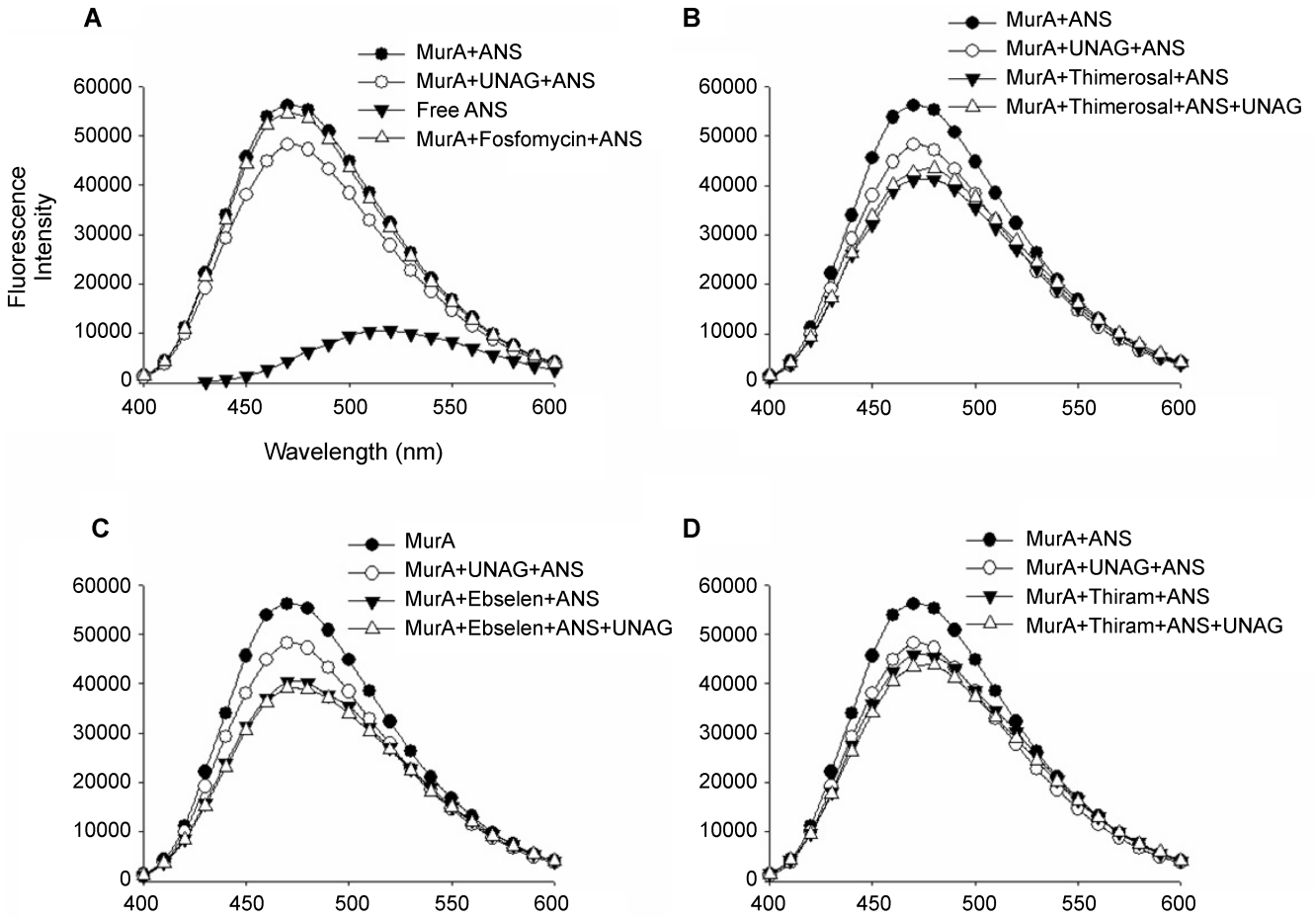

Fig. 4. The conformational change in MurA induced by the compounds.
To measure the fluorescence of ANS bound to *H. influenzae* MurA, 50 μM ANS was mixed with 1.5 μM MurA in 25 mM sodium–potassium phosphate, pH 6.9, and incubated for 20 min at 30°C. The fluorescence of ANS bound to MurA was measured after MurA was treated with 1 mM of fosfomycin (**A**), 10 μM of thimerosal (**B**), ebselen (**C**), thiram (**D**), or 1 mM UNAG for 20 min at 30°C. ANS fluorescence was exited at 366 nm and the emission spectra were monitored between 400 and 600 nm.

been used as antifungal or antiseptic agents, their inhibitory mechanism has not yet been established. The inhibitory activity of these compounds against MurA strongly suggests that the target molecule in the antibacterial activities of ebselen, thimerosal, and thiram is MurA. Thimerosal and thiram effectively inhibited the growth of various Gram-positive and Gram-negative strains with different degrees of susceptibility.

The inhibitory mechanism of the identified compounds was elucidated by examining their effects on the conformation of MurA and their reactivity toward the sulfhydryl group. Structural studies of *H. influenzae* MurA showed that the active site resides in between two domains covered by a lid-like loop, and C117 in this loop has a critical role in recruiting the substrate and releasing the reaction product from the active site. The open or closed conformations of *E. cloacae* or *E. coli* MurA can be observed using the fluorescence probe ANS. When ANS specifically binds near the active site of MurA in the open conformation, it displays a strong increase in fluorescence [23, 24]. Using this method, it was shown that ANS does not bind MurA when it is in the closed conformation (*i.e.*, when MurA forms a complex with UNAG) [24]. The structure of the *H. influenzae* MurA–fosfomycin complex differs from *E. cloacae* or *E. coli* MurA–fosfomycin, in that the *H. influenzae* MurA–fosfomycin complex has a partially or half-closed conformation [28]. The reduced fluorescence intensity of ANS from MurA modified by ebselen, thimerosal, or thiram indicated that these inhibitors transform MurA from an open conformation into a compact structure similar to the UNAG-bound conformation.

Fosfomycin covalently modified the active-site cysteine (Cys115) of *E. coli* and *E. cloacae* MurA in the closed conformation [4, 28]. In contrast, ebselen, thimerosal, and thiram covalently modified the Cys117 of *H. influenzae* MurA in the open conformation, and induced a conformational change to a compact conformation, which may block binding of UNAG at the active site. The reactivity of these

compounds to the sulfhydryl group appears much higher compared with that of fosfomycin. Fosformycin showed a low inhibitory activity (IC_{50}=40 μM) against *H. influenzae* MurA compared with ebselen, thimerosal, or thiram, indicating that the reactivity of the epoxide group in fosfomycin toward the sulfhydryl group is much lower than the reactivity of metal ions or the sulfide group in these inhibitors. However, it should be noted that fosfomycin has a higher selectivity to Cys117 among the other surface-exposed cysteine residues of MurA. Structural studies of the fosfomycin–MurA complex showed that Cys117 is the only cysteine residue that is covalent modified by fosfomycin [16]. This high selectivity of fosfomycin is probably due to the phosphate group that mimics the phosphate group of the substrate, phosphoenol pyruvate. A structural study of MurA bound to ebselen, thimerosal, or thiram will provide detailed information of the binding interface for the design of inhibitors with higher specificity and efficacy.

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