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Note

Structure-Activity Relationship Study of the Bacterial Actin-Like Protein MreB Inhibitors: Effects of Substitution of Benzyl Group in *S*-Benzylisothiourea

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We comprehensively investigated the effects of substitution of the benzyl group in *S*-benzylisothiourea derivatives on antibacterial activity, because we found previously that some substitutions enhanced it. A 2,4-Cl_2-derivative was found to be the most effective compound, it was stronger than the original one in Gram-negative rod shaped-bacteria such as *Escherichia coli* and *Salmonella typhimurium*.

Key words: A22; actin-like protein; benzylisothiourea; MreB; rod-shape determination

To overcome bacterial infectious diseases caused by multi-drug resistant pathogens, it is important to find a new drug that has a new target. For this purpose, we developed a new screening system for inhibitors of bacterial chromosome partitioning.[1)] The assay system is designed to detect production of chromosome-less cells (anucleate cells) caused by inhibition of chromosome partitioning in *E. coli*. Random screening for inhibitors of chromosome partitioning in *E. coli* was done using this assay system, and a novel *S*-benzylisothiourea derivative, *S*-(3,4-dichlorobenzyl) isothiourea, tentatively named A22, was found.[2)] A22 induced spherical cells and spherical anucleate cells in *E. coli*. Genetic analysis revealed that the target molecule of A22 was the actin-like cytoskeletal protein MreB in *Caulobacter crescentus* and *E. coli*.[3,4)] Using A22 as a probe, it was found that the actin-like MreB cytoskeleton was involved in chromosome segregation, at least in *C. crescentus* and *E. coli*. A22 is active only against Gram-negative rods, as tested so far. Since no antibacterial agents targeting bacterial cytoskeletal proteins have been introduced into the chemotherapeutic treatment of bacterial infectious diseases, A22 is expected to be a lead compound in developing new drugs effective against multi-drug resistant pathogens.

Our previous structure-activity relationship study showed that *S*-benzylisothiourea structure is necessary and sufficient to induce spherical cells, and that 3- and/or 4-chloro-substitution of the *S*-benzyl group enhance antibacterial activity.[5)] This led us to expect that modification of the benzyl group in *S*-benzylisothiourea would improve the antibacterial activity as well as the antibacterial spectrum of *S*-benzylisothiourea compounds. In this study, we investigated effects of substitutions of benzyl group in *S*-benzylisothiourea structure on antibacterial activity comprehensively. The results obtained in this study should be a hint for developing new bacterial actin-like protein inhibitors.

Chemical compounds, except for entry **1**, were synthesized by the method reported previously. Briefly, thiourea was suspended in dehydrated ethanol, and corresponding benzyl halide was added to the suspension. The mixture was heated at 130 °C to reflux for several h, and then cooled to room temperature. The reaction mixture was concentrated under vacuum and the residue was diluted with methanol. The product was purified by recrystallization from diethyl ether. The structure of the compound was confirmed by ^{1}H-NMR spectrometry and elemental analysis.

Minimal inhibitory concentrations (MIC) of the compounds for *E. coli* and several other bacteria were determined by the agar-dilution method using Mueller Hinton Broth (Becton Dickinson and Company, MD). The tested bacterial strains were *E. coli* MG1655, *Pseudomonas putida* NBRC14164 (the same as ATCC12633), *S. typhimurium* NBRC13245 (the same as LT2), *Bacillus subtilis* 168, and *Staphylococcus aureus* subsp. *aureus* NBRC15035 (the same as ATCC29213).

To examine the position effect of mono-substitution

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Abbreviations: MIC, minimal inhibitory concentration; PBP, penicillin-binding protein

Table 1. Summary of the Structure-Activity Relationship Study of *S*-Benzylisothiurea Derivatives

	Compounds[a]		MIC (μg/ml)[b]					
	R	X	*E. coli*	*P. putida*	*S. typhimurium*	*B. subtilis*	*S. aureus*	Shape[c]
1	Ph	Cl	100	>100	>100	100	>100	Sphere*
2	2-ClC_6H_4	Cl	12.5	>100	25	>100	>100	Sphere*
3	3-ClC_6H_4	Cl	50	>100	>100	50	>100	Sphere*
4	4-ClC_6H_4	Cl	3.13	>100	3.13	>100	>100	Sphere*
5	2-BrC_6H_4	Br	50	>100	50	>100	>100	Sphere*
6	3-BrC_6H_4	Cl	>100	>100	>100	>100	>100	Sphere**
7	4-BrC_6H_4	Br	3.13	>100	6.25	>100	>100	Sphere*
8	4-FC_6H_4	Cl	12.5	>100	12.5	50	50	Sphere*
9	4-IC_6H_4	Cl	12.5	>100	25	>100	>100	Sphere*
10	4-$NO_2C_6H_4$	Cl	25	>100	25	25	>100	Sphere*
11	4-$CH_3C_6H_4$	Cl	12.5	>100	25	>100	>100	Sphere*
12	4-$CF_3C_6H_4$	Cl	50	>100	>100	>100	>100	Sphere*
13	4-$CH_3OC_6H_4$	Cl	>100	>100	>100	>100	>100	Sphere**
14	4-$C_2H_5C_6H_4$	Cl	>100	>100	>100	>100	>100	Sphere**
15	2,3-$Cl_2C_6H_3$	Cl	25	>100	>100	>100	25	Sphere*
16	2,4-$Cl_2C_6H_3$	Cl	1.56	>100	1.56	>100	>100	Sphere*
17	2,5-$Cl_2C_6H_3$	Br	12.5	>100	25	>100	>100	Sphere*
18	2,6-$Cl_2C_6H_3$	Cl	12.5	>100	25	>100	>100	Sphere*
19	3,4-$Cl_2C_6H_3$ (A22)	Cl	3.13	100	3.13	100	>100	Sphere*
20	3,5-$Cl_2C_6H_3$	Cl	>100	>100	>100	>100	>100	Sphere**
21	2,4,6-$Cl_3C_6H_2$	Cl	100	>100	>100	100	>100	Sphere*

[a]Compounds were hydrochloride or hydrobromide salt.
[b]MICs were determined for *E. coli* MG1655, *P. putida* NBRC14164 (the same as ATCC12633), *S. typhimurium* NBRC13245 (the same as LT2), *B. subtilis* 168, and *S. aureus* subsp. *aureus* NBRC15035 (the same as ATCC29213).
[c]Cell shapes were determined from *E. coli* cultures treated with the compounds at 3× MIC (*) or at 300 μg/ml (**) at 30 °C for 16 h.

of the benzyl group, mono-chloro- and mono-bromo-substitutions at positions 2-, 3- and 4- of the benzyl group were done. All compounds showed activity inducing spherical cells in *E. coli*. Antibacterial activity against *E. coli* decreased in the order 4- > 2- > 3- in both chloro- and bromo-substitutions (Table 1, entries **2**–**7**). A similar tendency was also seen in *S. typhimurium*. These compounds did not show antibacterial activity against *P. putida*, but induced spherical cells at 100 μg/ml (data not shown). This is probably because of multi-drug efflux systems those are able to accommodate a variety of structurally unrelated antimicrobial agents in *P. putida*.[6] It is concluded that the most effective position of the benzyl group in *S*-benzylisothiourea is the 4-site.

To identify effective substituents, several 4-substituted compounds were synthesized and examined (Table 1, entries **4**, **7**–**14**). The most effective substituents were chloro-groups and bromo-groups for *E. coli*, but the bromo group was slightly less effective for *S. typhimurium*. The 4-Fluoro group, which is smaller than the chloro group, was less effective, while the 4-iodo group, which is lager than the bromo group, was also less effective. The order of antibacterial activity of 4-monohalogenized compounds was Cl ≥ Br > F ≥ I. It is suggested that a better molecular size-fitting in the supposed drug-biding pocket of the MreB protein is Cl- or Br-. Substitutions by alkyl groups (CH_3-, C_2H_5-) and several other substituents (CH_3O-, NO_2-, CF_3-) were all less effective.

Next, di- and tri-chloro-substituted compounds were synthesized and examined (Table 1, entries **15**–**21**). In the case of dichloro-substituted compounds, as expected from the results for mono-substituted compounds, 2,4-Cl_2-substituted compounds showed the most effective antibacterial activity against Gram-negative bacteria, and also induced spherical formation (Fig. 1C). Contrary to our expectations, a compound substituted at both *meta*-sites, 3,5-Cl_2-, was less effective than the non-substituted compound. It appears that the mono-*meta*-substituted compound is acceptable to the supposed drug-binding pocket of MreB, but simultaneous substitution in another meta-site prevents docking with MreB. The 2,4,6-Cl_3-substituted compound was less effective.

Most of the compounds tested in this study showed no antibacterial activity against *B. subtilis*, but NO_2- and CF_3-substituted compounds showed anti-*B. subtilis* activity, although weak (the MICs of NO_2- and CF_3- were 25 and 50 μg/ml respectively). However, they showed almost no effect on cell shape (Fig. 1E, and not shown). These compounds also showed antibacterial activity

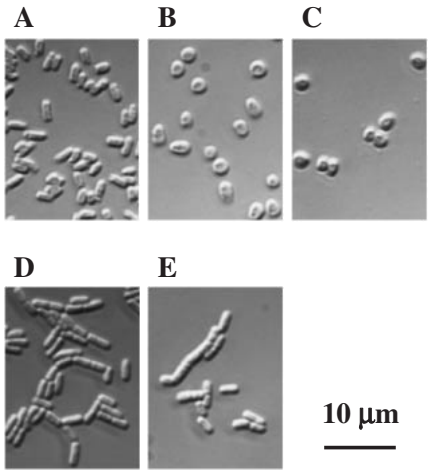

Fig. 1. Effects of *S*-Benzylisothiourea Derivatives on *E. coli* and *B. subtilis* Cell Shape.

E. coli MG1655 (A–C) and *B. subtilis* 168 (D, E) cells growing at 30 °C were treated for 4 h with: A, no treatment: B, 75 μg/ml of **10**: C, 4.58 μg/ml of **16**: D, no treatment: or E, 75 μg/ml of **10**. Differential interference contrast microphotographs are shown. Bar, 10 μm.

against *Staphylococcus aureus*, which does not possess the *mreB* homolog. Therefore, these phenomena were probably due to non-specific toxic effects of these compounds.

We found *S*-(2,4-dichlorobenzyl)isothiourea to be a more effective compound than the original A22 (3,4-Cl_2-). With respect to the antibacterial spectrum, we did not find a compound effective against Gram-positive rod *B. subtilis*. This problem remains to be solved.

It was recently found that the MreB protein is a direct target molecule of A22 by analysis of spontaneous A22-resistant mutants of *C. crescentus* and *E. coli*. MreB protein is known as a bacterial actin-like cytoskeltal protein, and MreB homologs are found in various non-coccal bacteria, such as rod-shaped bacteria, helical bacteria, and hyphal bacteria.[7–9] It is expected that *S*-benzylisothiourea derivatives will prove useful as leading compounds for developing novel antibacterial drugs, and as a molecular probes for analyzing the cellular functions of MreB.

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