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Structure-Activity Relationship of *S*-Benzylisothiourea Derivatives to Induce Spherical Cells in *Escherichia coli*

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We have previously reported that a novel *S*-benzylisothiourea derivative, *S*-(3,4-dichlorobenzyl)isothiourea, tentatively named A22, induced spherical cells in *Escherichia coli*. To elucidate the structural element(s) required for inducing these spherical cells, the biological activity of *S*-benzylisothiourea derivatives and related compounds toward *E. coli* cells was investigated. *S*-(4-Chlorobenzyl)isothiourea revealed spherical cell-inducing activity, although being slightly less potent than A22, and *S*-benzylisothiourea itself showed much less activity. *S*-Cyclohexylmethylisothiourea did not show antibacterial activity and had little effect on the cell shape. *S*-Heptylisothiourea showed antibacterial activity and induced elongated cells rather than spherical cells. Benzylisothiocyanate inhibited cell growth but did not induce spherical cells. *S*-Ethylisothiourea, benzylthiocyanate, benzylisocyanate, and *N*-phenylthiourea did not show any activity under the present experimental conditions. These results indicate that the *S*-benzylisothiourea structure was necessary and sufficient for inducing spherical cells and that 3- and/or 4-chloro-substitution of the *S*-benzyl group enhanced this activity.

Key words: benzylisothiourea; chromosome partitioning; rod-shape determination; spherical cell

The emergence of multi-drug-resistant pathogens has become a serious problem in the chemotherapy of bacterial infectious diseases. One of the strategies to overcome this problem is to find a new drug with a new molecular target. We developed for this purpose a new screening system for inhibitors of chromosome partitioning.[1)] The assay system was designed to detect the production of chromosome-less cells (anucleate cells) resulting from the inhibition of chromosome partitioning in *Escherichia coli*.

Random screening for inhibitors of chromosome partitioning in *E. coli* was carried out by 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*. The spherical cells induced by the treatment with A22 varied in size, and anucleate cells seemed to be more frequent among the smaller cells. These results suggest that loss of the rod shape in *E. coli* led to asymmetric cell division that resulted in the production of anucleate cells.[2)] The β-lactam antibiotic, mecillinam, is also known to induce spherical cells by inhibiting the penicillin-binding protein (PBP) 2 that is involved in side-wall peptidoglycan synthesis.[3–5)] However, an *in vitro* assay of ^{14}C-penicillin G binding has suggested that the target molecule of A22 was not PBP 2.[2)] A22 may act on a rod-shape-determining protein(s) other than PBP 2 such as RodA or MreB. Thus, A22 is expected to be a novel bioprobe for analyzing the shape-determination mechanism of *E. coli* as well as to be a lead compound for developing new antibacterial agents.

We investigated in this study the structure-activity relationship of *S*-benzylisothiourea derivatives and related compounds to define the structural element(s) required for inducing spherical cells in *E. coli*.

Materials and Methods

Anucleate cell blue assay. The anucleate cell blue assay was carried out as reported previously.[1,2)] Briefly, *E. coli* K-12 strain SH3210 (*ΔtrpE5 his λ* pXX747)[6)] was grown in a P medium containing 1% Polypepton and 0.5% NaCl (pH 7). Paper disks (ϕ 8 mm) each soaked with 20 μl of a sample solution were placed on P

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

medium agar plates (1.5% agar) containing 10^4 cells/ml of SH3210 and 40 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. The plates were incubated at 42 °C for 24 h, and the development of a blue color and the growth inhibitory zone around the paper disks were evaluated. The development of a blue color in this assay suggests the production of anucleate cells.

Microscopic observation. E. coli K-12 strain MG1655 (F^- λ^-)[7] was grown in Lennox broth containing 1% Polypepton, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.2). Cells spread on a slide glass were treated with methanol for 5 min and covered with poly-L-lysine. The fixed cells were observed by the differential interference contrast system through an Axioskop 2 microscope (Carl Zeiss Co., Ltd., Oberkochen, Germany). To observe the anucleate cells, cells were stained with a 4′,6-diamidino-2-phenylindole (DAPI) solution (5 μg/ml in saline) and then observed by the fluorescence phase-contrast combined method.[6]

Chemical compounds. *S*-(3,4-Dichlorobenzyl)isothiourea, *S*-(4-chlorobenzyl)isothiourea, *S*-cyclohexylmethylisothiourea and *S*-heptylisothiourea were synthesized as described later. *S*-Benzylisothiourea, benzylthiocyanate and benzylisocyanate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), benzylisothiocyanate from Avocado Research Chemicals Ltd. (Lancs, UK), thiourea, *N*-phenylthiourea and *S*-ethylisothiourea from Sigma-Aldrich Co. (St. Louis, Missouri, USA). 3,4-Dichlorobenzyl chloride, 4-chlorobenzyl chloride, bromomethylcyclohexane and 1-chloroheptane were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Synthesis of the compounds. Thiourea was suspended in dehydrated ethanol, and 3,4-dichlorobenzyl chloride was added to the suspension. The mixture was heated at 130 °C to reflux for several hours and then cooled to room temperature. The reaction mixture was concentrated under vacuum, and the resulting 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. *S*-(4-Chlorobenzyl)isothiourea, *S*-cyclohexylmethylisothiourea and *S*-heptylisothiourea were similarly synthesized by using 4-chlorobenzyl chloride, bromomethylcyclohexane and 1-chloroheptane, respectively, instead of 3,4-dichlorobenzyl chloride.

Other bacterial strains used. Bacillus subtilis 168 was from laboratory stock. *Pseudomonas putida* NBRC14164 (the same as ATCC12633), *Salmonella typhimurium* NBRC13245 (the same as LT2), and *Staphylococcus aureus* subsp. *aureus* NBRC15035 (the same as ATCC29213) were obtained from the National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan).

Determination of minimum inhibitory concentration (MIC). MICs were determined by using a standard 2-fold serial dilution format on Lennox agar plates (1.5% agar).

Results

In order to define the structural element(s) required for the activity to induce spherical cells in *E. coli*, the biological activity of two *S*-benzylisothiourea derivatives and seven related compounds, together with A22 (**1**), was examined in this study (Table 1).

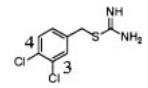
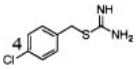
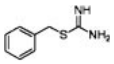
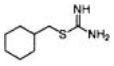
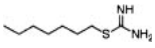
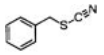
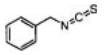
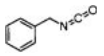
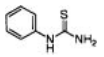
Since **1** had originally been found through screening by the anucleate cell blue assay,[1,2] the effect of each compound was first examined by this assay. The development of a blue color in the anucleate cell blue assay suggests the production of anucleate cells, and the formation of a growth inhibitory zone represents antibacterial activity.

S-(4-Chlorobenzyl)isothiourea (**2**) induced a deep blue zone around a growth inhibitory zone almost comparable to those of **1**. *S*-benzylisothiourea (**3**) also developed a blue zone just around the paper disk, but only a marginal growth inhibitory zone was detected. These indicate that 3- and/or 4-chloro-substitution of the *S*-benzyl group was not absolutely required for the activity to induce a blue zone in this assay, but did enhance the activity. *S*-Cyclohexylmethylisothiourea (**4**) induced neither a growth inhibitory zone nor blue zone in this assay. Interestingly, *S*-heptylisothiourea (**5**) induced a growth inhibitory zone as well as a blue zone, but, as mentioned later, did not induce spherical cells. *S*-Ethylisothiourea (**6**) induced neither a growth inhibitory zone nor blue zone in this assay. These results indicate that the *S*-benzyl group was necessary to show activity for inducing blue zones in this assay (Table 1).

Benzylthiocyanate (**7**) and benzylisocyanate (**9**) induced neither a growth inhibitory zone nor blue zone. Benzylisothiocyanate (**8**) induced a growth inhibitory zone, but no blue zone developed around the growth inhibitory zone in this assay. *N*-Phenylthiourea (**10**) induced neither a growth inhibition nor blue zone. These results suggest that the isothiourea group was also necessary for the activity to induce blue zones. Judging from all these results, the *S*-benzylisothiourea structure was necessary and sufficient for the activity to develop a blue color in this assay (Table 1).

The MIC of each compound for *E. coli* and other several bacteria was determined. **1** showed antibacterial activity toward *E. coli* and *S. typhimurium* at a relatively low concentration (3.13 μg/ml), but was less effective against *P. putida* (100 μg/ml) and Gram-positive bacteria (100 μg/ml for *B. subtilis* and more than 100 μg/ml for *S. aureus*).[2] **2** showed similar antibacterial activity to that of **1** against these bacteria (see Table 1). MIC of **3** was 100 μg/ml for *E. coli* and more than 100 μg/ml for the other bacteria tested. These results concur with those of the anucleate cell blue

Table 1. Summary of the Studies on the Structure-Activity Relationship of *S*-Benzylisothiourea Derivatives

	Compounds[a]	Anucleate cell blue assay (ϕ mm)[b]		MIC (μg/ml)[c]					Shape[d]
		Growth inhibition	Development of blue color	*E. coli*	*P. putida*	*S. typhimurium*	*B. subtilis*	*S. aureus*	
1		18	30	3.13	100	3.13	100	>100	Sphere*
2		17	30	3.13	>100	3.13	>100	>100	Sphere*
3		9	18	100	>100	>100	>100	>100	Sphere*
4		—	—	>100	>100	>100	>100	>100	Rod**
5		12	18	>100	>100	>100	>100	>100	Rod**
6		—	—	>100	>100	>100	>100	>100	Rod**
7		—	—	>100	>100	>100	>100	>100	Rod**
8		18	—	50	>100	100	25	12.5	Rod**
9		—	—	>100	>100	>100	>100	>100	Rod**
10		—	—	>100	>100	>100	>100	>100	Rod**

[a] **1**, A22, *S*-(3,4-dichlorobenzyl)isothiourea; **2**, *S*-(4-chlorobenzyl)isothiourea; **3**, *S*-benzylisothiourea; **4**, *S*-cyclohexylisothiourea; **5**, *S*-heptylisothiourea; **6**, *S*-ethylisothiourea; **7**, benzylthiocyanate; **8**, benzylisothiocyanate; **9**, benzylisocyanate; **10**, *N*-phenylthiourea.
[b] The anucleate cell blue assay was carried out as described in Materials and Methods section. Values represent the diameters of growth inhibitory zones or blue color zones.
[c] 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).
[d] Cell shape was examined for the *E. coli* MG1655 cultures treated with each compound at 3 × MIC (*) or at 300 μg/ml (**) at 30 °C for 16 h.

assay. Although **5** induced a growth inhibitory zone in the anucleate cell blue assay, MIC of **5** was more than 100 μg/ml for *E. coli* and for the other bacteria. The reason for this discrepancy is unclear, but it might have been due to the physical properties of **5** such as the diffusion efficiency of the compound in agar plates. **8** showed relatively strong antibacterial activity, this activity being stronger against Gram-positive bacteria than *E. coli*. This suggests that the mode of action of **8** differed from that of the *S*-benzylisothiourea derivatives. MICs of compounds **4**, **6**, **7**, **9** and **10** were each more than 100 μg/ml against all the bacteria tested (Table 1).

The effect of each compound on *E. coli* cell morphology was next examined. Exponentially growing *E. coli* MG1655 cultures were each treated with a compound at 3 × MIC (for those that showed antibacterial activity) or at 300 μg/ml (for those that did not show antibacterial activity) at 30 °C for 16 hrs. Only the compounds having an *S*-benzylisothiourea structure, **2** and **3** in addition to **1**, induced a spherical cell shape in *E. coli* (Figs. 1b–d). *E. coli* cells treated with **4** seemed to be shorter than corresponding untreated cells, but spherical cells were not induced (Fig. 1e). **5** induced somewhat elongated cells, but not spherical cells (Fig. 1f). This means that the action mechanism of **5** to induce a blue zone in the anucleate cell blue assay was different from that of the *S*-benzylisothiourea derivatives. All other compounds showed little effect on *E. coli* cell morphology (Figs. 1g–k). Spherical anucleate cells were only observed with those compounds having the *S*-benzylisothiourea structure (data not shown). This again concurs well with the results of the anucleate cell blue assay.

Discussion

We isolated the novel *S*-benzylisothiourea compound, A22, through screening by the anucleate cell blue assay.[1,2] A22 induced spherical cells and spherical anucleate cells in *E. coli*.[2] To define the essential structural element(s) required for inducing these phenomena in *E. coli*, the biological activities of two *S*-benzylisothiourea derivatives and seven related compounds, together with A22, were investigated in this study. It was only those compounds having the *S*-benzylisothiourea structure that showed activity to induce spherical cells in *E. coli*.

A22 seemed to be effective against Gram-negative rod-shaped bacteria. In fact, A22 induced spherical cells in *P. putida* and *S. typhimurium* as well as in *E. coli*, but

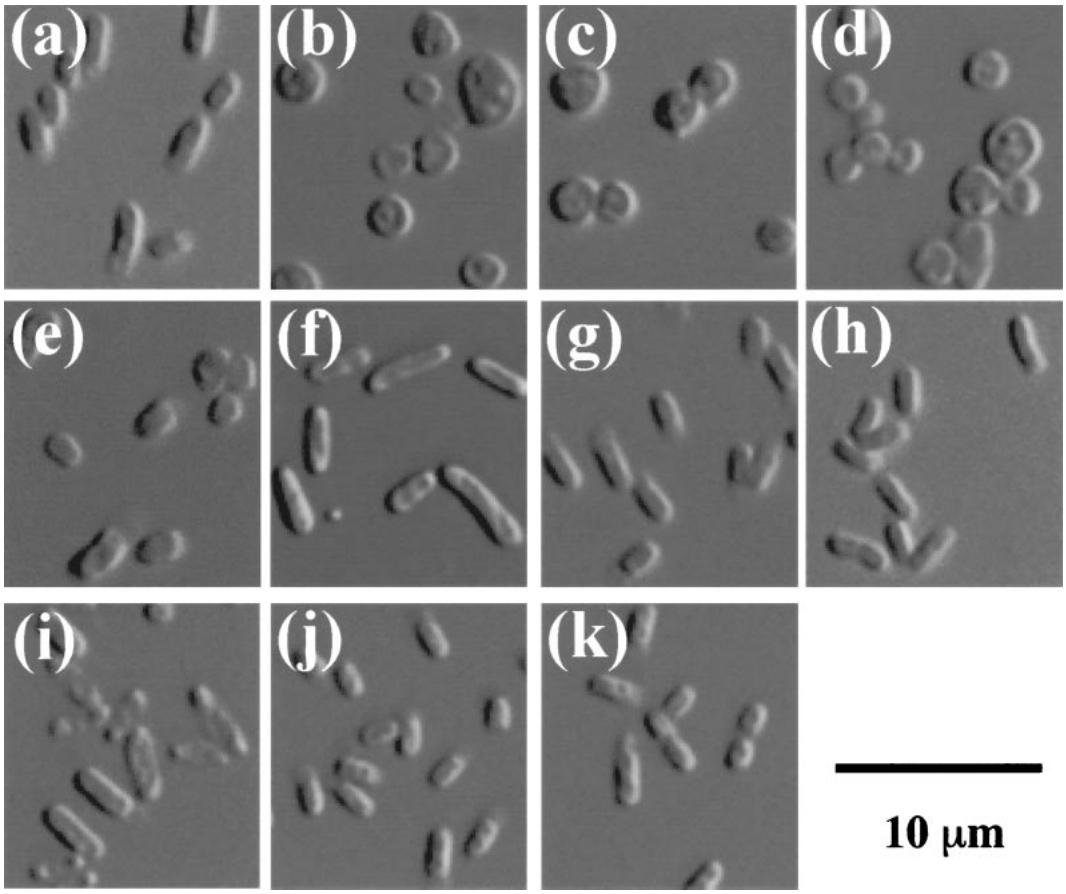


Fig. 1. Effects of *S*-Benzylisothiurea Derivatives on *E. coli* Cell Shape.

Differential interference contrast microphotographs are shown of *E. coli* MG1655 cells after (a) no treatment or treatment with (b) 9.39 μg/ml of **1**, (c) 9.39 μg/ml of **2**, (d) 300 μg/ml of **3**, (e) 300 μg/ml of **4**, (f) 300 μg/ml of **5**, (g) 300 μg/ml of **6**, (h) 300 μg/ml of **7**, (i) 150 μg/ml of **8**, (j) 300 μg/ml of **9** or (k) 300 μg/ml of **10** for 16 h. Bar: 10 μm.

did not in *B. subtilis* (data not shown). **2** also showed similar effects, suggesting that this was a common feature of this series of compounds, probably due to permeability or target specificity.

The activity to induce spherical cells concurred well with that to induce a blue color in the anucleate cell blue assay, suggesting that spherical cell formation was accompanied by anucleate cell production. Anucleate cell production has also been observed in the case of the amidinopenicillin, mecillinam.[2] Mecillinam induced spherical cells in *E. coli* by inhibiting PBP 2.[3–5] The spherical cells induced by these compounds varied in size. Asymmetric cell division has frequently been observed in cultures treated with A22 or mecillinam,[2] suggesting that the loss of a rod shape leads to asymmetric cell division which results in anucleate cell production.

Genetic analyses have so far identified five genes, *pbpA* (a structural gene of PBP 2), *rodA*, *mreB*, *mreC* and *mreD*, that are involved in the process of rod shape formation in *E. coli*.[8–10] Preliminary biochemical and physiological analyses have suggested that the target molecule of A22 was not PBP 2.[2] A22 may therefore act on a rod-shape-determining protein(s) other than PBP2. It has recently been reported that the MreB protein had an actin-like structure and functioned as a cytoskeletal protein in the rod shape-determination mechanism.[11–13] The functions of *rodA*, *mreC* and *mreD* are unknown as yet. A22 and other derivatives inducing spherical cells will be useful tools for analyzing the rod shape-determination mechanism in *E. coli*.

Studies on the structure-activity relationship revealed the essential active structure for inducing spherical cells and spherical anucleate cells in *E. coli* to be *S*-benzylisothiourea. It also seems that 3- and/or 4-chloro-substitution on *S*-benzyl group in *S*-benzylisothiourea enhanced the activity. It will be interesting to examine the effects of other modifications to the *S*-benzyl group. Since no antibacterial agent, except mecillinam, that acts on the rod shape-determination mechanism is known, *S*-benzylisothiourea is an attractive lead compound for developing new antibacterial agents having a new molecular target.

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