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New Class of Bacterial Phenylalanyl-tRNA Synthetase Inhibitors with High Potency and Broad-Spectrum Activity

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Phenylalanyl (Phe)-tRNA synthetase (Phe-RS) is an essential enzyme which catalyzes the transfer of phenylalanine to the Phe-specific transfer RNA ($tRNA^{Phe}$), a key step in protein biosynthesis. Phenyl-thiazolylurea-sulfonamides were identified as a novel class of potent inhibitors of bacterial Phe-RS by high-throughput screening and chemical variation of the screening hit. The compounds inhibit Phe-RS of *Escherichia coli*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, with 50% inhibitory concentrations in the nanomolar range. Enzyme kinetic measurements demonstrated that the compounds bind competitively with respect to the natural substrate Phe. All derivatives are highly selective for the bacterial Phe-RS versus the corresponding mammalian cytoplasmic and human mitochondrial enzymes. Phenyl-thiazolylurea-sulfonamides displayed good in vitro activity against *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* strains, reaching MICs below 1 μg/ml. The antibacterial activity was partly antagonized by increasing concentrations of Phe in the culture broth in accordance with the competitive binding mode. Further evidence that inhibition of $tRNA^{Phe}$ charging is the antibacterial principle of this compound class was obtained by proteome analysis of *Bacillus subtilis*. Here, the phenyl-thiazolylurea-sulfonamides induced a protein pattern indicative of the stringent response. In addition, an *E. coli* strain carrying a *relA* mutation and defective in stringent response was more susceptible than its isogenic $relA^+$ parent strain. In vivo efficacy was investigated in a murine *S. aureus* sepsis model and a *S. pneumoniae* sepsis model in rats. Treatment with the phenyl-thiazolylurea-sulfonamides reduced the bacterial titer in various organs by up to 3 log units, supporting the potential value of Phe-RS as a target in antibacterial therapy.

Resistance to multiple antibiotics is spreading throughout the world, seriously challenging our ability to treat bacterial infections (42). *Staphylococcus aureus*, for instance, is one of the leading causes of threatening nosocomial infections (18), especially since the increasing reliance on vancomycin has led to the emergence of isolates exhibiting intermediate or even high-level glycopeptide resistance (27, 37). A further example is *Streptococcus pneumoniae*, an important pathogen in community-acquired respiratory tract infections that has developed a high degree of β-lactam and macrolide resistance (2). In addition to the requirement for a more judicious use of our current collection of antibiotics, there is a critical need for compounds with no cross-resistance to commonly prescribed antibiotics.

Aminoacyl-RSs (aa-RSs) attracted interest as potential novel targets in bacterial protein synthesis, because they are indispensable for the highly specific translation of the mRNA template into protein via specific transfer RNAs (tRNAs) as adapter molecules (16, 36). In the present study we focused on phenylalanyl (Phe)-tRNA synthetase (Phe-RS), which is responsible for coupling the amino acid Phe to the corresponding Phe-specific tRNA ($tRNA^{Phe}$) (4). The catalytic reaction proceeds in two steps and includes, first, the activation of Phe by adenylation, yielding Phe-AMP as an intermediate, and, subsequently, the transfer of the amino acid to the 3′ end of the cognate $tRNA^{Phe}$ (4). The charged Phe-$tRNA^{Phe}$ is then able to interact with the elongation factor Tu and with the ribosomal A site to elongate the nascent protein chain.

Most organisms possess 20 aa-RSs, one for each amino acid, which are classified into two structurally distinct classes on the basis of typical consensus motifs (39). Phe-RS shows some unique characteristics. First, it is one of the largest aa-RSs known and has an exceptional $\alpha_2\beta_3$ quaternary structure, which appears only in Phe-RS and Gly-RS (28). Furthermore, Phe-RS is the only class II enzyme which attaches the amino acid to the 2′OH group of the tRNA's 3′-terminal ribose (14). Analysis of the crystal structure of Phe-RS alone and in complex with its substrates and a Phe-AMP analog revealed that the α-subunit contains the catalytic center and that the β-subunit is involved in binding of the tRNA (17, 28, 31). The sequences of bacterial Phe-RSs are well conserved (39) but differ significantly from those of their eukaryotic counterparts (32). As Phe-RS is indispensable for growth in all organisms, this suggests an interesting target for antibacterial therapy.

Only one aa-RS inhibitor, the Ile-RS inhibitor mupirocin, is currently marketed as an antibacterial agent (Bactoban). Due to its labile ester bond, the use of mupirocin (also known as pseudomonic acid) (15) has been limited to the treatment of topical infections (3). However, no Phe-RS inhibitor useful as an antibiotic agent against systemic infections has been described so far. Studies from the 1970s report a 50% inhibitory

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concentration (IC_{50}) value for *N*-benzyl-D-amphetamine of 140 nM for the Phe-RS of *Escherichia coli* and 20-fold-lower activity against the corresponding enzyme from rat liver (1, 35). For ochratoxin, the food-contaminating mycotoxin, inhibition of Phe-RS was initially discussed as a possible mechanism of toxicity (23). Subsequent investigators question this interpretation, nevertheless, as the concentration of ochratoxin in *Bacillus subtilis* appears to be too low to significantly compete with phenylalanine for the binding site of Phe-RS (33). For other aa-RS enzymes several inhibitors have been patented and reported in the literature over the years (6, 16, 40), but none of them has been developed as an antibacterial agent so far.

Here we describe the phenyl-thiazolylurea-sulfonamides as a novel class of Phe-RS inhibitors. These compounds inhibit Phe-RSs of gram-positive and gram-negative bacteria, with IC_{50}s in the nanomolar range and high levels of specificity for the bacterial versus the mammalian cytoplasmic and the mitochondrial proteins. In vitro and in vivo data support the potential use of this compound class and the value of Phe-RS as an antibacterial target.

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MATERIALS AND METHODS

Strains and antibiotics. *S. aureus* 133, *S. pneumoniae* G9A, *S. pneumoniae* 1707/4, *Haemophilus influenzae* Spain 7, and *Moraxella catarrhalis* 489 are clinical isolates (purified and identified according to standard procedures) from human infections. *S. aureus* 133 is deposited with the number DSM11832 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *E. coli* CP78 and CP79 are an isogenic pair of laboratory strains (a *relA*$^+$ strain and a strain carrying a mutated *relA* gene, respectively) (12). Phenyl-thiazolylurea-sulfonamides were synthesized at Bayer, and mupirocin was provided by Glaxo-Smith-Kline. All other antibiotics used in this study were obtained from Sigma-Aldrich.

Susceptibility testing. MICs were determined by broth microdilution in synthetic glucose-salt medium with an inoculum of 10^5 CFU/ml. After incubation for 18 h at 37°C, MICs were read as the lowest concentrations of compounds that prevented visible bacterial growth. Streptococci and *Haemophilus* strains were incubated in the presence of 10% CO_2; all other strains were incubated in ambient air. For *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, C-DEN medium similar to the streptococcal medium described previously (24) but without any complex ingredients to adjust the Phe concentration was used. The C-DEN medium contained the following ingredients (per liter): 6.3 g of K_2HPO_4, 0.28 g of KH_2PO_4, 1.2 g of sodium acetate, 2.05 g of NaCl, 0.22 g of $MgCl_2$, 1.9 g of glucose, 0.23 g of sucrose, 0.25 g of pyruvate, 0.09 g of alanine, 0.1 g of arginine, 0.05 g of asparagine, 0.19 g of aspartic acid, 0.04 g of cysteine, 0.57 g of glutamic acid, 0.03 g of glutamine, 0.05 g of glycine, 0.08 g of histidine, 0.2 g of isoleucine, 0.25 g of leucine, 0.23 g of lysine, 0.08 g of methionine, 0.3 g of proline, 0.15 g of serine, 0.12 g of threonine, 0.04 g of tryptophan, 0.02 g of tyrosine, 0.19 g of valine, 2.3 mg of $CaCl_2$, 0.014 mg of $MnSO_4$, 19 mg of adenosine, 19 mg of uridine, 5 mg of choline, 0.02 mg of biotin, 0.6 mg of nicotinic acid, 0.7 mg of pyridoxine HCl, 2.5 mg of calcium pantothenate, 0.7 mg of thiamine HCl, and 0.3 mg of riboflavin. For *S. pneumoniae*, the medium was supplemented with 10 g of choline/liter and 20 mg of yeast extract/liter; for *H. influenzae*, the medium was supplemented with 10 g of IsoVitale X (Becton-Dickinson)/liter and 10 g of hemin/liter. For *S. aureus* and *E. coli*, a somewhat simpler minimal medium with the following ingredients (per liter) was used: 3.3 g of Na_2HPO_4, 1 g of KH_2PO_4, 1 g of NaCl, 0.5 g of NH_4Cl, 0.34 g of $MgSO_4$, 10 g of glucose, 1.2 mg of nicotinic acid, 0.03 mg of thiamine, 0.003 mg of biotin, and 25 μg of each amino acid (except phenylalanine)/ml. For *E. coli* CP78 and CP79, the medium was supplemented with 4 g of brain heart infusion/liter, 4 mg of thiamine/liter, and 25 mg of polymyxin B nonapeptide (Sigma-Aldrich)/liter as permeabilizer of the outer membrane. For MIC testing in the presence of various Phe concentrations, cultures were grown to the exponential growth phase in medium containing 8.3 mg of Phe/liter and then diluted into fresh medium with the desired Phe concentration.

Time-kill study. *S. pneumoniae* G9A was grown in C-DEN medium supplemented with 10 g of choline/liter and 20 mg of yeast extract/liter at 37°C in the presence of 10% CO_2. The medium was inoculated with 3×10^6 CFU/ml, and the number of CFU was monitored for 6 h after the addition of compound. Colony counts were determined by plating culture aliquots on compound-free Columbia blood agar.

Cytotoxicity assay. Chinese hamster ovary cells (5×10^4 cells/ml) were cultured in 200 μl of RPMI 1640 medium with glutamic acid (Gibco)–10% fetal calf serum in 96-well microtiter plates for 24 h at 37°C and 5% CO_2. Cells were then incubated for 24 h in fresh medium supplemented with the test compounds in a serial twofold-dilution series. After removal of the compounds, cells were grown in fresh medium for a further 48 h. Subsequently, cells were stained by adding 80 μl of a solution of 4′,6-diamidino-2-phenylindole-dihydrochloride (Sigma-Aldrich) (2.5 μg/ml in water) and incubated for 1 additional hour at 37°C and 5% CO_2. Cell viability was determined by fluorescence measurement at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

Isolation of Phe-RS proteins. Cultures of *S. aureus* 133 were grown in Luria broth supplemented with 0.2% (wt/vol) glucose. *S. pneumoniae* G9A and *H. influenzae* Spain 7 were cultured in C-DEN medium and incubated in the presence of 10% CO_2. For preparations of subcellular fractions, cultures were grown at 37°C up to an optical density at 578 nm of 0.5. Cells were harvested by centrifugation (5,000 × *g*, 7 min, 4°C), washed three times in 50 ml of preparation buffer (10 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 30 mM NH_4Cl, 4 mM 2-mercaptoethanol) and centrifuged again (8,000 × *g*, 10 min, 4°C). Cells were resuspended in 1 ml of preparation buffer per gram (wet weight). Phenylmethanesulfonyl fluoride (0.5 mM) was added to avoid protein degradation. Cells were disrupted by four French Press Cell (Sim Aminco SLM Instruments Inc.) passages at 14,000 lb/in^2. The cell lysate was precipitated by centrifugation (8,000 × *g*, 10 min, 4°C). The supernatant was subjected to centrifugation (9,000 × *g*, 30 min, 4°C) followed by a second centrifugation for 18 h at 3,000 × *g*. The supernatant was decanted and dialyzed three times for 1 h with 1 liter of preparation buffer. All enzymes were shock frozen in liquid nitrogen and stored at −80°C. *E. coli* protein purified to homogeneity was obtained from M. Ibba (Ohio State University, Columbus). Human mitochondrial Phe-RS was isolated according to Bullard et al. (8), and rabbit reticulocyte lysate was purchased from Promega.

Phe-RS inhibition studies. The Phe-tRNA aminoacylation reaction was determined by ethanol precipitation of ^{14}C-labeled $tRNA^{Phe}$ (9, 29). The assay was performed by incubation of [^{14}C]Phe with cell extracts in 180 mM HEPES (pH 7.6)–20 mM magnesium acetate–10 mM glutathione–7.5 mM ATP–0.2 mM CTP–0.1 U of $tRNA^{E.coli}$ (Sigma-Aldrich)–1% dimethyl sulfoxide in a final volume of 75 μl for 20 min at room temperature. The reaction product ([^{14}C]Phe-tRNA) was separated from the [^{14}C]Phe by precipitation with 200 μl of ethanol followed by 30 min of incubation at 4°C and subsequent filtration through a GF/C 96-well plate (Packard). Filter-bound radioactivity was detected with a scintillation counter. [^{14}C]Ile was used instead of [^{14}C]Phe for control experiments with mupirocin. IC_{50}s correspond to the concentration at which half of the enzyme activity is inhibited by the compound.

Proteome analysis. *B. subtilis* 168 was grown in Belitzky minimal medium in the presence of compound 1 at concentrations of 0.25 and 0.5 μg/ml, representing 0.5 and 1 times the MIC, respectively. At 10 and 20 min after the addition of the antibiotic, samples were pulse labeled with [^{35}S]methionine. Growth and labeling conditions, as well as the procedure for two-dimensional polyacrylamide gel electrophoresis and data analysis, were described previously (5).

Systemic infection. For the *S. aureus* 133 sepsis procedure, an exponentially growing culture was diluted in 5% mucin–0.9% NaCl to the final challenge dose of 10^6 bacteria per mouse. Female CFW1 mice were infected by injecting the bacterial suspension into the peritoneal cavity and were treated once intravenously (i.v.) 0.5 h after infection. Organs were removed aseptically 3 h after infection and homogenized, and the bacterial titer was determined by plating. One group of mice was fed on a phenylalanine-free diet (FSS333905; Ssniff, Soest, Germany) for 5 days prior to infection, reducing the phenylalanine concentration in the plasma to one-fifth of the normal level (i.e., that seen with mice on a normal diet) (FSS333906; Ssniff). Determination of the Phe level in the murine plasma was performed according to the method of Liu (25). For the *S. pneumoniae* 1707/4 sepsis investigations, young Wistar rats were infected intra-

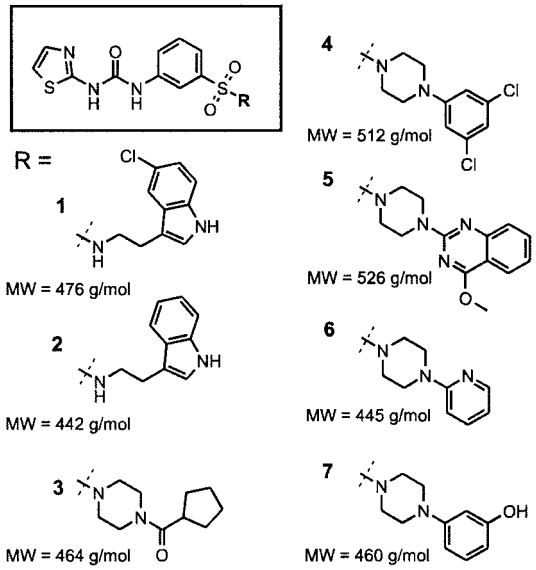

FIG. 1. Chemical structures of the phenyl-thiazolylurea-sulfonamides discussed in this study.

peritoneally (i.p.) with a challenge dose of 3×10^5 bacteria per animal and were treated twice i.p. 0.5 and 3 h after infection. The bacterial load in the organs was determined 6 h after infection.

RESULTS

Compound discovery and enzymatic activity against the target. High-throughput screening of our compound library identified several inhibitors of the *E. coli* Phe-RS enzyme. During the lead optimization process, the compounds were tested for inhibition of Phe-RS derived from different gram-negative pathogens (*E. coli* and *H. influenzae*) as well as from gram-positive pathogens (*S. aureus* and *S. pneumoniae*). Chemical optimization of initially detected compounds resulted in the phenyl-thiazolylurea-sulfonamides as strong Phe-RS inhibitors (Fig. 1). These compounds demonstrated strong activity against Phe-RS derived from gram-negative organisms with IC_{50}s in the nanomolar range (Table 1). For example, compound 7 showed IC_{50}s of <5 and 10 nM with the enzymes from *E. coli* and *H. influenzae*, respectively. With the Phe-RS of gram-positive pathogens, these compounds were slightly less potent. For instance, the IC_{50} values of the most potent compounds (compounds 4 and 5) with Phe-RS derived from *S. pneumoniae* were 50 nM and 40 nM, respectively (Table 1). Exemplary congeners were also tested with Phe-RS in cell extracts of *S. aureus*. Compounds 2 and 3 yielded IC_{50}s of 50 and 80 nM, respectively.

Selectivity. Both the mammalian cytoplasmic Phe-RS and the mitochondrial Phe-RS were investigated independently to determine selectivity. To analyze the effect on the cytoplasmic Phe-RS, we used a rabbit reticulocyte lysate (Table 2). No inhibition was detected at compound concentrations of up to 200 μM. In the case of purified human-mitochondrial Phe-RS, inhibition was observed only at high phenyl-thiazolylurea-sulfonamide concentrations with IC_{50}s of ≥10 μM (Table 2). In addition, when compounds 2, 5, and 6 were analyzed in a cytotoxicity assay with Chinese hamster ovary cells, IC_{50}s were above the highest concentrations tested (160 μg/ml).

TABLE 1. Inhibition of different bacterial Phe-RS enzymes by the phenyl-thiazolylurea-sulfonamides

Enzyme and compound or drug	IC_{50} (μM)[a]		
	E. coli	*H. influenzae*[b]	*S. pneumoniae*[b]
Phe-RS			
1	0.007	0.02	0.2
2	0.008	0.008	0.2
3	0.015	0.015	0.3
4	0.015	0.015	0.05
5	0.025	0.06	0.04
6	0.02	0.02	0.2
7	<0.005	0.01	0.3
Ile-RS			
Mupirocin	0.0014[b]	0.0014	0.0014

[a] IC_{50}s were determined for Phe-RS derived from different gram-negative and gram-positive pathogens. Inhibition of Ile-RS by mupirocin was measured in the presence of [^{14}C]Ile instead of [^{14}C]Phe; the results are included for comparison.
[b] Values are the results of investigations performed using cell extracts.

Phe-RS binding mode. In enzyme kinetic measurements with purified *E. coli* Phe-RS, the phenyl-thiazolylurea-sulfonamides inhibited the aminoacylation of $tRNA^{Phe}$ competitively with respect to the substrate Phe (Fig. 2A). The Michaelis-Menten constant (K_m = 1.9 μM) with Phe in the concentration range from 1 to 16 μM was close to the previously reported value (K_m = 1.5 μM) (21). We measured the inhibitory constant K_i of compound 2 as a representative of the phenyl-thiazolylurea-sulfonamides. The K_i value (21 nM) was 2 orders of magnitude lower than the K_m value of Phe. In contrast, noncompetitive binding was observed with respect to the second substrate, ATP. Increasing ATP concentrations did not reverse the inhibitory effect of compound 2 (Fig. 2B).

Antibacterial activity. The phenyl-thiazolylurea-sulfonamides demonstrated significant antibacterial activity (Table 3). For example, compound 4 in a synthetic medium without Phe possessed submicromolar MICs for *S. aureus* (MIC = 0.4 μg/ml), *S. pneumoniae* (MIC = 0.8 μg/ml), *H. influenzae* (MIC = 0.4 μg/ml), and *M. catarrhalis* (MIC = 0.4 μg/ml). The antibacterial activity was partly antagonized by the addition of Phe to the culture broth. MICs of all compounds tested in the presence of 50 μM Phe increased by a factor of 2 to 4 for *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. For *S. aureus*,

TABLE 2. Selectivity of the phenyl-thiazolylurea-sulfonamides

Compound	IC_{50} (μM)[a]		
	E. coli Phe-RS	Mammalian cytoplasmic Phe-RS	Human mitochondrial Phe-RS
1	0.007	>200	60
2	0.008	>200	>200
3	0.015	>200	>200
4	0.015	>200	25
5	0.025	>200	10
6	0.02	>200	90
7	<0.005	>200	90

[a] Values shown indicate concentrations of 50% inhibition of *E. coli* Phe-RS compared to those of inhibition of mammalian cytoplasmic Phe-RS (rabbit reticulocyte lysate) and human mitochondrial Phe-RS.

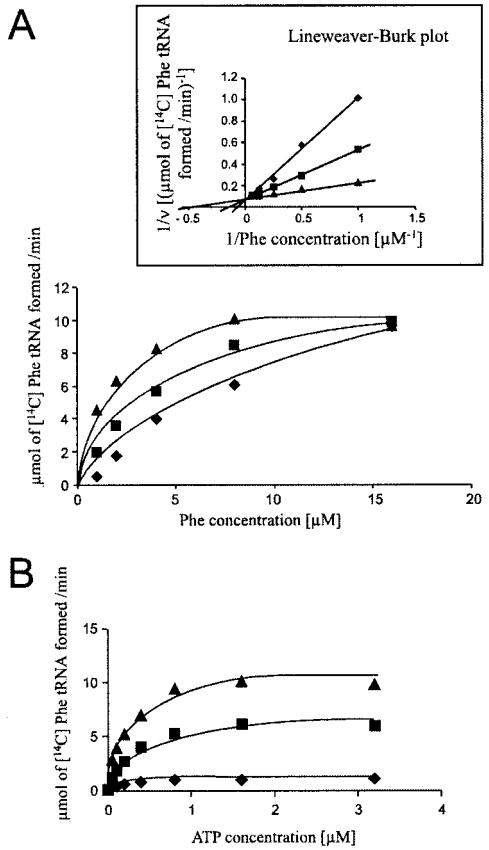

FIG. 2. Inhibition of *E. coli* Phe-RS by compound 2. The rate of Phe-tRNA formation was determined at room temperature over 20 min. (A) Competitive binding with respect to Phe. Compound 2 concentrations were as follows: 0 μM (▲), 0.04 μM (■), and 0.156 μM (◆). The inset shows results in the form of a Lineweaver-Burk plot (K_m for Phe = 1.9 μM; K_i for compound 2 = 21 nM). (B) Noncompetitive binding with respect to ATP. Compound 2 concentrations were as follows: 0 μM (▲), 0.01 μM (■), and 0.04 μM (◆).

this antagonism was more pronounced, with an ~8-fold increase in MIC (Table 3). The effect of increasing the Phe concentrations in the culture medium on the antibacterial activity of the phenyl-thiazolylurea-sulfonamides was investigated in more detail (Fig. 3). The addition of up to 250 μM Phe antagonized the activity against the gram-negative pathogens *H. influenzae* and *M. catarrhalis* and the gram-positive bacterium *S. pneumoniae* only slightly, but a stronger effect was observed for *S. aureus*, especially at Phe concentrations above 50 μM (e.g., for compounds 4 and 5, MIC = 3 μg/ml at 50 μM Phe and MIC = 100 μg/ml at 100 μM Phe). Time-kill studies with *S. pneumoniae* demonstrated that the phenyl-thiazolylurea-sulfonamides act by a bacteriostatic mechanism (Fig. 4).

The MIC increase seen in the presence of extracellular Phe already suggested that the antibacterial mechanism of the phenyl-thiazolylurea-sulfonamides is based on inhibition of Phe-RS. To confirm the interference with this enzyme inside of the intact bacterial cell, we performed proteome analysis of *B. subtilis* after treatment with compound 1. Cytoplasmic proteins synthesized by the bacterium in response to the presence of the phenyl-thiazolylurea-sulfonamide were analyzed by two-dimensional polyacrylamide gel electrophoresis, and the results were compared with the proteome pattern of an untreated control (Table 4). The proteome signature of compound 1 included the induction of many proteins previously identified during norvaline (11) or mupirocin (5) treatment of *B. subtilis*, e.g., Ald, MinD, Spo0A, SpoVG, YurP, and YvyD. These proteins are known to be positively controlled by the stringent response in this organism (11). In addition, even the direct target, the α-subunit of Phe-RS (PheS), was induced in phenyl-thiazolylurea-sulfonamide-treated cells. This is in contrast to the results seen with mupirocin, which (as expected) did not induce PheS but did induce the corresponding IleS and additional proteins of isoleucine-valine biosynthesis (5). In a further experiment, an *E. coli* strain carrying a *relA* mutation was used to investigate the effect of the stringent response on the activity of our compounds (Table 5). Mupirocin and all phenyl-thiazolylurea-sulfonamides were more potent with the relaxed strain (the *relA* mutant; strain CP79) incapable of the induction of the stringent response (12) than with the corresponding isogenic wild-type strain (*relA*$^+$; strain CP78). For example, MICs of compound 3 with strains CP79 and CP78 were 0.003 and 0.2 μg/ml, respectively. This effect was even more pronounced for our Phe-RS inhibitors than for the Ile-RS inhibitor. In contrast, other protein synthesis inhibitors that did not induce the stringent response showed equal activities against

TABLE 3. Antimicrobial activity of selected phenyl-thiazolylurea-sulfonamides against major respiratory tract pathogens

Compound or drug	MIC (μg/ml)[a]							
	S. aureus 133		*S. pneumoniae* G9A		*H. influenzae* Spain 7		*M. catarrhalis* 489	
	−Phe	+50 μM Phe	−Phe	+50 μM Phe	−Phe	+50 μM Phe	−Phe	+50 μM Phe
1	0.39	3.13	6.25	6.25	0.39	1.56	0.39	1.56
2	1.56	12.5	12.5	12.5	0.39	1.56	1.56	3.13
3	0.78	25	12.5	12.5	0.39	1.56	3.13	6.25
4	0.39	3.13	0.78	1.56	0.39	0.78	0.39	0.78
5	0.78	3.13	0.39	0.78	0.39	0.78	0.39	0.78
6	1.56	12.5	6.25	12.5	0.2	0.78	0.78	1.56
7	3.13	25	6.25	12.5	0.2	0.78	0.39	1.56
Mupirocin	0.1	0.1	0.1	0.1	0.024	0.024	1.56	1.56
Chloramphenicol	6.25	6.25	6.25	6.25	0.78	0.78	0.78	0.78
Methicillin	NT	NT	0.39	0.39	6.25	6.25	6.25	6.25

[a] MICs were determined in a synthetic medium either without Phe or supplemented with 50 μM Phe. NT, not tested. For comparison with the IC_{50} data presented above, the molecular mass values were in the range of 500 g/mol (Fig. 1). Thus, 1 μg/ml corresponds to approximately 2 μM.

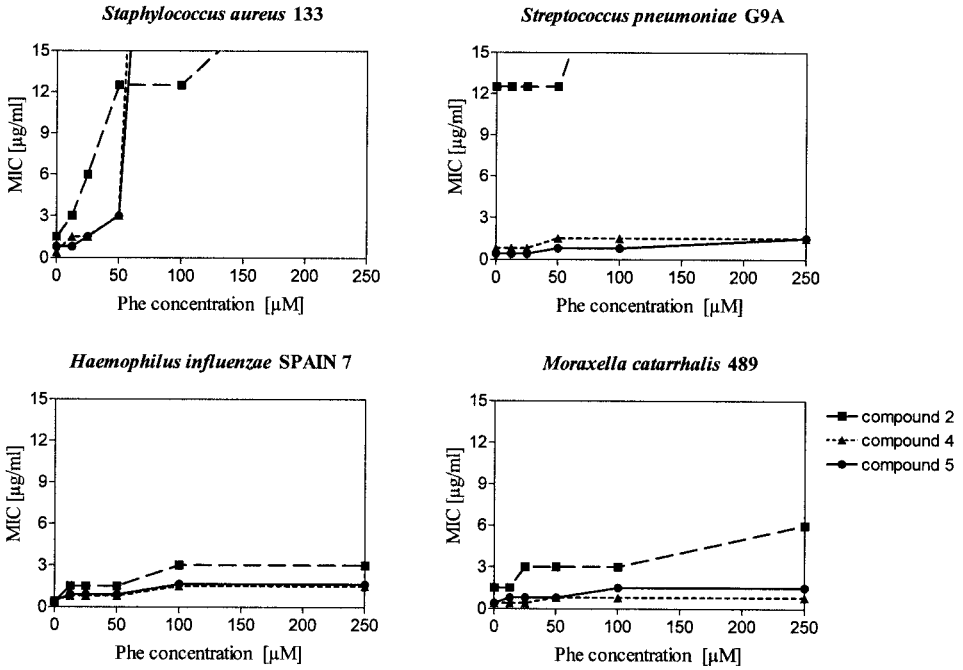

FIG. 3. Effect of increasing Phe concentrations in the culture broth on the antibacterial activity of the phenyl-thiazolylurea-sulfonamides in vitro.

both strains (Table 5). These results prove the nature of the anticipated mechanism of action. By inhibition of Phe-RS, the phenyl-thiazolylurea-sulfonamides lead to the accumulation of uncharged $tRNA^{Phe}$, which in turn triggers the induction of the stringent response.

In vivo potency. To evaluate the influence of the Phe concentration in the blood on the activity of the phenyl-thiazolylurea-sulfonamides, mice were fed on a Phe-free diet and the concentration of free Phe in the plasma was monitored. The Phe level decreased from 70 µM at day 0 to 15 µM at day 5. When compound 2 (i.v. treatment with 100 mg/kg of body weight) was investigated in the murine *S. aureus* sepsis model with mice fed on a Phe-free diet for 5 days prior to infection, the bacterial titer in liver and spleen of these mice was significantly lower than the level seen with untreated control animals (Fig. 5A). A smaller decrease in CFU numbers was observed for regularly fed mice with a normal Phe blood level (70 µM), which is in accordance with the strong Phe antagonism against *S. aureus* observed with compound 2 in vitro.

Less-pronounced Phe antagonism was predicted from the in vitro data for the phenyl-thiazolylurea-sulfonamides and the pathogen *S. pneumoniae* (Table 3 and Fig. 3). Thus, we employed an *S. pneumoniae* sepsis model for further in vivo studies. In addition, we used a rat sepsis model (instead of a murine sepsis model) to circumvent the exceptionally high level of affinity of the phenyl-thiazolylurea-sulfonamides for mouse serum albumin. (MICs were lower in medium containing rat serum albumin than in medium containing mouse serum albumin and human serum albumin; data not shown.) When compound 4 was tested in rats with Phe blood levels of 63 µM and fed with regular food, after i.p. treatment with 100 mg/kg at 0.5 and 3 h after infection a decrease in CFU of 2 to 3 $\log_{10}$ in the lung, kidney, and spleen was observed (Fig. 5B).

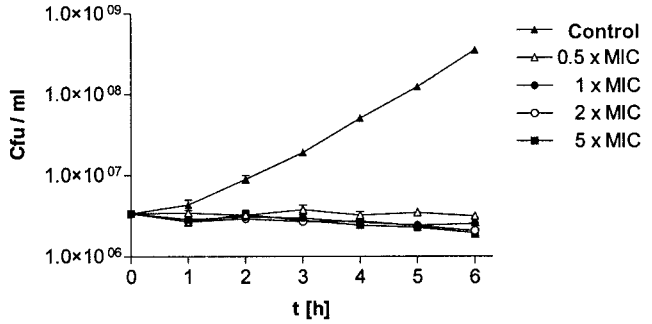

FIG. 4. Time-kill study. Effect of increasing concentrations of compound 5 on the number of CFU of *S. pneumoniae* G9A.

DISCUSSION

aa-RSs are essential proteins in all living organisms (7). When one aa-RS is inhibited, the corresponding tRNA is not charged and is therefore unavailable for translation. This leads to protein synthesis inhibition, which in turn causes cell growth arrest. Consequently, each compound that inhibits any of the aa-RSs could be a potential antibacterial agent. We selected Phe-RS as a target, because the structures of the bacterial Phe-RSs are well conserved (39) but differ significantly from their eukaryotic counterparts (32). Phe-RS adopts a $\alpha_2\beta_3$ tetrameric structure in all prokaryotes and eukaryotic cytoplasmic sources known (13), and both subunits are required for aminoacylation of the tRNA. An exception to the tetrameric structures is the mitochondrial Phe-RS (34). The human mitochon-

TABLE 4. Proteome analysis of *B. subtilis*[a]

Protein[b]	Translational induction factor[c]		Function and/or similarity[d]
	0.5× MIC, 20 min	1× MIC, 10 min	
Ald	2.4	2.4	L-Alanine dehydrogenase
Dps	3.1	3.2	Stress- and starvation-induced gene controlled by sigmaB
FbaA	1.4	4.4	Fructose-1,6-bisphosphate aldolase
MinD	2.8	3.6	Cell-division inhibitor (septum placement)
PheS	2.3	1.7	Phenylalanyl-tRNA synthetase (alpha subunit)
Spo0A	3.2	2.4	Two-component response regulator central for the initiation of sporulation
SpoVG	1.8	3.7	Required for spore cortex synthesis
Tpx	1.8	3.7	Probable thiol peroxidase
YceE	2.7	>10	Unknown; similar to tellurium resistance protein
YocJ	2.4	2.7	Unknown; similar to acyl-carrier protein phosphodiesterase
YodC	1.4	2.4	Unknown; similar to nitroreductase
YtxH	1.9	>10	Unknown; similar to general stress protein
YurL	2.1	2.3	Unknown; similar to ribokinase
YurP[e]	10.8	8.6	Unknown; similar to glutamine-fructose-6-phosphate transaminase
YvyD	1.9	>10	Unknown; similar to sigma-54 modulating factor of gram-negative bacteria

[a] Cytoplasmic proteins produced by *B. subtilis* in response to treatment with compound 1 were separated by two-dimensional gel electrophoresis in the pI range of 4 to 7.

[b] Proteins that were induced more than twofold in both experimental series and that accumulated to amounts sufficient for determination of their identity by peptide mass fingerprinting are listed.

[c] Induction factors represent the ratio of protein synthesis results for the antibiotic-treated versus the control culture. Experiments were performed in duplicate. During each experimental series, two treatment conditions (0.5 times the MIC for 20 min and 1 times the MIC for 10 min) were analyzed. The induction factors correspond to the results of one representative experiment.

[d] Functional annotation according to data from the SubtiList web server (http://genolist.pasteur.fr/SubtiList/).

[e] In the antibiotic-treated as well as the control culture, YurP migrated in several spots on the gels, possibly due to protein modifications. The amount of all YurP spots increased in the presence of the antibiotic. The numbers refer to the YurP spot with the highest induction factor.

drial Phe-RS enzyme is a small protein that is active as a monomer (8).

The aminoacylation reaction catalyzed by *E. coli* Phe-RS was used to screen our compound library. After initial hit identification, lead optimization yielded very potent phenyl-thiazolylurea-sulfonamides with IC_{50}s for *E. coli* and *H. influenzae* proteins in the low nanomolar range. IC_{50}s for Phe-RS derived from gram-positive bacteria (*S. aureus* and *S. pneumoniae*) were higher but still submicromolar. For comparison, mupirocin has IC_{50}s of 1.4 to 3 nM against Ile-RS derived from this panel of bacteria. Further investigation of the binding mode of the phenyl-thiazolylurea-sulfonamides to the *E. coli* enzyme demonstrated that these compounds bind competitively with respect to Phe. The inhibitory constant of compound 2 was 100-fold lower than the Michaelis-Menten constant (K_i = 21 nM; K_m = 1.8 μM), demonstrating that the phenyl-thiazolylurea-sulfonamides are potent inhibitors of the Phe-RS enzyme.

TABLE 5. Effect of the phenyl-thiazolylurea-sulfonamides on the susceptibility of a *relA*$^+$ strain and its isogenic strain carrying a *relA* mutation

Compound or drug	MIC (μg/ml)	
	E. coli CP78	*E. coli* CP79
1	0.78	0.05
2	0.39	0.1
3	0.2	0.003
4	0.39	0.05
5	0.78	0.2
6	0.2	0.012
7	0.2	0.006
Mupirocin	0.2	0.05
Chloramphenicol	0.1	0.1
Kanamycin	0.05	0.05
Erythromycin	0.003	0.003
Tetracycline	0.05	0.05
Rifampicin	0.0008	0.0008
Cerulenin	0.05	0.05

[a] MICs were compared for the *relA*$^+$ strain *E. coli* CP78, which is able to induce the stringent response cascade, versus the isogenic *relA* CP79, which is not. The culture broth was supplemented with polymyxin B nonapeptide to facilitate passage of the compounds across the outer membrane. In the absence of polymyxin B nonapeptide, no activity was detected for the phenyl-thiazolylurea-sulfonamides.

That we detected competitive inhibitors by our high-throughput screening might have been a consequence of the screening format employed (incorporation of radiolabeled Phe into ethanol-precipitable Phe-tRNA). This format dictated the use of limiting amounts of labeled Phe; consequently, the assay was relatively sensitive with respect to inhibitors which bind to the Phe-RS in a region that overlaps the Phe binding site. To circumvent potential selectivity issues with other ATP binding proteins, an excess of ATP was used in the high-throughput screening to minimize the number of compounds that bind to the ATP binding pocket of Phe-RS. Indeed, competition experiments demonstrated that ATP and phenyl-thiazolylurea-sulfonamides do not compete for the same binding site. The hypothesis that the phenyl-thiazolylurea-sulfonamides target the Phe binding site on the Phe-RS but not the ATP pocket was further strengthened by the results of experiments with a mutated *E. coli* Phe-RS enzyme in which Ala_{294} was mutated to Gly (20, 22). This mutated protein binds para-chloro-Phe and para-bromo-Phe as substrates, whereas the wild-type protein does not bind these bulky Phe analogues. The authors speculate that Ala_{294} is part of the Phe binding pocket and that the mutation enlarges the Phe binding cavity, a hypothesis which is supported by crystallographic data (26, 28). We used this mutant protein to test compounds in which the thiazolyl group was replaced by enlarged ring systems. A dramatic de-

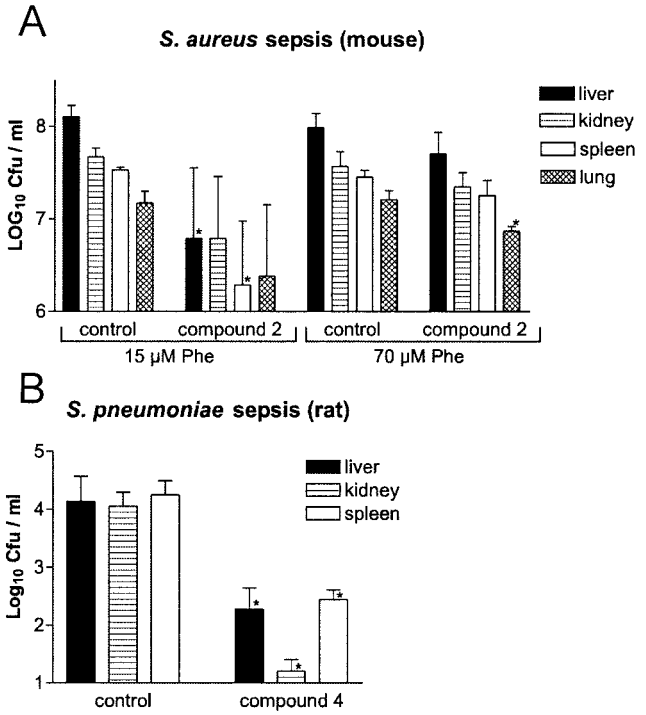

FIG. 5. In vivo activity of the phenyl-thiazolylurea-sulfonamides. (A) *S. aureus* 133 sepsis in the mouse. Bacterial loads in the organs after i.v. treatment with 100 mg of compound 2/kg of body weight at 0.5 h after infection are indicated. Antibacterial activity was compared in mice on normal or phenylalanine-free diets, resulting in blood phenylalanine concentrations of 70 or 15 μM, respectively. (B) *S. pneumoniae* 1707/4 sepsis in regularly fed rats without Phe limitation and with normal Phe blood levels (63 μM). Bacterial titers in the organs after i.p. treatment with 100 mg of compound 4/kg at 0.5 and 3 h after infection are indicated. Five animals were employed per group. The results of statistical analyses performed using the Mann-Whitney test were calculated using GraphPad Prism software (version 3.02). *, statistically significant ($P < 0.05$).

crease in potency, with IC_{50}s in the millimolar range, was observed for piperazine and pyrimidine derivatives tested with the wild-type protein. The decrease in potency was less pronounced with the mutant protein, with IC_{50}s still in the submicromolar range (data not shown).

The phenyl-thiazolylurea-sulfonamides demonstrated high levels of selectivity for the bacterial Phe-RS proteins compared with the results seen with the mammalian counterparts. Human-mitochondrial Phe-RS was inhibited only at high concentrations, with selectivity indices ranging from 400 to >25,000 compared to the results seen with *E. coli* enzyme. The mammalian cytoplasmic Phe-RS was not inhibited at compound concentrations of up to 200 μM.

The growth of a broad spectrum of bacteria, including gram-positive and gram-negative pathogens, was prevented by the presence of the phenyl-thiazolylurea-sulfonamides. Drug MICs below 1 μg/ml were measured for *Staphylococcus*, *Streptococcus*, *Haemophilus*, and *Moraxella* strains in synthetic medium in the absence of Phe. In accordance with the competitive binding mode, the antibacterial activity was partially antagonized by the addition of Phe to the culture broth. The physiological Phe concentration in human blood ranges from 38 to 115 μM (10), with an average of 48 μM (30, 38). In the presence of physiological Phe concentrations, MICs of the phenyl-thiazolylurea-sulfonamides increased marginally for *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*; however, stronger antagonism was seen against *S. aureus*. Increases in MICs were also observed for mupirocin and the corresponding amino acid Ile (19, 41). For instance, Wilson and coworkers reported substantial antagonism after the addition of 1 mM Ile in investigations using the *S. aureus* strain Russel (MIC without Ile in the medium, 0.06 μg/ml; MIC in the presence of 1 mM Ile, 2 μg/ml) (41).

To assess the value of Phe-RS as a potential antibacterial target, it was important to validate that the observed antibacterial effect of the phenyl-thiazolylurea-sulfonamides was indeed mediated by the inhibition of Phe-RS activity in the intact bacterium. The MIC increase in the presence of external Phe, the induction of the stringent response in gram-positive bacteria, and the susceptibility increase of a gram-negative *relA* mutant demonstrate that inhibition of Phe-RS is the basis of the promising antibacterial in vitro activity. To evaluate in vivo efficacy, the activity of the compounds was investigated in two different infection models. The compounds showed activity in an *S. aureus* sepsis model with mice having reduced Phe blood levels and remarkable potency in an *S. pneumoniae* sepsis model in rats with physiological Phe concentrations.

In summary, the phenyl-thiazolylurea-sulfonamides are the most potent Phe-RS inhibitors described to date. They demonstrate good in vitro activity against a broad spectrum of bacterial pathogens and a high level of selectivity with respect to the mammalian Phe-RS enzymes. The potential use of the phenyl-thiazolylurea-sulfonamides as antibacterial agents and the value of Phe-RS as a target in antibacterial therapy are supported by the in vivo activity of this class of compounds.

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