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Carba-sugars Activate the glmS-Riboswitch of *Staphylococcus aureus*

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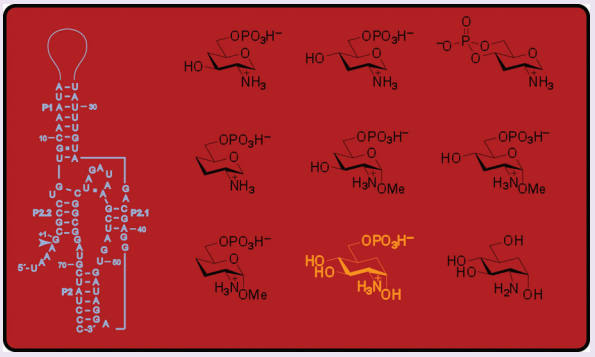
Supporting Information

ABSTRACT: The glmS-riboswitch is unique among riboswitch families as it represents a metabolite-dependent ribozyme that undergoes self-cleavage upon recognition of glucosamin-6-phosphate. The glmS-riboswitch is located in the 5′-untranslated region of bacterial genes involved in cell wall biosynthesis. Therefore, this riboswitch represents a promising target for developing new antibiotics. We describe the metabolite-dependent glmS-riboswitch of pathologically relevant and vancomycin-resistant *Staphylococcus aureus* and the discovery and synthesis of a carba-sugar with potency similar to that of the native metabolite glucosamine-6-phosphate in modulating riboswitch activity. This compound represents a valuable lead structure for the development of antibiotics with a novel mode of action.



Riboswitches are RNA elements found in the 5′-UTR of bacterial mRNA molecules. Upon binding to metabolites they undergo conformational changes that, in turn, result in the modulation of gene expression.[1−3] Up to 4% of all bacterial genes appear to be under control of riboswitch entities, and therefore this regulatory mechanism represents a fundamental way to control bacterial metabolism.[4] With exception of the *thi*-box riboswitch that has been described to control alternative splicing in *Arabidopsis thaliana*, such RNA elements have not yet been identified in eukaryotes.[5,6] Moreover, some riboswitches control the expression of essential genes necessary for bacterial survival.[7] These findings predestine riboswitches as potential targets for the development of antibiotics with a novel mode of action. In this way, several endeavors have been undertaken aiming at the identification of artificial molecules that modulate riboswitch function, documented by the development of either HTS-based screening assays or the synthesis of metabolite analogues.[8−12] Until now, however, only a few examples of molecules are known that have been strategically designed to modulate riboswitch function. Those are mainly based on purine analogues presumably acting on guanine-responsive riboswitches.[13−15]

Here, we describe the design, synthesis, and characterization of compounds that activate the glmS-riboswitch of the vancomycin-resistant *Staphylococcus aureus* strain Mu50. GlmS-riboswitches are RNA elements found in the 5′-UTR of the glmS mRNA in Gram-positive bacteria.[16] Upon binding to glucosamine-6-phosphate (GlcN6P), the riboswitch undergoes activation of a self-cleavage pathway that ultimately results in the destabilization of the mRNA (Figure 1).[17−20] In this process, GlcN6P is assumed to be directly involved in catalysis rather than functioning as an allosteric effector.[21−24] The *GLMS* gene product glutamine-fructose-6-phosphate amidotransferase is involved in early steps of the bacterial cell wall biosynthesis where it catalyzes the reaction of fructose 6-phosphate with glutamine yielding GlcN6P.[25] GlcN6P, in turn, is an important building block of the bacterial cell wall, and reduced expression levels of the transferase result in the formation of unstable cell walls and, thus, limited bacterial growth and turnover.[25] Therefore, the glmS-riboswitch is an attractive target for the development of novel antibiotics.

Although the existence of the glmS-riboswitch in *Staphylococci* was postulated on the basis of *in silico* homology data,[26] it has, to the best of our knowledge, not yet been experimentally validated. For that reason we synthesized the full-length 246 nt riboswitch emanating from genomic DNA, using PCR followed by *in vitro* transcription and RNA purification (Supporting Information). The resultant RNA was radioactively labeled, and GlcN6P-dependent self-cleavage was investigated similarly as previously described.[9] Albeit the glmS-riboswitch from *S. aureus* bears an insertion of 50 nucleotides after P1 (Figure 1), it has characteristics comparable to those of the homologous riboswitch found in *B. subtilis* (Table 1 and Supplementary Figure 1). The main observed differences during *in vitro* self-cleavage concerned the requirement of Mg^{2+}-ion concentrations that appeared to be optimal at 9 mM compared to 5 mM for the *B. subtilis* glmS-riboswitch (Table 1).[16] We also found that, in contrast to the *B. subtilis* glmS-riboswitch, Co^{2+}-ions cannot replace Mg^{2+}-ions during *S. aureus* glmS-riboswitch self-cleavage (Table 1 and

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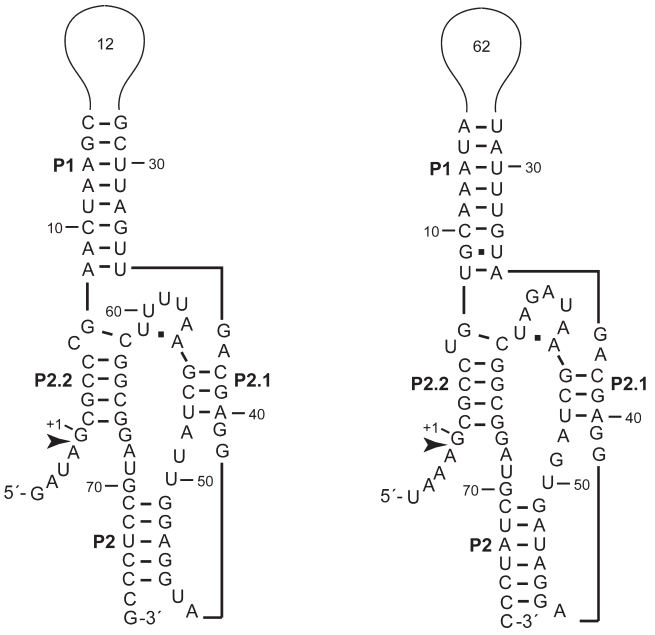

Figure 1. Secondary structure of the minimal catalytic core (P1 to P2) of the glmS-riboswitches of *B. subtilis* (left) and *S. aureus* (right). The arrowheads indicate self-cleavage sites after activation with glucosamine-6-phosphate.

Table 1. Comparison of *in Vitro* Self-Cleavage Parameters of *S. aureus* and *B. subtilis*[16] glmS-Riboswitches

parameter	*S. aureus* Mu50	*B. subtilis*
optimal pH	7	7
optimal Mg^{2+} conc.	9 mM	5 mM
divalent cations accepted	Mg^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}	Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Sr^{2+}
activating metabolites	GlcN6P, GlcN, GlcN6S, GlcNAc6P	GlcN6P, GlcN, GlcN6S, GlcNAc6P
cleavage temp	37 °C	37 °C

Supplementary Figure 1).[16] Having shown that the *S. aureus* glmS-riboswitch has a very similar behavior as its homologues from other Gram-positive bacteria, we started to search for novel compounds that could replace GlcN6P in riboswitch activation.

We previously described a fluorescence polarization-based high-throughput-compatible screening assay to monitor GlcN6P-dependent glmS-riboswitch self-cleavage of the minimal riboswitch from *B. subtilis* (Figure 1).[9] Using this assay, we performed screening of a diverse library of small molecules with more than 5000 drug-like compounds. This library was assembled to include mainly compounds that follow the Lipinski's rule of five. Interestingly, these efforts did not yield any compounds that activate the glmS-riboswitch. It has been demonstrated for other riboswitches that metabolite analogues can be effective in riboswitch activation.[7,11] Thus, we simultaneously started an alternative approach in which we designed and synthesized the novel GlcN6P analogues **1**−**9** (Figure 2A and Supporting Information). These analogues were subsequently investigated for their ability to induce self-cleavage of the 5′-^{32}P-labeled glmS-riboswitch.

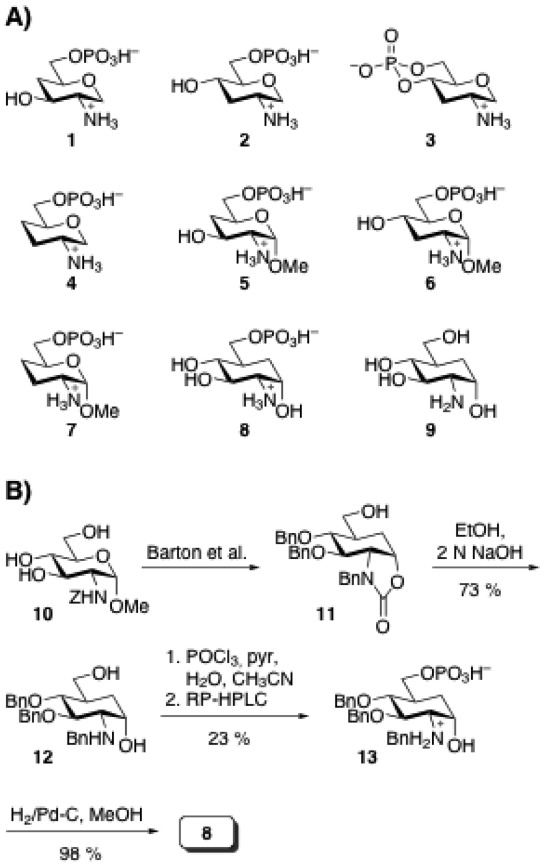

Figure 2. (A) Glucosamine-6-phosphate analogues designed, synthesized, and characterized in this study. (B) Synthesis scheme of carba-sugar **8**.

1-Deoxyglucosamine-6-phosphate has been shown previously to activate the glmS-riboswitch from *B. cereus*.[27] Emanating from this finding we designed compounds **1**−**4** as well as the methyl glycosides **5**−**7**. These allow investigation of the importance of the C-3 and C-4 hydroxy groups, in regard to *S. aureus* glmS-riboswitch activation. Additionally, we synthesized carba-sugars **8** and **9** that make it possible to address the role of the ring oxygen for riboswitch activation. The synthesis of carba-sugar **8** is depicted in Figure 2B. Methyl glycoside **10** was converted to carba-cycle **11** as described by Barton et al.[28] Basic cleavage of the cyclic carbamate gave diol **12** that could be selectively phosphorylated at the primary OH group to yield **13**. Catalytic hydrogenation gave carba-sugar **8**. The derivatives of 1-deoxy-GlcN6P lacking one or two further equatorial hydroxy groups are shown to not be active any more (Figure 3A). Similarly, variants with a cyclic phosphate or a methyl glycoside functionality are also not effective in inducing glmS-riboswitch hydrolysis. In contrast, compound **8** (carba-GlcN6P) was shown to induce glmS-riboswitch self-cleavage very efficiently (Figure 3A). While the overall cleavage rates of the *S. aureus* glmS-riboswitch were found to be slower than those reported for glmS-riboswitches from other bacteria (Table 2),[16] the activation by compound **8** was less pronounced in the glmS-riboswitch of *B. subtilis* (Supplementary Figure 2). The rate constants (k_{obs}) of metabolite-induced cleavage of the *S. aureus* glmS-riboswitch were found to be comparable for GlcN6P and compound **8** (Table 2). These data indicate that the potential of compound **8** for riboswitch

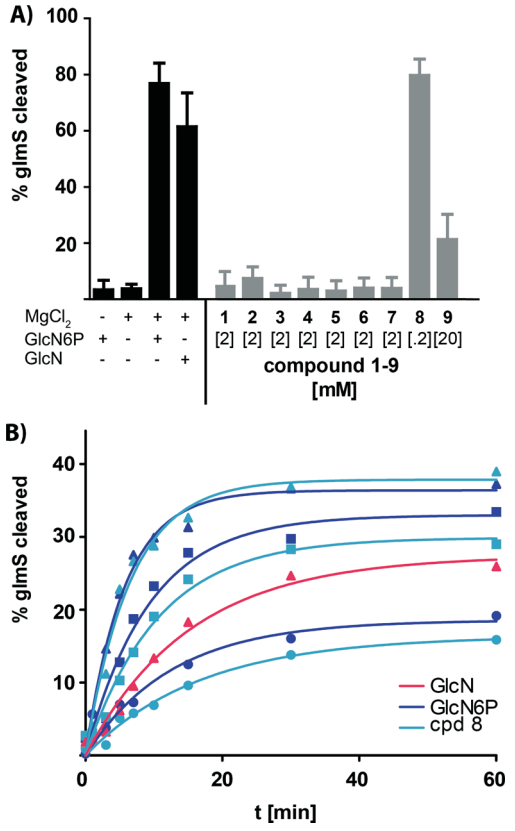

Figure 3. Performance of glucosamine-6-phosphate and derivatives in the metabolite-dependent glmS-riboswitch cleavage assay. (A) Cleavage of the glmS-riboswitch induced by GlcN6P and GlcN [0.2 mM] (black bars) compared to a panel of compounds **1**−**9** [concentration as indicated] (gray bars). (B) Cleavage rates of the glmS-riboswitch at different concentrations [circles 2 μM; squares 20 μM; triangles 200 μM] of GlcN6P (blue), compound **8** (cyan), and GlcN (red). Plots of the fraction cleaved as a function of time are shown. Experiments were carried out in triplicate. Error bars omitted from the graphs for clearer representation. For error bars please refer to Supplementary Figure 3A.

activation is very similar to that of the natural metabolite (Figure 3B). This finding is also reflected by concentration dependent analysis of riboswitch activation that revealed an EC_{50} value of 6.24 ± 0.66 μM of compound **8** (carba-GlcN6P) compared to 3.61 ± 0.39 μM found for GlcN6P (Supplementary Figure 3B). Glucosamine (GlcN) has been shown previously to activate the *B. subtilis* glmS-riboswitch.[9,16] This could also be observed with the riboswitch from *S. aureus*, and an EC_{50} value of 189 ± 22 μM was determined (Supplementary Figure 3B). In contrast, compound **9** (carba-GlcN) induced glmS-riboswitch self-cleavage only at very high concentrations (Figure 3A).

In summary, we provide for the first time biochemical data on the glmS-riboswitch from a vancomycin-resistant *S. aureus* strain. It is shown that this glmS-riboswitch behaves very similar to homologues from other Gram-positive bacteria. However, it has a higher intrinsic requirement of Mg^{2+}-ions and is less tolerant toward bivalent cation replacement *in vitro*. Employing novel synthetic GlcN6P analogues, we probed the ligand-binding specificity of this riboswitch and could demonstrate that it is very sensitive regarding modification of GlcN6P. Precisely, the data point out that more than a single modification dramatically diminishes glmS-riboswitch activation. We discovered a novel

Table 2. Rate Constants (k_{obs}) of *S. aureus* glmS-Riboswitch Cleavage Induced by GlcN6P or Compound 8 (carba-GlcN6P)

	S. aureus, k_{obs} [min^{-1}]	
concentration [μM]	GlcN6P	compound **8**
200	0.177	0.153
20	0.107	0.095
2	0.068	0.060

carba-analogue of GlcN6P that is able to activate the metabolite-dependent glmS-riboswitch from *S. aureus* with almost the same potency as does the native metabolite. These findings are in accordance with recent cocrystal structures of cleavage-inhibited glmS-riboswitches and GlcN6P from *B. anthracis* and *T. tengcongensis*. In these, the ring oxygen and the phosphate group are shown to be required for stabilization of the interaction of the glmS-riboswitch with GlcN6P rather than being involved in the catalysis reaction.[22,29] Destabilization of these interactions as reflected by the carba-analogue of GlcN6P is tolerated to some extend. However, more pronounced modifications, obviously, lead to a significant loss of affinity and thus activity.

Since the glmS-riboswitch is involved in the regulation of early steps of cell wall biosynthesis[25] and interference with cell wall biosynthesis represents a valuable strategy to inhibit bacterial growth, this compound or a prodrug thereof[30] serves as important lead structure for developing novel antibiotics.

METHODS

Synthesis and Preparation of RNA. PCR amplification of *S. aureus* glmS-riboswitch from genomic DNA was accomplished by Pfu DNA-polymerase (annealing temperature 60 °C, 30 cycles) and 5′ primers containing the T7 promoter. After phenol/chloroform extraction and NaOAc precipitation, the PCR product was used for *in vitro* transcription with T7 RNA polymerase (37 °C, o/n). The transcription product was treated with DNase and purified by PAGE followed by gel extraction and precipitation. This procedure was followed by RNA dephosphorylation using calf intestine alkaline phosphatase (CIAP, Promega). Finally, glmS-RNA was radioactively labeled by phosphorylation at its 5′ end using the T4 polynucleotide kinase (PNK, NEB) and γ-^{32}P-ATP (10 mCi mL^{-1} NEN, Zaventem, Belgium). The reaction mixture was prepared on ice; incubation occurred at 37 °C for 30 min. The product was desalted using a G25 column (GE Healthcare) that had been equilibrated with DEPC-treated H_2O.

Metabolite-Induced Self-Cleavage Assay. The radioactively labeled glmS-riboswitch was incubated in the presence of 10 mM $MgCl_2$, 50 mM HEPES (pH 7.5), 200 mM KCl with or without metabolite (e.g., glucosamine-6-phosphate) for 30 min at 37 °C. The reaction was stopped by adding PAGE loading buffer, followed by separation on 17% denaturing polyacrylamide gel for analysis. Detection took place *via* autoradiography on a phosphorimager FLA 3000; data evaluation was done using AIDA and GraphPad Prism software. Rate constants for ribozyme cleavage were determined using trace amounts of ^{32}P-labeled RNA incubated at 37 °C using standard conditions (10 mM $MgCl_2$, 50 mM HEPES (pH 7.5), 200 mM KCl) and indicated concentrations of GlcN6P, GlcN, or compound **8**. Aliquots were withdrawn at various time points, and the reaction was terminated by the addition of PAGE loading buffer (95% formamide, 10 mM EDTA, 0.1% (v/v) xylene cyanol and 0.1% (v/v) bromophenol blue). The products were analyzed using denaturing polyacrylamide gel electrophoresis (PAGE), and the rate constants for RNA cleavage were determined by plotting the

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fraction cleaved against time. Curves were fitted according to pseudofirst order association kinetics.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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