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Phosphatase-Inert Glucosamine 6-Phosphate Mimics Serve as Actuators of the glmS Riboswitch

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ABSTRACT: The glmS riboswitch is unique among gene-regulating riboswitches and catalytic RNAs. This is because its own metabolite, glucosamine-6-phosphate (GlcN6P), binds to the riboswitch and catalytically participates in the RNA self-cleavage reaction, thereby providing a novel negative feedback mechanism. Given that a number of pathogens harbor the glmS riboswitch, artificial actuators of this potential RNA target are of great interest. Structural/kinetic studies point to the 2-amino and 6-phosphate ester functionalities of GlcN6P as being crucial for this actuation. As a first step toward developing artificial actuators, we have synthesized a series of nine GlcN6P analogs bearing phosphatase-inert surrogates in place of the natural phosphate ester. Self-cleavage assays with the Bacillus cereus glmS riboswitch give a broad SAR. Two analogs display significant activity, namely, the 6-deoxy-6-phosphonomethyl analog (5) and the 6-O-malonyl ether (13). Kinetic profiles show a 22-fold and a 27-fold higher catalytic efficiency, respectively, for these analogs vs glucosamine (GlcN). Given their nonhydrolyzable phosphate surrogate functionalities, these analogs are arguably the most robust artificial glmS riboswitch actuators yet reported. Interestingly, the malonyl ether (13, extra O atom) is much more effective than the simple malonate (17), and the “sterically true” phosphonate (5) is far superior to the chain-truncated (7) or chain-extended (11) analogs, suggesting that positioning via Mg coordination is important for activity. Docking results are consistent with this view. Indeed, the viability of the phosphonate and 6-O-malonate ether mimics of GlcN6P points to a potential new strategy for artificial actuation of the glmS riboswitch in a biological setting, wherein phosphatase-resistance is paramount.

Riboswitches are found in noncoding regions of mRNAs, and gene expression is modulated when a metabolite binds directly to the RNA. Many riboswitches, once liganded, repress expression of associated or adjacent genes involved in the synthesis of the metabolite, providing an efficient feedback mechanism of genetic control.1,2 The glmS riboswitch resides upstream of the glmS gene in B. subtilis and in a number of other Gram-positive bacteria.3 It is an essential gene that encodes for the GlmS enzyme, glucosamine 6-phosphate synthase, which uses fructose-6-phosphate (Fru6P) and glutamine to generate glucosamine-6-phosphate (GlcN6P).4 This reaction is the first committed step in the pathway that produces UDP-N-acetylg glucosamine, key for bacterial cell wall biosynthesis.5,6 The glmS riboswitch is found in high profile bacterial targets, including B. anthracis, Clostridium difficile, and Staphylococcus aureus, motivating efforts to develop artificial actuators.6–8 Indeed, structure—activity relationship (SAR) studies of other riboswitches and how they interact with their cognate metabolites have enabled rational design of artificial agonists and ultimately antibiotics.8,9

The glmS riboswitch consists of four paired regions P1–P4 that exhibit a high level of sequence conservation, particularly within the catalytic core where nucleotide identities are >95% conserved (Figure 1).3,10 The glmS riboswitch is mechanistically unique as it is the only known riboswitch for which catalytic activity provides the basis of genetic regulation, and it is the only known ribozyme that depends upon a “coenzyme” or actuator, namely its metabolite GlcN6P, for self-cleavage.11,12 The glmS riboswitch/ribozyme selectively binds GlcN6P, thereby accelerating the cleavage reaction by at least 6 orders of magnitude.3,12 This results in the release of a large downstream fragment, including the coding region of the mRNA that is degraded by RNase J1.13 In the presence of GlcN6P but not UDP-N-acetylg glucosamine, the final product of this pathway, the intracellular concentration of the GlmS enzyme, is decreased, but expression is not completely inhibited.4

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The picture that has emerged is one in which the 2-amino group of GlcN6P serves as both a general base and a general acid, a mechanistic postulate that is well preceded in both related protein\textsuperscript{23,26} and nucleic acid chemistry.\textsuperscript{27,28} This proposal aligns nicely with Raman difference crystallography studies\textsuperscript{29} and computational simulations,\textsuperscript{30} indicating that upon riboswitch binding, the pK\textsubscript{a} of the GlcN6P amino group is lowered to align with the optimal reaction pK\textsubscript{a}.

Beyond this, specific sugar hydroxyl groups also are advantageous for binding/catalysis\textsuperscript{12,15–18,20,24,31} with the 4-hydroxyl group serving as a hydrogen-bond donor. Interestingly, a carba-sugar analog of GlcN6P has been reported to promote glmS ribozyme self-cleavage with activity similar to that of the natural metabolite, suggesting that the ring oxygen is not essential.\textsuperscript{31,32} Finally, the riboswitch selectively binds to the α-anomer of GlcN6P, pointing to possible involvement of the anomeric hydroxyl in binding and/or catalysis.\textsuperscript{33}

In addition to the structural constraints articulated above, available X-ray crystallographic structures suggest that the phosphate ester functionality is important for positioning the GlcN6P actuator in the riboswitch active site via chelation to Mg\textsuperscript{2+}. Given the susceptibility of phosphate esters to cleavage by digestive phosphatases, it was a principal goal of this study to develop riboswitch actuators that are phosphatase-inert. The simplest design might be to replace the bridging phosphate monoester oxygen with a carbon, to generate phosphonate analogs.

There is currently great interest in naturally occurring phosphonates, as antibiotics,\textsuperscript{34} including the widely used fosfomycin,\textsuperscript{35,36} a PEP analog that inactivates UDP-GlcNAc-3-enolpyruvyl transferase (MurA), a critical enzyme in bacterial cell wall biosynthesis. More recently, fosfomycin has shown promise as a potential antituberculosis lead compound, as it potently inhibits DXR in the committed step for the nonmammalian isoprenoid biosynthetic pathway.\textsuperscript{37} Indeed, Metcalf and co-workers have dubbed this “an underexplored family of secondary metabolites.”\textsuperscript{38}

The Berkowitz group has a longstanding interest in the synthesis of unnatural phosphonates\textsuperscript{39–45} and in their use to probe active sites\textsuperscript{46} and glycoprotein receptor binding pockets,\textsuperscript{47,48} for example. Therefore, we initially set about to construct a set of phosphonate mimics of GlcN6P as potential artificial actuators of the glmS ribozyme.

**RESULTS AND DISCUSSION**

**Design and Synthesis of GlcN6P Analogs.** Synthetically, the key strategy undertaken was to develop a synthetic route into a viable glucosamine-6-O-triflate, for installation of various phosphate surrogate functionalities. In earlier work, we had established the utility of sugar triflate displacement with C-nucleophiles in general,\textsuperscript{49} and toward sugar phosphonates in particular.\textsuperscript{42–46,50} However, prior to this work, triflates derived from amino-sugars had not been synthesized or examined for displacement with this chemistry. A significant challenge was to install an amino protecting group (PG) that would be amenable to primary sugar triflate preparation. Initial forays into N-acetyl protection met with little success at the triflate installation stage. On the other hand, more strongly electron-withdrawing N-sulfonyl PGs proved successful, with the (2′-trimethylsilyl)-ethanesulfonyl (SES) PG being optimal.

The synthetic route begins with the glucal 1 and introduces the 2-amino functionality via glycal iodo-sulfonamidination (Figure 3). Then the amino group “rolls over” from the 1- to

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**Figure 1.** Secondary structure of the *Bacillus cereus* glmS riboswitch/ribozyme. The highly conserved core sequence of the glmS ribozyme is shown in red (P2.1 and P2.2), while requisite structural elements (P1 and P2) and peripheral structural elements (P3–P4) are also displayed with nucleotide detail. The arrowhead denotes the site of self-cleavage.

**Figure 2.** Illustration of the dual general base/acid mechanism for GlcN6P-promoted strand scission in the glmS ribozyme. GlcN6P is proposed to promote 2′,3′-cyclic phosphodiester formation by completing a circuit of proton transfer events that includes (indirect) deprotonation of the nucleophilic 2′-OH of the A-1 riboside and protonation of the 5′-O leaving group. Other active site bases that have been implicated in proton transfer or H-bonding roles here include the O2′ of A58, N1 of G33, N1 of G32, and O4 of U43.

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fact, it has been demonstrated that glmS self-cleavage is dependent upon the pK\textsubscript{a} of the amine functionality of GlcN6P and other actuators,\textsuperscript{12} with a Brønsted β value of 0.7 having been observed in a recent study, suggesting that the general base role resides in the GlcN6P ligand itself.\textsuperscript{23} GlcN6P analogs that lack the amine functionality do not support glmS self-cleavage; those with elevated amine pK\textsubscript{a} values support self-cleavage activity at elevated reaction pK\textsubscript{a} values,\textsuperscript{12} and those that remove the N lone pair via quaternization or sequestration through amide protection are also ineffective.\textsuperscript{24}
the 2-position, via a presumed N-sulfonyl aziridine intermediate.\textsuperscript{51,52} With further protection of the nitrogen and removal of 6-O-silyl PG, the free alcohol \textsuperscript{3} was prepared and stored in large quantity. The reaction between \textsuperscript{3} and triflic anhydride under basic conditions gave the glucosamine 6-O-triflate \textsuperscript{4} expeditiously (91\% yield). Direct displacement with lithium dibenzyl phosphonate\textsuperscript{53} proceeded smoothly and was followed by SES deprotection and subsequent catalytic hydrogenation to afford the phosphonate \textsuperscript{5}, the targeted GlcN6P surrogate, for the first time.

In addition, we set out to explore tether length in such phosphonate-based analogs (Figure 4). S\textsubscript{N}2 substitution of the key intermediate triflate \textsuperscript{4} by dibenzyl phosphate anion afforded compound \textsuperscript{6} in 80\% yield. Following the standard deprotection method, “truncated” phosphonate \textsuperscript{7} was synthesized. Triflate displacement by a malonate anion followed by a decarboxylation/reduction sequence built the “elongated” alcohol \textsuperscript{9}. However, the extended triflate was unstable even at low temperature (−40 °C). Thus, an iodide intermediate was instead employed. Displacement with a phosphite anion and then sequential N-SES and global benzyl deprotection provided compound \textsuperscript{11} as an “elongated” phosphonate analog.

Motivated by recent studies on a malonate-based analog that binds to a sugar phosphate-binding site in phosphomannose isomerase,\textsuperscript{54} we next targeted three carboxylate-bearing GlcN6P surrogates. One of these, namely the malonyl ether \textsuperscript{13}, was constructed by Rh(II)-mediated O−H insertion of the carbenoid species derived from dibenzyl diazomalonate (Figure 5). The dicarboxylic acid derivative \textsuperscript{17} and the monocarboxylic acid derivative \textsuperscript{18}, in turn, could be accessed from intermediate \textsuperscript{14}, which was efficiently formed in the reaction of triflate \textsuperscript{4} and lithiodibenzyl malonate.

The GlcN6P-like phosphoramidate was next targeted as the aza-analog of the parent phosphate monoester. The common precursor triflate \textsuperscript{4} was displaced by azide in 89\% yield. Cleavage of the N-sulfonyl linkage with nucleophilic fluoride and then Staudinger reduction conveniently produced compound \textsuperscript{20}. The primary amine was then reacted with dibenzyl chlorophosphate to install the phosphoramidate. Global debenzylation gave phosphoramidate analog \textsuperscript{22} (Figure 6).

Because of the key role postulated for the 2-amino group in catalysis, N-methylation was next explored.\textsuperscript{24} Thus, silyl ether \textsuperscript{2} was methylated and deprotected with TBAF. Following triflate displacement, deprotection of the SES amide and global benzylation afforded N-monomethylated phosphonate \textsuperscript{27} (Figure 7). The SES-deprotected compound \textsuperscript{25} could be further methylated with methyl iodide, to yield the N,N-dimethyl analog \textsuperscript{28}, after deprotection.
Self-Cleavage Assays with the \textit{glmS} Riboswitch. The \textit{Bacillus cereus} glmS ribozyme was utilized in self-cleavage kinetic assays performed in the presence of various synthesized GlcN6P analogs. Nine analogs were tested for their ability to support \textit{glmS} self-cleavage (Figure 8). Seven of the nine analogs contain an unmodified amine functionality. The remaining two analogs combine the “sterically true” phosphonate ester with N-methylated amino groups. Mono- or dimethylation of the amine still retains the lone pair on nitrogen, which would, in principle, still allow the amine to function as a general base/acid.

Cleavage assays and data are presented in Figure 8 and Table 1, as well as in the SI. Of the nine analogs tested, five exhibited significant self-cleavage rates ($k_{\text{obs}}$), namely, the sterically true phosphonate (5) and its N-methyl (27) and N,N-dimethyl (28) congeners, as well as the malonyl ether (13) and the phosphoramide (22). While GlcN6P is the natural actuator/ligand for the \textit{glmS} riboswitch, the self-cleavage rate is difficult to measure accurately (see range given in Table 1), with reported values for the $k_{\text{obs}}$(GlcN6P)/$k_{\text{obs}}$(GlcN) ratio ranging from 37:1.12 to 143:1.23. Therefore, for experimental benchmarking, we have chosen to compare our results to the ligand analog GlcN. The observed pseudo-first-order rate constants ($k_{\text{obs}}$) for riboswitch cleavage with the artificial actuators at pH 7.3 are collected in the table.

As can be seen from the primary data (see SI), the other four analogs, namely the chain-truncated phosphonate (7), the chain-elongated phosphonate (11), the simple malonate (17), and the monocarboxylate (18), show only very modest induction of \textit{glmS} riboswitch self-cleavage (Note: Owing to limited quantities of material, compounds 11 and 18 were tested at 1 mM concentrations, rather than the 10 mM concentrations used for the other analogs).

Follow-up kinetic assays were conducted on the five most active analogs, the sterically true phosphonate (5) and its N-methyl (27) and N,N-dimethyl (28) congeners, as well as the malonyl ether (13) and the phosphoramide (22). These experiments were undertaken to estimate pseudo-second-order rate constants (i.e., $k_{\text{cat}}/K_m$) at subsaturating cofactor concentrations, according to the approach of Fedor and Viladoms.23 Pleasingly, the “sterically true” phosphonate analog (5) and the 6-O-malonyl ether (13) exhibited the greatest catalytic efficiency, 22-fold and 27-fold more reactive than glucosamine, respectively (Table 1).

These results provide strong support for the notion that the phosphate is critical to GlcN6P positioning in the riboswitch “active site.” This is seen in the phosphonate series, in which...
only the analog (5) possessing a single methylene (CH$_2$) unit in place of the bridging phosphate oxygen is effective. Deletion of this methylene (in 7) or insertion of an additional methylene (in 11) all but abolishes this activity. In addition to this apparent positioning constraint, a diaionic end group also appears to be advantageous. This can be seen in the carboxylate mimic series, wherein the monocarboxylate (18) is nearly inactive.

Further modifications to the best phosphonate analog (5), namely N-mono- and dimethylation, did not lead to improved activity. The measured $pK_a$ for the monomethylated analog (27) is 8.2, whereas the $pK_a$ for the dimethylated analog (28) is 7.8. So, based upon acid–base chemistry considerations alone, one might predict that the latter analog would be the superior analog. Indeed, while 28 does outperform 27, given that 5 displays a nearly 40-fold better $k_{cat}/K_m$ relative to both 27 and 28, it would appear that in this series, deleterious steric factors are the dominant factor.

We set about to examine the binding of the most promising analogs more closely via molecular docking. There are currently six published X-ray crystal structures of the glmS riboswitch precleavage complex with GlcN6P bound, four from *Thermoanaerobacter tengcongensis* (2Z74, 2Z7S, 3B4B, 3B4C)$^{15,17,18}$ and two from *Bacillus anthracis* [3G8T (G33A, 2NZ4 (2′OMe-A-1))]$^{16,20}$ The latter structure was chosen as the *B. anthracis* riboswitch displays 98% identity with the *B. cereus* riboswitch employed in the cleavage assays in this work. As can be seen in Figure 9, deletion of the bound GlcN6P, followed by molecular docking (Autodock 4—see SI for details) of the diaionic form of phosphonate 5 supports a model in which Mg$^{2+}$-coordination projects the 2-amino group appropriately for its putative general acid/base function in the cleavage reaction.

To better assess ligand charge, titrations for the most interesting analogs were carried out, and the results are tabulated in the SI. Of potential significance, the second $pK_a$ of phosphonate analog 5 was determined to be ~7.4 from titration curves (SI: Figure S2A). Therefore, under the cleavage assay conditions (pH 7.3), only ~50% of the phosphonate moiety is diaionic, although modeling suggests that this form is required for optimal binding through magnesium ion chelation. Therefore, the diminished activity of 5 relative to the native GlcN6P ligand ($pK_a$ ~ 6.2)$^{12}$ and to the malonate ether (13) may be at least in part due to the elevated second $pK_a$ of phosphonate.

Interestingly, the simple malonate analog, 17, is ~300-fold less active than the malonyl ether analog 13. Titration of these 1,3-dicarboxylic acids indicates that $pK_a$ of the malonate analog (17) is 5.3 and that of the malonyl ether (13) is 3.9 (SI: Figure S2). Therefore, both of these bis-carboxylates are in their diaionic forms under the conditions of the assay. Given this, each analog was docked to the glmS riboswitch in diaionic form. The results are striking (Figure 10). The malonyl ether analog is well positioned in the active site. However, in contrast to the native GlcN6P ligand or its phosphonate analog 5, the bis-carboxylate moiety appears to permit coordination to two magnesium ions in the active site (Figure 10). A similar bis-chelation motif is observed for the docked malonate (17) as well. However, for the latter ligand, docking suggests that the 2-amino group is improperly positioned for catalysis, interacting with the A42−U43 phosphodiester bond rather than with the susceptible phosphodiester linkage between A−1 and G1. Structural biology studies (e.g., cocystalization) may be able to shed further light on this molecular-docking-based model for malonate ether/malate binding to the riboswitch in the future (Note: In response to a reviewer suggestion, a preliminary screen of self-cleavage rate vs Mg$^{2+}$ concentration was carried out with the P1−P4 construct of the glmS riboswitch and indicates that for both GlcN and GlcN6P, across a range of 1−100 mM Mg$^{2+}$, reaction rates are fairly constant, whereas the rate of the reaction with the malonate ether (13) from 1 to 10 mM Mg$^{2+}$ increases 4.5-fold and then remains fairly constant from 10 to 100 mM).

In examining the literature on malonate-type surrogates for biological phosphates, one certainly finds cases in which simple malonates make for good analogs, in sharp contrast with our findings here, e.g., the aforementioned malonate analog of M6P (phosphomannose isomerase).$^{34}$ Indeed, in one head-to-head case carefully examined by Frost and co-workers, the malonate substrate analog displayed an order of magnitude superior activity to its malonyl ether counterpart in the inhibition of 3-dehydroquinate synthase.$^{55}$ Moreover, we previously saw a
similar preference for simple malonates over malonyl ether mimics of M6P in binding to the M6P-insulin-like growth factor II receptor.48

On the other hand, perhaps the best case of a particularly active malonyl ether phosphate mimic described heretofore was in a classic study by Sikorski et al. at Monsanto toward the development of novel EPSP synthase inhibitors.26 However, this may be the first indication of the potential advantage of a malonyl ether in bis-carboxylate positioning for metal ion coordination, indeed possibly for bis-Mg coordination.

Taken together, the results of this study suggest that both the malonyl ether (13) and the sterically true phosphate (5) mimics of GlcN6P are promising starting points for the development of phosphatase-resistant artificial actuators for the glmS riboswitch as is critical for in vivo application. We note that these actuators are only ~1/7 as active as the natural ligand GlcN6P, and therefore there is potential for improvement. Future studies will be guided by the observations reported herein and will build upon these two promising lead platforms.

## METHODS

### Synthesis of GlcN6P Analogs

The construction of the phosphate analogs exploited the triflate displacement chemistry previously reported45—57 where possible. A detailed description of the synthesis of all nine analogs and spectral data for each intermediate can be found in the Supporting Information.

### Preparation of RNA

Templates for transcription were prepared by primer extension and PCR amplification using synthetic DNA corresponding to ribozyme sequence. Ribozymes were prepared by in vitro transcription using T7 RNA polymerase and 32P-labeled by incorporation of [α-32P]-UTP. Transcription products were separated by denaturing 10% polyacrylamide gel electrophoresis (PAGE), and ribozymes were eluted in solution containing 50 mM HEPES (pH 7.3 at 22 °C) and 200 mM NaCl, precipitated with ethanol, and redissolved in water.12

### Self-Cleavage Assay

Ribozyme reactions were performed as previously described.12,23 Briefly, reactions contained a ligand analog as indicated and were performed under standard conditions consisting of incubation at 22 °C in solution containing 50 mM HEPES pH 7.3. A saturating concentration of MgCl2 was used in order to avoid a slow folding step and to allow for the formation of native glmS RNA structure.20,58 The [α-32P]-UTP-labeled glmS ribozyme (<250 nM) was prefolded in 50 mM HEPES pH 7.5, 0.1 mM EDTA, and 50 mM MgCl2 at 22 °C. Reactions were started by adding coenzyme at varying concentrations (10 mM—10 μM final concentration) in 50 mM HEPES pH 7.3 buffer. Reactions were terminated by the addition of a gel loading dye containing 10 M urea, 50 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Products were separated by denaturing 10% PAGE and analyzed using a PhosphorImager and IMAGEQUANT software (Molecular Dynamics). kobs values for self-cleavage were derived by plotting the natural logarithm of the fraction of uncleaved ribozyme versus time and establishing the negative slope of the resulting line. Stated values represent the average of at least three replicate assays. First order cleavage rate constants were obtained at different concentrations of coenzyme in the linear range of a Michaelis–Menten plot (ligand concentration ~20% of apparent Kc value) and were fit by linear regression to obtain apparent second-order rate constants kobs/Kc.23

## ASSOCIATED CONTENT

### Supporting Information

Details of the synthesis of all compounds, of analog titration, biological assays and molecular docking are available. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


