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Synthesis of PEG-modified Guanine for Modulation of Transcription via xpt-pbuX Riboswitch

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Abstract

Riboswitches are RNA molecules which respond to specific stimuli by turning on or off transcription or translation. The aptamer domain of the \textit{xpt-pbuX} riboswitch in \textit{B. subtilis} has a three-dimensional structure with a specific binding site for guanine. When guanine is bound, the expression platform forms a three-dimensional structure which inhibits transcription of downstream purine synthesis genes. In this work, polyethylene glycol (PEG) was conjugated to position nine of guanine. This will potentially increase the solubility of guanine in solvents such as dimethyl sulfoxide, making it easier to use for assays. The PEGylated guanine will be used to probe the xpt-pbuX riboswitch in an \textit{in vivo} assay.
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INTRODUCTION

Gene expression is the process by which information, stored in the form of nucleic acid sequences in cell nuclei, is used as instructions to synthesize a gene product: protein or RNA. Gene expression consists of transcription and translation. The first step of transcription is initiation. An enzyme, RNA polymerase, binds to a promoter DNA sequence and DNA’s helix structure is unwound to expose the template DNA strand. In the next step of transcription, elongation, RNA is synthesized from the 5’ to 3’ end from the template DNA. The previously unwound and transcribed DNA then reforms its helix as the RNA polymerase moves along the DNA. Upon termination, RNA and RNA polymerase are released.

There are five kinds of RNA. Messenger RNA (mRNA) determines the amino acid sequence of a polypeptide (Figure 1). Transfer RNA (tRNA) brings amino acids to the ribosome for translation. Ribosomal RNA (rRNA), with proteins, make up ribosomes which are responsible for translation of mRNA into polypeptides. Transfer-messenger RNA (tmRNA) found in bacteria has tRNA and mRNA properties. Small nuclear RNA (snRNA), with proteins, forms complexes that are used in RNA processing in eukaryotes.

**Figure 1** Mature mRNA structure. Figure from Wikipedia [16].
Translation begins when the ribosome’s small subunit docks onto a Shine-Dalgarno sequence just before the start codon of an mRNA molecule (Scheme 1). Then, tRNA molecules attached to amino acids dock at the corresponding triplet codon sequence (anticodon) on the mRNA molecule. This repeats many times with multiple tRNAs docking and connecting successive amino acids into a growing chain that elongates out of the ribosome. When the ribosome encounters a stop codon, it falls off the mRNA molecule and releases the protein.

**Scheme 1** Bacterial translation initiation. Purple: mRNA. Green: ribosome. Figure adapted from Khan Academy [17].

It was long believed that the only role of RNA was to convert the information stored in DNA into proteins. However, many other functions of RNA have been elucidated, including enzymatic activity and regulation of gene expression. Riboswitches are *cis*-encoded, *cis*-acting regulatory segments of messenger RNA, found in many species of bacteria, which work by signal-dependent structure changes. Riboswitches are found in the 5′ untranslated region of messenger RNA and most consist of an aptamer domain and an expression platform. The aptamer region is highly conserved. Upon recognition of a signal by the aptamer domain,
typically a small ligand, a confirmation change occurs in the expression platform. The RNA configuration can modulate transcription or translation and thus gene expression. Riboswitches were initially discovered due to gene regulation without any apparent enzymatic activity, and could be evolutionary descendants of a pre-enzyme regulatory system.

The largest class of riboswitches is metabolite-binding riboswitches, which bind a specific ligand with high selectivity. This class of riboswitches uses a feedback mechanism, regulating concentration of a specific molecule. While the majority of riboswitches function to repress enzymatic synthesis when the product of an enzyme is in high concentration, others control metal ion concentrations by repressing import pathways or triggering export pathways. However, riboswitches do not solely detect small molecules. Some have been found to respond to specific tRNA molecules as well as other parameters such as pH and temperature [1].
**Scheme 2** Modulation of gene expression via riboswitch. Figure from University of Colorado Boulder [18].

**Scheme 3** Transcription attenuation mechanism. Black lines represent DNA. RNAP is RNA polymerase. Green and orange lines are RNAs that base-pair to form the terminator in the presence of the signal (left: transcription on) and the antiterminator in absence of the signal (right: transcription off). Figure from Breaker et al. [8]

Riboswitch aptamers bind to their target ligands with dissociation coefficients as low as the mid picomolar range [8]. Upon recognition of the appropriate signal, a riboswitch will shift
from the “on” confirmation, which permits gene expression, to the “off” confirmation, which inhibits gene expression. Most riboswitches modulate gene expression via either transcription attenuation (cotranscriptional recognition of signal) or translation initiation (signal recognition can be co- or posttranscriptional). Many of those which act on transcription form a terminator structure in the transcription elongation complex (TEC) which causes RNA polymerase to detach from DNA, impeding transcription of the downstream sequence. In the absence of the physiological signal, the riboswitch sequence forms an antiterminator and transcription continues.

Riboswitches which act on translation form a sequestrator helix, occluding the Shine-Dalgarno sequence and preventing ribosome binding. This kind of regulation is advantageous in that it can quickly react to its stimuli and is viable for the lifetime of the transcribed messenger RNA. In the absence of the signal, the riboswitch sequence forms an antisequestrator, the ribosome binds and translation occurs. Primer extension inhibition assays can be used to detect modulation of translation initiation. Rarer classes of riboswitches do not act by transcription attenuation or translation initiation, but rather affect accessibility of the RNA molecule to degradation enzymes.

Various biochemical techniques can be employed to uncover riboswitch sequences and structures. In determining whether a riboswitch acts on transcription attenuation or translation initiation, purified \textit{in vitro} RNA can be used to detect structure change and transcription attenuation upon binding of ligands. Ligand-RNA binding can be studied by techniques such as
isothermal calorimetry. In-line probing, which relies on reduction of RNA cleavage at the nucleotides which bind a ligand, can determine where in a sequence a ligand binds. Modulation of gene expression via a riboswitch mechanism can be studied in bacteria cells through reporter gene assays such as the β-galactosidase assay (Miller Assay). In this assay, the riboswitch gene is inserted upstream of a lacZ operon which includes the gene for the enzyme β-galactosidase. *Ortho*-Nitrophenyl-β-D-galactoside reacts with β-galactosidase to form a yellow compound, *ortho*-nitrophenol, which is easily quantifiable by UV-Vis absorbance. Riboswitches are indispensable to the progress of biochemistry in that small molecules can be used to covalently switch on and off gene expression. Transcription and translation are obligatory for cell survival, making targeting these processes a promising antibiotic technique.

From the 1930s to the 1960s, many antibiotics were being discovered. This period saw a large number of people cured of previously untreatable, and some fatal, infections. However, decades later, antibiotics are causing grave problems in healthcare. People are overusing antibiotics, as medication as well as in agriculture. Multidrug-resistant bacteria are emerging spreading. There are many factors contributing to this resistance including overpopulation, enhanced international migration, increased use of antibiotic drugs, and lack of new antibiotic drugs attributable to time-consuming regulations and scarce funding [9]. Mechanisms of bacterial resistance include enzymatic modification of antibiotic compounds, cell wall modification to inhibit uptake, and transfer (via horizontal gene transfer) of resistance genes between bacteria [10].
In designing new antibacterial compounds, it is a challenge to identify pathways to target that are critical as well as sufficiently widespread and conserved in bacteria. Many known antibiotics bind to the 16S ribosomal RNA component of the bacterial ribosome. Riboswitch RNAs are also highly conserved, making them potential candidates for antibiotic drug targets. Riboswitch-modulated repression of genes necessary to synthesize or import essential metabolites may inhibit bacterial growth or promote bacterial cell death. Riboswitches that bind thiamin pyrophosphate, lysine and flavin mononucleotide have all been shown to impede bacterial growth via binding of ligand analogs [5]. Additionally, because the genes they regulate are involved in fundamental metabolic pathways in certain bacterial pathogens, riboswitches which bind guanine may be viable targets for the development of novel antibiotics.

One such guanine-binding riboswitch is the xpt-pbuX riboswitch in B. subtilis (Figures 2-4). This riboswitch has a highly characterized sequence and three-dimensional structure, making it a useful scaffold for studying metabolite-binding riboswitches. The xpt-pbuX riboswitch uses a transcription attenuation mechanism regulates genes crucial to purine biosynthesis and transport.
Figure 2 Guanine (spheres) bound to \textit{xpt-pbuX} riboswitch aptamer domain (sticks).

Figure adapted from Jain, et al. 2015 [12]

Figure 3 Sequence and secondary structure of \textit{xpt-pbuX} riboswitch from \textit{B. subtilis}.

Circled: nucleotides that undergo reduction of spontaneous cleavage during in-line probing reactions containing guanine concentrations above the $K_D$. Shaded: alternative pairing to form
anti-terminator stem when ligand is absent. Asterisks: nucleotides added to facilitate *in vitro* transcription. Figure adapted from Breaker, et al. [6]

**Figure 4** Guanine in the binding pocket of the *xpt-pbuX* riboswitch aptamer. Black ovals represent spaces where guanine could be modified without too much interference with RNA-ligand binding. Figure from Kim, et al. [5]
Figure 5 Equilibrium dissociation constants for C2- and C6-modified guanine analogs.

Figure from Kim, et al. [5]

RNA crystal structures of the xpt-pbuX riboswitch aptamer domain with guanine bound to the binding pocket, as well as RNA-ligand docking software, facilitate guanine analog design. Many of the guanine analogs designed by Kim, et al. (Figure 5), notably G4 and G11, do not repress growth (determined by OD600 of B. subtilis). However, in-line probing assays indicate that these compounds bind relatively tightly to the xpt-pbuX aptamer. One of the several possible reasons for this is low bioavailability, which is proportional to the area under the curve of concentration versus time. The metabolites may have difficulty entering the cell or its nucleus, or
could be converted to another molecule, making it unable to bind to the highly specific riboswitch aptamer sequence.

Guanine is difficult to use in experiments compared to other nucleic acid bases due to its very low solubility. One problem with analogs G7 and G15 is their insolubility at concentrations near their respective minimum inhibitory concentrations. The attempt by Breaker’s group to generate G7- and G15-resistant *B. subtilis* strains were not successful due to this insolubility [5]. Guanine is sparingly soluble in water and aprotic solvents and insoluble in other common solvents such as alcohol, ether, chloroform and benzene. Despite this, guanine and derivatives play many roles in biochemical pathways and it is imperative to study them. Since discovery of antiviral activity of 9-[(2-hydroxy-ethoxy)methyl]guanine (acyclovir) in the 1960s, synthetic chemists have attempted to synthesize and test the effects of many N9-substituted guanine and guanosine analogs. Acyclovir and ganciclovir are N9-modified guanine analogs which inhibit DNA polymerase and are widely used to treat herpes simplex types one and two, as well as HIV.

In modifying guanine, appropriate protecting groups are necessary as there are five nitrogen atoms and one oxygen atom in the molecule. While 2-amino-6-alkoxypurines have been used to enhance selectivity for N9-coupling to guanine, O6 diphenylcarbamoylation has also been used [2,3].

Polyethylene glycol (PEG) is a polyether with a variety of applications in manufacturing and medicine. PEG-modification (PEGylation) of a drug can render it more effective.
PEG-modification improves drug solubility, decreases immunogenicity, increases drug stability and retention time, and reduces proteolysis and renal excretion, allowing a reduced dosing frequency. PEG is non toxic and can additionally act as a micelle or nanoparticle to aid in delivery of a drug compound in the body. Here, progress was made toward modifying guanine with a short polyethylene glycol at N9 in order to increase solubility of guanine in common solvents and test the N9-PEG-modified guanine as a potential ligand to bind the xpt-pbuX riboswitch aptamer.
MATERIALS AND METHODS

Reporter Gene Assay

A construct encompassing the entire xpt-pbuX guanine riboswitch was placed in a pDG1661 plasmid upstream of a β-galactosidase (lacZ) reporter gene by the Breaker group at Yale University. This vector was transformed into the amyE locus of B. subtilis strain 1A1. Cells were grown in glucose minimal media as described previously [11]. β-galactosidase expression after incubation with various guanine analogs at 700 µM was measured using standard Miller assays [5]. Miller units were calculated using the formula: 1 Miller unit = ((1000)*Abs420nm)-((1.75)*(Abs550nm)) / (t*v*Abs600nm) where t = time in minutes since addition of ortho-Nitrophenyl-β-D-galactoside and v = volume of cell culture in mL.

Kirby Bauer Assay

LB agar plates were prepared with 5 µg/mL chloramphenicol prepared in methanol, as described previously [11]. B. subtilis cells were spread on LB agar plates using glass beads. Plates were divided into quadrants and a sterile paper disc containing 10 µL of antibiotic or a ligand (5 mM in DMSO) was placed in each quadrant. Plates were grown overnight at 37°C.
PEGylation of Guanine

Scheme 4 Synthetic scheme for N9-PEGylation of guanine.

Under inert conditions, a stirred suspension of 236.1 mg (1 mmol) of 2-N,9-diacetylguanine in 348 µL of EtN(i-Pr)$_2$ and 4.8 mL of dry (distilled with KOH and molecular sieves) pyridine was treated with 257 mg (1.1 mmol) of Ph$_2$NCOCl for 1 h at ambient temperature. H$_2$O (400 µL) was added and stirring continued for 10 min. Evaporation in vacuo was followed by heating (with reflux) the residue with 12 mL of 50% EtOH/H$_2$O for 1.5 h. Cooling, vacuum filtration, washing (EtOH), and drying gave 179 mg (46%) of 1. $^1$H NMR sample was prepared in DMSO-$d_6$ and 16 scans were run.
70.66 mg (0.269 mmol) of triphenylphosphine was added to a two-neck round bottom flask with a condenser, under inert conditions. 43.12 µL (0.269 mmol) of triethylene glycol monomethyl ether was added. Solution was stirred. 100 mg (0.257 mmol) of 1 dissolved in 4.7 mL of anhydrous THF was added. 53.05 µL (0.269 mmol) of diisopropyl azodicarboxylate was added. Reaction refluxed and stirred at 70°C for 6 h. A second equivalent of triethylene glycol monomethyl ether, triphenylphosphine, and diisopropyl azodicarboxylate were added. Reaction refluxed and stirred at 70°C for 6 h. Reaction was cooled and poured into 6 mL of saturated NaCl solution. Mixture was extracted with dichloromethane (11 mL x 3). Combined organic layers were washed (H₂O) and dried (magnesium sulfate). Evaporation in vacuo gave 362 mg crude 2. ¹H NMR sample was prepared in DMSO-­d₆ and 16 scans were run. Yield cannot be determined. Analysis of 2 (dissolved in ethyl acetate) by TLC was performed using 0.5:1.5:8 H₂O/H₃COH/ethyl acetate.
RESULTS

Reporter Gene Assay

Miller assay was performed to quantify β-galactosidase production. If a ligand is bound to the riboswitch aptamer domain (Scheme 1 and Figure 2), the expression platform will adopt the “off” configuration, impeding RNA polymerase and switching off transcription of the downstream genes. Because the riboswitch gene was inserted upstream of the gene encoding β-galactosidase production, less β-galactosidase should be produced by those cells treated with ligands capable of binding to the riboswitch’s aptamer domain. Figure 6 shows relative Miller units, proportional to absorbance of 420 nm light and therefore production of β-galactosidase, for different guanine analog ligands. All ligands show less β-galactosidase production than the control, DMSO. It is impossible, from these results alone, to determine the mechanism of inhibition of β-galactosidase production by the ligands. However, these results can be used as evidence to support the riboswitch mechanism of transcription attenuation.

These results are not in agreement with literature in that guanine normally decreases β-galactosidase production by at least 80% compared to the control when tested in this assay [5, 11, 12]. Additionally, 6-thioguanine is known to incorporate itself into DNA as a base and is toxic to cells [7]. Therefore, it is possible that the low β-galactosidase production by cells incubated with 6-thioguanine is due to this toxicity, rather than the binding of 6-thioguanine to the riboswitch aptamer.
Figure 6 β-galactosidase expression for *B. subtilis* following incubation with purine ligands (700 µM) normalized to expression observed with DMSO. Error bars represent standard error between replicates ($n_{\text{DMSO}} = 5$; $n_{\text{guanine}} = 3$; $n_{\text{guanosine}} = 5$; $n_{\text{thioguanine}} = 4$).

**Kirby Bauer Assay**

Kirby Bauer disc assay was performed to assess cell growth inhibition by various guanine analogs (Figure 7). While the Miller assay gave information regarding gene expression, the
Kirby Bauer assay was done to look for any effect of the ligands on bacterial growth, as well as any correlation between gene expression inhibition and growth inhibition. None of the guanine analogs showed any zones of inhibition around the discs, implying that bacterial growth was not impeded. Taken with the results of the Miller assay, it can be concluded that reduction of β-galactosidase expression does not necessarily lead to growth inhibition. For both guanine and 6-thioguanine, β-galactosidase expression was reduced in presence of the ligand although cell growth was not. Therefore, the cell can recuperate and grow normally even when β-galactosidase production is hindered.

Figure 7 Kirby Bauer disc assay. N: Neomycin Trisulfate Salt Hydrate antibiotic (Zone of inhibition = 1 cm). T: Thioguanine. D: DMSO. G: Guanine. All compounds at 5 mM.
PEGylation of Guanine

$^1$H NMR of the starting material, 2-N,9-diacylguanine, was taken to verify its identity (Figure 8). The peaks and integrations are in agreement with literature values [4, 14].

**Figure 8** $^1$H NMR spectrum (400 MHz, DMSO-$d_6$) of starting material, 2-N,9-diacylguanine: white solid. δ 2.17 (s, 3H, C13-H$_3$), 2.78 (s, 3H, C16-H$_3$), 8.42 (s, 1H, H-8), 11.53 (s, 1H, N10-H), 11.99 (s, 1H, N1-H).
$^1$H NMR was performed to verify formation of 1 (Figure 9). Peaks and integrations are consistent with literature values [4, 13]. The appearance of a broad singlet at 13.53 ppm is evidence of the removal of the acetyl group from the N9 position of 2-N,9-diacetylguanine. Additionally, the appearance of two multiplets, integrating to ten hydrogens, confirms the presence of the diphenyl group. It is possible that the peaks at 11.92 ppm and 12.08 ppm are the N1-H from the starting material [14].
Figure 9 $^1$H NMR spectrum (400 MHz, DMSO-$d_6$) of 1: purple solid. $\delta$ 2.12 (s, 3H, CH$_3$), 7.25-7.45 (m, 10H, Ph$_2$), 8.37 (s, 1H, H-8), 10.60 (s, 1H, H-10), 13.53 (s, 1H, H-9).

$^1$H NMR was performed to verify formation of 2 (Figure 10). Loss of the singlet at 13.53 ppm (Figure 9) suggests that the polyethylene glycol was attached at the N9 position. It is unclear if peaks at 10.25 ppm and 8.50 ppm are from 1, or if they are something else. The exact shifts do not show peaks in the 1H NMR spectrum of 1 (Figure 9). The C6-H peak could not be identified. It is most likely masked by the phenyl proton peaks in the 7.0-7.25 ppm range.
**Figure 10** $^1$H NMR spectrum (400 MHz, DMSO-$d_6$) for 2: yellow oil. $\delta$ 2.20 (s, 3H, C13-H), 3.17 (s, 3H, OCH$_3$), 3.36-3.62 (m, 8H, 2x CH$_2$OCH$_2$), 3.82 (t, 2H, CH$_2$CH$_2$N), 4.34 (t, 2H, CH$_2$OCH$_3$), 7.4-7.7 (m, 10H, Ph$_2$), 8.88 (s, 1H, H-8), 10.68 (s, 1H, N10-H).

**Chromatography**

In order to purify 2, a silica column was run in a micropipet with 0.5:1.5:8 water/methanol/ethyl acetate. This solvent system was chosen due to solubility of the crude material in this system as well as the clear separation afforded via TLC. Fractions were collected and spotted on TLC plates next to the crude material in order to identify components of the crude product (Figure 11). Six clear spots were visible in the TLC of the crude material, meaning there
are six different compounds present. The top three spots (the three least polar compounds) were collected in the 18 fractions. While running the column, no apparent bands were visible moving down the silica. Most likely, the three bottom spots of the crude material (the three most polar compounds) were stuck on the polar silica of the column.

Figure 11: TLC separation of 2 (dissolved in ethyl acetate). Eluent: 0.5:1.5:8 H$_2$O/H$_3$COH/ethyl acetate. C: crude 2, pre-column. $R_f$ compound 1: 0.90 (Fractions 5-7); $R_f$ compound 2: 0.68 (Fractions 11-13); $R_f$ compound 3: 0.45 (Fractions 15-18).
DISCUSSION

Reporter Gene Assay

Miller assay results varied greatly, which could be a result of poor solubility of the ligands. The assay should be repeated with much lower concentrations of ligands. This could also be the result of biofilm formation in the *B. subtilis* cultures. The cells are grown in growth minimal media, spiked with ligands, which may cause the cells stress, leading to biofilm formation. This poses a problem in transferring the cells from the test tube to the microcentrifuge tube used for the assay because it is very difficult to not bring up any of the biofilm into the pipet. In the future, this problem should be further investigated. If biofilm production can be prohibited, the amount of cells in each tube would be more uniform.

Kirby Bauer Assay

Fractions from the micropipet column (Figure 11) should be analyzed by \( ^1\text{H} \) NMR to determine the identity of each compound. Additionally, another silica column with the same eluent should be run on a much larger scale as the minute fractions were difficult to work with. When proper separation of all six compounds present in the crude 2 is achieved, more 2 should be generated and flash chromatography should be employed. This could give much better purification than running a silica column by hand. After this purification, 2 would be verified again via TLC and \( ^1\text{H} \) NMR to be sure there is nothing else present.
Upon purifying 2, the final deprotection step (Scheme 4) can be performed via reflux in ammonia/methanol to afford crude 3. The protocol, adapted from the N9-alkylation protocol of Ciesielski et al., calls for a final purification via silica gel [15]. This could also potentially be done by flash chromatography after preliminary TLC analysis and subsequent micropipet column using a small amount of crude 3.

Considering the three-dimensional structures of the riboswitch, it is possible that the N9-PEGylated guanine may not bind as tightly as guanine to the binding pocket of the xpt-pbuX riboswitch due to steric hindrance. Figure 4 shows optimal positions for modification, which do not include the N9 position. However, x-ray crystal structures misrepresent the aptamer domain of the riboswitch as being static, when it is in fact flexible and capable of rearrangement. This, in conjunction with the free rotation about the sigma bonds of polyethylene glycol, make it plausible for the N9-PEGylated guanine to stick to the binding pocket of the xpt-pbuX riboswitch aptamer domain. Future studies of this PEGylated analog should include in vivo β-galactosidase assay as well as in vitro binding studies with the purified xpt-pbuX aptamer domain. Binding studies can provide a $K_D$ value for PEGylated guanine, while the β-galactosidase assay will expose repression of gene expression, whether through this riboswitch mechanism or another pathway. In-line probing could also be done with this new analog.

The most important applications of riboswitches are creation of ligand-controlled genetic constructs, design of new ligand analogs for natural riboswitches, and engineering of designer
riboswitches. *Xpt-pbuX* riboswitch is a viable model system for riboswitch-mediated control of transcription and gene expression. However, *B. subtilis* is not known to cause disease. These methods should be used to study repression of gene expression and bacterial growth in pathogenic bacteria, especially those which show resistance to broad-spectrum antibiotics.
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