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Modulation of the *E. coli rpoH* Temperature Sensor with Triptycene-Based Small Molecules

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Abstract: *Regulation of the heat shock response (HSR) is essential in all living systems. In E. coli, the HSR is regulated by an alternative σ factor, σ^{32}, which is encoded by the rpoH gene. The mRNA of rpoH adopts a complex secondary structure that is critical for the proper translation of the σ^{32} protein. At low temperatures, the rpoH gene transcript forms a highly structured mRNA containing several three-way junctions, including a rare perfectly paired three-way junction (3WJ). This complex secondary structure serves as a primitive but highly effective strategy for the thermal control of gene expression. In this work, the first small-molecule modulators of the E. coli σ^{32} mRNA temperature sensor are reported.*

Temperature is a universal stress factor for all living organisms, and a rapid response to temperature fluctuations is essential for cell survival. The heat shock response (HSR) is a cellular process characterized by the increased synthesis of a set of heat shock proteins (HSPs) in response to stress, such as temperature.[1,2] The *Escherichia coli* (*E. coli*) HSR is regulated by an alternative σ factor, σ^{32}, encoded by the *rpoH* gene. An increase in temperature from 30 °C to ≥ 37 °C results in the increased synthesis and stability of σ^{32}, leading to the transcription of σ^{32}-dependent genes involved in the HSR.[3–11] Translational control is a common strategy for the modulation of the HSR in both eukaryotes and prokaryotes. Morita and co-workers have found that the σ^{32} mRNA secondary structure acts as a thermosensor, crucial for the induction of σ^{32}, in the *E. coli* HSR pathway (Figure 1).[12–16] Intramolecular base-pairing interactions in the first 229 nucleotides control the translation efficiency of σ^{32}. Analysis of a series of deletions and mutations shows the presence of two regulatory elements that fold into a complex structure, preventing the initiation of translation at low temperatures. The first regulatory element is a 15 nucleotide downstream box (region A) near the AUG start codon that allows for binding of the 30S ribosome. The second regulatory element, stem III (Figure 1b), blocks the downstream box. The AUG start codon is then blocked by nucleotides present in stem I. Base pairing of the start codon and the downstream box by stems I and III prevents ribosome binding at low temperatures. Primer-extension inhibition (toeprinting) experiments have demonstrated that thermal stress disrupts the RNA secondary structure, leading to ribosome binding and increased translation.[15] These experiments directly correlated the degree of ribosome binding to RNA stability.[4–8] Very few small molecules have been developed for direct prokaryotic or eukaryotic translational control at the RNA level.[17–19] Small-molecule probes with the ability to stabilize

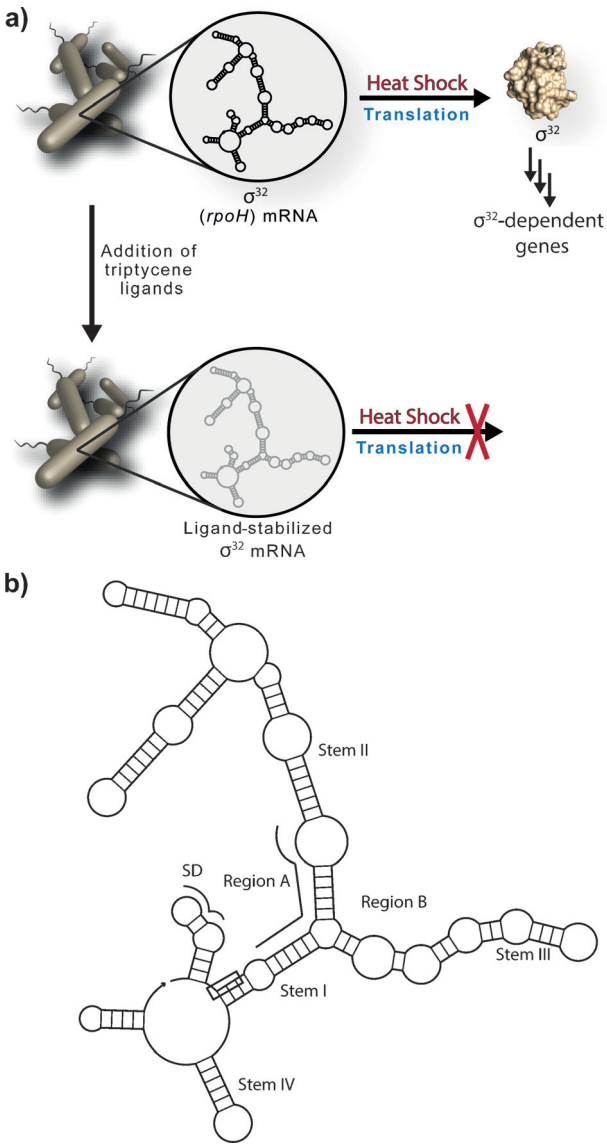

Figure 1. a) The heat shock response in *E. coli* and a strategy for small-molecule modulation at the mRNA level. b) The overall secondary structure of the 5′-end of the σ^{32} mRNA regulatory element. Important regions are shown, with the boxed area corresponding to the AUG start codon.

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the σ^{32} mRNA secondary structure could be useful probes for studying the HSR pathway as well as potential antibacterial agents or adjuvants.

Chemical and enzymatic probing of the 5′-end of the σ^{32} mRNA secondary structure reveals that the regulatory regions (regions A and B) within the RNA structure form a perfectly paired three-way junction (3WJ).[16] Recently, we developed a new class of nucleic acid junction binders based on the triptycene scaffold.[20–22] Herein, we report the first triptycene-based small molecules that are able to modulate the stability of the σ^{32} mRNA. We determined the ability of these ligands to modulate the structure of σ^{32} RNA by UV thermal melting, circular dichroism (CD), and fluorescence quenching experiments. Furthermore, we demonstrate the in vivo modulation of the heat shock response using a σ^{32}–GFP fusion protein reporter system in *E. coli*.

We initiated our studies with a model system corresponding to the regulatory junction present in the *rpoH* mRNA (Figure 2). UV melting experiments were performed to determine the ability of Trip **1** and **2** (Figure 2a,c) to stabilize the model system. In the absence of ligand, the RNA melted at 51.6 °C. Thermal stabilization was observed in the presence of Trip **1** and **2**, with ΔT_m values of 11.3 and 13.7 °C, respectively (Figure 2c). CD spectroscopy was also performed to investigate the interaction of Trip **1** and **2** with the RNA. CD spectra of the model system in the presence and absence of Trip **1** and **2** at 4 °C are consistent with A-form RNA, displaying a maximum at 266 nm, a large minimum at 210 nm, and a smaller minimum around 240 nm (Supporting Information, Figure S1). As the temperature increases from 4 °C to 80 °C, the maximum at 266 nm decreased and the minimum at 210 nm became less negative. These changes are indicative of the melting of the helical segments. Temperature-dependent CD spectroscopy in the presence of Trip **1** and Trip **2** gave the same trend, but the change was more gradual, particularly between 50 °C and 80 °C (Figure S1). This is consistent with ligand-induced stabilization as observed in the UV experiment. CD spectra in the presence of increasing concentrations of the triptycenes show slight signal changes (Figure S2). A more negative signal is observed at 210 nm as well as a decrease and slight shift at 220 nm. These changes are not consistent with intercalation or groove-binding modes, rather they are suggestive of native helical structural stabilization through a non-helix-perturbing binding event.[23–25]

A fluorescence quenching experiment was used to further support the modulation of the σ^{32} RNA (Figure 2d). The oligonucleotide was labeled with a fluorophore on the 5′-end and a quencher on the 3′-end. Once the RNA is folded, little to no fluorescence is observed as the fluorophore and quencher are in close proximity. Upon addition of a 16 base pair oligonucleotide complementary to the 5′-end, an increase in fluorescence is observed, indicating that both ends are further apart in space owing to the formation of an open state (Figure 2e). The addition of Trip **1** and **2** to this open-state structure results in a decrease in fluorescence, which is consistent with reformation of the folded 3WJ state (Figure 2f). The apparent K_d values of Trip **1** and Trip **2** were determined to be 2.5 μM and 1.5 μM, respectively.

Having characterized the interactions of Trip **1** and **2** with the model system by fluorescence quenching, we turned our attention to the full 5′-region of the σ^{32} mRNA (−19 to +229). The 5′-end of the σ^{32} mRNA was transcribed in vitro for characterization by temperature-dependent UV and CD

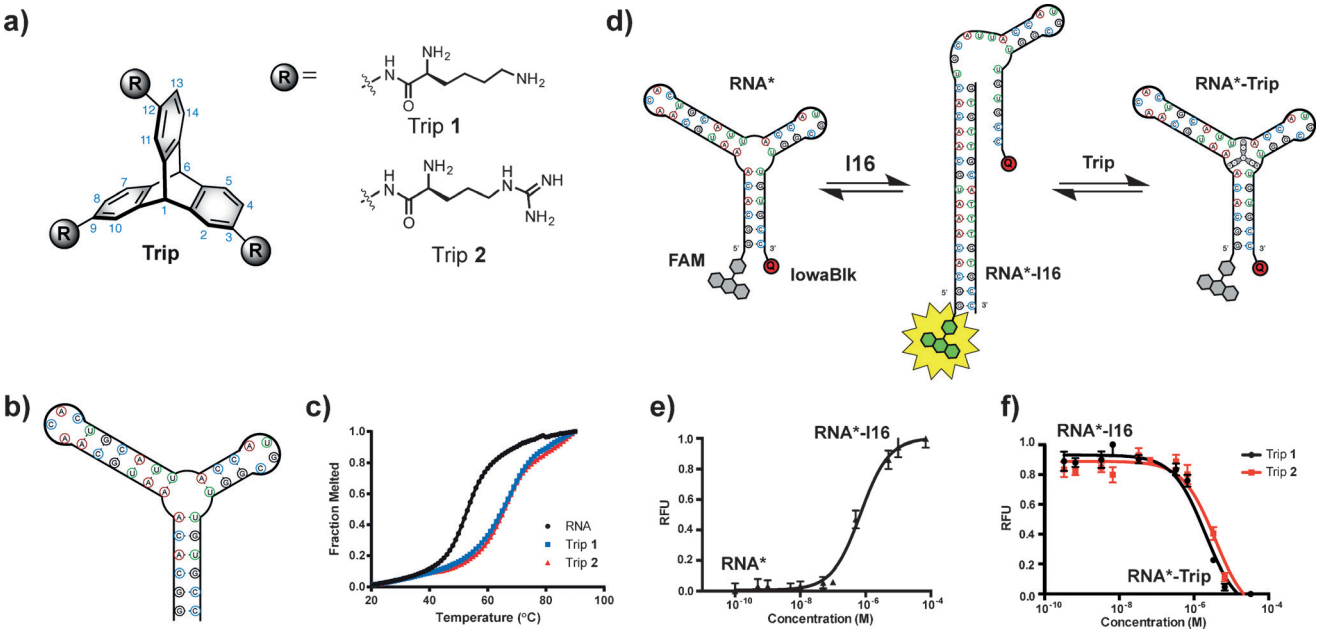

Figure 2. Stabilization of a model-system RNA by triptycene derivatives **1** and **2**. a) Structures of the triptycene derivatives Trip **1** and Trip **2**. b) The RNA oligonucleotide used as a model system, corresponding to a minimal sequence for junction formation. c) UV thermal melting plots in the presence and absence of the triptycenes. d) Schematic representation of the fluorescence quenching experiment. e) Titration of inhibitor 16 (I16) results in an increase in fluorescence. f) Titration of Trip **1** or Trip **2** to the RNA*–I16 complex results in a decrease in fluorescence. The apparent K_d values of Trip **1** and Trip **2** were determined to be 2.5 μM and 1.5 μM, respectively.

techniques. UV thermal melting experiments in the absence of the triptycenes showed a double inflection, indicating that portions of the RNA melt at different temperatures, with the most critical structural changes occurring below 42 °C. The first inflection, with an initial onset below 42 °C, is consistent with the temperature-dependent translation previously observed for *rpoH* mRNA. In the presence of Trip **1** and Trip **2**, thermal stabilization of the full-length σ^{32} mRNA (−19 to +229, Figure 3a) is observed. The shift in thermal stabilization is especially prominent between 30 and 60 °C. The CD spectra in the absence and presence of Trip **1** and **2** are consistent with an A-form RNA structure (Figure S3).

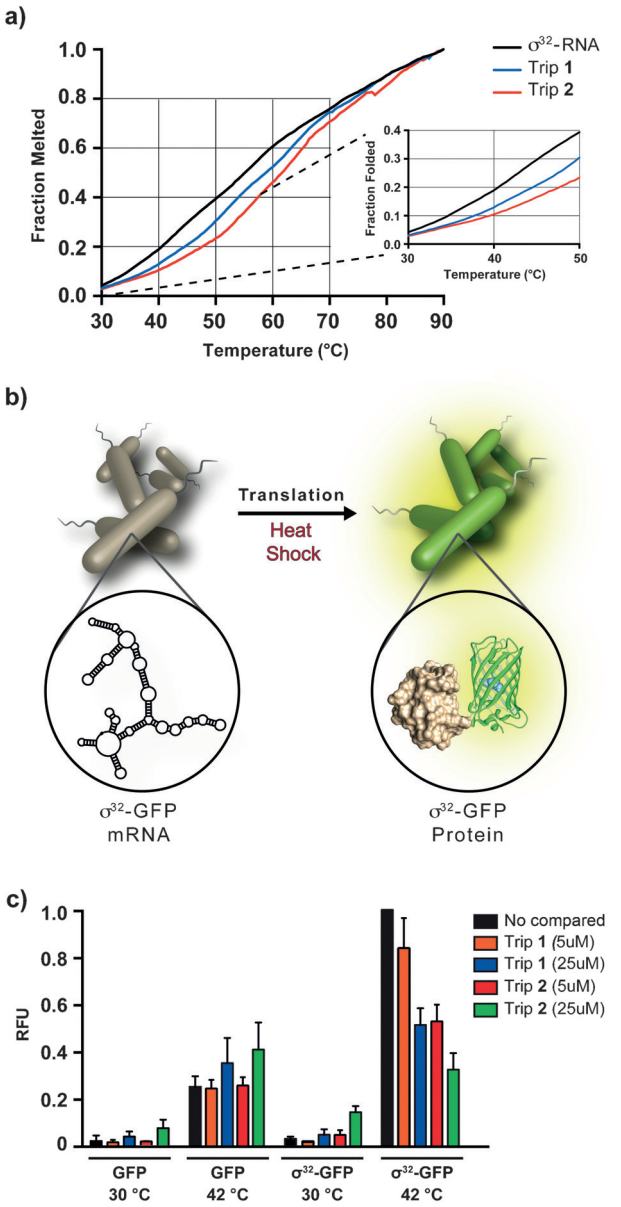

Figure 3. Modulation of σ^{32} mRNA (−19 to +229) by triptycene derivatives and targeting σ^{32} in *E. coli*. a) UV thermal melting plots in the absence and presence of Trip **1** and Trip **2**. b) Targeting *rpoH* using a σ^{32}–GFP fusion protein. c) Relative fluorescence intensities of the GFP control and the σ^{32}–GFP fusion protein at 30 °C and 42 °C in the presence and absence of Trip **1** and Trip **2**.

The melting of the helical regions of the RNA was confirmed by temperature-dependent CD spectroscopy. The addition of Trip **1** or **2** resulted in a more negative peak at 210 nm and a slightly lower maximum at 266 nm, which is indicative of a structural change in the RNA. Thermal stabilization is maximally observed between 40 °C and 50 °C and between 60 °C and 80 °C in the presence of the triptycenes.

A reporter assay based on a σ^{32}–GFP fusion protein was developed and used to monitor the responses to cellular stress in *E. coli* (Figure 3b). The *rpoH* gene, which codes for the σ^{32} protein, along with its promoters, was PCR-amplified from the genomic DNA of *E. coli* and inserted into a plasmid encoding GFP. Cells were grown at 30 °C for several hours in the presence or absence of various triptycene derivatives, followed by heat shock at 42 °C (Figure S4). Cells containing the control GFP plasmid (no σ^{32}) showed low relative GFP fluorescence when grown at 30 °C (Figure 3c). An increase in temperature to 42 °C resulted in a slight increase in fluorescence in the absence and presence of triptycene derivatives using the GFP control plasmid. As expected, cells that contained the σ^{32}–GFP fusion protein and were grown at 30 °C showed low fluorescence similar to the GFP control plasmid. However, those grown at 42 °C displayed a large increase in GFP fluorescence in the absence of these compounds. Upon triptycene addition, a decrease in fluorescence was observed to varying degrees at 42 °C. The most significant decrease in fluorescence was observed in the presence of Trip **1** or Trip **2** compared to Trip **3**, **4**, and **5** (Figure S4). The addition of a 5 μM solution of Trip **1** or Trip **2** resulted in a slight decrease in the relative fluorescence intensity at 42 °C. This decrease was more significant at triptycene concentrations of 25 μM. The relative fluorescence intensities were similar to those observed with the control GFP plasmid, indicating a loss of the heat shock response in the presence of both triptycenes, although Trip **2** appeared to be more potent. A concentration-dependent decrease in the signal at 42 °C was also observed (Figure S5). This is consistent with thermal stabilization of the mRNA by the triptycenes, where upon an increase in temperature, the structure is more folded and stable, suppressing translation of the σ^{32}–GFP fusion protein. The increased translational inhibition with Trip **2** over Trip **1** could be due to a combination of affinity, cell permeability, or various non-specific interactions.

Non-specific inhibition of translation was evaluated using a control GFP-only plasmid in the presence of Trip **1** and Trip **2**. Interestingly, we observed an approximately fourfold increase in translation going from the GFP control plasmid at 42 °C to the σ^{32}–GFP plasmid at 42 °C (Figure 3c). This is reflective of increased translation upon incorporation of the heat-shock-responsive σ^{32} RNA element, which promotes translation at higher temperatures. Furthermore, we observed a mild increase in translation upon treatment with Trip **1** and **2**, except in the case of σ^{32}–GFP at 42 °C. Polyamines have been shown to enhance translation in certain cases, and this could be the origin, although the effect is small.[26] Bacterial-growth experiments in the presence of the triptycenes indicate that Trip **1** and Trip **2** are moderately inhibitory at high concentrations (Figure S6). Furthermore, we conducted

qRT-PCR experiments to gauge the amount of transcriptional inhibition induced by Trip **1** and Trip **2**. Overall, the mRNA levels of σ^{32} were not affected by Trip **1** and only moderately affected at high concentrations of Trip **2**, indicating little inhibition of transcription or differential mRNA stabilization, except with high levels of Trip **2** (Figure S7).

In summary, we have described triptycene-based molecules that modulate the 5′-region of the σ^{32} mRNA temperature sensor from *E. coli*. Trip **1** and Trip **2** thermally stabilize a model system consisting of the critical central three-way junction that is present in the σ^{32} mRNA and responsible for regulation of the heat shock response as determined by UV thermal melting experiments and temperature-dependent CD spectroscopy. UV thermal melting experiments on the full 5′-region of the σ^{32} mRNA also show thermal stabilization. This stabilization was corroborated by temperature-dependent CD spectroscopy in the presence of ligands. To determine the effect of the triptycenes on the heat shock response in *E. coli*, a σ^{32}–GFP fusion protein assay was utilized. In the absence of the triptycenes, an increase in fluorescence was observed when the cells were heat-shocked at 42 °C, indicating σ^{32} protein translation. However, the addition of Trip **1** or Trip **2** suppresses the fluorescence, which is consistent with a decrease in σ^{32} protein translation. This new class of small molecules may be useful for studying the effects of the heat shock response in *E. coli*. Furthermore, modulation of the temperature-sensing RNA regulatory elements in bacteria could lead to the development of novel methods for targeting pathogens or potentiating current antibiotics. Studies are underway to investigate these unique applications.

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