Bioorganic & Medicinal Chemistry Letters 26 (2016) 3958–3962

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Synthesis and polymerase incorporation of β,γ-modified α-L-threofuranosyl thymine triphosphate mimics

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ARTICLE INFO

*Article history:*
Received 2 May 2016
Revised 30 June 2016
Accepted 2 July 2016
Available online 4 July 2016

*Keywords:*
TNA
Nucleosides
Triphosphate mimics
Polymerases
Primer extension

ABSTRACT

Three β,γ-modified α-L-threofuranosyl nucleoside triphosphates were synthesized. The β,γ-modified tTTPs undergo a single incorporation event with HIV RT but undergo multiple incorporations to form full-length product with engineered thermophilic polymerases.



Alpha-L-threofuranosyl nucleic acid (TNA) is an artificial genetic polymer that has been proposed as a possible biological precursor to DNA and RNA.[1–3] In addition to its potential role in early forms of life, TNA has received great interest due to its unique chemical and biological properties. For example, the 2′–3′ backbone linkage found in TNA makes it highly resistant to cleavage by nucleases,[4] but still allows TNA to form stable Watson–Crick duplexes with DNA or RNA.[5] Furthermore, TNA can be transcribed from a DNA template using engineered versions of the thermophilic polymerases Deep Vent (exo-), 9°N, and KOD, which are capable of accepting α-L-threofuranosyl nucleoside triphosphates tNTPs as substrates.[6–9] These polymerases also retain their ability to utilize deoxyribose nucleoside triphosphates dNTPs, suggesting that the mutations made to the polymerases serve to relax their substrate specificity.

Considering the ability of tNTPs to be selectively accepted by polymerases having relaxed substrate specificity or lack of exonuclease editing ability, TNA provides a potentially useful scaffold for the design of anti-viral therapeutics. In 2003, Chaput and Szostak carried out DNA primer extension experiments using HIV reverse transcriptase (HIV RT). Interestingly, they found that the enzyme will incorporate two sequential tTTP monomers, but this appears to then cause chain termination.[6] In a subsequent study, Herdewijn and coworkers demonstrated that 2′-deoxythreosyl phosphonates show anti-HIV activity, presumably from incorporation by HIV RT, resulting in chain termination.[10]

Given the encouraging results from these studies, we were curious to explore the biological activity of tTTP analogues having modifications at the β,γ-bridging oxygen of the triphosphate (Fig. 1). β,γ-Modified dNTPs have been widely used as chemical probes to study the catalytic mechanisms of DNA polymerases, as the leaving group capability of the β,γ-diphosphate unit can be fine-tuned by changing the electron withdrawing capability of the unnatural bridging group.[11–13] Additionally, β,γ-modified dNTPs have been reported to serve as substrates for a variety of DNA polymerases. For example, Krayevsky and coworkers reported that dTTP having a $CF_2$ or CHF group replacing the β,γ-bridging oxygen are substrates for human DNA polymerases α and β, as well as Avian Myeloblastosis Virus (AMV) reverse transcriptase.[14] Taking a slightly different approach, Wang and coworkers synthesized and studied a series of modified AZT triphosphate mimics. Unsurprisingly, they found that modification of α,β-linkage led to poor inhibition of HIV RT. However, an AZT analogue having a β,γ-$CF_2$ modification and a non-bridging α-$BH_3$ modification showed a $K_i$ value comparable to that of AZT, but with significantly enhanced stability in serum and cell extract.[15]

Despite these encouraging results, β,γ-modified triphosphates have yet to be widely explored as potential therapeutics, as nucleoside triphosphates are typically not capable of penetrating the cell membrane. However, Dinh and coworkers have reported a prodrug approach aimed at overcoming this limitation through acylation of the γ-phosphate with a fatty acid or cholesterol analogue.[16,17]

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http://dx.doi.org/10.1016/j.bmcl.2016.07.008
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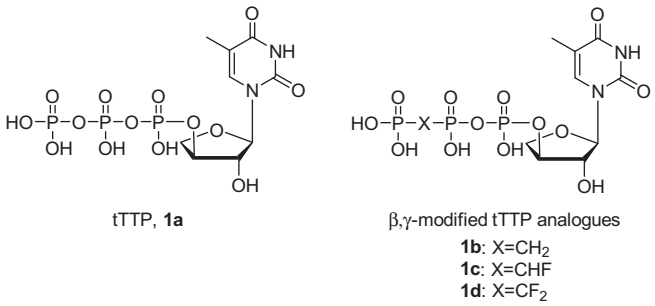



**Figure 1.** Chemical structure of tTTP **1a** and β,γ-modified tTTP analogues **1b–d**.

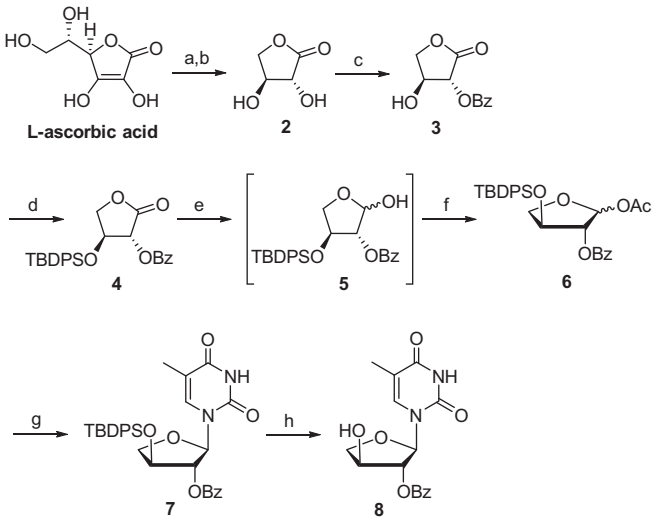
Taking a similar approach, Meier and coworkers have recently reported the successful cellular delivery of nucleoside triphosphates having a lipophilic acyloxybenzyl group at the γ-phosphate, which can be cleaved by intracellular esterases to reveal the nucleoside triphosphate therapeutic.[18,19] These recent developments suggest that viable prodrug approaches could be implemented for β,γ-modified nucleoside triphosphates, which encouraged us to further explore their ability to be incorporated by DNA polymerases.

Here we report the synthesis and study of three β,γ-modified tNTP analogues, which represents the first example of β,γ-modifications in the context of a non-native carbohydrate scaffold. We synthesized 'wild-type' tTTP **1a** and tTTP analogues having a $CH_2$, CHF, or $CF_2$ group replacing the β,γ-bridging oxygen (**1b–d**), and compared their incorporation efficiencies in primer extension reactions using a variety of polymerases. Excitingly, we find that the β,γ-modified tTTPs are substrates for HIV RT, and unlike **1a**, which requires two incorporations before chain termination, **1b–d** show no further elongation after only a single incorporation event. Additionally, we demonstrate that a mutant KOD polymerase is capable of generating full length product in a primer extension experiment using β,γ-$CF_2$ tTTP **1d**. Together, these experiments explore new chemical space by combining carbohydrate modifications with triphosphate modifications, and demonstrate that this approach can lead to enhanced inhibition of a viral reverse transcriptase.

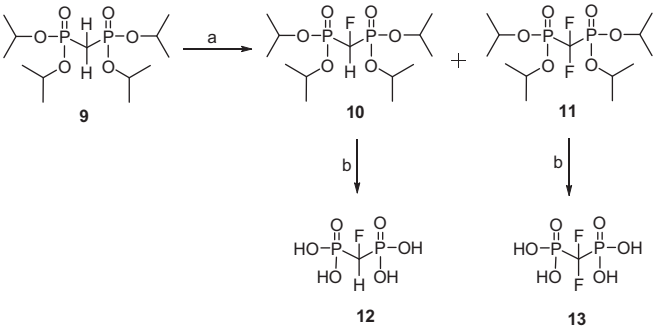
To synthesize tTTPs **1a–d**, we began by following a recently published procedure for generating 2′-*O*-Bz protected α-L-threose nucleoside **8** (Scheme 1).[20] Starting from L-ascorbic acid, we were able to synthesize **8** in high yield over 8 steps, providing the mono-protected nucleoside precursor necessary for elaboration into tTTP **1a** and analogues **1b–d**.

In parallel, we synthesized pyrophosphate analogues (fluoromethylene)bisphosphonic acid **12** and (difluoromethylene)-bisphosphonic acid **13** starting from the commercially available tetraisopropyl methylenediphosphonate (**9**) according to a previously reported procedure (Scheme 2).[11] While synthesis of deoxyribose nucleoside 5′ triphosphates is relatively straightforward, synthesis of threose nucleoside 3′ triphosphates (tNTPs) is much more difficult, owing to the increased steric hindrance of the 3-hydroxyl group.[21] Thus, once all of our key intermediates were in hand, we chose to explore both one-pot and stepwise synthetic routes to access the target compounds tTTP **1a** and β,γ-bridging oxygen modified tTTP analogues **1b–d**.

To investigate the possibility of a one-pot synthesis of tTTP **1a** and analogues **1b–d** from nucleoside intermediate **8**, we first focused on the synthesis of dichlorophosphoridate **14**, which we envisioned as a key intermediate (Scheme 3). The widely used method for synthesizing nucleoside 5′ triphosphates involves formation of a dichlorophosphoridate intermediate via reaction of the 5′ hydroxyl with $POCl_3$ in $PO(OMe)_3$ using Yoshikawa's procedure.[22] However, this approach failed to yield the desired

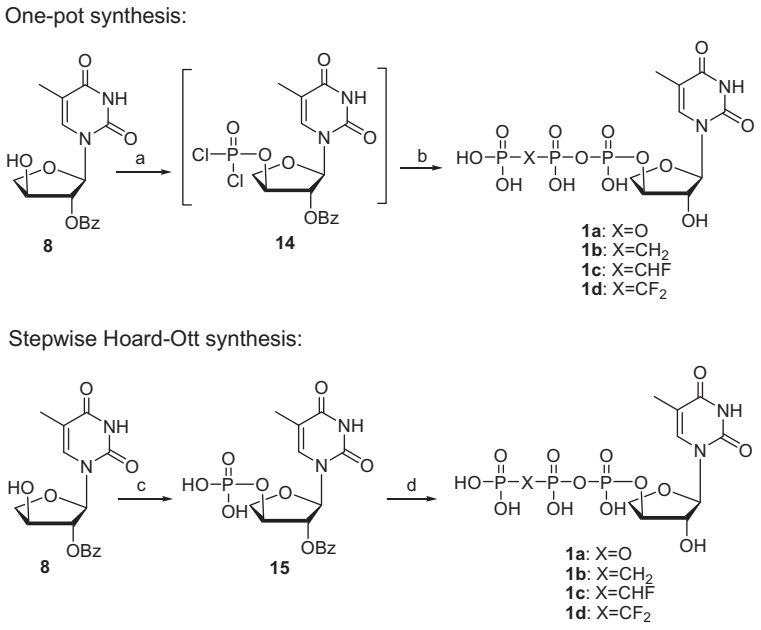



**Scheme 1.** Synthesis of 2′-O-Bz protected thymine nucleoside **8**. Reagents and conditions: (a) (i) 30% $H_2O_2$, $CaCO_3$, $H_2O$, 18 h, 0 °C-rt; (ii) active charcoal, 70 °C, 2 h, 80%; (b) 10% $H_2SO_4$ in $H_2O$, pTsOH, $CH_3CN$, 2 h, reflux, 90%; (c) BzCl, pyridine, $CH_2Cl_2$; 0 °C, 0.5 h, 75%; (d) TBDPS-Cl, imidazole, DMAP (cat), $CH_3CN$, 18 h, 0 °C-rt 86%.; (e) DIBAL-H, THF, −78 °C, 0.5 h; (f) $Ac_2O$, DMAP, pyridine, $CH_2Cl_2$, −78 °C-rt, 1.5 h, 90% from **4**; (g) (i) thymine, BSA, $CH_3CN$, 60 °C, 15 min; (ii) TMSOTf, 60 °C, 2 h, 82%; (h) TBAF, THF, 0 °C, 1 h, 86%.




**Scheme 2.** Synthesis of modified diphosphate building blocks **12** and **13**. Reagents and conditions: (a) (i) NaH, THF, DMF, 0 °C-rt 1 h; (ii) Selectfluor, rt, 3 h; (iii) $NH_4Cl$, $H_2O$, 18% (**10**) and 30% (**11**); (b) $CH_2Cl_2$, TMSBr, rt, 48 h, 70–85%.

intermediate **14** when applied to the sterically hindered 3′ hydroxyl of threose nucleoside **8**. To compensate for the increased steric hindrance of **8**, we investigated the use of a variety of organic bases in conjunction with the $POCl_3$ and $PO(OMe)_3$, and observed that this significantly improved the efficiency of the phosphorylation reaction. Specifically, we screened a series of nitrogen-containing organic bases including pyridine, $Et_3N$, $Bu_3N$, DMAP, and proton sponge (1,8-bis(dimethylamino)natpthalene), and found that proton sponge provided the highest yield of **14**, with 80% conversion observed by HPLC. Moreover, the hydrophobicity of the proton sponge base enabled it to be easily removed at the conclusion of the reaction sequence via extraction with $Et_2O$ under weakly basic aqueous conditions. Having optimized conditions for generating intermediate **14**, we next turned to investigating the remainder of the one-pot synthetic method. To couple the β,γ-diphosphate unit, we added to the reaction mixture tributylamine and either tributylammonium pyrophosphate, methylenediphosphonic acid, **12**, or **13**, which yielded the benzoyl protected precursors to **1a–d**, respectively. The benzoyl group was then removed using concentrated ammonium hydroxide, and **1a–d** were each purified via aqueous extraction followed by reverse phase HPLC using a




**Scheme 3.** Synthesis of tTTP **1a** and analogues **1b–d** using a one-pot or stepwise method. Reagents and conditions: (a) $POCl_3$, $PO(OMe)_3$, proton sponge; (b) (i) tributylammonium pyrophosphate, methylenediphosphonic acid, **12**, or **13**, $Bu_3N$, DMF; (ii) 1 M TEAB buffer, 15 min; (iii) $NH_4OH$, rt, 4–6 h. Yields for **1a–d** ~ 5%. (c) (i) $POCl_3$, $PO(OMe)_3$, proton sponge, 0 °C, 2–4 h, 80%; (d) (i) CDI, DMF, rt, 4–6 h; (ii) tributylammonium pyrophosphate, methylenediphosphonic acid, **12**, or **13**, $Bu_3N$, DMF, rt, 16–48 h; (iii) 1 M TEAB buffer, 15 min; (iv) $NH_4OH$, rt, 4–6 min. Yields from **8**: 46% for **1a**; 41% for **1b**; 36% for **1c**, and 30% for **1d**.

$C_{18}$ column. Together, the one-pot synthesis provided the desired nucleoside triphosphates **1a–d**, but the isolated yield of each product was lower than expected at approximately 5%. We hypothesize that this low yield can be attributed to the high sensitivity of dichlorophosphoridate intermediate **14** to trace water, as this may have led to hydrolysis or other unwanted byproducts.

Although the one-pot synthetic method described above did not produce the desired triphosphates in high yield, our observation that proton sponge could efficiently catalyze the mono-phosphorylation reaction was useful toward developing a stepwise synthetic route. Our stepwise route was inspired by the Hoard-Ott method, in which a nucleoside monophosphate is prepared, then activated using carbonyldiimidizole (CDI) to promote subsequent coupling with pyrophosphate.[23] As shown in Scheme 3, we utilized the same conditions from our one-pot method to generate **14** in situ, but this was hydrolyzed to nucleoside 3′ monophosphate **15** via aqueous workup under basic conditions. Monophosphate **15** was then activated using CDI, and reacted with either tributylammonium pyrophosphate, methylenediphosphonic acid, **12**, or **13**, providing the benzoyl protected precursors to **1a–d**. Similar to our one-pot method, the benzoyl group was deprotected using ammonium hydroxide. The crude tTTPs were then precipitated from ethanol, purified by reverse phase HPLC using a $C_{18}$ column, and characterized by HPLC, $^1H$ NMR, $^{19}F$ NMR, $^{31}P$ NMR and mass spectrometry. The stepwise synthetic route provided **1a–d** with overall reaction yields of 30–46% from nucleoside intermediate **8**, representing a significant improvement over the one-pot method.

Once we had tTTP **1a** and β,γ-modified analogues **1b–d** in hand, we set out to investigate their ability to serve as substrates for polymerase enzymes. Given that DNA polymerases vary significantly in their discrimination of modified nucleoside triphosphate substrates, we chose to examine four polymerases across two categories: (1) HIV reverse transcriptase, for which tTTP had previously been shown to stop chain elongation, and (2) Kod RI, 9°N RI, and Therminator, which are each able to polymerize TNA on a DNA template using 'wild type' tNTPs.

Considering the previously demonstrated ability of tTTP and β,γ-modified AZT analogues to inhibit HIV RT, we were curious to investigate the activity of our β,γ-modified tTTP analogues **1b–d**. To survey primer extension efficiency, we utilized a previously described assay format[24] in which a DNA primer labeled with a 5′ fluorescein (FAM) was annealed to a template having the complement of the primer sequence followed by an $A_7$ sequence. This duplex was then combined with 50 μM of either dTTP, tTTP **1a**, or one of the β,γ-modified tTTP analogues **1b-d**, along with the HIV RT enzyme. After incubating for 30 min at 37 °C, each primer extension reaction was quenched by the addition of denaturing gel loading buffer, and run on a 20% denaturing PAGE gel. The gel was imaged for FAM emission using a laser gel scanner to determine the length of the primer sequence after primer extension using each nucleoside triphosphate.

As shown in Figure 2 (lanes 2–6), all of the nucleoside triphosphates tested were capable of incorporation by HIV RT. As anticipated, dTTP provided the full length primer extension product (lane 2), indicating processive incorporation, and tTTP provided a primer extension product having two nucleotides added (lane 3), consistent with the previous observation from Chaput and Szostak.[6] However, we observed that each of the β,γ-modified tTTP analogues undergo a single incorporation event, then block further polymerization (lanes 4–6). Of the three tTTP analogues, β,γ-$CH_2$-tTTP (**1b**, lane 4) appears to be the best potential inhibitor of HIV

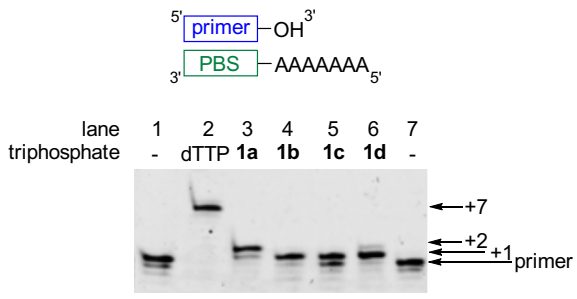



**Figure 2.** Primer extension assay using HIV reverse transcriptase to analyze chain termination by **1a–d**. PBS = primer binding site.

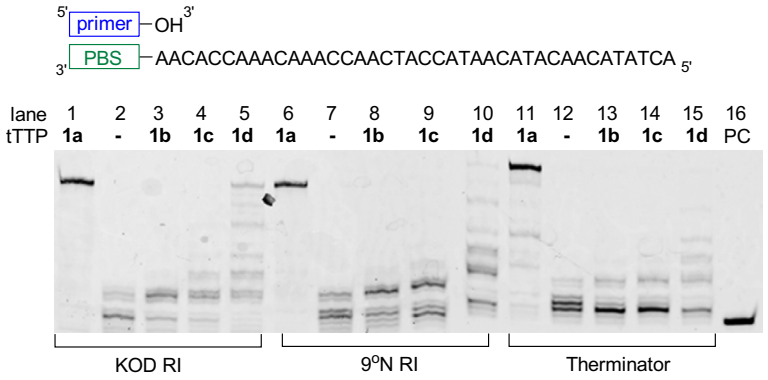



**Figure 3.** Primer extension assay using engineered thermophilic polymerases to analyze incorporation of **1a**–**d**. PBS = primer binding site; PC = primer control.

RT, as incorporation to give the +1 primer extension product is nearly quantitative, but no +2 product is observed on the gel. In comparison, β,γ-CHF-tTTP (**1c**, lane 5) shows less efficient incorporation, with a significant amount of unreacted primer remaining, and β,γ-$CF_2$-tTTP (**1d**, lane 6) gives primarily the +1 product, but a small amount +2 product can also be observed. Together, these results demonstrate that β,γ-modification can enhance the ability of unnatural nucleoside triphosphates to stop chain elongation, and the magnitude of this effect can be tailored by changing the structure of the bridging group at the β,γ-position.

In addition to testing the activity of **1b–d** with HIV RT, we were curious to explore whether the β,γ-modified tTTP analogues could serve as substrates for the engineered thermophilic polymerases that have been shown to transcribe TNA polymers from DNA templates. For these experiments, we utilized a mixed-sequence DNA template composed of nucleotides T, C, and A (Fig. 3). This template sequence was chosen because TNA polymerization on G-rich templates is known to be challenging, and the use of a template having >50% A would provide a rigorous test for the enzymatic polymerization of our modified tTTP analogues. To survey primer extension efficiency, we annealed the template with a FAM-labeled primer in a 2:1 molar ratio in 1× Thermopol buffer with 1 μM $MnCl_2$ and KOD RI, 9°N RI, or Therminator polymerase.[9] We then added to each reaction mixture 100 μM of tTTP **1a** or β,γ-modified tTTP **1b–d**, along with 100 μM each of tATP, tGTP, and tCTP. The reaction mixtures were incubated for 8 hours at 55 °C, then quenched with stop buffer (1× TBE, 20 mM EDTA, 7 M urea) and analyzed via 12% denaturing PAGE gel. For comparison, control reactions were performed having no tTTP (lanes 2, 7, and 12). As shown in Figure 3, all of the β,γ-modified tTTP analogues provide some degree of primer extension compared to the control lanes having tTTP omitted, although with lower efficiency compared to 'wild type' tTTP **1a**. Of the three modified tTTP analogues, incorporation efficiency follows the trend of **1d** > **1c** > **1b**, which is not surprising given the increased leaving group ability of the diphosphates having electron withdrawing fluorine atoms. We also observed that primer extension efficiency followed the trend of KOD RI > 9°N RI > Therminator with regard to enzyme identity. While these results show that analogues **1b–d** are less efficient than **1a** for transcription of TNA polymers, we are encouraged that the combination of $CF_2$-modified analogue **1d** with KOD RI polymerase does provide some full length product in the primer extension reaction (lane 5). The differences in incorporation efficiency of the β,γ-modified triphosphate mimics using engineered thermophilic polymerases can be explained using transition state theory,[11–13] as the observed trend of incorporation efficiency with $CH_2$ < CHF < $CF_2$ mirrors the increase in leaving group ability of the pyrophosphate as increasingly electronegative atoms are added at the β,γ-carbon.[12]

In conclusion, we report the synthesis and polymerase compatibility of three novel β, γ-modified tTTP analogues **1b–d**. The modified 3′ nucleoside triphosphates were prepared in high yield using the Hoard–Ott method, and were fully characterized by HPLC, $^{1}$H NMR, $^{19}$F NMR, $^{31}$P NMR and mass spectrometry. We envision that this synthetic approach can be easily extended to enable the future synthesis of β,γ-modified analogues of tATP, tGTP and tCTP. Primer extension assays using HIV RT revealed that **1b–d** are more efficient than 'wild type' tTTP **1a** at stopping chain elongation, as termination is observed after a single incorporation event. Additionally, we found that the modified tTTPs could serve as substrates for the KOD RI, 9°N RI, or Therminator polymerases that have been previously reported for DNA-dependent TNA polymerization. In this case, we find that the modified triphosphates are less efficiently incorporated than tTTP. However, full length primer extension product can be formed using β,γ-$CF_2$ triphosphate **1d** in conjunction with the KOD RI polymerase. Together, these studies are the first to explore the synthesis and enzymatic activity of nucleoside triphosphates having both a non-ribose sugar and modifications to the triphosphate unit. We anticipate future expansion of this chemical space to investigate additional β,γ-modifications and nucleobases, as well as prodrug modifications to the β,γ-modified triphosphates. We envision that this will provide additional insight into the substrate preferences of polymerase enzymes and further promising leads for anti-viral drug development.

## Acknowledgments

This work was supported by the DARPA Folded Non-Natural Polymers with Biological Function (Fold F(x)) Program under award number N66001-14-2-4054. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of DARPA. We are thankful to Prof. John C. Chaput and laboratory for the generous gift of plasmids for KOD RI and 9°N RI, and for many helpful conversations.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.07.008.

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