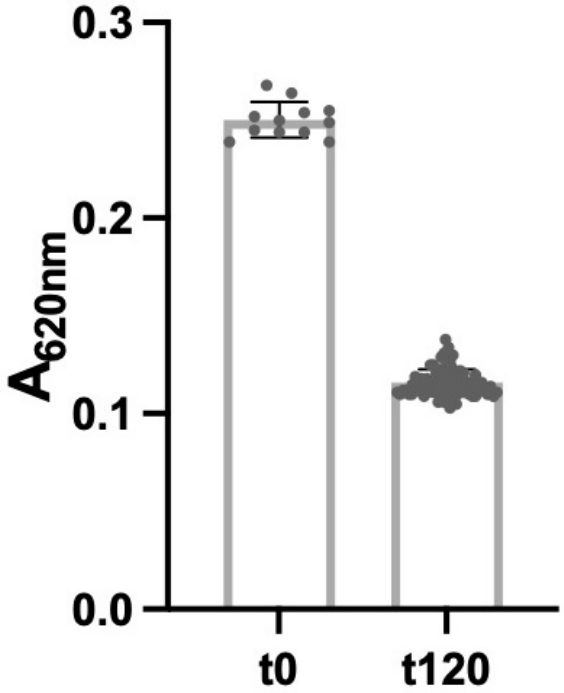
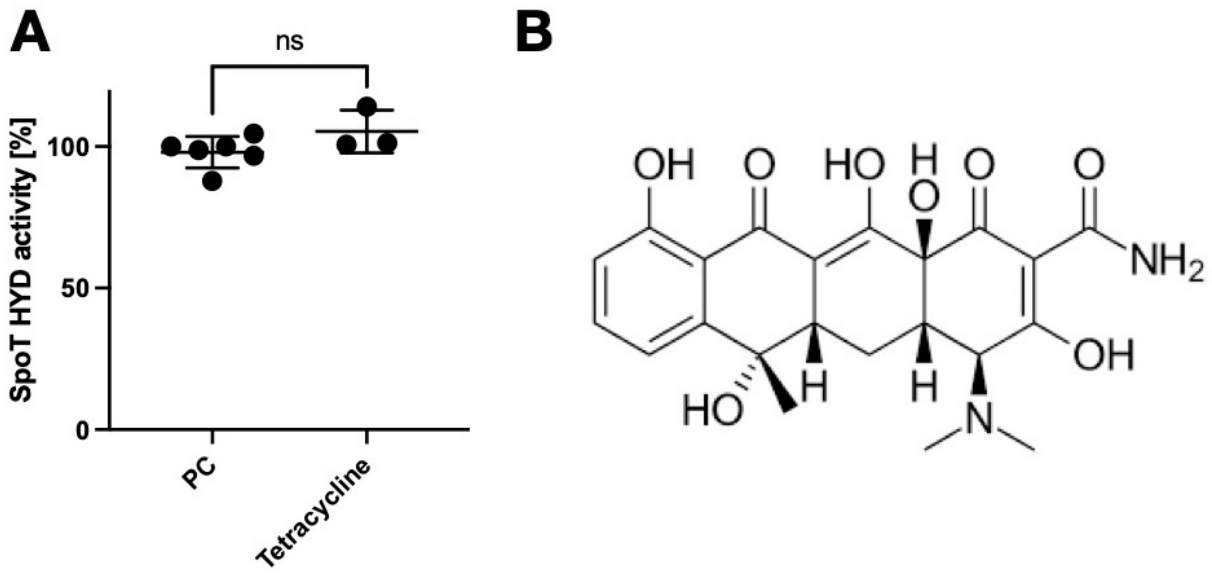
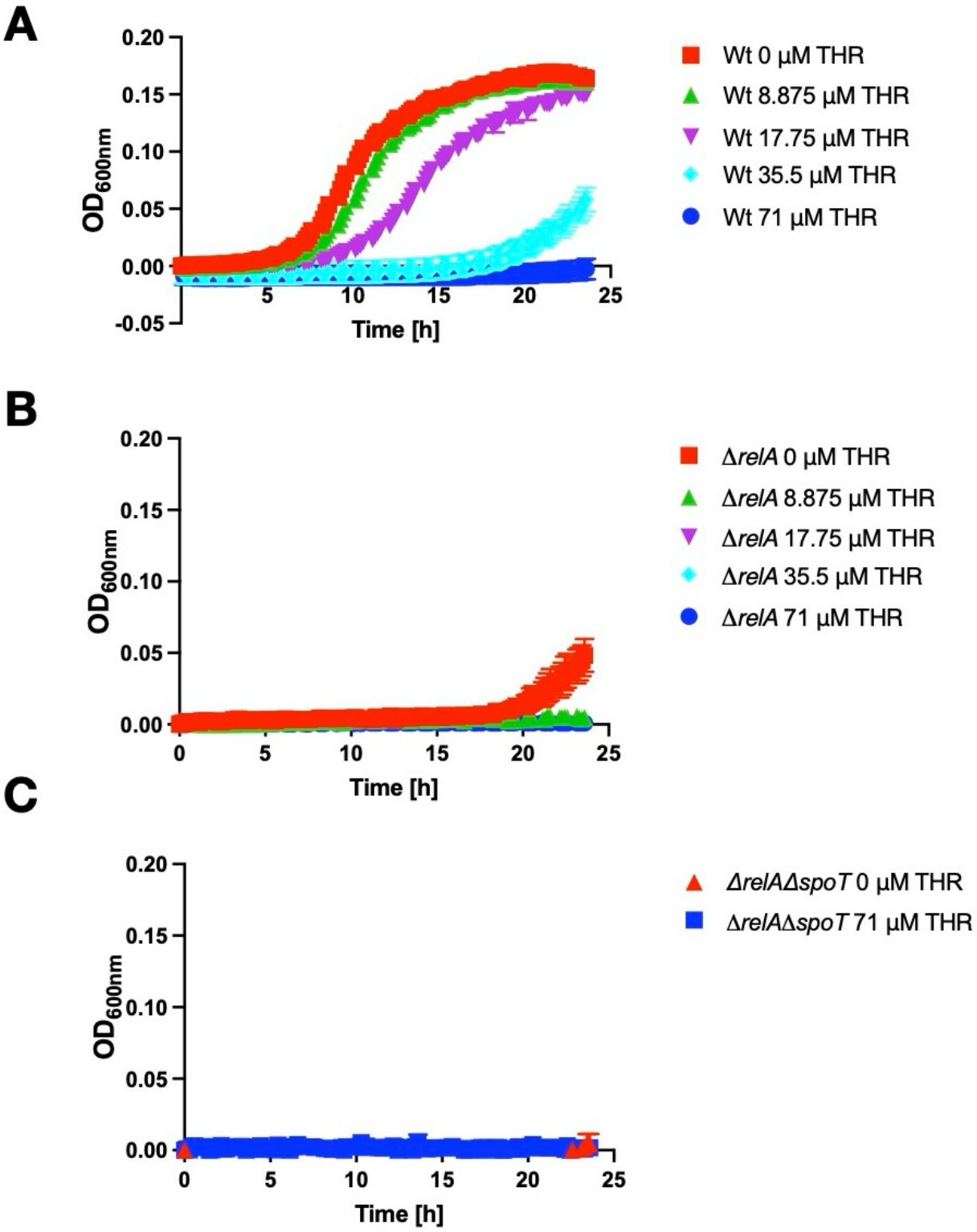
Supplementary figures and legends



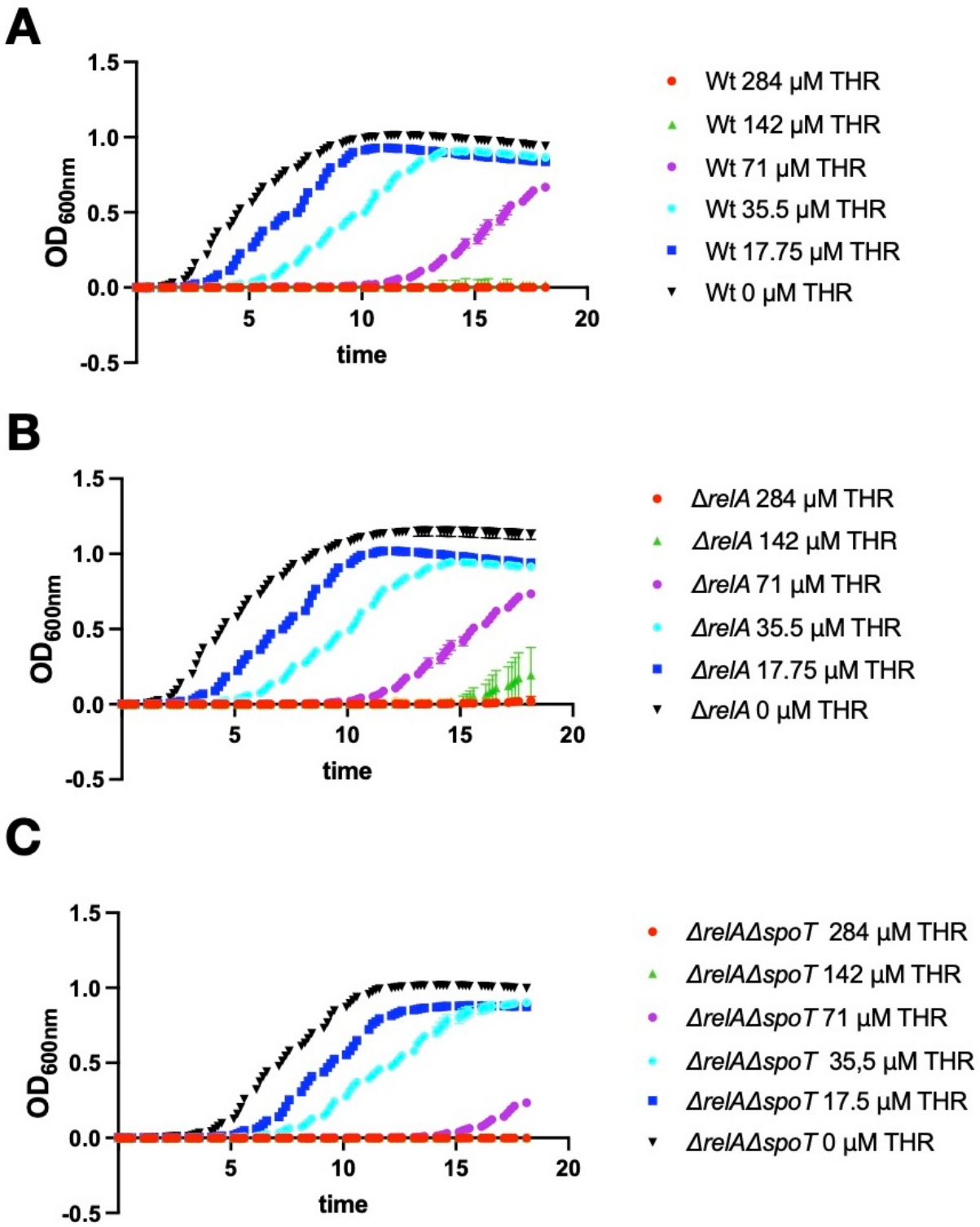
Supplementary Figure S1. Variation-test of the malachite green assay for assessing the hydrolase activity of SpoT in a 96-well plate format. Error bars represent 12 replicates for t0 (SpoT added just before stopping the reaction) and 84 replicates for t120 (the SpoT reaction occurred for 120 min).



Supplementary Figure S2. (**A**) **Tetracycline had no inhibitory effect on SpoT HYD activity.** The tested concentration was 35.6 μM of tetracycline HCl, and the percentage activity was normalized to the reaction without tetracycline (PC). The data represent the mean and standard deviation of three replicates. ns, not significant via unpaired t-test. (**B**) chemical structure of tetracycline.

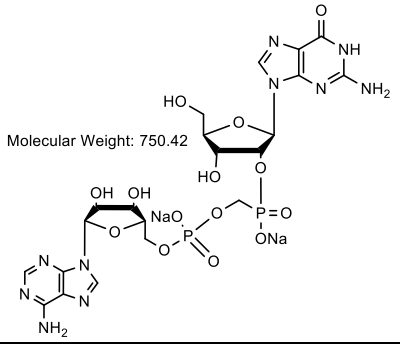
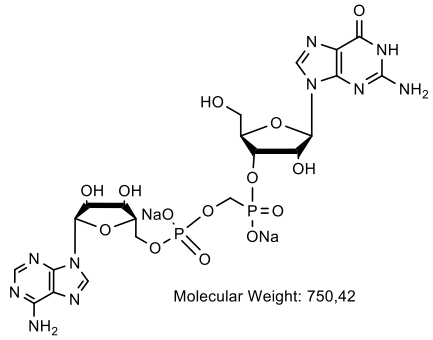
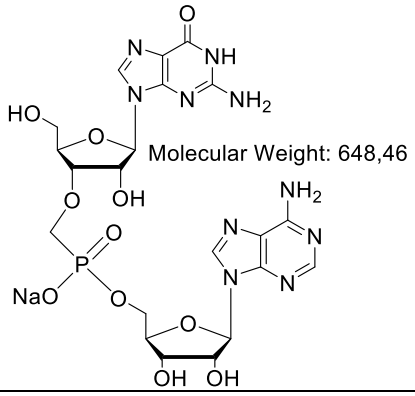


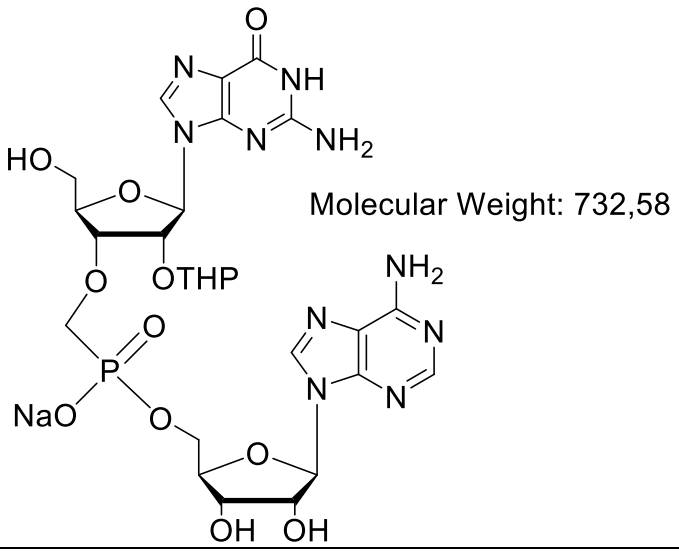
Supplementary Figure S3. The growth of Δ*relA* in M9-SMG medium was not restored by thermorubin. The *E. coli* K12 MG1655 strains (**A**) Wt (**B**) Δ*relA* (**C**) Δ*relA*Δ*spoT* were grown in M9Glc medium supplemented with SMG and varied concentrations (µM) of thermorubin (THR). The data shown represent the mean and SD of three biological replicates.

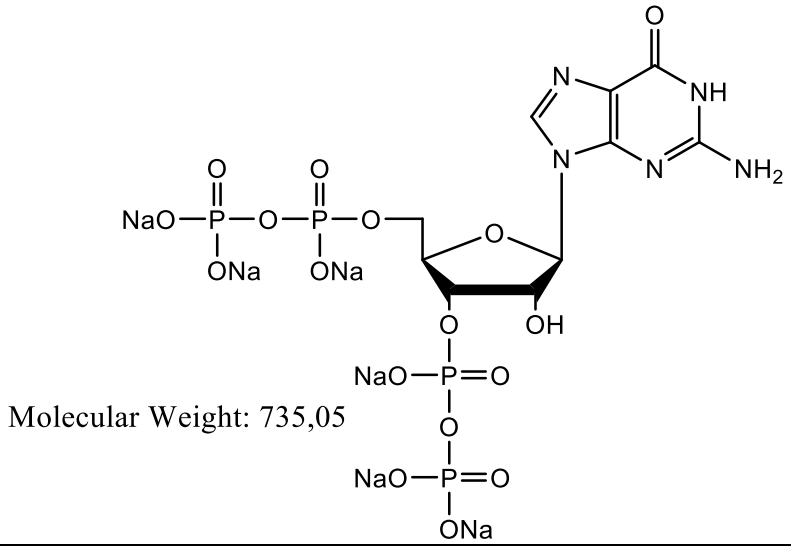
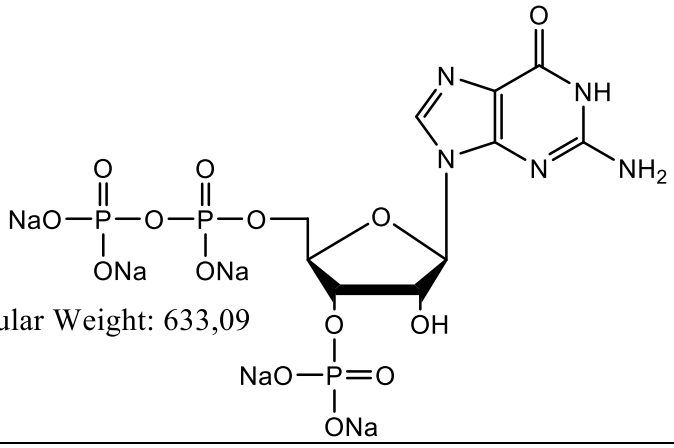
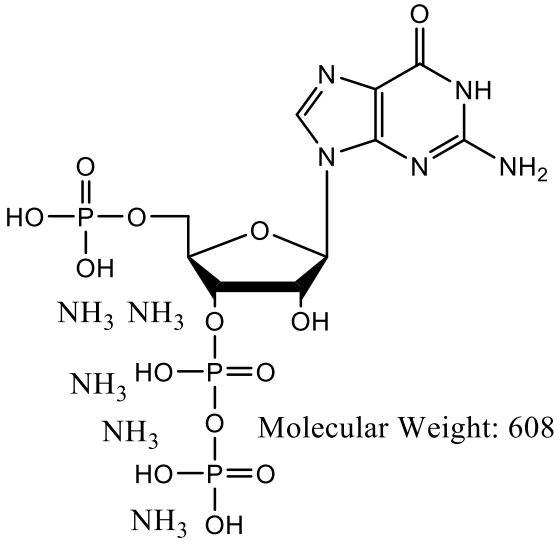


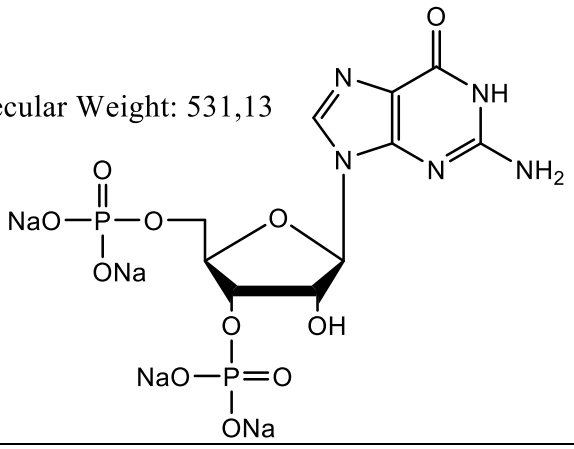
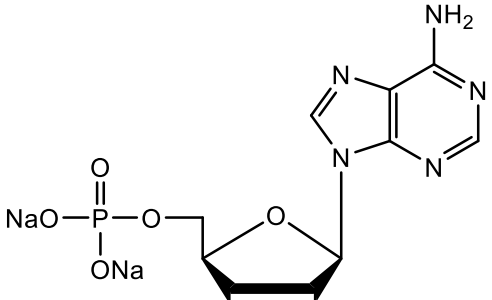
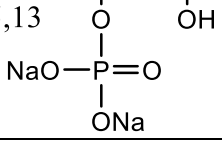
Supplementary Figure S4. The growth curves of three tested strains in LB in the presence of varied thermorubin. The growth was assessed in LB medium at 37oC for (**A**) Wt (**B**) Δ*relA* (**C**) Δ*relAΔspoT* strains with the indicated concentrations of thermorubin (THR, µM). The data shown represent the mean and SD of three biological replicates.

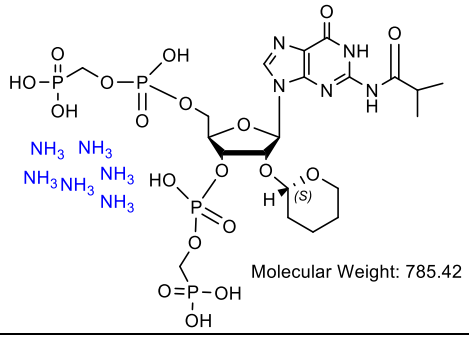
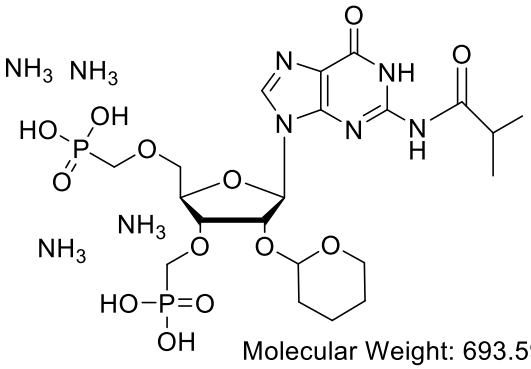
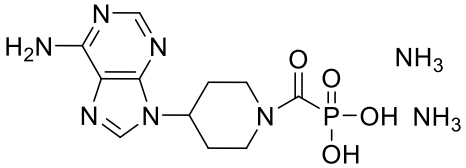
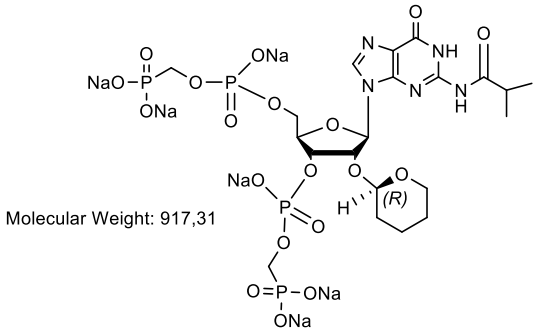
Supplementary document S1 The ppGpp analogues screened in this study

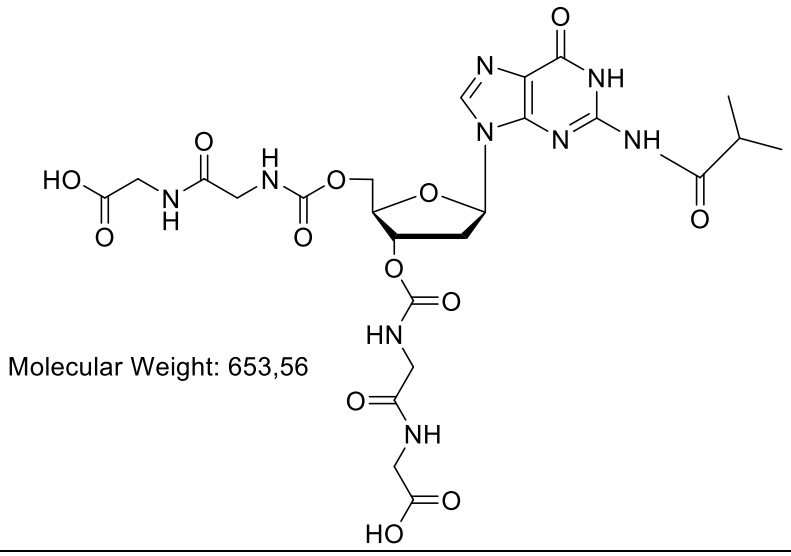
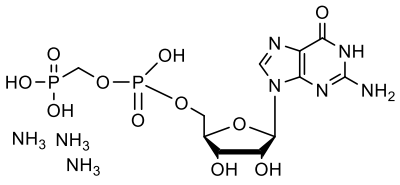
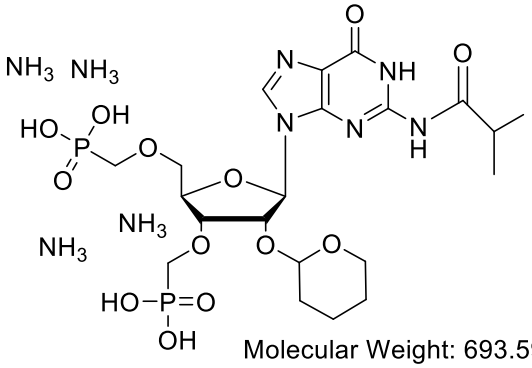
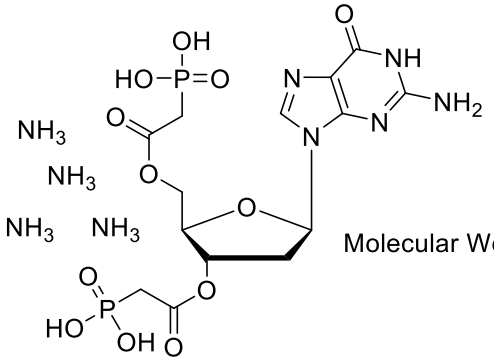
Code amount	Structure	**comment**
DR-6902B 3.9 mg		**A transition state analogue of ppGpp synthesizing enzymes (e.g. RelA)**
DR-6902A 3.5 mg		**A transition state analogue of ppGpp synthesizing enzymes (e.g. RelA)**
DR-6330A 4.9 mg		**A transition state analogue of ppGpp synthesizing enzymes (e.g. RelA)**

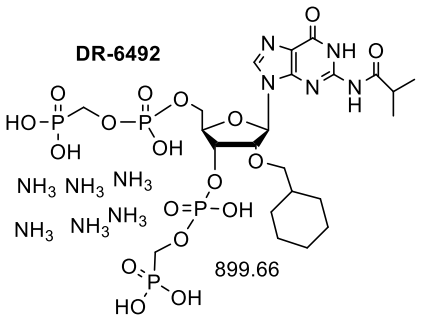
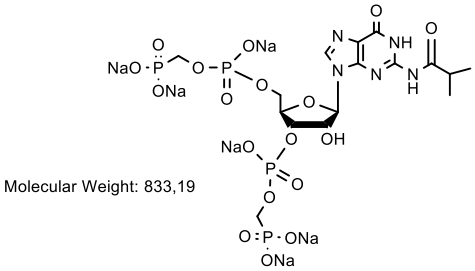
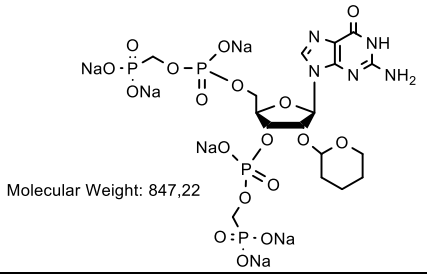
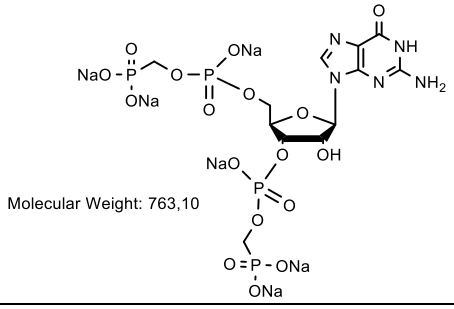
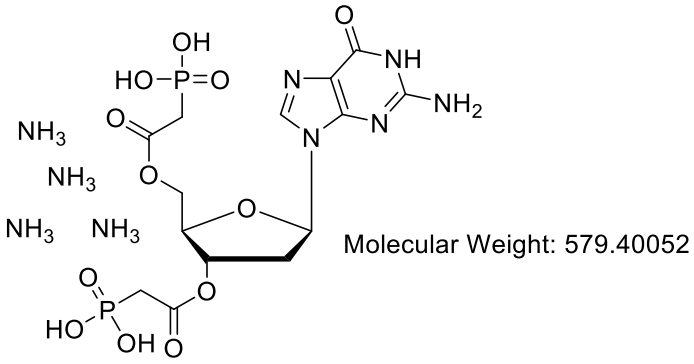
DR-6330B 2.9 mg	 Molecular Weight: 732,58	**A transition state analogue of ppGpp synthesizing enzymes (e.g. RelA)**

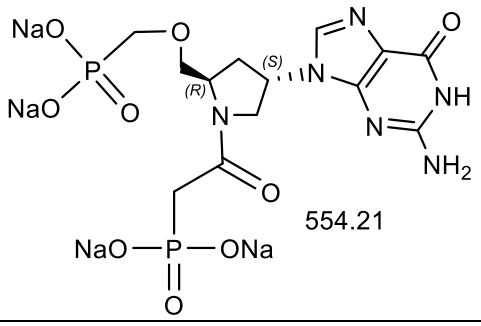
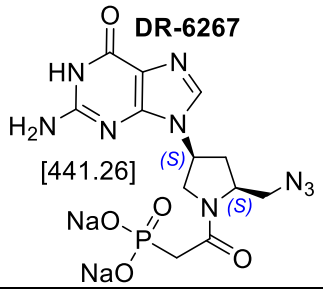
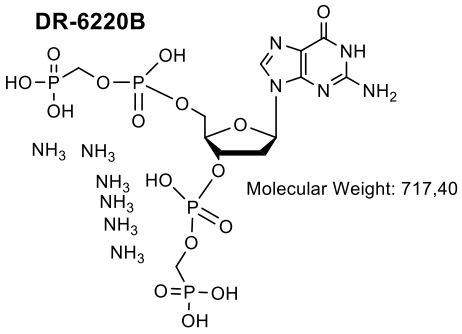
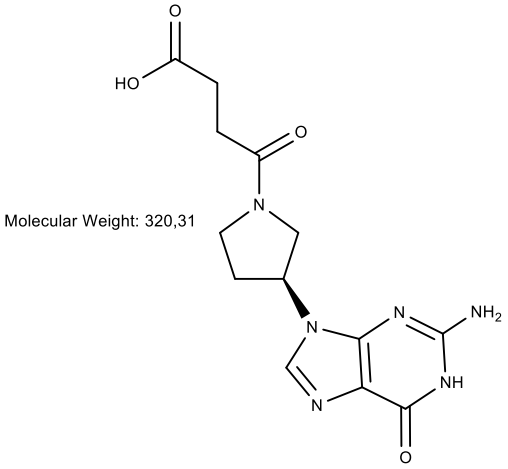
ppGpp Na^+ DR-6975 12.6 mg	 Molecular Weight: 735,05	
ppGp Na^+ 8.5 mg	 Molecular Weight: 633,09	
pGpp NH_4^+ 4.9 mg	 Molecular Weight: 608,34	

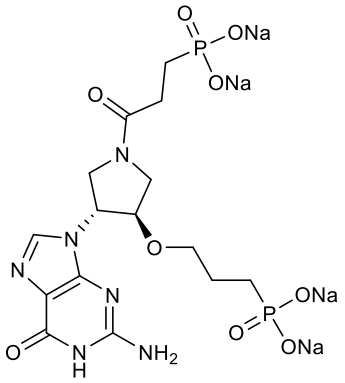
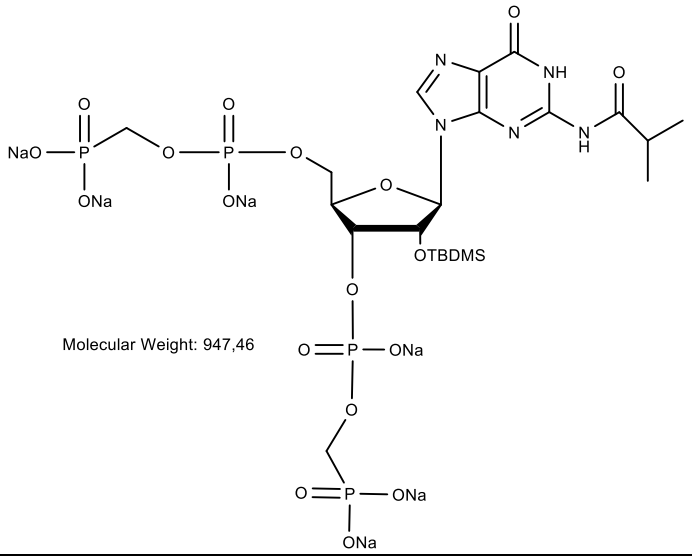
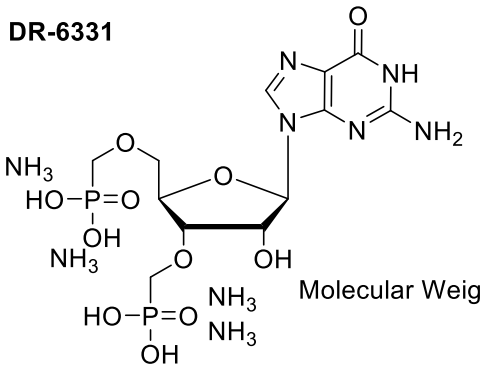
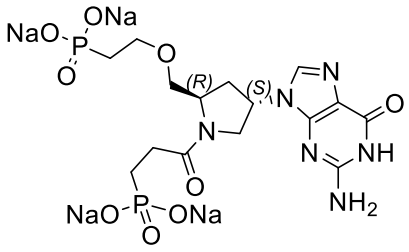
pGp Na^+ 15.6 mg	Molecular Weight: 531,13 	
pAp Na^+ 8.8 mg	 Molecular Weight: 515,13 	

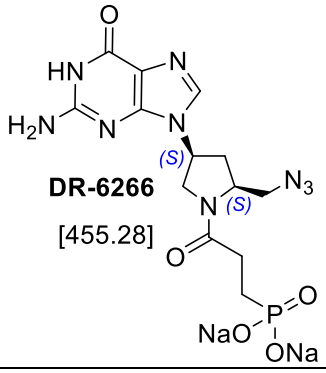
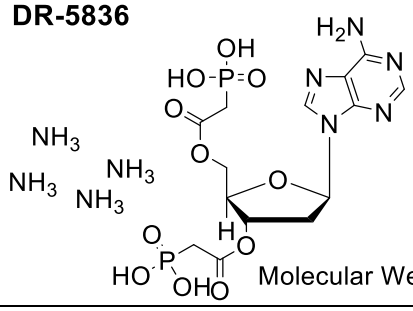
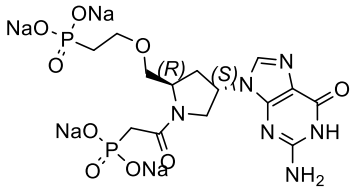
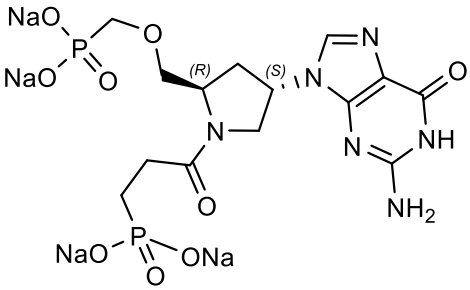
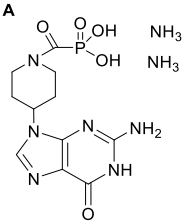
Code amount	Structure	**comment**
DR-6436 4.1 mg (DR-3688		
DR-6404 5.8 mg		ppGpp analogue
DR-5994A 4.8 mg		**Piperidine phosphonate**
DR-6410A 11.9 mg		ppGpp analogue

RELACIN 23 mg	 Molecular Weight: 653,56	
DR-6222 10 mg	 Molecular Weight: 508,32152	**GDP analogue**
DR-6406 6.5 mg	 Molecular Weight: 693.59	ppGpp analogue
DR-5839B 7.8 mg	 Molecular Weight: 579.40052 Chemical Formula: $C_{14}H_{19}N_5O_{12}P_2$ Exact Mass: 511.05054	ppGpp analogue

DR-6492 8 mg		ppGpp analogue
DR-6392 4 mg		ppGpp analogue
DR-6393 3.2 mg		ppGpp analogue
DR-6397 5.3 mg		ppGpp analogue
DR-5839A 8.9 mg		ppGpp analogue

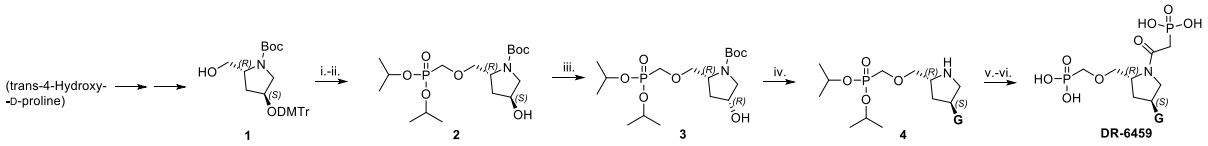
DR-6459 5.5 mg		**Prolinol bisphosphonate**
DR-6267 9 mg		**Prolinol phosphonate**
DR-6220B 7 mg		ppGpp analogue
DR-6549 8 mg		**Pyrrolidine nucleoside carboxylate**

DR-6272 8.3 mg	 Molecular Weight: 582,26	**Pyrrolidine bisphosphonate**
DR-6506 10.6 mg	 Molecular Weight: 947,46	ppGpp analogue
DR-6331 7.1 mg	**DR-6331**  Molecular Weight: 539.37952	ppGpp analogue
DR-6541 4.1 mg	 Chemical Formula: $C_{15}H_{20}N_6Na_4O_9P_2$ Molecular Weight: 582.26460	**Prolinol bisphosphonate**

DR6266 4.5 mg	 **DR-6266** [455.28]	**Prolinol phosphonate**
DR-5836 15.2 mg	**DR-5836**  Molecular Weight: 563.40	**ppApp analogue**
DR-6542 5.6 mg	 Chemical Formula: $C_{14}H_{18}N_6Na_4O_9P_2$ Molecular Weight: 568.23760	**Prolinol bisphosphonate**
DR-6468 16.6 mg	 568,24	**Prolinol bisphosphonate**
DR-6011A 4.4 mg	**DR-6011A**  Chemical Formula: $C_{11}H_{15}N_6O_5P$ Exact Mass: 342.08415	**Piperidine phosphonate**

Supplementary document S2 Synthesis of compound DR-6459

Compound **DR-6459** was synthesized according to scheme 1 starting from *trans*-4-hydroxy-D-proline. Detailed description of synthesis of compound **DR-6459** will be published elsewhere together with its derivatives.



Scheme 1 Synthesis of prolinol nucleotide **DR-6459**. G stands for guanin-9-yl

Experimental

Synthesis

General conditions and used materials: Unless stated otherwise, all used solvents were anhydrous. TLC was performed on silica gel pre-coated aluminium plates TLC Silica gel 60 F_{254} (Supelco), and compounds were detected by UV light (254 nm), by heating (detection of dimethoxytrityl group, orange color), by spraying with 1% solution of ninhydrine to visualize amines, and by spraying with 1% solution of 4-(4-nitrobenzyl)pyridine in ethanol followed by heating and treating with gaseous ammonia (blue color of mono- and diesters of phosphonic acid). Preparative column chromatography was carried out on silica gel (40–63μm, Fluorochem), and elution was performed at the flow rate of 60–80 mL/min. The following solvent systems were used for TLC and preparative chromatography: toluene-ethyl acetate 1:1 (T), chloroform-ethanol 9:1 (C1), ethyl acetate-acetone-ethanol-water 6:1:1:0.5 (H3), ethyl acetate-acetone-ethanol-water 4:1:1:1 (H1). The concentrations of solvent systems are stated in volume percents (%, *v*/*v*). The purity of the final compounds was greater than 95%. Purity of prepared compounds was determined by LC-MS performed on Waters AutoPurification System with 2545 Quarternary Gradient Module and 3100 Single Quadrupole Mass Detector using LUNA C18, column (Phenomenex, 100×4.6 mm, 3 μm) at flow rate 1 mL/min. Typical conditions: mobile phase, A – 50mM NH_4HCO_3, B – 50 mm NH_4HCO_3 in 50% *aq.* CH_3CN, C – CH_3CN, A→B/10 min, B→C/10 min, C/5 min. Preparative RP HPLC was performed on LC5000 Liquid Chromatograph (INGOS-PIKRON, CR) using Luna C18 (2) column (250×21.2 mm, 5 μm) at flow rate of 10 mL/min by a gradient elution of methanol in 0.1M TEAB pH 7.5 (A = 0.1M TEAB, B = 0.1M TEAB in 50% aq. methanol, C = methanol) or without buffer. All final compounds were lyophilized from water. Mass spectra were recorded on LTQ Orbitrap XL (Thermo Fisher Scientific) using ESI ionization. Infrared (IR) spectra were recorded on a Thermo Scientific

Nicolet 6700 spectrometer. Absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}). Specific rotation values were determined with an Autopol IV (Rudolph Research Analytical, USA, 2001) polarimeter. Specific rotation values $[\alpha]_D^{20}$ were measured in H_2O (concentration units: g/100 mL). Compounds NMR spectra were measured on Bruker AVANCE III™ HD 400 MHz (^1H at 400.1 MHz, ^{13}C at 100.6 MHz and ^{31}P at 162.0 MHz), Bruker Avance III™ HD 400 MHz Prodigy (^1H at 401.0 MHz, ^{13}C at 100.8 MHz and ^{31}P at 162.0 MHz), Bruker Avance III™ HD 500 MHz (^1H at 500.0 MHz, ^{13}C at 125.7 MHz and ^{31}P at 202.4 MHz) and JEOL JNM-ECZR 500 MHz (^1H at 500.2 MHz, ^{13}C at 125.8 MHz and ^{31}P at 202.5 MHz) spectrometers. D_2O (reference (dioxane) = ^1H 3.75 ppm, ^{13}C 69.3 ppm. Chemical shifts (in ppm, δ scale) were referenced to TMS as internal standard, coupling constants (*J*) are given in Hz. Complete assignment of protons and carbons was done by analysis of correlated homonuclear 2D-COSY and heteronuclear ^1H-^{13}C HSQC and ^1H-^{13}C HMBC spectra. Relative configuration was checked using DPFGSE-NOE and 2D-ROESY techniques. All intermediates were determined by LC-MS.

General method (i) Reaction with diisopropyl tosyloxymethanephosphonate

The mixture of starting material (1 mmol) and diisopropyl tosyloxymethanephosphonate (1.5 mmol) was co-evaporated with toluene (2x 20 mL) and dissolved in DMF (10 mL). Sodium hydride (2 mmol) was added at 0 °C (an ice bath) under argon atmosphere and the reaction mixture was stirred overnight at rt. The mixture was cooled to 0 °C (an ice bath) and acetic acid (2 mmol) was added. The mixture was stirred at rt for 20 min and concentrated in vacuo. The product was obtained using column chromatography on silica gel using linear gradient of ethyl acetate in toluene.

General method (ii) Removal of DMTr protecting group

Starting material (1 mmol) was dissolved in chloroform (10 mL) and 4% TFA in chloroform was added (10 mL). The reaction mixture was stirred at rt for 15 min and then neutralized with solid $NaHCO_3$. Solids were removed by filtration and the filtrate was concentrated in vacuo. The product was obtained by column chromatography on silica gel using linear gradient of ethanol in chloroform.

General method (iii) Inversion of configuration

The mixture of hydroxy derivative (1 mmol), triphenylphosphine (2.5 mmol), lutidine (1.5 mmol), and 4-nitrobenzoic acid (1.3 mmol) was co-evaporated with THF (2x 10 mL) and dissolved in the same solvent (10 mL/mmol). DIAD (2.5 mmol) was added under argon atmosphere, and the reaction mixture was stirred at rt overnight. The reaction mixture was concentrated in vacuo and 4-nitrobenzoic acid ester with inverted configuration was obtained by column chromatography on silica gel using linear gradient of ethanol in chloroform. The product was dissolved in methanol and the solution was saturated with gaseous ammonia at 0 °C. The mixture was left aside at rt overnight and concentrated in vacuo. The desired hydroxy derivative with inverted configuration was obtained by column chromatography on silica gel using linear gradient of ethanol in chloroform.

General method (iv) Mitsunobu nucleosidation with subsequent Boc group removal and nucleobase hydrolysis

DIAD (3.5 mmol) was added to the solution of diphenylpyridylphosphine (3.5 mmol) in THF (5 ml/mmol) and the mixture was stirred at rt under argon atmosphere for 30 min. The mixture was then added to the mixture of substrate (1 mmol) and 2-amino-6-chloropurine (1.5 mmol) (co-evaporated prior to the reaction with THF (2x10mL) in THF (5 ml/mmol). The reaction mixture was stirred under argon atmosphere at rt overnight. The reaction mixture was concentrated in vacuo and the chloropurine product was obtained by column chromatography on silica gel using linear gradient of ethanol in chloroform.

Protected chloropurine derivative (1 mmol) was stirred with EtOH (10 mL/mmol) and 3M aq. HCl (10 mL/mmol) at 80 °C overnight. The reaction mixture was diluted with water:EtOH 1:1 (20 mL/mmol) and applied on column of Dowex 50 in H^+ form (20 mL/mmol). The Dowex was washed with 50% aq. ethanol (50 mL/mmol) and the crude product was eluted with 3% ammonia in 50% aq. ethanol. After evaporation the product was used in the crude form for the next reaction step or purified using HPLC on reversed phase using linear gradient of MeOH in water.

General method (v) attachment of phosphonoacetic acid

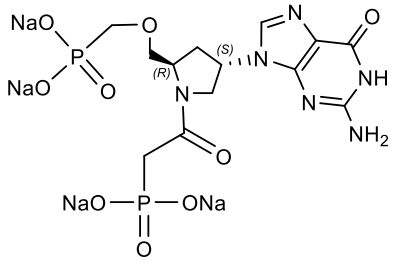
EDC (3 mmol) was added to the mixture of starting material (1 mmol) and diisopropyl phosphonoacetic acid (1.2 mmol) in DMF (10 mL/mmol), and the reaction mixture was stirred under argon atmosphere at 90 °C for 4 h. The reaction mixture was concentrated in vacuo, and the desired product was obtained by column chromatography on silica gel using linear gradient of H1 system in ethyl acetate.

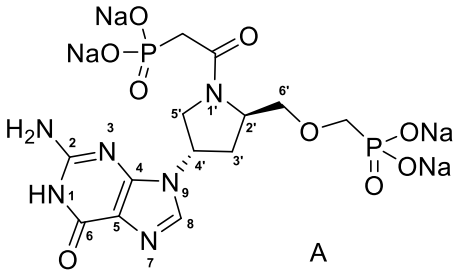
General method (vi) de-esterification of phosphonates

Tetra ester (1 mmol) was dissolved in MeCN (10 mL/mmol). TMSBr (7 mmol) was added and the reaction mixture was stirred under argon atmosphere at rt overnight. The solvent was removed in vacuo, the residue was dissolved in 2M aq. TEAB (5 mL/mmol) and EtOH (5 mL/mmol) and again concentrated. The target compound was obtained using preparative HPLC on reversed phase using linear gradient of MeOH in 0.1M aq. TEAB. Fractions containing the desired product (according to LCMS) were combined and evaporated. The residue was co-evaporated with MeOH (3x 10 mL/mmol) to remove all remaining TEAB. Finally, the product was converted to sodium salt by passing its aq. solution through column of Dowex 50 in Na^+ form. The final product was lyophilized from water to form a white solid.

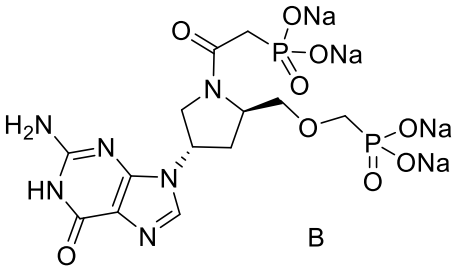
[2*R*,4*S*]-4-Guanin-9-yl-4-(phosphonomethoxymethyl)-1-*N*-(2-phosphonoacetyl)-pyrrolidine DR-6459

Compound **DR-6459** was prepared from **1** according to General procedures **(i)-(vi)** in overall yield 120 mg, 10 %.





A



B

A mixture of rotamers A:B ~ 2:1

^{1}H NMR (500.0 MHz, D_2O, ref(*t*-BuOH) = 1.24 ppm): 2.52 – 2.71 (m, 4H, H-3′-A,B); 2.75 – 2.95 (m, 3H, $COCH_2P$-A, $COCH_a\mathbf{H_b}P$-B); 3.20 (dd, 1H, $J_{H,P}$ = 19.8, J_{gem} = 13.8, $COC\mathbf{H_a}H_bP$-B); 3.63 – 3.72 (m, 4H, OCH_2P-A,B); 3.75 (dd, 1H, J_{gem} = 9.9, $J_{6'b,2'}$ = 3.0, H-6′b-A); 3.78, 3.82 (2 × dd, 2 × 1H, J_{gem} = 10.2, $J_{6',2'}$ = 4.9, H-6′-B); 3.83 (ddd, 1H, J_{gem} = 12.6, $J_{5'b,4'}$ = 6.8, $J_{H,P}$ = 1.3, H-5′b-B); 3.87 (dd, 1H, J_{gem} = 9.9, $J_{6'a,2'}$ = 5.1, H-6′a-A); 3.98 (dd, 1H, J_{gem} = 11.2, $J_{5'b,4'}$ = 6.6, H-5′b-A); 4.00 (ddd, 1H, J_{gem} = 12.6, $J_{5'a,4'}$ = 8.3, $J_{H,P}$ = 2.1, H-5′a-B); 4.32 (dd, 1H, J_{gem} = 11.2, $J_{5'b,4'}$ = 7.6, H-5′a-A); 4.50 (m, 1H, H-2′-A); 4.76 (m, 1H, H-2′-B); 5.17 – 5.26 (m, 2H, H-4′-A,B); 7.84 (s, 1H, H-8-A); 7.90 (s, 1H, H-8-B).

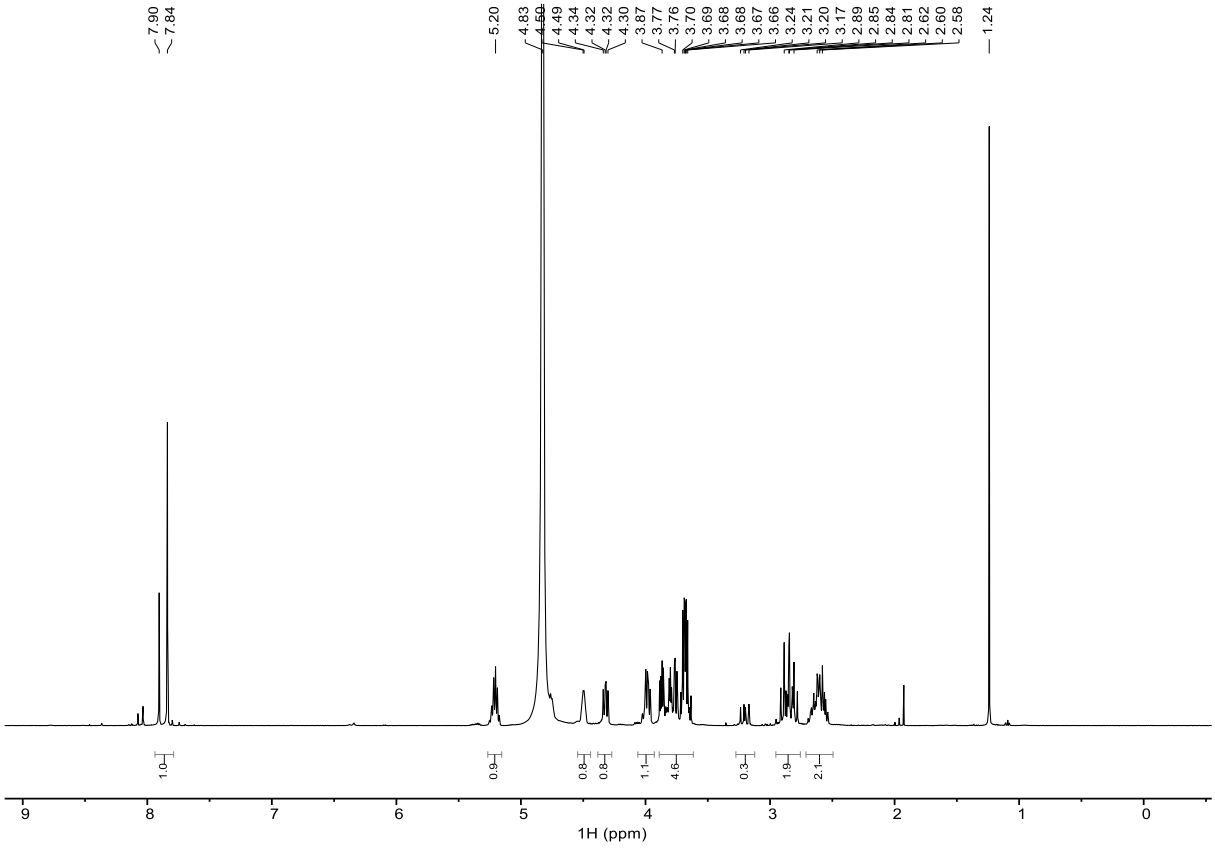
^{13}C NMR (125.7 MHz, ref(*t*-BuOH) = 30.29 ppm): 33.51 (CH_2-3′-A); 34.78 (CH_2-3′-B); 37.09 (d, $J_{C,P}$ = 117.6, $CO\mathbf{C}H_2P$-B); 38.27 (d, $J_{C,P}$ = 117.4, $CO\mathbf{C}H_2P$-A); 51.24 (CH_2-5′-B); 52.43 (CH-4′-B); 53.35 (CH-4′-A); 53.37 (CH_2-5′-A); 56.95 (CH-2′-A); 58.21 (CH-2′-B); 68.49 (d, $J_{C,P}$ = 155.5, OCH_2P-A); 68.79 (d, $J_{C,P}$ = 155.3, OCH_2P-B); 73.03 (d, $J_{C,P}$ = 10.4, CH_2-6′-A); 75.01 (d, $J_{C,P}$ = 11.7, CH_2-6′-B); 116.57 (C-5-B); 116.62 (C-5-A); 138.29 (CH-8-A); 138.45 (CH-8-B); 151.99 (C-4-A,B); 154.24 (C-2-A); 154.26 (C-2-B); 159.43 (C-6-A,B); 170.37 (d, $J_{C,P}$ = 5.9, $\mathbf{C}OCH_2P$-A); 170.55 (d, $J_{C,P}$ = 5.8, $\mathbf{C}OCH_2P$-B).

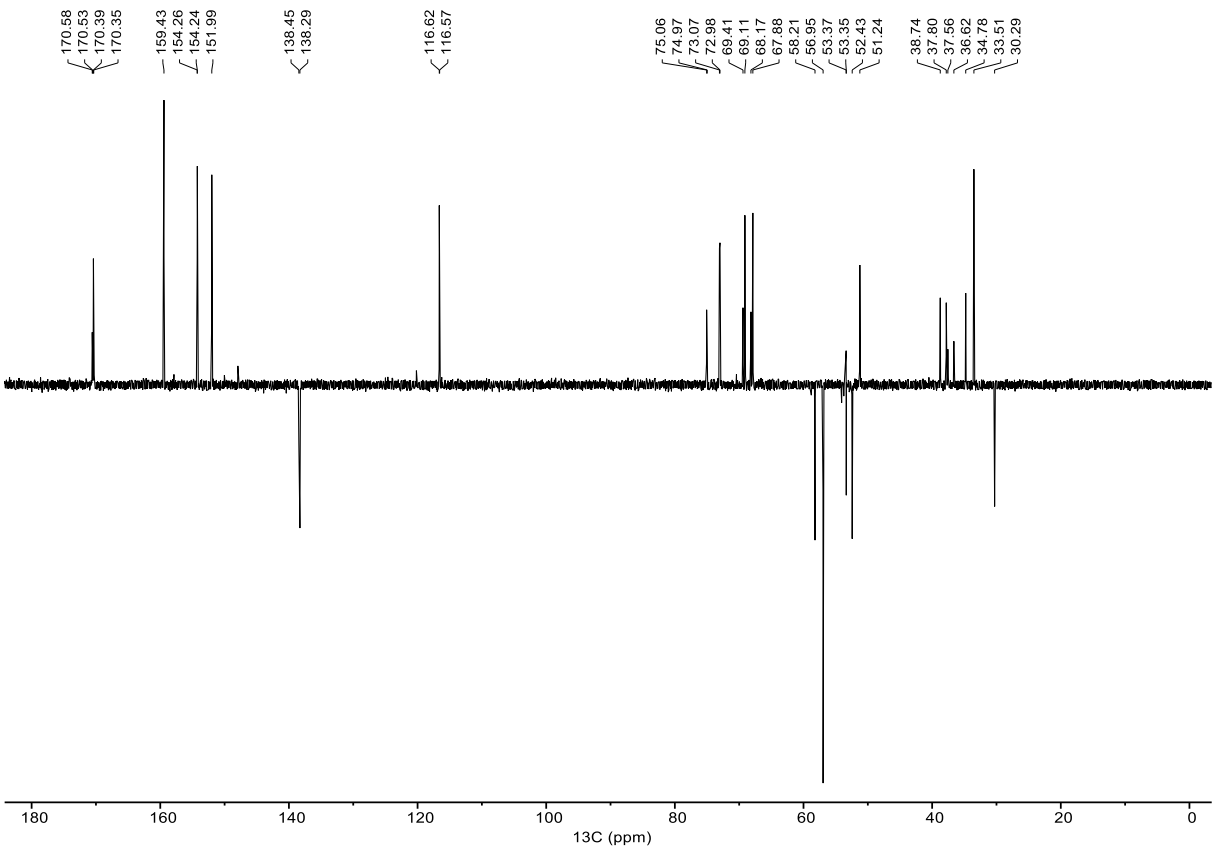
$^{31}P\{^{1}H\}$ NMR (202.4 MHz, D_2O): 13.67 (PCH_2CO-A); 13.79 (PCH_2CO-B); 15.80 (PCH_2O-B); 16.34 (PCH_2O-A).

IR ν_{max} (KBr) 3420 (vs, vbr), 3320 (vs, sh), 3119 (s, br), 2779 (m, br), 2368 (m, vbr), 1693 (vs), 1612 (s, br), 1570 (m, sh), 1481 (m, sh), 1409 (m), 1321 (vw), 1118 (m, sh), 1075 (s, br), 909 (m, br), 782 (w), 695 (w), 641 (w).

HR-ESI $C_{13}H_{19}O_9N_6P_2$ $(M-H)^-$ calcd 465.06942, found 465.06927.

$[\alpha]_D^{20}$ +18.2 (c 0.318, H_2O)

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38.74
37.80
37.56
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140
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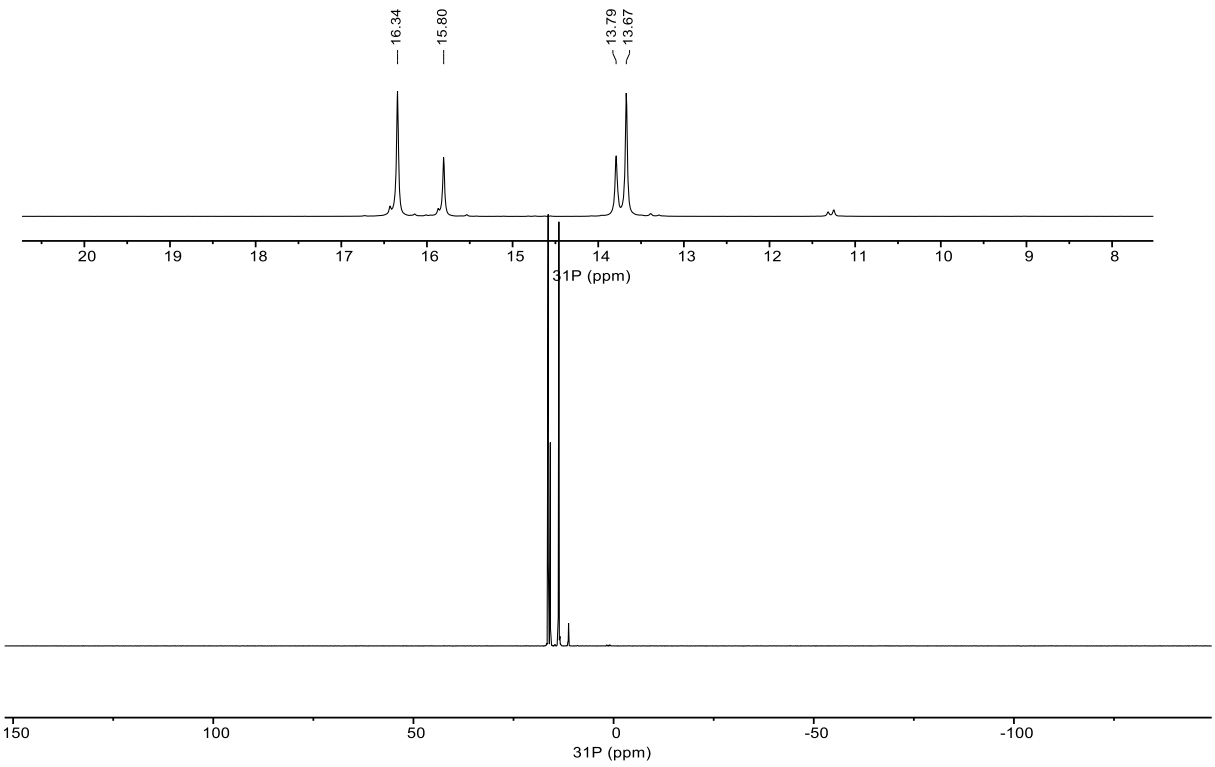
100
80
60
40
20
0
13C (ppm)


16.34
15.80
13.79
13.67
20
19
18
17
16
15
14
13
12
11
10
9
8
31P (ppm)
150
100
50
0
-50
-100
31P (ppm)

Supplementary Table 1.

Table of the final readings at 620 nm for the ppGpp analogues' effect on the SpoT HYD activity. One representative of the screening data is shown. The negative controls were the normal SpoT HYD reactions without supplemented ppGpp analogue (A_{620nm}= 0.207 ± 0.023, five replicates), while the positive controls did not contain the SpoT protein (A_{620nm}= 0.419 ± 0.053, five replicates). The two analogues showing the greatest inhibition of SpoT HYD activity are in bold.

analogue nr.	A 620nm	analogue nr.	A 620nm
DR-6902B	0.2	DR-6397	0.273
DR-6902A	0.339	**DR-5839A**	**0.384**
DR-6330A	0.291	**DR-6459**	**0.344**
DR-6330B	0.193	DR-6267	0.261
pAp Na+	0.258	DR-6220B	0.228
DR-6436	0.251	DR-6549	0.268
DR-6404	0.257	DR-6272	0.237
DR-5994A	0.202	DR-6506	0.278
DR-6410A	0.251	DR-6331	0.224
DR-6222	0.196	DR-6541	0.247
DR-6406	0.198	DR6266	0.226
DR-5839B	0.25	DR-5836	0.233
DR-6492	0.275	DR-6542	0.247
DR-6392	0.254	DR-6468	0.25
DR-6393	0.258	DR-6011A	0.211

Supplementary Table 2

The list of 60 known metabolites/antibiotics (at the final concentration of 11.1 μg/ml) produced by actinomycetes which were tested with their potential effects on SpoT HYD activity.

amicetin	NAI-112	NAI-113	NAI-808	α-823	butenolide	pentenomycin	chrolactomycin	6-ammino penicillanic acid	Ramoplanin
actinomycin D	purpuromycin	GE23077	NAI-107	streptolydigin	α-698 (p4)	α-698 (p7)	amphotericin B	gentamycin	GE2270
luxomycin	thermorubin	vancomycin	tetracycline	viomycin	Cl-tetracycline	colistin	A40926	penicillinG	teicoplanin
virginiamycin	nosiheptide	kirromycin	salinomycin	clindamycin	metronidazole	nystatin	Cloramphenicol	thiostrepton	monensin
fusidic acid	novobiocin	streptomycin	neomycin	panosialin	gramicidin	spectinomycin	kasugamycin	tunicamycin	lincomycin
lidicamycin	daunorubicin	gardimycin	NAI-857	erithromycin	rifampicin	NAI- 414	NAI-491	Rifamycin O	Rifamycin S