nature | methods

Virtual terminator nucleotides for next-generation DNA sequencing

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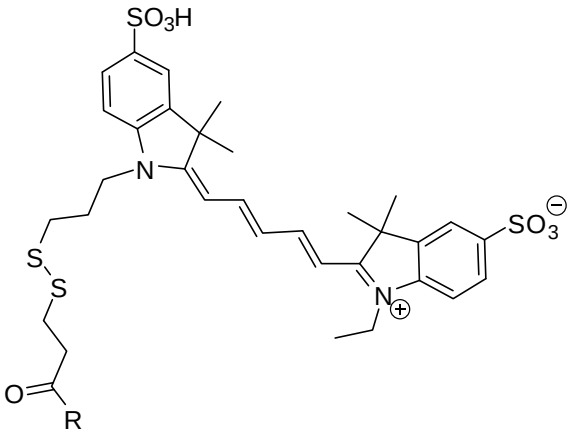
Supplementary figures and text:

Supplementary Figure 1. Cy5-12ss-dNTP analogs



25, R = C5-propargylamino-2'-deoxyuridine-5'-triphosphate

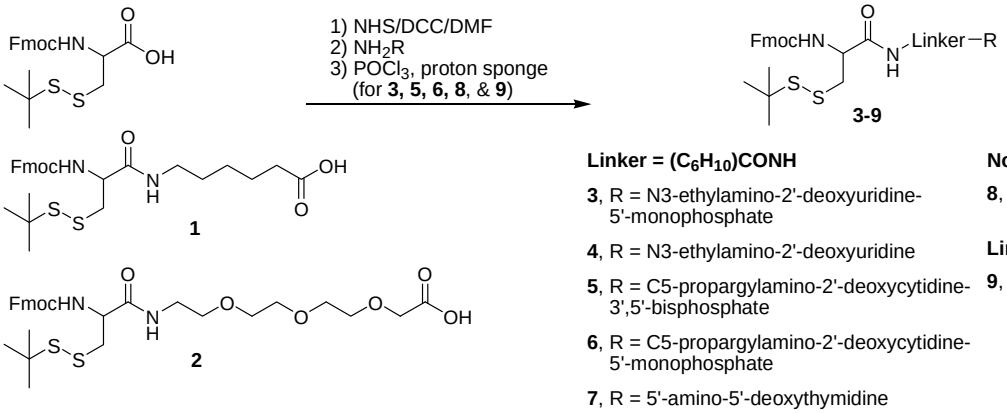
26, R = C5-propargylamino-2'-deoxycytidine-5'-triphosphate

27, R = C7-propargylamino-2'-deoxy-7-deazaguanosine-5'-triphosphate

28, R = C7-propargylamino-2'-deoxy-7-deazaadenosine-5'-triphosphate

Nature Methods: doi:10.1038/nmeth.1354

Supplementary Figure 2. Scheme 1 for nucleotide analog synthesis (Compounds 17-24, 29, 30, & 32)



Linker = $(C_6H_{10})CONH$

3, R = N3-ethylamino-2'-deoxyuridine-5'-monophosphate

4, R = N3-ethylamino-2'-deoxyuridine

5, R = C5-propargylamino-2'-deoxycytidine-3',5'-bisphosphate

6, R = C5-propargylamino-2'-deoxycytidine-5'-monophosphate

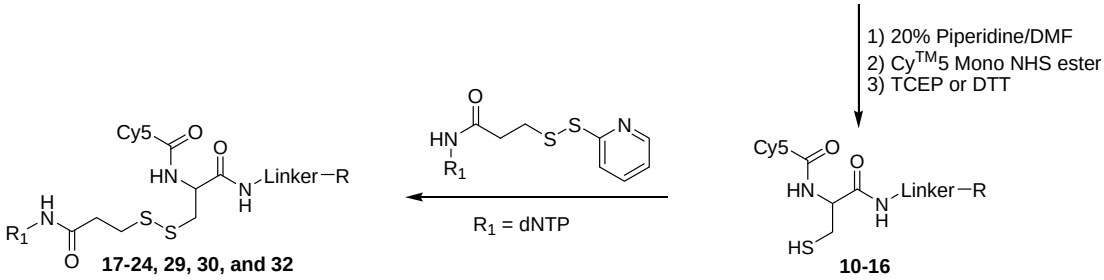
7, R = 5'-amino-5'-deoxythymidine

No linker

8, R = C5-aminoallyl-2'-deoxyuridine-5'-monophosphate

Linker = $(C_2H_4O)_3CH_2CONH$

9, R = C5-aminoallyl-2'-deoxycytidine-5'-monophosphate



Linker = $(C_6H_{10})CONH$

10, R = N3-ethylamino-2'-deoxyuridine-5'-monophosphate

11, R = N3-ethylamino-2'-deoxyuridine

12, R = C5-propargylamino-2'-deoxycytidine-3',5'-bisphosphate

13, R = C5-propargylamino-2'-deoxycytidine-5'-monophosphate

14, R = 5'-amino-5'-deoxythymidine

No linker

15, R = C5-aminoallyl-2'-deoxyuridine-5'-monophosphate

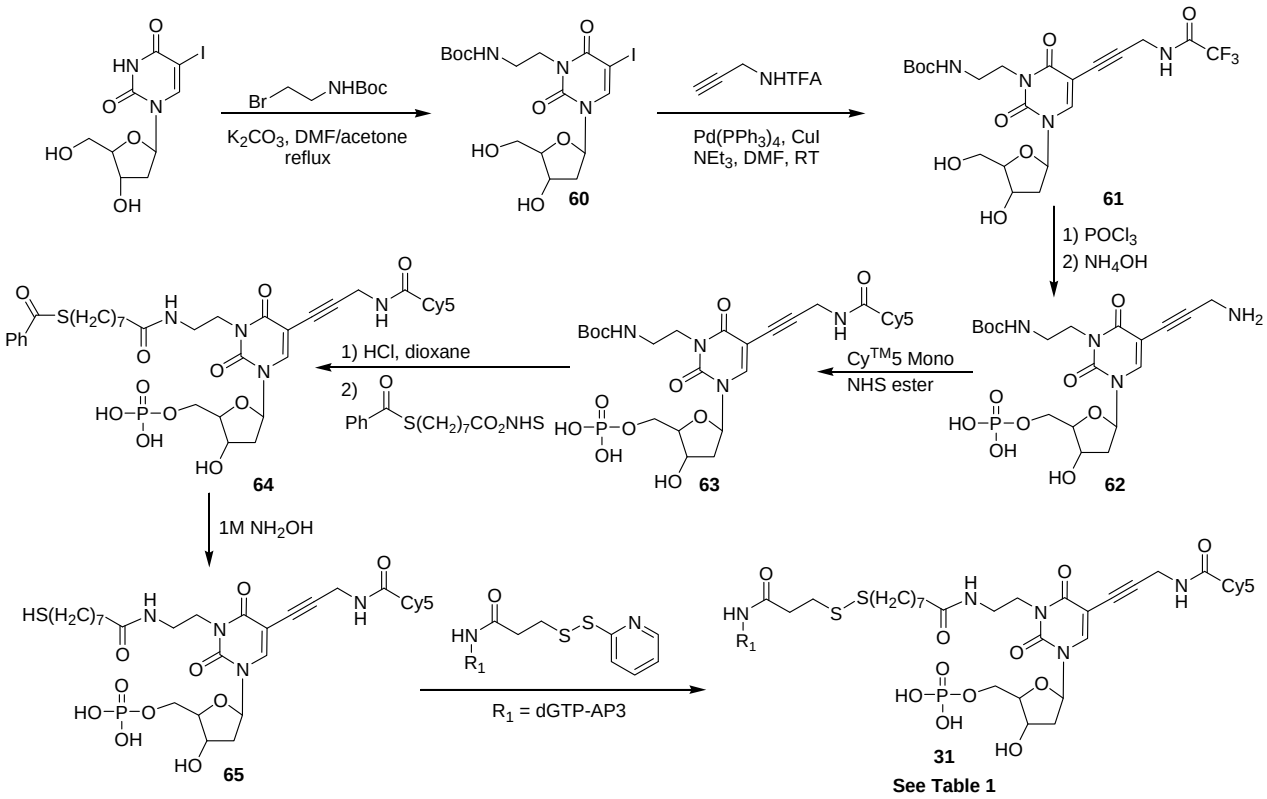
Linker = $(C_2H_4O)_3CH_2CONH$

16, R = C5-aminoallyl-2'-deoxycytidine-5'-monophosphate

See Table 1 for R and R_1 substituents

Nature Methods: doi:10.1038/nmeth.1354

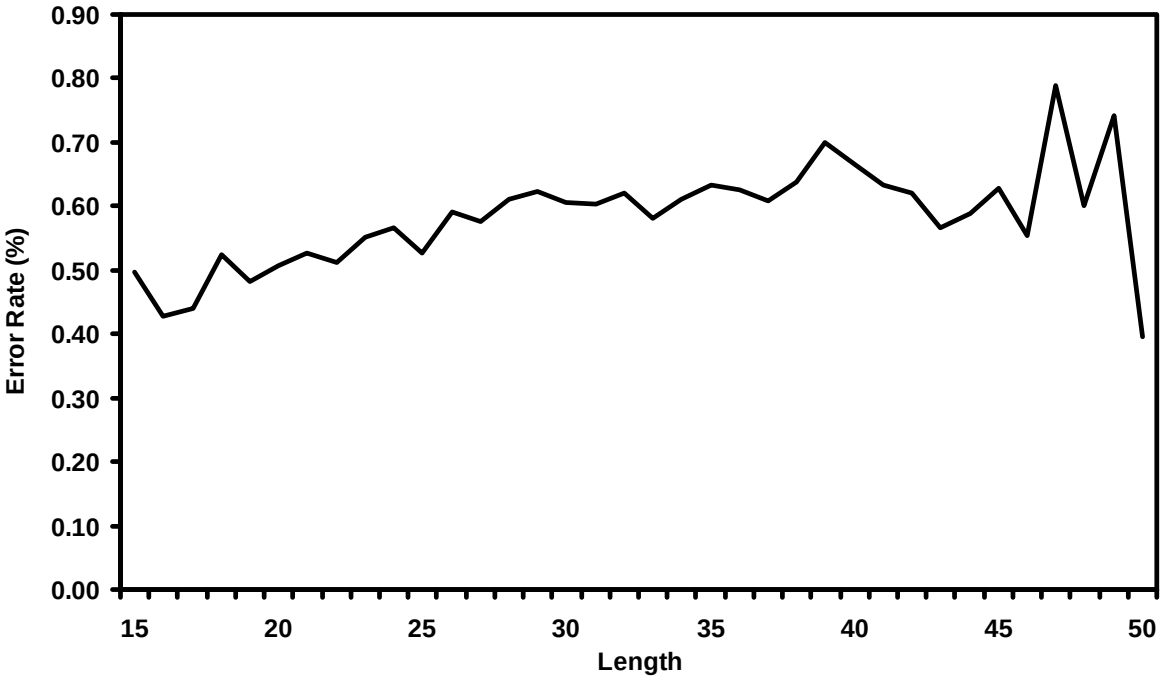
Supplementary Figure 3. Scheme 2 for nucleotide analog synthesis (Compound 31)



Nature Methods: doi:10.1038/nmeth.1354

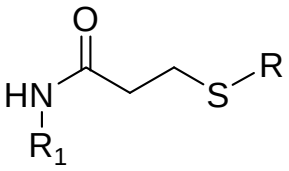
Supplementary Figure 4: Error Rate

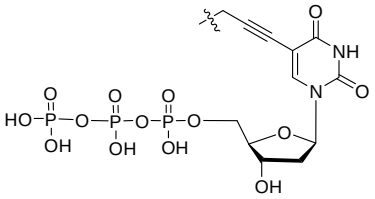
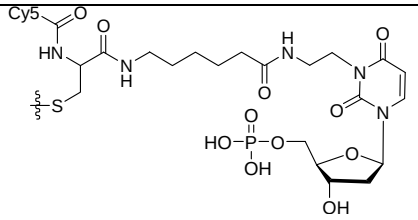
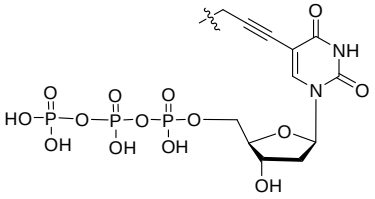
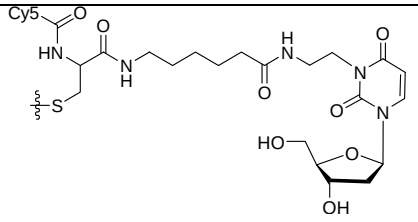
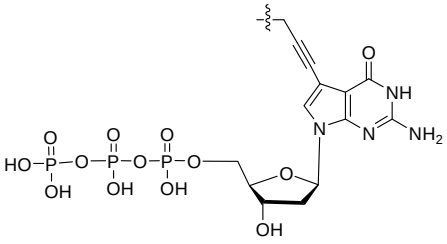
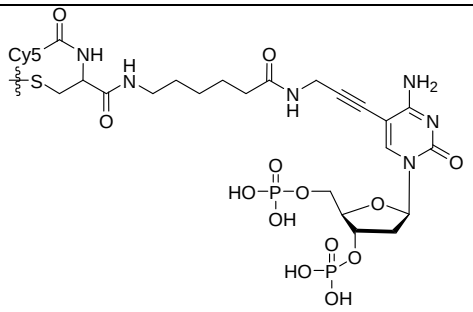
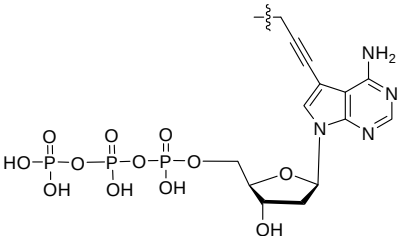
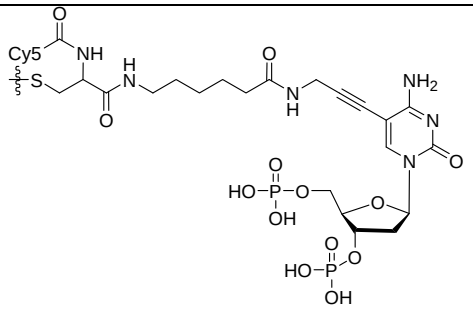
The error rate as a function of read length is shown for all reads for which two pass sequences were obtained. This is essentially invariant due to the random nature of the errors. The error rate reduction achieved with multiple pass sequencing is determined by the square of the single pass error rate.



Nature Methods: doi:10.1038/nmeth.1354

Supplementary Table 1. Nucleotide analogs



Compound Number	R_1	R
17		
18		
19		
20		

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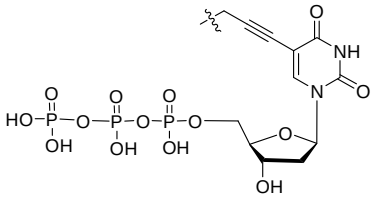
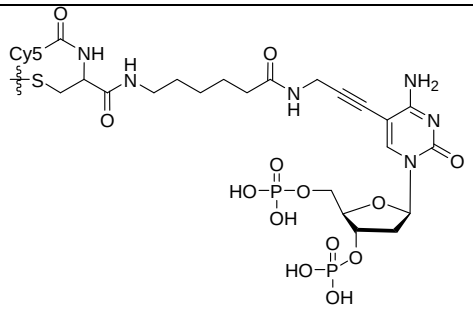
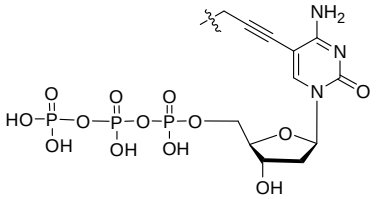
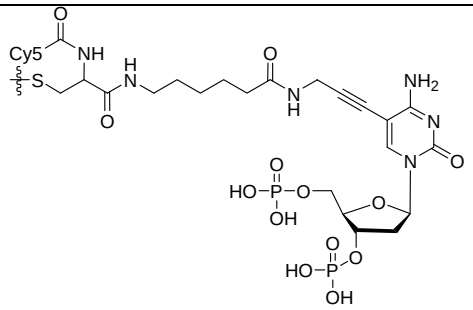
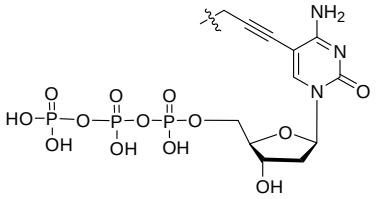
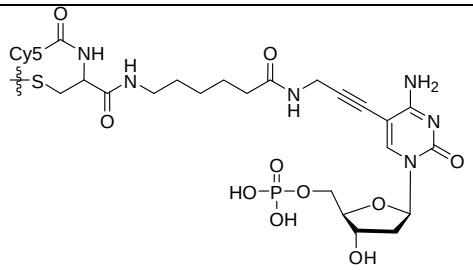
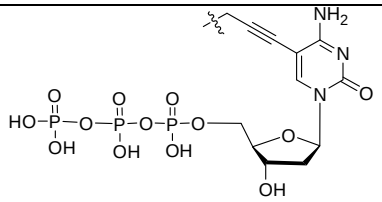
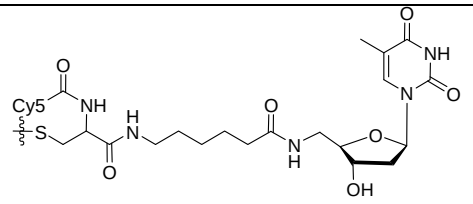
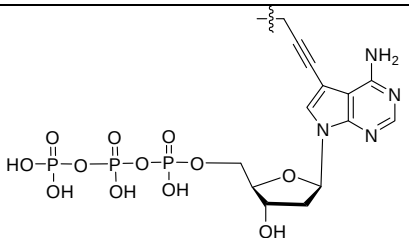
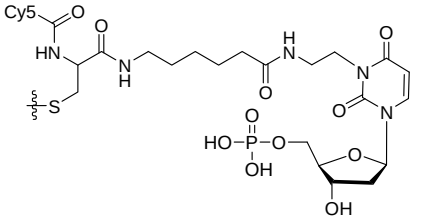
21		
22		
23		
24		
29		

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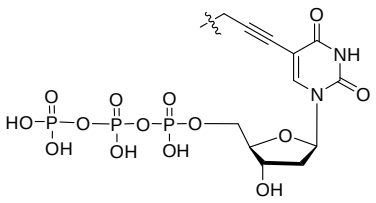
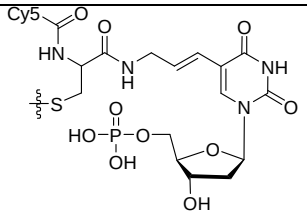
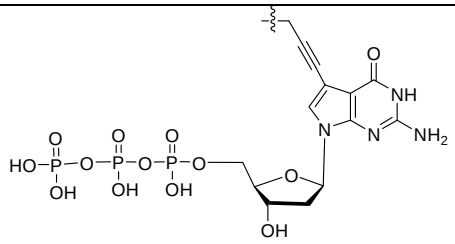
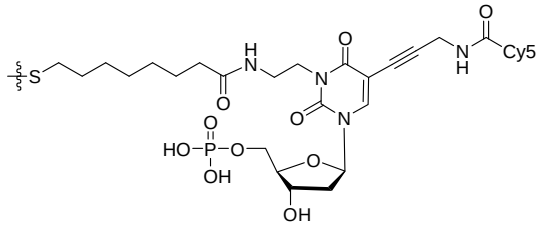
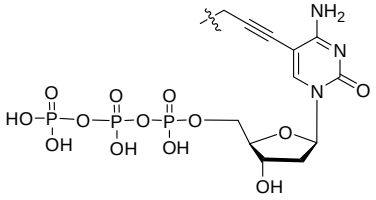
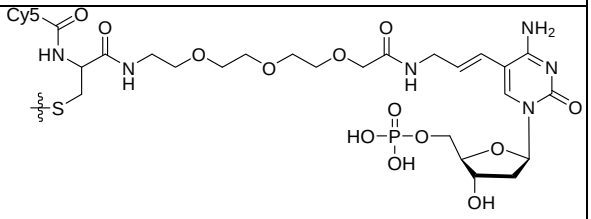
30		
31		
32		

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Supplementary Table 2 Misincorporation rates for two analogs

The incorportation rates for the standard Cy5-12ss-dNTPs analog are compared to one of the analogous Virtual Terminator nucleotides for all possible pairings. The specificity of incorporation for the proper base pair versus the possible mispairs is shown (with specificity defined as the rate of incorporation of the correct nucleotide divided by the rate of misincorporation). In all cases, analogs showed similar fidelity (correct incorporation rate divided by the misincorporation rate) to that of the Cy5-12ss-dUTP analog. Similar fidelity results were observed for other analogs (data not shown).

25 (12ss-dUTP)			**17 (U*pU)**		
DNA	Rate	Specificity	DNA	Rate	Specificity
TA	0.42	-	TA	0.14	-
TG	2.6e-3	162	TG	8.5e-4	164
TT	2.5e-3	168	TT	1.5e-4	933
TC	9.9e-5	4242	TC	8.9e-5	1573

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Supplementary Table 3 Termination ability of analogs to prevent second base incorporation

The rate of incorporation is shown for both the first and second base in a homopolymer run. For most reactions, nucleotides were at 100 nM. However, some k1 and k2 rates were slow so concentrations for those analogs were increased to 250 nM (marked by superscript "a"). Compound structures are shown in Supplementary Table 1 and Supplementary Figure 1. The "type" column is a shorthand nomenclature which indicates the incorporated nucleotide connected via the tether (*) to the inhibitory component. Different tethers with some analogs are signified by a different number of asterisks.

Analog	Type	$k_1 (s^{-1})$	$k_2 (s^{-1})$	k_1/k_2
17	U*pU	0.015	$6.0e^{-5}$	250
18	U*U	0.05	$5.6e^{-3}$	8.9
19	G*pCp	0.06[a]	$2.5e^{-5}$	2115
20	A*pCp	0.03[a]	$<1e^{-6}$	$>3e^{4}$
21	U*pCp	0.02[a]	$<1e^{-6}$	$>2e^{4}$
22	C*pCp	0.03[a]	$<1e^{-6}$	$>3e^{4}$
23	C*pC	0.04	$3.0e^{-4}$	117
24	C*T	0.08	$1.6e^{-2}$	5
25	12ss-dUTP	0.064	$3.8e^{-2}$	1.7
26	12ss-dCTP	0.098	$3.6e^{-2}$	2.7
27	12ss-dGTP	0.078	$4.3e^{-3}$	18.1
28	12ss-dATP	0.12	$1.2e^{-2}$	10
29	A*pU	0.02	$4.8e^{-5}$	396
30	U**pU	0.02	$6.4e^{-5}$	359
31	G***pU	0.04	$1.7e^{-5}$	2118
32	C****pC	0.03[a]	$1.3e^{-4}$	246

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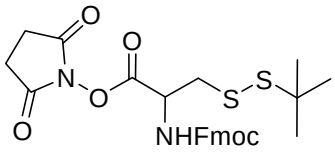
Supplementary Note

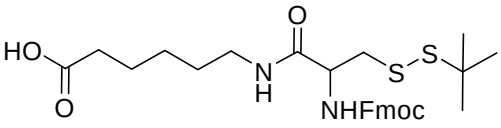
Experimental Procedures

Compound 33



Fmoc-Cys(S*t*Bu)-OH (2.0 g, 4.63 mmol, 1 equiv) was dissolved in MeCN (10mL). DCC (1.2 g, 5.81 mmol, 1.26 equiv) was added, followed by NHS (0.70 g, 6.08 mmol, 1.31 equiv) and the reaction was stirred at RT for an hour. White precipitate (DCU) began forming within five minutes. The reaction mixture was transferred to eppendorf tubes and centrifuged to remove the white precipitate. The supernatant was then used in subsequent reactions without further purification: LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{26}H_{28}N_2O_6S_2$ 527, found 527, R_f = 0.83 (10% MeOH/CH_2Cl_2).

Compound 1



6-Aminohexanoic acid (0.60 g, 4.57 mmol, 1 equiv) was dissolved in 1:1 H_2O:DMF (6 mL total). DIPEA (0.016 mL) was added to keep the pH ~ 8. NHS ester **33** (4.63 mmol in 10 mL MeCN, 1.01 equiv) was added to the reaction mixture in 1 mL

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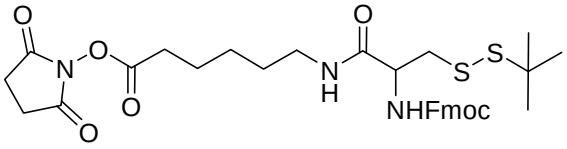
aliquots over ~10 min. DIPEA (0.02 mL) was added after each aliquot to keep the reaction basic. After the first aliquot of **33** was added, the reaction became cloudy, and addition of extra H_2O (0.2 mL) was needed to clear up the solution. The reaction was stirred at RT for two hours, then quenched with 20 mL 10% HCl (aq). The aqueous phase was extracted 2 × 50mL CH_2Cl_2. The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield a brown oil. Purification by flash column chromatography (100% CH_2Cl_2 to 5% MeOH/CH_2Cl_2) afforded the desired acid as a white foam (2.14 g, 87%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{28}H_{36}N_2O_5S_2$ 543, found 543, R_f = 0.41 (10% MeOH/CH_2Cl_2).

Compound 34



Acid **1** (0.99 g, 1.82mmol, 1 equiv) was dissolved in MeCN (10 mL). DCC (0.46 g, 2.23 mmol, 1.23 equiv) was added, followed by NHS (0.28 g, 2.43 mmol, 1.34 equiv) and the reaction was stirred at RT for an hour. White precipitate (DCU) began forming within five minutes. The reaction mixture was transferred to eppendorf tubes and centrifuged to remove the white precipitate. The supernatant was then used in subsequent reactions without further purification: LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{32}H_{39}N_3O_7S_2$ 640, found 640, R_f = 0.62 (10% MeOH/CH_2Cl_2).

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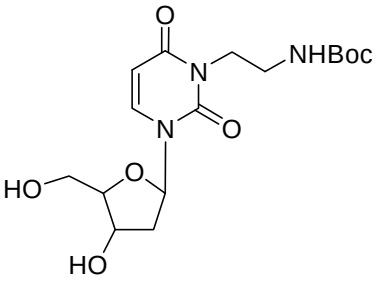
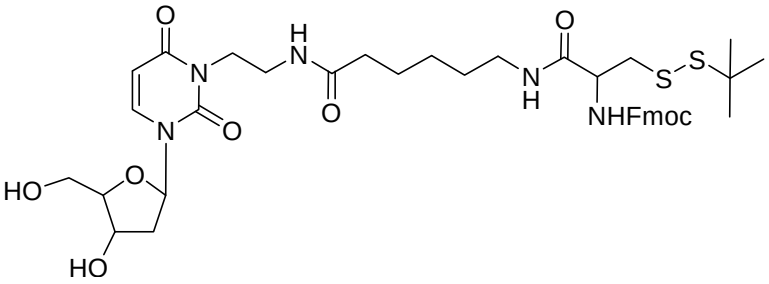
Compound 35



2'-Deoxyuridine (0.40 g, 1.75 mmol, 1 equiv) and 2-(boc-amino)ethyl bromide (0.59 g, 2.63 mmol, 1.50 equiv) were dissolved in 1:1 DMF:acetone (3.4 mL total). K_2CO_3 (0.41 g, 2.97 mmol, 1.69 equiv) and tetrabutylammonium iodide (0.07 g, 0.19 mmol, 0.11 equiv) were added, and the reaction was heated at 60 °C under an Ar atmosphere for 12 hours. After cooling to RT, the reaction was diluted with EtOAc (50 mL) and washed with brine (2 × 50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield the desired carbamate, which was used without further purification (0.50 g, 77%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{16}H_{25}N_3O_7$ 370, found 370, R_f = 0.19 (10% MeOH/CH_2Cl_2).

Compound 4



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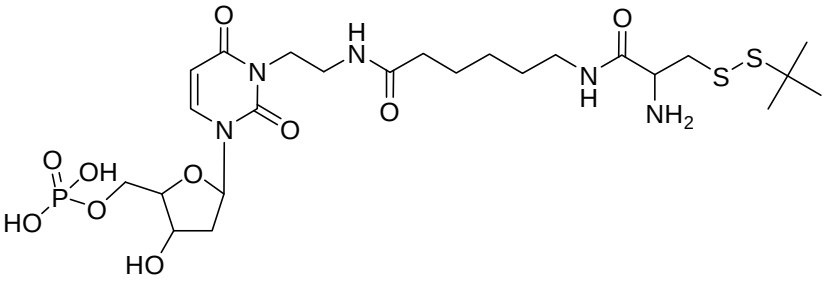
Carbamate **35** (0.31 g, 0.83 mmol, 1 equiv) was dissolved in THF (3mL). HCl (1.7 mL, 6.8 mmol, 4.0 M in dioxane, 8.2 equiv) was added, causing formation of white precipitate within five min. After stirring at RT for 4 hours, the reaction was cooled to 0 °C and DIPEA (3.5 mL, 20.1 mmol, 24.2 equiv) was added slowly to neutralize the excess acid and liberate the amine. The reaction was then warmed to RT and a solution of NHS ester **34** (0.54 g, 0.84 mmol, 1.01 equiv) in MeCN (5 mL) was added. After stirring for two hours, the reaction was diluted with EtOAc (50 mL) and washed with brine (2 × 50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (2% to 10% MeOH/CH_2Cl_2) afforded alcohol **4** as a white foam (0.45 g, 67%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{39}H_{51}N_5O_9S_2$ 797, found 797, R_f = 0.22 (10% MeOH/CH_2Cl_2).

Compound 36



Proton sponge and alcohol **4** were dried over P_2O_5 under vacuum overnight prior to use. A solution of alcohol **4** (0.25 g, 0.31 mmol, 1 equiv) and proton sponge (0.10 g, 0.47 mmol, 1.52 equiv) in trimethyl phosphate (1 mL) was stirred over 4 Å molecular sieves at 0 °C for 30 minutes. Neat $POCl_3$ (0.045 mL, 0.48 mmol, 1.55

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equiv) was added dropwise over 15 minutes to the colorless solution (see note 1). The reaction became purple immediately after the first drop of $POCl_3$ was added. After two hours the reaction was quenched with 0.05M TEAB buffer (7 mL), which caused formation of a milky precipitate and loss of the purple color. MeCN (2 mL) was added to improve the solubility, and the reaction was warmed to RT. After stirring for 2-3 hours the reaction cleared up. The solution was then concentrated under reduced pressure to yield crude monophosphate **3**.

Next the residue was treated with 20% piperidine/MeCN (5 mL) for 30 minutes to remove the Fmoc protecting group. The solvent was removed under reduced pressure, and the residue was dissolved in H_2O (~10 mL), causing formation of copious white precipitate (proton sponge and dibenzylfulvene). The mixture was transferred to eppendorf tubes and centrifuged to remove the precipitate. The supernatant was then HPLC purified (Phenomenex C18 preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 5 min, then 3% B/min, buffer A 0.1M TEAB, buffer B MeOH, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amine **36** as a white foam (35 μmol, 11%, ε_{289} = 13000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{24}H_{42}N_5O_{10}PS_2$ 654, found 654.

Notes: 1. Monophosphate formation should be monitored by LCMS (0.5 μL aliquot plus 40 μL water). Initially 1.2 equivalents of $POCl_3$ are added, and more is added slowly until <10% starting material remains.

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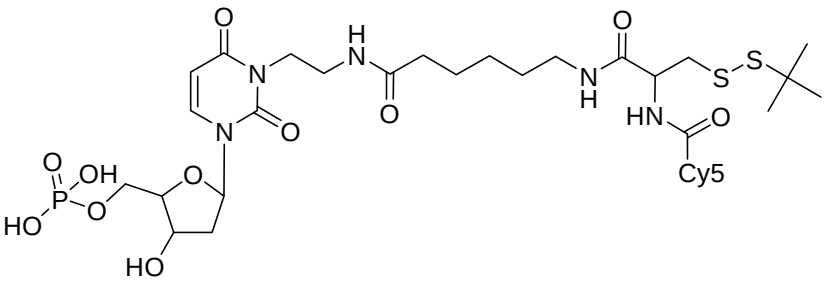
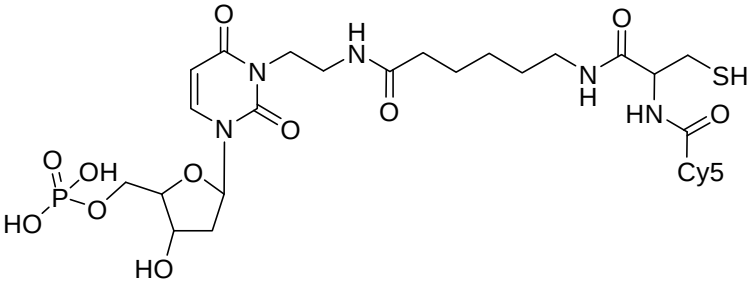
Compound 37



Cy5 Mono NHS Ester (1.05 mL, 0.069 mmol, 0.066 M in anhydrous DMF, 1.73 equiv) was added to a solution of amine **36** (0.026 g, 0.040 mmol, 1 equiv) in H_2O (5 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by LCMS or HPLC, the reaction was lyophilized to yield pU-N3-Cy5-SS-tBu as a bright blue solid which was used without purification or quantification for the subsequent reaction: LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{57}H_{81}N_7O_{17}PS_4^+$ 646, found 646.

Compound 10



A solution of disulfide **37** (0.051g, 0.040 mmol, 1 equiv) in H_2O was treated with TCEP (2.75 mL, 1.37 mmol, 0.5M in H_2O, 34 equiv) in an aluminum foil covered flask. After 30 minutes the crude reaction was HPLC purified (Phenomenex C18

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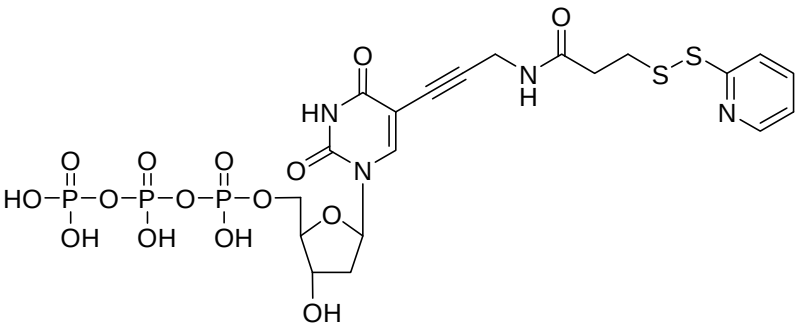
preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired thiol were pooled and used immediately for the subsequent displacement reaction without removing the solvent (18 μmol, 45%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{53}H_{73}N_7O_{17}PS_3^+$ 602, found 602.

Compound 38



SPDP (0.39 mg, 1.25 μmol, 1.25 equiv) in anhydrous DMF (0.025 mL), was added to a solution of dUTP-AP3* (0.52 mg, 1.0 μmol, 1 equiv) in H_2O (0.090 mL) buffered to pH ~8.5 with 1M $NaHCO_3$ (0.010 mL) and allowed to stand at RT. After disappearance of the starting dNTP (monitored by HPLC or LCMS, generally ~20 min), the crude reaction mixture was HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield the product as a white foam (0.80 μmol, 80%, ε_{289} = 13000): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{20}H_{25}N_4O_{15}P_3S_2$ 717, found 717.

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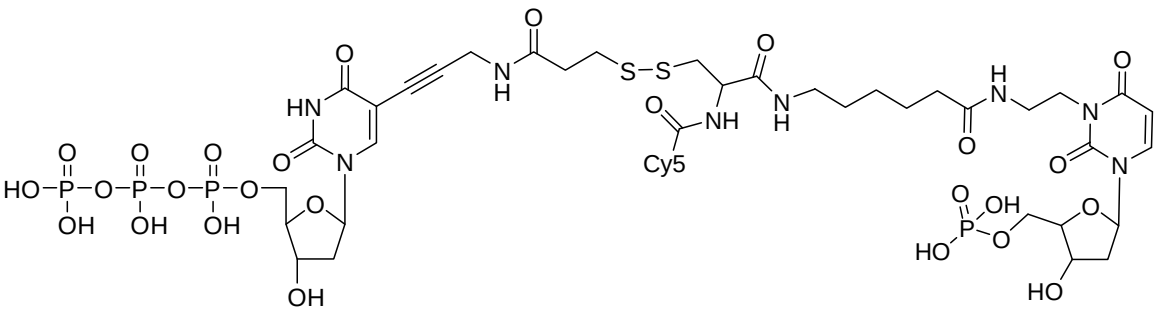
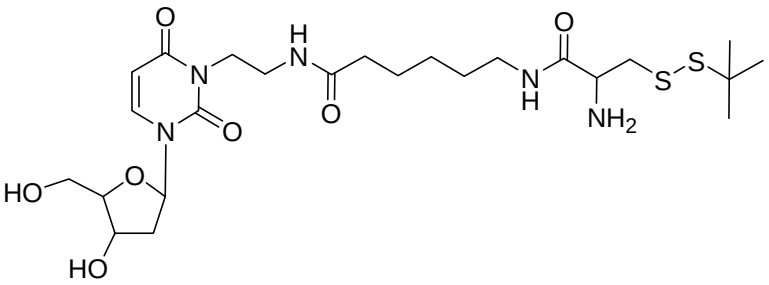
Compound 17



HPLC fractions containing thiol **10** (0.52 mg, 0.43 μmol, 1 equiv) were mixed with difulfide **38** (0.57 mg, 0.80 μmol, 1.9 equiv) in MeCN (0.25 mL) and H_2O (0.25 mL) in an aluminum foil covered flask. After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **17** as a bright blue solid (0.12 μmol, 27%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{68}H_{93}N_{10}O_{32}P_4S_4^+$ 905, found 905.

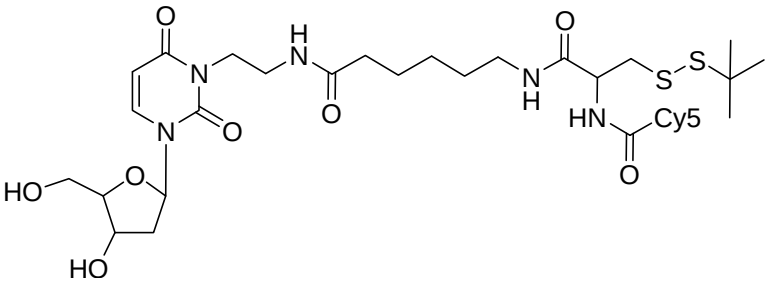
Nature Methods: doi:10.1038/nmeth.1354

Compound 39



Compound **4** (0.015 g, 0.019 mmol) was treated with a solution of 20% piperidine (5 mL) in MeCN for ~30 minutes. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (10% MeOH/DCM) to yield amine **39** (0.004 g, 37%): R_f = 0.10 (10% MeOH/DCM).

Compound 40



Cy5 Mono NHS Ester (0.13 mL, 0.009 mmol, 0.066 M in anhydrous DMF, 2.2 equiv) was added to a solution of amine **39** (2.30 mg, 0.004 mmol, 1 equiv) in 1M K_2HPO_4 (0.5 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by LCMS, the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.05M TEAB, buffer B MeCN, 10 mL/min flow). Fractions

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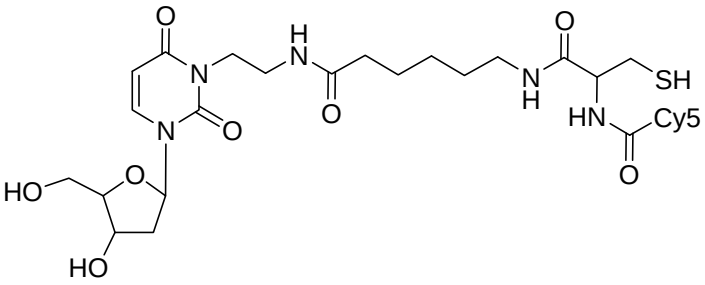
containing the desired were pooled and lyophilized to yield compound **40** as a bright blue solid (2.8 μmol, 70%, ε_{289} = 250000): LCMS(ES-) *m/z* calcd for [(M-2H)/2]$^-$ $C_{57}H_{80}N_7O_{14}S_4^+$ 606, found 606.

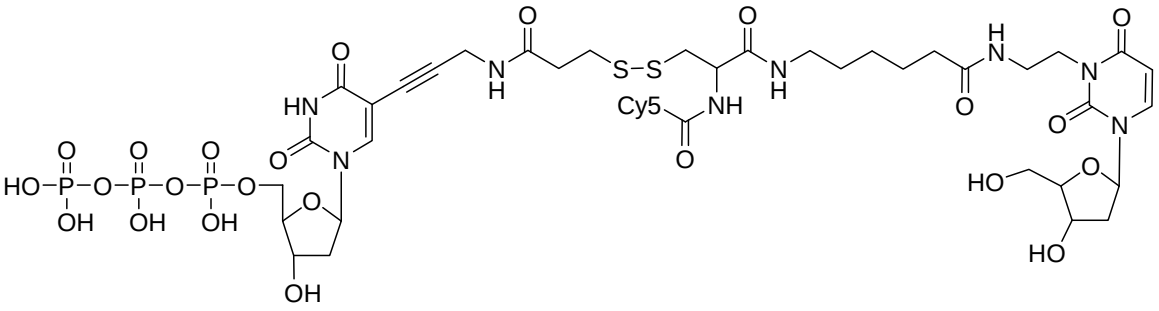
Compound 11



A solution of disulfide **40** (2.43 mg, 0.002 mmol, 1 equiv) in 20% MeCN/H_2O (1 mL) was treated with TCEP (0.15 mL, 0.075 mmol, 0.5M in H_2O, 37 equiv) in an aluminum foil covered flask. After 30 minutes the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.05M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired thiol **11** were pooled and used immediately for the subsequent displacement reaction without removing the solvent (1.0 μmol, 50%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for [(M-2H)/2]$^-$ $C_{53}H_{72}N_7O_{14}S_3^+$ 562, found 562.

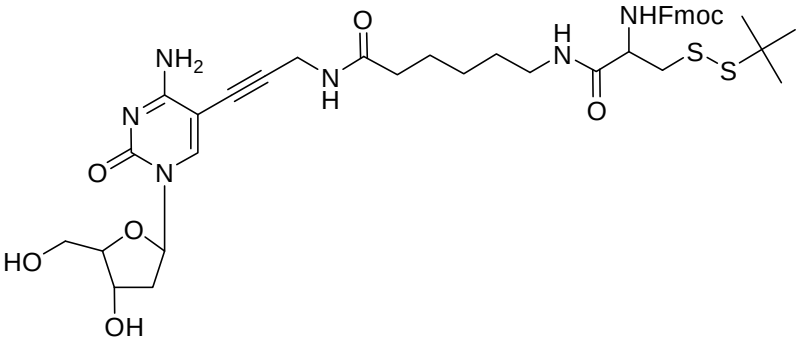
Nature Methods: doi:10.1038/nmeth.1354

Compound 18



HPLC fractions containing thiol **11** (1.13 mg, 1.0 μmol, 1 equiv) were mixed with disulfide **38** (0.001 g, 1.4 μmol, 1.4 equiv) in MeCN (0.25 mL) and H_2O (0.25 mL) in an aluminum foil covered flask. After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **18** as a bright blue solid (0.80 μmol, 80%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{68}H_{92}N_{10}O_{29}P_3S_4^+$ 865, found 865.

Compound 41



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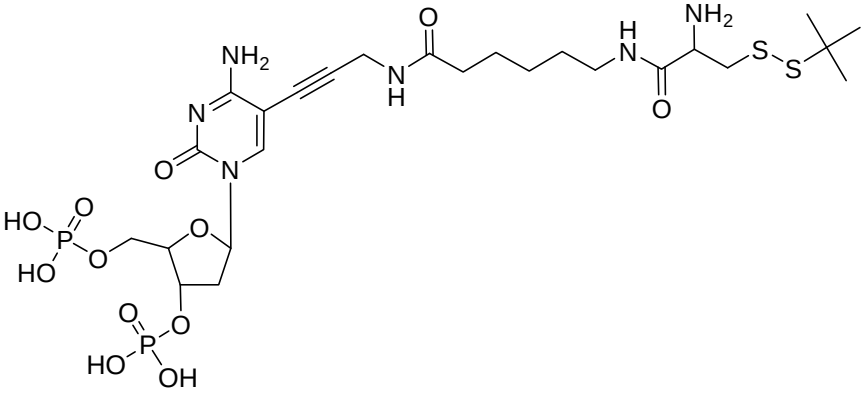
A solution of NHS ester **34** (0.57 g, 0.89 mmol, 1.08 equiv) in MeCN (5 mL) was added to 2'-deoxycytosine-AP3 (0.23 g, 0.82 mmol, 1 equiv), followed by DIPEA (0.96 g, 7.4 mmol, 9.0 equiv). After stirring at room temperature for one hour, the reaction was diluted with EtOAc (30 mL) and washed with brine (2 × 30 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (20% MeOH/EtOAc) afforded **41** as a white foam (0.62 g, 94%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{40}H_{50}N_6O_8S_2$ 805, found 805, R_f = 0.73 (50% MeOH/EtOAc).

Compound 42



Alcohol **41** was dried over P_2O_5 under vacuum overnight prior to use. A solution of alcohol **41** (0.40 g, 0.50 mmol, 1 equiv) and proton sponge (0.25 g, 1.17 mmol, 2.3 equiv) in trimethyl phosphate (1.4 mL) was stirred over 4 Å molecular sieves at 0 °C for 30 minutes. Neat $POCl_3$ (0.30 mL, 3.21 mmol, 6.4 equiv) was added dropwise over 15 minutes. After four hours the reaction was quenched with 0.05M

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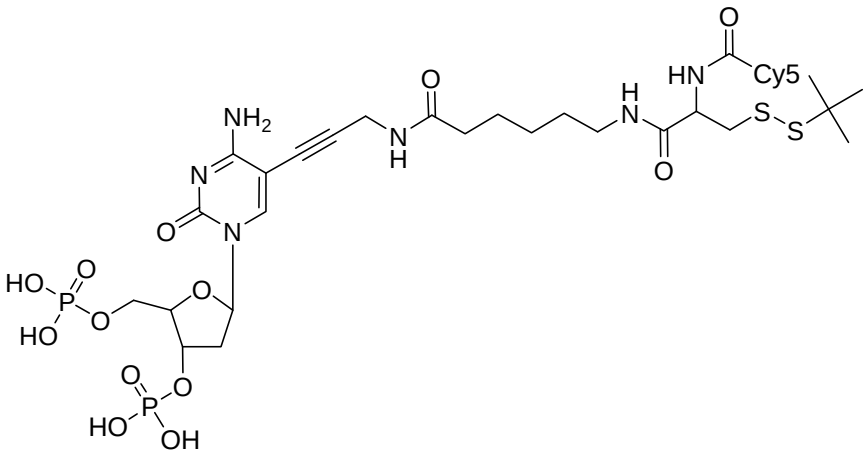
TEAB buffer (10 mL) and stirred at RT for three hours. The solution was then concentrated under reduced pressure to yield crude bisphosphate **5**.

Next the residue was treated with 20% piperidine/MeCN (8 mL) for 50 minutes to remove the Fmoc protecting group. The solvent was removed under reduced pressure, and the residue was dissolved in H_2O (~10 mL), causing precipitation of dibenzylfulvene. The mixture was transferred to eppendorf tubes and centrifuged to remove the precipitate. The supernatant was then HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeOH, 15 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amine **42** as a white foam (30 μmol, 6%, ε_{294} = 9300): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{25}H_{42}N_6O_{12}P_2S_2$ 743, found 743.

Compound 43



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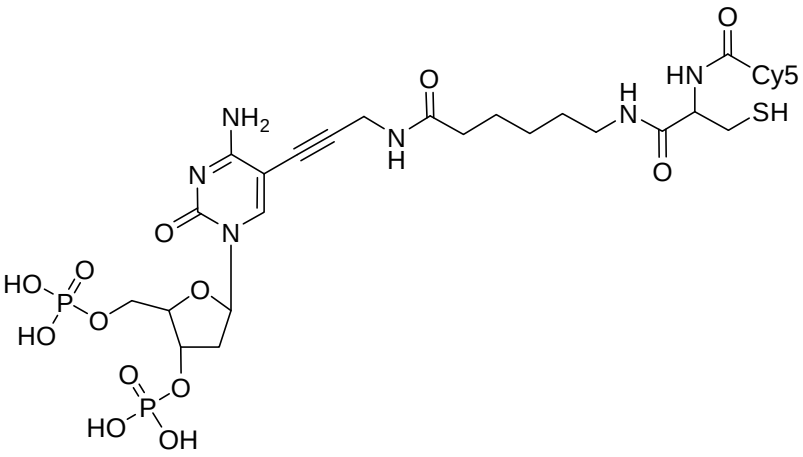
Cy5 Mono NHS Ester (0.20 mL, 0.013 mmol, 0.066 M in anhydrous DMF, 1.3 equiv) and 1M K_2HPO_4 (0.18 mL) were added to a solution of amine **42** (7.44 mg, 0.010 mmol, 1 equiv) in H_2O (0.66 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by HPLC, the reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.05M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **43** as a blue solid (4.18 μmol, 42%, ε_{650} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{58}H_{81}N_8O_{19}P_2S_4^+$ 690, found 690.

Compound 12



A solution of disulfide **43** (5.53 mg, 4.0 μmol, 1 equiv) in H_2O was treated with TCEP (0.45 mL, 0.23 mmol, 0.5M in H_2O, 56 equiv) and 1M K_2HPO_4 (0.4 mL) in an aluminum foil covered flask. After 30 minutes the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient:

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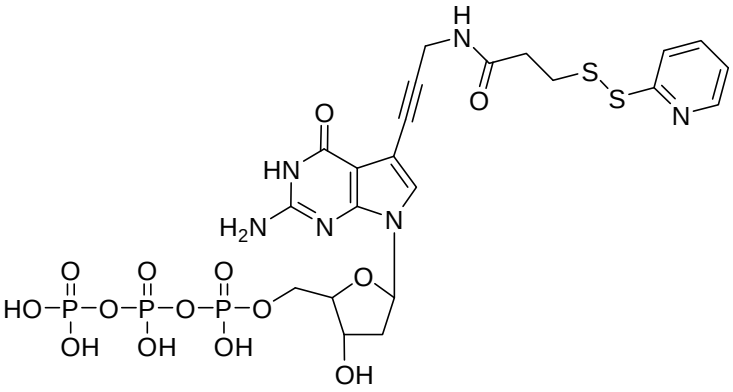
100% A for 5 min, then 3% B/min, buffer A 0.05M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired thiol **12** were pooled and used immediately for the subsequent displacement reaction without removing the solvent: LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{54}H_{73}N_8O_{19}P_2S_3^+$ 646, found 646.

Compound 44



SPDP (0.60 mL, 0.05 M in anhydrous DMF, 1.77 equiv) was added to a solution of dGTP-AP3* (0.095 g, 0.017 mmol, 1 equiv) in H_2O (1.5 mL) buffered to pH ~8.5 with K_2HPO_4 (0.17 mL) and allowed to stand at RT. After disappearance of dGTP-AP3 (monitored by HPLC or LCMS, generally ~20 min), the crude reaction mixture was HPLC purified (Phenomenex C18 preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing compound **44** were pooled and used for subsequent reactions without removing the solvent: LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{22}H_{27}N_6O_{14}P_3S_2$ 755, found 755.

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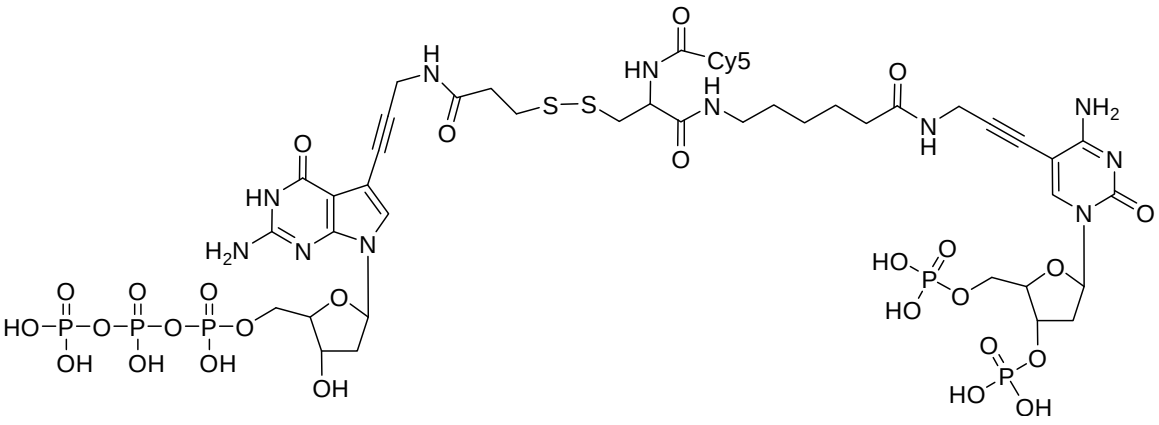
Compound 19



HPLC fractions containing thiol **12** (~2.0 μmol, 1.4 equiv) were mixed with disulfide **44** (1.06 mg, 1.40 μmol, 1 equiv) in H_2O (1 mL) in an aluminum foil covered flask. After disappearance of SPDP-dGTP as determined by HPLC the reaction was lyophilized, then HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.05 M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **19** as a bright blue solid (0.18 μmol, 13%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{71}H_{95}N_{13}O_{33}P_5S_4^+$ 970, found 970.

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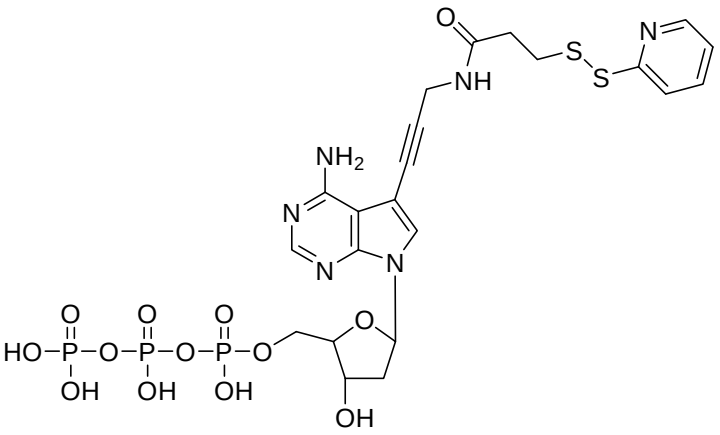
Compound 45



SPDP (0.50 mL, 0.1 M in anhydrous DMF, 1.25 equiv) was added to a solution of dATP-AP3* (0.022 g, 0.04 mmol, 1 equiv) in H_2O (1.35 mL) buffered to pH ~8.5 with K_2HPO_4 (0.15 mL) and allowed to stand at RT. After disappearance of dATP-AP3 (monitored by HPLC or LCMS, generally ~20 min), the crude reaction mixture was HPLC purified (Phenomenex C18 preparative column, 250 x 15.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing compound **45** were pooled and used for subsequent reactions without removing the solvent: LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{22}H_{27}N_6O_{13}P_3S_2$ 739, found 739.

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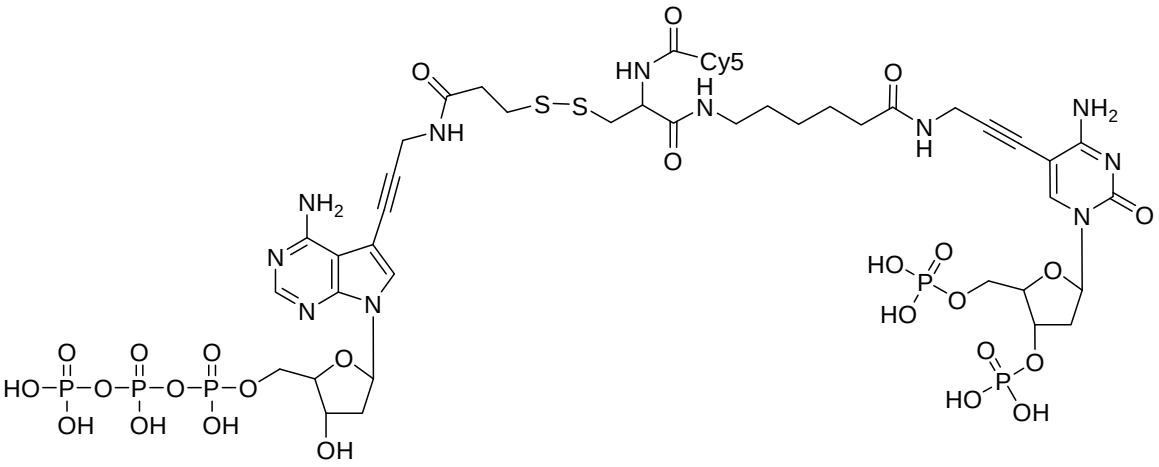
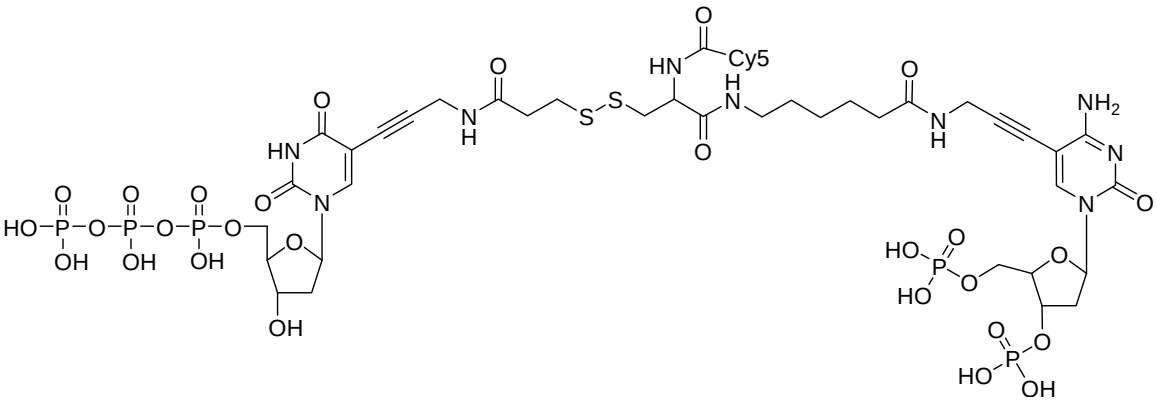
Compound 20



HPLC fractions containing thiol **12** (~1.0 μmol, 1.0 equiv) were mixed with disulfide **45** (1.06 mg, 1.50 μmol, 1.5 equiv) in H_2O (2 mL) in an aluminum foil covered flask. After disappearance of SPDP-dATP as determined by HPLC the reaction was lyophilized, then HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.05 M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **20** as a bright blue solid (0.33 μmol, 33%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{71}H_{95}N_{13}O_{32}P_5S_4^+$ 961, found 961.

Nature Methods: doi:10.1038/nmeth.1354

Compound 21



HPLC fractions containing thiol **12** (~0.67 μmol, 1.7 equiv) were mixed with disulfide **38** (0.29 mg, 0.40 μmol, 1 equiv) in H_2O (0.20 mL) in an aluminum foil covered flask. After disappearance of SPDP-dUTP as determined by HPLC the reaction was lyophilized, then HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.05 M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **21** as a bright blue solid (0.15 μmol, 37%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{69}H_{93}N_{11}O_{34}P_5S_4^+$ 950, found 950.

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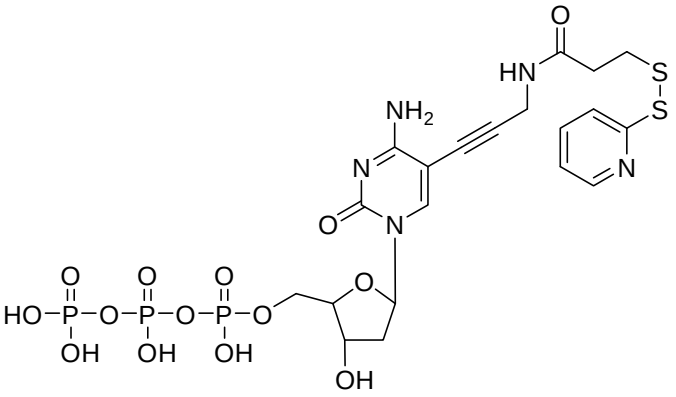
Compound 46



SPDP (0.0016 g, 0.005 mmol, 1.7 equiv, 0.05 M in anhydrous DMF) was added to a solution of dCTP-AP3* (0.0015g, 0.0028 mmol, 1 equiv) in H_2O (0.25 mL) buffered to pH ~8.5 with K_2HPO_4 (0.06 mL) and allowed to stand at RT. After disappearance of the starting dNTP (monitored by HPLC or LCMS, generally ~20 min), the crude reaction mixture was HPLC purified (Phenomenex C18 semi-preparative column, 250 x 10.00 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing compound **46** were pooled and lyophilized to yield the product as a white foam (0.0014g, 0.0019 mmol, 68%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{20}H_{26}N_5O_{14}P_3S_2$ 716, found 716.

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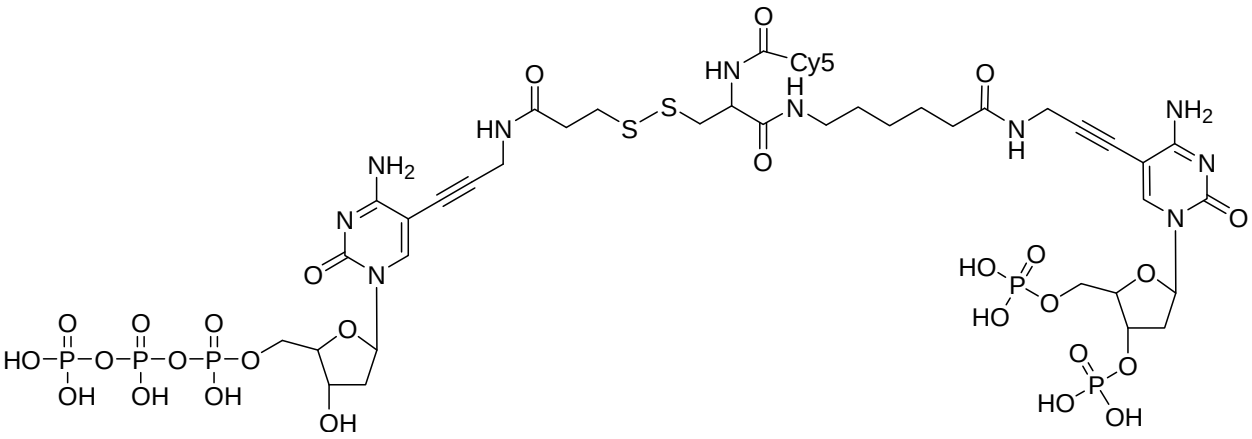
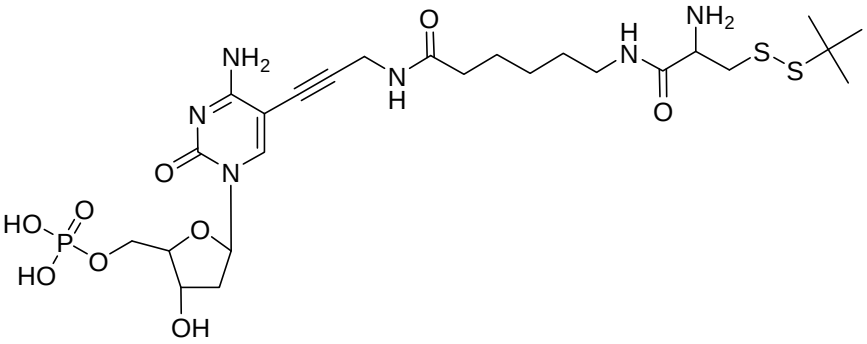
Compound 22



HPLC fractions containing thiol **12** (~0.52 μmol, 1 equiv) were mixed with disulfide **46** (0.29 mg, 1.6 μmol, 3.1 equiv) in H_2O (0.30 mL) in an aluminum foil covered flask. After disappearance of **12** as determined by HPLC the reaction was lyophilized, then HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.05 M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **22** as a bright blue solid (0.10 μmol, 19%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{69}H_{93}N_{11}O_{34}P_5S_4^+$ 950, found 950.

Nature Methods: doi:10.1038/nmeth.1354

Compound 47



Alcohol **41** was dried over P_2O_5 under vacuum overnight prior to use. A solution of alcohol **41** (0.25 g, 0.31 mmol, 1 equiv) in trimethyl phosphate (0.7 mL) was stirred over 4 Å molecular sieves at 0 °C for 30 minutes. Neat $POCl_3$ (0.037 mL, 0.40 mmol, 1.3 equiv) was added dropwise over 15 minutes to the colorless solution (see note 1). After two hours the reaction was quenched with 0.05 M TEAB buffer (20 mL), then warmed to RT and stirred for 2-3 hours. The solution was then concentrated under reduced pressure to yield crude monophosphate **6**.

Next the residue was treated with 20% piperidine/MeCN (5 mL) for 30 minutes to remove the Fmoc protecting group. The solvent was removed under reduced pressure, and the residue was dissolved in H_2O (~5 mL), causing precipitation of dibenzylfulvene. The mixture was transferred to eppendorf tubes and centrifuged to remove the precipitate. The supernatant was then HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 15 mL/min flow). Fractions containing amine **47** were pooled and lyophilized to yield the product as a white

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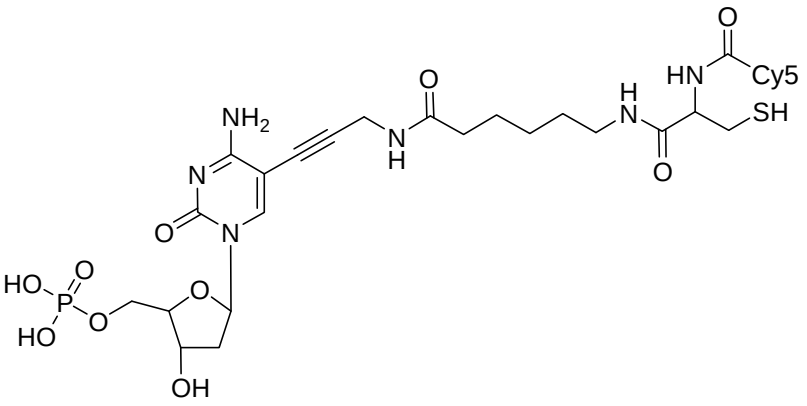
foam (5.3 μmol, 2%, ε_{294} = 9300): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{25}H_{41}N_6O_9PS_2$ 664, found 664.

Notes: 1. Monophosphate formation should be monitored by LCMS (0.5 μL aliquot plus 40 μL water). Initially 1.2 equivalents of $POCl_3$ are added, and more is added slowly until <10% starting material remains.

Compound 13



Cy5 Mono NHS Ester (0.10 mL, 0.066 mmol, 0.066 M in anhydrous DMF, 1.24 equiv) and 1M K_2HPO_4 (0.05 mL) were added to a solution of amine **47** (3.5 mg, 5.32 μmol, 1 equiv) in H_2O (0.5 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by HPLC, the crude disulfide **48** was treated with TCEP (0.27 mL, 0.13 mmol, 0.5M in H_2O, 24 equiv). After 30 minutes the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 15 mL/min flow). Fractions containing thiol **13** were pooled and used immediately for the subsequent displacement reaction

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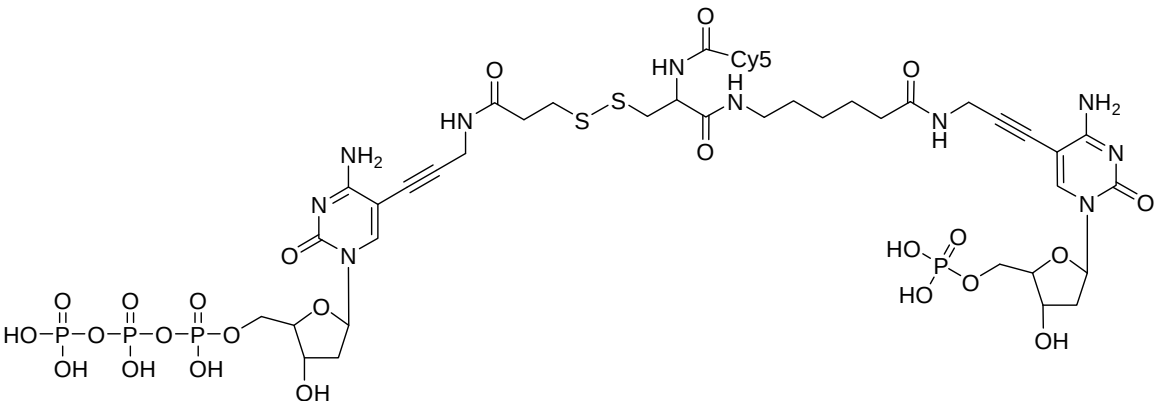
without removing the solvent: LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{54}H_{72}N_8O_{16}PS_3^+$ 607, found 607.

Compound 23



HPLC fractions containing thiol **13** (~4.86 mg, 4.0 μmol, 1 equiv) were mixed with disulfide **46** (0.002 g, 2.8 μmol, 0.70 equiv) in H_2O (0.25 mL) in an aluminum foil covered flask. After 30 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Phenomenex C18 preparative column, 250 × 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **23** as a bright blue solid (0.93 μmol, 23%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{69}H_{93}N_{12}O_{30}P_4S_4^+$ 909, found 909.

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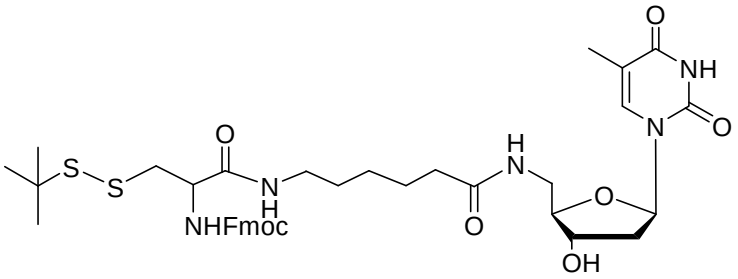
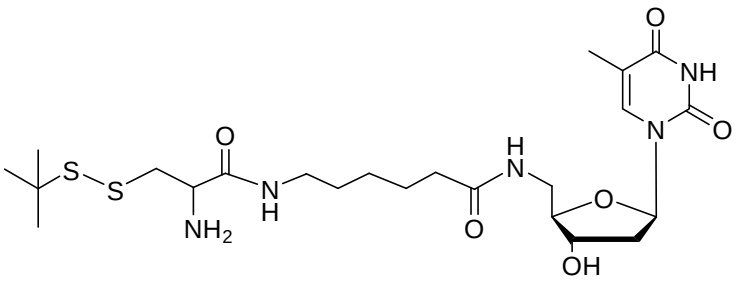
Compound 7



A solution of NHS ester **34** (0.058 g, 0.091 mmol, 1.0 equiv) in DMF (0.5 mL) was added to a solution of 5'-amino-5'-deoxythymidine (0.025 g, 0.091 mmol, 1 equiv) in 0.1N $NaHCO_3$ (0.3 mL) and DMF (0.3 mL). After stirring at room temperature for one hour, the reaction was acidified and extracted with DCM (3 × 10 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (5% MeOH/DCM) afforded compound **7** (0.03 g, 43%): R_f = 0.43 (10% MeOH/DCM).

Compound 49



Compound **7** (0.030 g, 0.039 mmol) was treated with a solution of 20% piperidine in DMF for ~20 minutes. The solvent was removed under reduced pressure, and the

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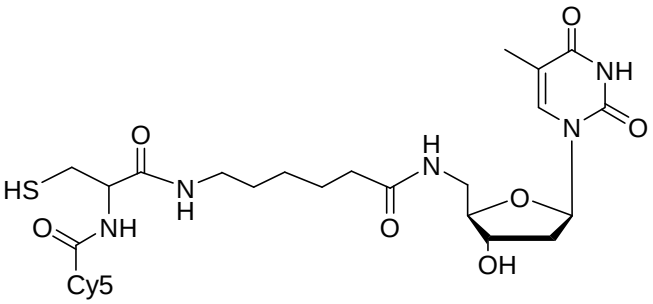
residue was purified by flash column chromatography (5% MeOH/DCM) to yield amine **49** (0.020 g, 92%): R_f = 0.12 (10% MeOH/DCM).

Compound 14



Cy5 Mono NHS Ester (0.35 mL, 0.006 mmol, 0.016 M in anhydrous DMF, 1.1 equiv) was added to a solution of amine **49** (2.73 mg, 0.005 mmol, 1 equiv) in 0.1N $NaHCO_3$ (0.30 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by HPLC, the crude disulfide **50** was treated with dithiothreitol (10 mL, 0.50 mmol, 0.05 M in 0.1N $NaHCO_3$). After 60 minutes the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 10.0 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.05M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **14** as a bright blue solid (2.25 μmol, 45%, ε_{649} = 250000).

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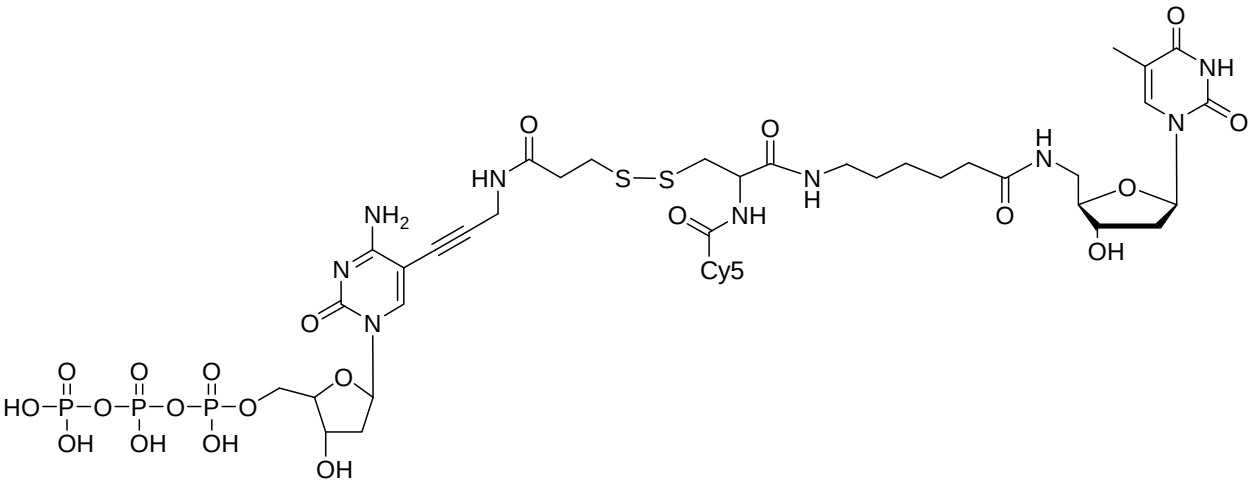
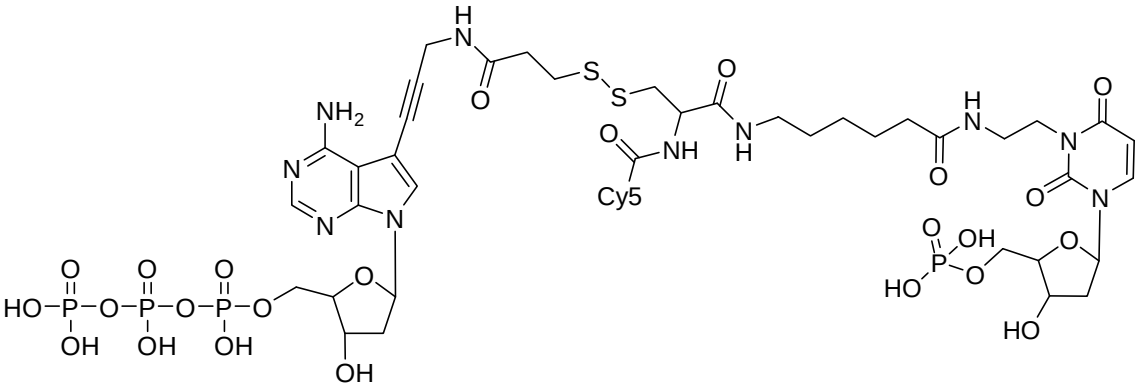
Compound 24



A solution of thiol **14** (~1.5 μmol, 1 equiv) in 50% ACN/H_2O (0.60 mL) was added to disulfide **46** (0.8 μmol, 0.53 equiv) in 50% ACN/H_2O (0.50 mL) and 1M K_2HPO_4 (0.10 mL) in an aluminum foil covered flask. After disappearance of SPDP-dCTP as determined by HPLC the reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 10.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.05 M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **24** as a bright blue solid (0.57 μmol, 70%, ε_{649} = 250000).

Nature Methods: doi:10.1038/nmeth.1354

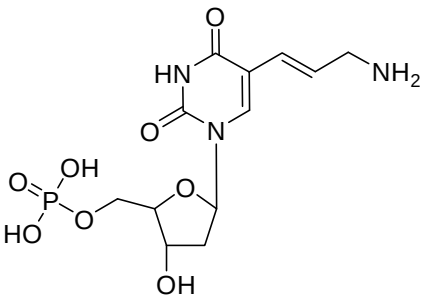
Compound 29



HPLC fractions containing thiol **10** (0.022 g, 0.018 mmol, 1 equiv) were mixed with hplc fractions containing difulfide **45** (0.015 g, 0.020 mmol, 1.11 equiv) in an aluminum foil covered flask. After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Phenomenex C18 preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **29** as a bright blue solid (10.3 μmol, 57%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{70}H_{95}N_{12}O_{30}P_4S_4^+$ 917, found 917.

Nature Methods: doi:10.1038/nmeth.1354

Compound 51



Proton sponge and 5-aminoallyl-2'-deoxyuridine-5'-OH were dried over P_2O_5 under vacuum overnight prior to use. A solution of 5-aminoallyl-2'-deoxyuridine-5'-OH (0.03 g, 0.071 mmol, 1 equiv) and proton sponge (0.018 g, 0.084 mmol, 1.18 equiv) in trimethyl phosphate (0.2 mL) was stirred at 0 °C for 30 minutes. Neat $POCl_3$ (0.024 mL, 0.26 mmol, 3.6 equiv) was added dropwise over ten minutes to the colorless solution. After 1.5 hours the reaction was quenched with 0.05M TEAB buffer (5 mL) and the reaction was warmed to RT. After stirring for one hour, NH_4OH (5 mL) was added to remove the protecting groups. The solution was lyophilized after two hours to yield crude monophosphate **51**, which was then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeOH, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield **51** as a white foam (43 μmol, 60%, ε_{294} = 9300): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{12}H_{18}N_3O_8P$ 362, found 362.

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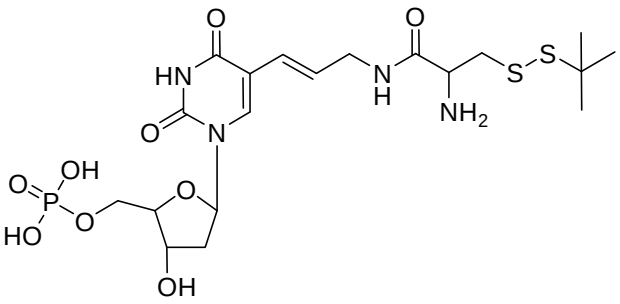
Compound 52.



A solution of NHS ester **33** (10 μmol, 1.11 equiv) in MeCN (0.5 mL) was added to a solution of monophosphate **51** (9 μmol, 1 equiv) in H_2O (0.42 mL) buffered to pH ~8.5 with 1M K_2HPO_4 (0.08 mL). After 30 minutes, the solution containing crude amide **8** was treated with piperidine (0.2 mL) to remove the Fmoc protecting group. The solvent was removed under reduced pressure after an hour, and the residue was dissolved in H_2O (~2 mL), causing formation of a white precipitate (dibenzylfulvene). The mixture was transferred to eppendorf tubes and centrifuged to remove the precipitate. The supernatant was then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1.5% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amine **52** as a white foam (1.8 μmol, 20%, ε_{294} = 9300): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{19}H_{31}N_4O_9PS_2$ 553, found 553.

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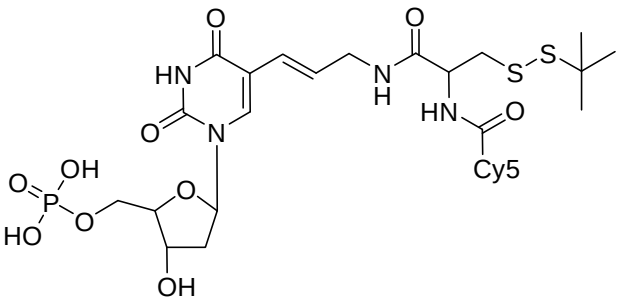
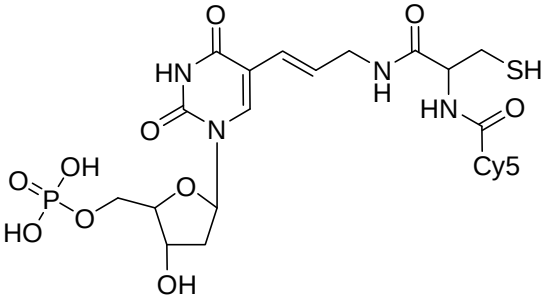
Compound 53.



Cy5 Mono NHS Ester (1.05 mL, 0.069 μmol, 0.066 mM in anhydrous DMF, 1.73 equiv) was added to a solution of amine **52** (40 μmol, 1 equiv) in H_2O (5 mL). After one hour the crude reaction was HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amide **53** as a bright blue solid (32 μmol, 80%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{52}H_{70}N_6O_{16}PS_4^+$ 595, found 595.

Compound 15.



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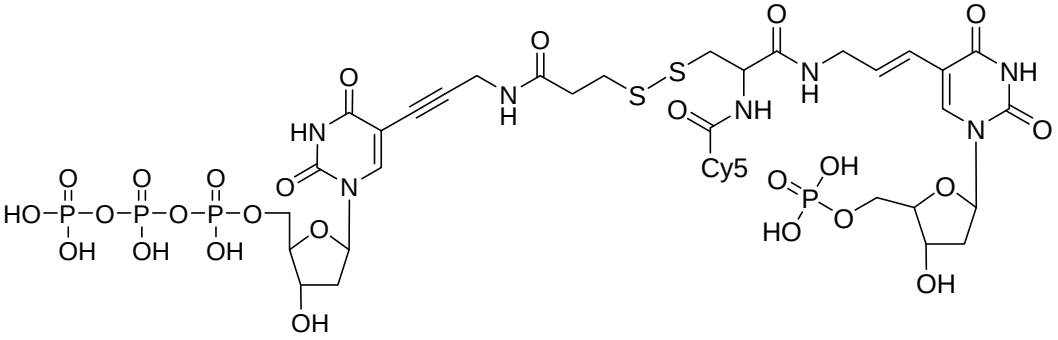
A solution of amide **53** (32 μmol, 1 equiv) in H_2O was treated with DTT (2.75 mL, 1.37 mmol, 0.5M in H_2O, 34 equiv). After 30 minutes the crude reaction was HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing thiol **15** were pooled and used immediately for the subsequent displacement reaction without removing the solvent (18 μmol, 56%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{48}H_{62}N_6O_{16}PS_3^+$ 551, found 551.

Compound 30.



HPLC fractions containing thiol **15** (17 μmol, 1 equiv) were mixed with HPLC fractions containing SPDP-dUTP **38** (24 μmol, 1.4 equiv). After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 15.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **30**

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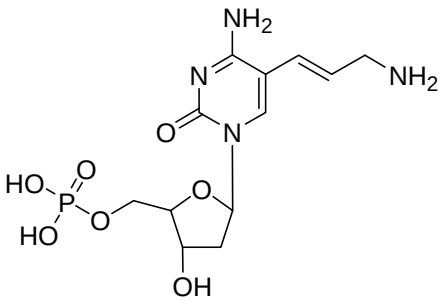
as a bright blue solid (10 μmol, 59%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{63}H_{82}N_9O_{31}P_4S_4^+$ 855, found 855.

Compound 54



Proton sponge and 5-aminoallyl-2'-deoxycytidine-5'-OH were dried over P_2O_5 under vacuum overnight prior to use. A solution of 5-aminoallyl-2'-deoxycytidine-5'-OH (0.03 g, 0.071 mmol, 1 equiv) and proton sponge (0.018 g, 0.084 mmol, 1.18 equiv) in trimethyl phosphate (0.2 mL) was stirred at 0 °C for 30 minutes. Neat $POCl_3$ (0.024 mL, 0.26 mmol, 3.6 equiv) was added dropwise over ten minutes to the colorless solution (see note 1). After 1.5 hours the reaction was quenched with 0.05 M TEAB buffer (5 mL) and the reaction was warmed to RT. After stirring for one hour, NH_4OH (5 mL) was added to remove the protecting groups. The solution was lyophilized after two hours to yield crude monophosphate, which was then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeOH, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield **54** as a white

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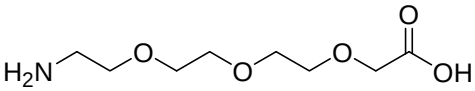
foam (43 μmol, 60%, ε_{290} = 5041): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{12}H_{19}N_4O_7P$ 361, found 361.

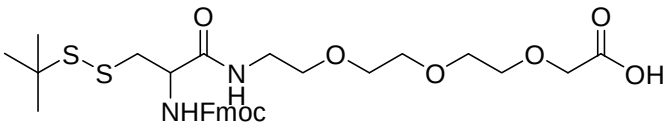
Notes: 1. Monophosphate formation should be monitored by LCMS (0.5 μL aliquot plus 40 μL water). Initially 1.2 equivalents of $POCl_3$ are added, and more is added slowly until <10% starting material remains.

Compound 55



Boc-mini-PEG-3 (0.46 g, 1.50 mmol, 1 equiv) was stirred with 1:1 CH_2Cl_2:TFA (4 mL total) at RT for 30 min. The solvent was removed under reduced pressure, then co-evaporated with CH_2Cl_2 (2 × 4 mL) and MeCN (2 × 4 mL) to afford amine **55**, which was used without further purification or characterization.

Compound 56



A solution of NHS ester **33** (0.53 g, 1.0 mmol, 1.59 equiv) in MeCN (4 mL) was added to a solution of amine **55** (0.13 g, 0.63 mmol, 1 equiv) in MeCN (2 mL) and 0.1M $NaHCO_3$ (1.2 mL). The reaction pH was corrected to 7.5 by addition of neat

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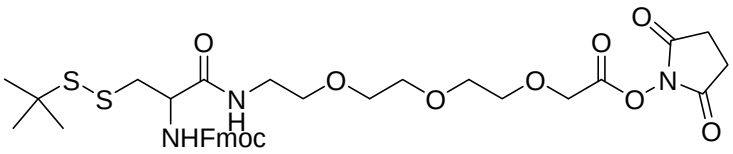
DIPEA (0.070 mL) in small aliquots. After disappearance of the starting amine by TLC (ninhydrin was used to visualize the amine), the MeCN was removed under reduced pressure. The reaction was then diluted with CH_2Cl_2 (20 mL) and washed with H_2O (2 × 25 mL). The organic layer was dried over Na_2SO_4 and concentrated to yield 0.42 g of crude material. Purification by flash column chromatography (100% CH_2Cl_2 to 50% MeOH/CH_2Cl_2) afforded acid **56** as a white solid (0.245 g, 62%): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{30}H_{40}N_2O_8S_2$ 619, found 619.

Compound 57



Acid **56** (0.15 g, 0.24 mmol, 1 equiv) was dissolved in MeCN (1.3mL). DCC (0.06 g, 0.29 mmol, 1.21 equiv) was added, followed by NHS (0.036 g, 0.31 mmol, 1.29 equiv) and the reaction was stirred at RT for an hour. White precipitate (DCU) began forming within five minutes. The reaction mixture was transferred to eppendorf tubes and centrifuged to remove the white precipitate. The supernatant was then used in subsequent reactions without further purification.

Compound 58

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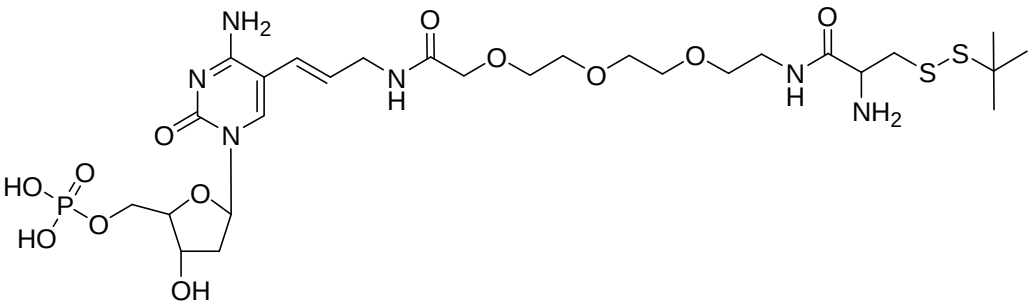

A solution of NHS ester **57** (0.0072 g, 0.010 mmol, 1.11 equiv) in MeCN (0.5 mL) was added to a solution of monophosphate **54** (0.0032 g, 0.009 mmol, 1 equiv) in H_2O (0.42 mL) buffered to pH ~8.5 with 1M K_2HPO_4 (0.08 mL). After 30 minutes, the solution containing crude amide **9** was treated with piperidine (0.2 mL) to remove the Fmoc protecting group. The solvent was removed under reduced pressure after an hour, and the residue was dissolved in H_2O (~2 mL), causing formation of a white precipitate (dibenzylfulvene). The mixture was transferred to eppendorf tubes and centrifuged to remove the precipitate. The supernatant was then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 21.20 mm 10 micron, gradient: 100% A for 5 min, then 1.5% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amine **58** as a white foam (1.8 μmol, 20%, ε_{294} = 9300): LCMS(ES-) *m/z* calcd for $(M-H)^-$ $C_{27}H_{47}N_6O_{12}PS_2$ 741, found 741.

Compound 59

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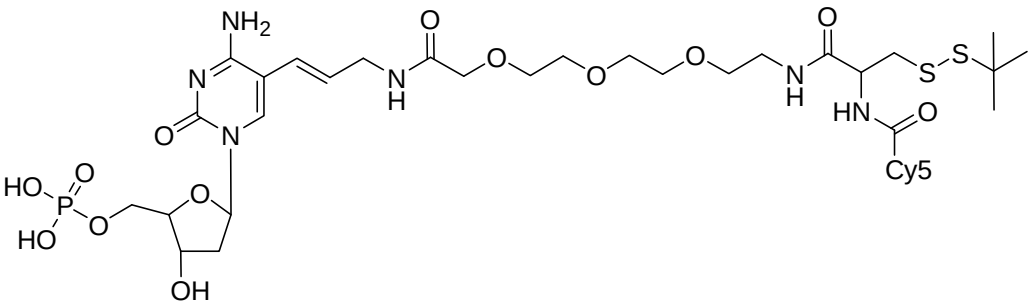
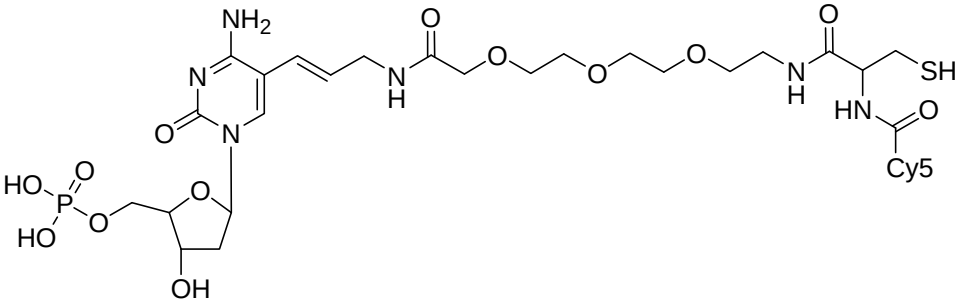


Cy5 Mono NHS Ester (0.0028g, 0.055 mL, 0.0036 mmol, 0.066 mM in anhydrous DMF, 4.0 equiv) was added to a solution of amine **58** (0.67 mg, 0.9 μmol, 1 equiv) in H_2O (0.25 mL) buffered to pH 8.5 with 1M K_2HPO_4 (0.03 mL) in an aluminum foil covered flask. After disappearance of the starting amine as determined by LCMS or HPLC, the crude reaction mixture was used without purification or quantification for the subsequent reaction. LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{60}H_{86}N_8O_{19}PS_4^+$ 689, found 689.

Compound 16



A solution of crude amide **59** (0.0012 g, 0.9 μmol, 1 equiv) in H_2O/DMF (5/1, 0.335 mL) was treated with TCEP (0.10 mL, 0.050 mmol, 0.5 M in H_2O, 56 equiv) in an aluminum foil covered flask. After 20 minutes the crude reaction was HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18

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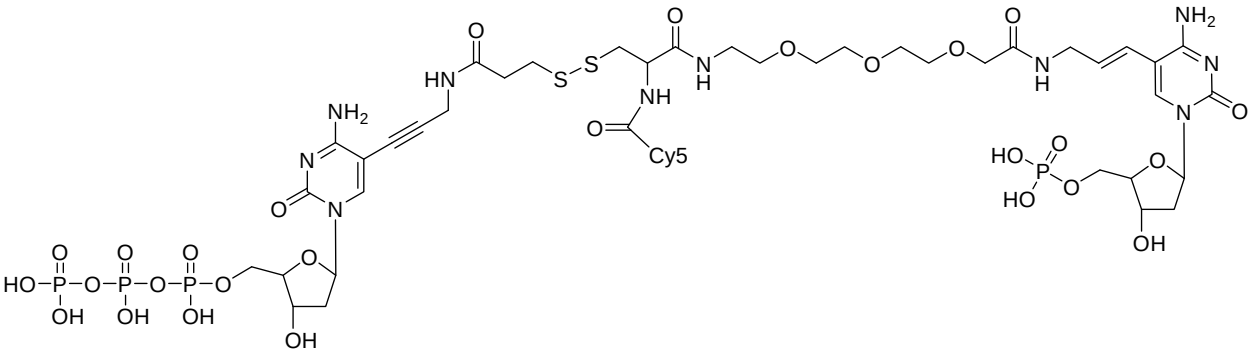
semi-preparative column, 250 x 10.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing thiol **16** were pooled and used immediately for the subsequent displacement reaction without removing the solvent or quantifying. LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{56}H_{78}N_8O_{19}PS_3^+$ 645, found 645.

Compound 32



HPLC fractions containing thiol **16** (1.2 mg, 0.9 μmol, 1 equiv) were mixed with HPLC fractions containing disulfide **46** (0.86 mg, 0.0012 mmol, 1.33 equiv) in an aluminum foil covered flask. After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 semi-preparative column, 250 x 10.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 5 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **32** as a bright blue solid (0.49 μmol, 54%, ε_{649} = 250000). LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{71}H_{99}N_{12}O_{33}P_4S_4^+$ 948, found 948.

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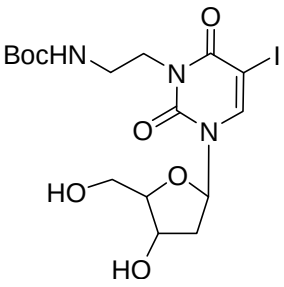
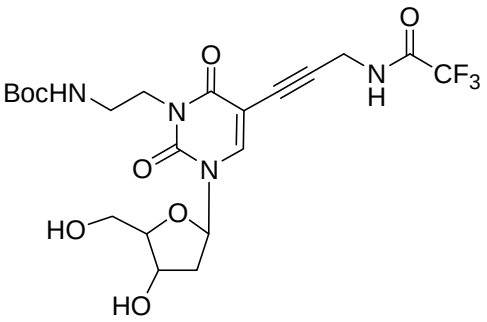
Compound 60



5-Iodo-2'-Deoxyuridine (0.25 g, 0.71 mmol, 1 equiv) and 2-(boc-amino)ethyl bromide (0.23 g, 1.03 mmol, 1.44 equiv) were dissolved in 1:1 DMF:acetone (1.4 mL total). K_2CO_3 (0.16 g, 1.16 mmol, 1.63 equiv) and tetrabutylammonium iodide (0.03 g, 0.081 mmol, 0.11 equiv) were added, and the reaction was heated at 55 °C under an Ar atmosphere for 3 hours. After cooling to RT, the reaction was diluted with EtOAc (20 mL) and washed with brine (2 × 20 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to yield carbamate **60** as a pale yellow foam that was used without further purification (0.35 g, 99%).

Compound 61



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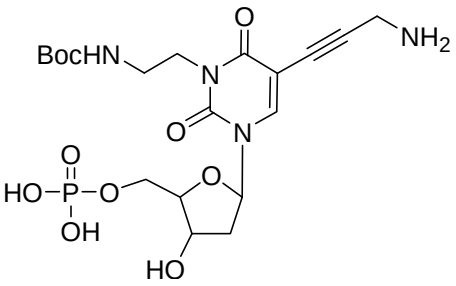
2, 2, 2-trifluoro-*N*-prop-2-ynyl-acetamide (0.35 g, 2.32 mmol, 3.0 equiv), NEt_3 (0.33 g, 3.23 mmol, 4.25 equiv), and CuI (0.070 g, 0.37 mmol, 0.50 equiv) were added to a solution of iodide **60** (0.38 g, 0.76 mmol, 1 equiv) in anhydrous DMF (3 mL) under an Ar atmosphere. $Pd(PPh_3)_4$ (0.16 g, 0.14 mmol, 0.18 equiv) was then added to the clear yellow solution, immediately causing the solution to become dark brown. After stirring the reaction at RT in the dark for 12 hrs, most of the DMF was removed under reduced pressure. The crude material was then purified by flash column chromatography (1% MeOH/CH_2Cl_2 to 5% MeOH/CH_2Cl_2) to afford compound **61** as a brown solid (0.22 g, 55%).

Compound 62



Proton sponge and alcohol **61** were dried over P_2O_5 under vacuum overnight prior to use. A solution of alcohol **61** (0.070 g, 0.13 mmol, 1 equiv) and proton sponge (0.050 g, 0.33 mmol, 2.5 equiv) in trimethyl phosphate (0.65 mL) was stirred at 0 °C under an Ar atmosphere for 15 minutes. Neat $POCl_3$ (0.032 mL, 0.34 mmol, 2.6 equiv) was added dropwise over 15 minutes (see note 1). After one hour the reaction was quenched with 0.05 M TEAB buffer (5 mL), then warmed to RT and stirred for one hour. The crude reaction mixture was then transferred to eppendorf

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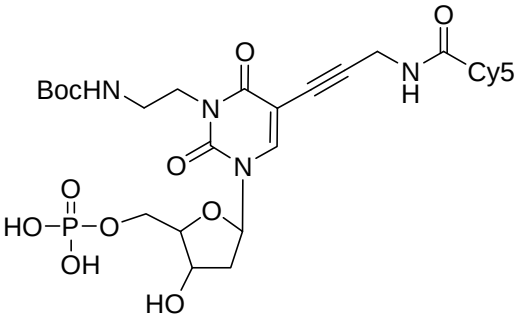
tubes and centrifuged to remove the precipitate (proton sponge). The supernatant was then HPLC purified (Phenomenex C18 preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 5 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Next the fractions containing the desired monophosphate were pooled and partially concentrated, then treated with NH_4OH (6 mL) for one hour to remove the TFA group. After removing the solvent, the material was again HPLC purified (Phenomenex C18 preparative column, 250 × 15.00 mm 10 micron, gradient: 100% A for 5 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield amine **62** as a white foam (11 μmol, 8%, ε_{289} = 13000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{19}H_{29}N_4O_{10}P$ 503, found 503.

Notes: 1. Monophosphate formation should be monitored by LCMS (0.5 μL aliquot plus 40 μL water). Initially 1.2 equivalents of $POCl_3$ are added, and more is added slowly until <10% starting material remains.

Compound 63



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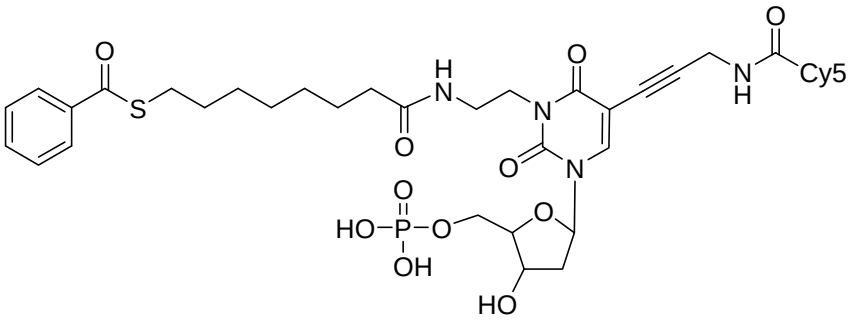
Cy5 Mono NHS Ester (0.24 mL, 0.016 mmol, 0.066 M in anhydrous DMF, 1.45 equiv) and 1M K_2HPO_4 (0.20 mL) were added to a solution of amine **62** (5.5 mg, 11 μmol, 1 equiv) in H_2O (4 mL). After disappearance of the starting amine as determined by LCMS, the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 15.0 mm 10 micron, gradient: 100% A for 3 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **63** as a bright blue solid (9.7 μmol, 88%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{52}H_{68}N_6O_{17}PS_2^+$ 570, found 570.

Compound 64



HCl (0.80 mL, 3.2 mmol, 4.0 M in dioxane) was added to a solution of carbamate **63** (4.5 μmol, 1 equiv) in H_2O (0.5 mL), and the reaction was stirred at RT until the starting material had disappeared as determined by LCMS (~3 hrs). The reaction was then cooled to 0 °C and DIPEA (1 mL, 5.74 mmol) was added slowly to neutralize the excess acid and liberate the amine. The reaction was next warmed to RT and a solution of 8-benzoylsulfanyl-octanoic acid NHS ester (0.020 g, 0.053

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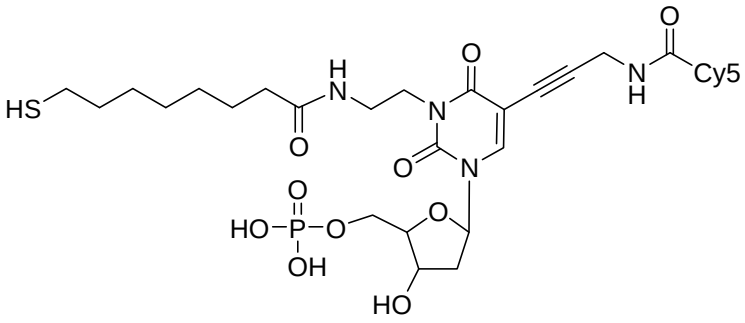
mmol, 12 equiv) in DMF (0.40 mL) was added. After disappearance of the free amine as determined by LCMS, the crude reaction was HPLC purified (Phenomenex C18 preparative column, 250 × 15.0 mm 10 micron, gradient: 100% A for 3 min, then 2% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **64** (3.35 μmol, 74%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{62}H_{78}N_6O_{17}PS_3^+$ 651, found 651.

Compound 65



A solution of thiobenzoate **64** (1.45 μmol) in H_2O (0.5 mL) was treated with 1 M NH_2OH (2.0 mL, pH ~7). The reaction was stirred at RT for one hr, then immediately HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 15.0 mm 10 micron, gradient: 100% A for 3 min, then 2% B/min, buffer A 0.1 M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired thiol **65** were used immediately for subsequent reactions without quantifying or concentrating. LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{55}H_{74}N_6O_{16}PS_3^+$ 599, found 599.

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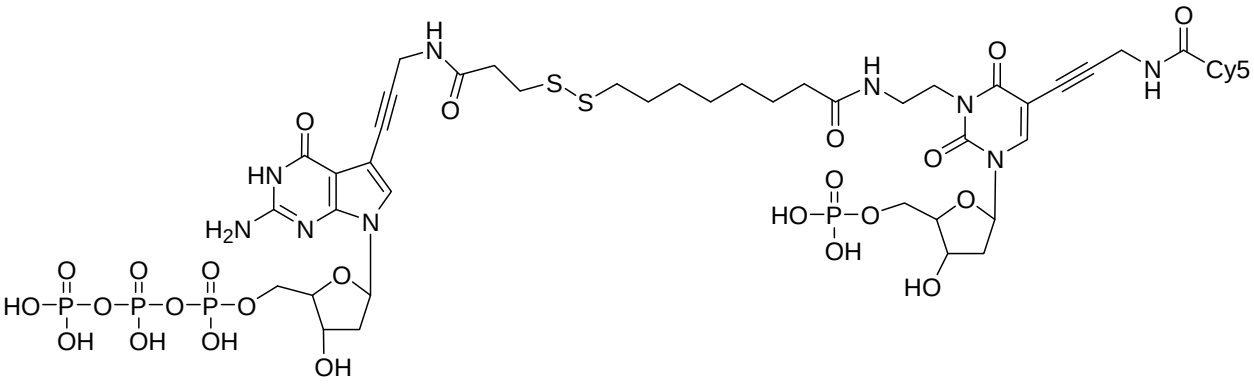
Compound 31



HPLC fractions containing thiol **65** (1 μmol, 1 equiv) were mixed with HPLC fractions containing disulfide **44** (1.2 μmol, 1.2 equiv). After 15 minutes the reaction was partially concentrated under reduced pressure to remove MeCN, then HPLC purified (Waters Delta 600 pump and 2487 Dual λ Absorbance Detector, Phenomenex C18 preparative column, 250 x 15.00 mm 10 micron, gradient: 100% A for 3 min, then 1% B/min, buffer A 0.1M TEAB, buffer B MeCN, 10 mL/min flow). Fractions containing the desired were pooled and lyophilized to yield compound **31** as a bright blue solid (0.7 μmol, 70%, ε_{649} = 250000): LCMS(ES-) *m/z* calcd for $[(M-2H)/2]^-$ $C_{72}H_{96}N_{11}O_{30}P_4S_4^+$ 922, found 922.

***** dNTP-AP3's were prepared according to: F.W. Hobbs, Jr. and A.J. Cocuzza, Alkynylamino-nucleotides. US Patent 5047519, 1991 with the following modifications: a) Pyrophosphate and tributylamine were added to the reaction mixture rather than vice versa.; b) After pyrophosphate addition the reaction was quenched within 15 min.; c) Sephadex chromatography was replaced by preparative HPLC.

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