Protein Domain Specific Covalent Inhibition of Human DNA Polymerase β

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General Methods

Modified oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 oligonucleotide synthesizer. Oligonucleotide synthesis reagents including 5’-phosphorylation reagent (Solid CPR II), SIMA HEX (dichloro diphenyl fluorescein) phosphoramidite, THF abasic site analogue (dSpacer), TAMRA phosphoramidite, and BHQ phosphoramidite were purchased from Glen Research (Sterling, VA). Oligonucleotides containing only native nucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) (See below). Oligonucleotides were characterized using a Bruker AutoFlex III MALDI-TOF/TOF system.

Compound 1 was obtained from Dr. Dumitru Arian. All chemicals were purchased from Sigma Aldrich, Fisher, or Alfa and were used without further purification. Small quantities of all library compounds (Chart S1) were purchased from Sigma Aldrich but were from a variety of vendors. Prior to use, all solvents were distilled over calcium hydride, with the exception of THF, which was distilled over sodium metal. Trypsin, dNTPs, terminal deoxynucleotide transferase, Klenow exo-, and T4 polynucleotide kinase were obtained from New England Biolabs. Radionuclides were from Perkin Elmer. Poly-Prep columns were from BioRad. C18-Sep-Pak cartridges were obtained from Waters. Zip-Tips were purchased from Millipore. Photolyses were carried out in a Rayonet photoreactor filled with 16 lamps having a maximum output at 350 nm. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager 840 equipped with ImageQuant.

UPLC-MS/MS analyses were carried out on a Waters Acquity/Xevo-G2 UPLC-MS system equipped with an ACQUITY UPLC HSS T3 Column (100 Å, 1.5 μm, 2.1 mm x 100 mm). Masses were obtained via deconvolution using MassLynx 4.2 software or BioPharmaLynx 1.3.2 software.
Well plates used for organic solvents and photolyses were obtained from VWR. Well plates used for fluorescence assays were obtained from Corning (CLS3825, 384 well plates, for homogenous luminescent and HTRF assays). Fluorescence data were collected on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a well plate attachment. Fluorescence anisotropy measurements were conducted using an AVIV Biomedical Model ATF 107 spectrofluorometer at the Center for Molecular Biophysics at Johns Hopkins University.

The following items were generous gifts from colleagues. Plasmids for the 8 kDa and 31 kDa domains were from Dr. Sam Wilson, NIH. The Pol β domains were expressed and isolated using previously reported conditions.²,³

All small molecules synthesized were characterized using a Bruker Avance 400 MHz Spectrometer or an Avance Neo 800 MHz spectrometer at Johns Hopkins University.
All $R_f$ values provided in TLC information correspond to the product unless otherwise explicitly stated.

**Scheme S1. Synthesis of 3**

![Reaction Scheme]

**Preparation of S1**

Thymidine (3.08 g, 12.7 mmol, 1 eq) was azeotropically dried with pyridine (2 x 8 mL). DMAP (159 mg, 1.27 mmol, 0.2 eq) was added to a flask and flushed with Argon. The reagents were dissolved in distilled DCM (6.7 mL) and cooled to 0 °C. Pyridine (12.5 g, 12.8 mL, 158.8 mmol, 12.5 eq) was added slowly to the solution. Acetic anhydride (3.82 g, 6 mL, 63.6 mmol, 5 eq) was then added dropwise. After 3.5 h, TLC (3:2 EtOAc/Hex, $R_f = 0.8$, stained with PAA) confirmed that the reaction was complete. The reaction was quenched with sat. NaHCO$_3$ until neutral pH was reached. The mixture was diluted with EtOAc (15 mL) and washed with water (2 x 15 mL). The aqueous layer was extracted with EtOAc (2 x 30 mL) and the combined organic layers were washed with brine (1 x 50 mL), dried over Na$_2$SO$_4$, and evaporated to dryness under vacuum. The crude residue was purified by column chromatography (1:1 Hex/EtOAc) to yield 3.8 g (88%) of S1. $^1$H NMR (400 MHz, CDCl$_3$) δ 10.10 (s, 1H), 7.21 (s, 1H), 6.24 (dd, $J = 4.2$, 8.4 Hz, 1H), 5.13 (d, $J = 6.8$ Hz, 1H), 4.28 (dd, $J = 4.2$, 12.1 Hz, 1H), 4.23 (dd, $J = 3.3$, 12.1 Hz, 1H), 4.15 (dd, $J = 3.3$, 6.8 Hz, 1H), 2.37 (dd, $J = 6.0$, 8.4 Hz, 1H), 2.13 (m, 1H), 2.03 (d, $J = 6.0$ Hz, 6H), 1.83 (s, 3H).
**Preparation of S2**

Compound S1 (785.8 mg, 2.44 mmol, 1 eq) was mixed with NBS (693.9 mg, 3.9 mmol, 1.8 eq) and AlBN (64 mg, 0.39 mmol, 0.18 eq) in benzene (16 mL). The mixture was sparged with Ar for 20 min. The reaction was heated to reflux (80 °C) and stirred for 30-45 min, during which the mixture turned red. At that time, TLC (2:1 EtOAc/Hex, Rf = 0.6, UV active and stained with PAA) showed the starting material was mostly consumed without the formation of side products. The mixture was immediately filtered through a glass frit and concentrated under vacuum to give a yellow residue. The residue was redissolved in DMF (12 mL, 200 mM). NaN₃ (904 mg, 4.88 mmol, 2 eq) and added to the solution, which turned brown immediately. The reaction was heated to 60 °C and stirred for 1-2 h. At that time, TLC (2:1 EtOAc/Hex, Rf = 0.2, UV active and stained with PAA), showed the disappearance of the intermediate. The reaction was cooled and diluted with EtOAc (30 mL), washed with water (2 x 40 mL), and brine (2 x 40 mL). The aqueous layer was extracted with EtOAc (2 x 80 mL) and the combined organic layers were dried over Na₂SO₄ and evaporated to dryness under vacuum to give a red crude residue. The residue was purified by column chromatography in 1:1 Hex/EtOAc to give 628 mg (55%) of S2. ¹H NMR (400 MHz, CDCl₃) δ 7.57 (s, 1H), 6.29 (dd, J = 5.6, 8.5 Hz, 1H), 5.22 (dt, J = 1.9, 8.5 Hz, 1H), 4.40 (dd, J = 4.2, 12.1 Hz, 1H), 4.31 (dd, J = 4.2, 12.1 Hz, 1H), 4.28 – 4.26 (m, 1H), 4.22 (dd, J = 1.0, 14.4 Hz, 1H), 4.12 (dd, J = 1.0, 14.4 Hz, 1H), 2.52 (ddd, J = 1.9, 5.6, 14.4 Hz, 1H), 2.30 – 2.18 (m, 1H), 2.12 (d, J = 5.6 Hz, 6H).

**Preparation of S3**

Compound S2 (300 mg, 0.82 mmol) was dissolved in dioxane (12 mL, 68 mM). Concentrated NH₄OH (29% in water, 12 mL, 14.5 M) was added to the reaction ([S2] = 34 mM, [NH₄OH] = 9 M). The reaction was stirred for 18 h. Analysis by TLC (4:1 EtOAc/Hex, Rf = 0.05, UV active and stained with PAA) confirmed the
absence of starting material. The reaction was evaporated to dryness under vacuum and purified by column chromatography (4:1 EtOAc/Hex -> 100 % EtOAc, dry loaded) to yield 260 mg (90%) of S3. ¹H NMR (400 MHz, CD₃OD) δ 8.09 (s, 1H), 6.21 (t, J = 6.6 Hz, 1H), 4.36 (td, J = 3.4, 6.6 Hz, 1H), 4.03 (s, 2H) 3.89 (dd, J = 3.4, 6.2 Hz, 1H), 3.76 (dd, J = 3.4, 12.1 Hz, 1H), 3.69 (dd, J = 3.7, 12.1 Hz, 1H), 2.26 (dd, J = 3.7, 6.2 Hz, 1H), 2.19 (m, 1H).

Preparation of S4

Compound S3 (895.5 mg, 3.16 mmol) was azeotropically dried with pyridine (2 x 3 mL). DMT-Cl (1.32 g, 3.79 mmol, 1.2 eq) was added and reactants were dissolved in pyridine (6.4 mL, 500 mM) at 0 °C. The mixture turned orange and was monitored by TLC (5:1 EtOAc/Hex, Rf = 0.3, UV active and stained orange then blue with PAA). After 2 h, a side product (presumably the bis-tritylated product) was observed via TLC. Therefore, the reaction was concentrated under vacuum. The residue was dissolved in EtOAc (30 mL) and washed with sat. sodium bicarbonate (1 x 30 mL) and H₂O (1 x 30 mL). The aqueous layer was extracted with EtOAc (2 x 30 mL) and the combined organic layers were washed with brine (1 x 50 mL), dried over Na₂SO₄, and evaporated to dryness under vacuum. The crude material was purified by column chromatography (5:1 EtOAc/hexanes) to yield 1.078 g (60%) of S4. ¹H NMR (400 MHz, CDCl₃) δ 8.56 (s, 1H), 7.82 (s, 1H), 7.65 (m, 1H), 7.36 (m, 1H), 7.30 – 7.22 (m, 5H), 6.80 (m, 4H), 6.37 (d, J = 5.8 Hz, 1H), 4.57 (t, J = 3.0 Hz, 1H), 4.06 (s, 2H), 3.76 (s, 6H), 3.52 (d, J = 13.7 Hz, 1H), 3.45 (d, J = 3.2 Hz, 1H), 3.40 – 3.34 (dd, J = 3.2, 13.7 Hz, 2H), 3.32 (d, J = 13.5 Hz, 2H), 2.44 (ddd, J = 3.0, 5.8, 13.5 Hz, 1H), 2.34 – 2.24 (m, 1H).
Preparation of 3

Compound S4 (452.2 mg, 0.77 mmol, 1 eq) was azeotropically dried with pyridine (3 x 1 mL). DMF (2.6 mL, 300 mM) dissolved S4. Imidazole (330. mg, 4.64 mmol, 6 eq) and TBDMSCl (377 mg, 2.32, 3 eq) were added to the mixture. The reaction was heated at 50 °C and stirred overnight. When confirmed complete by TLC (2:1 Hex/EtOAc, Rf = 0.25, UV active and stained with PAA), the reaction was cooled and diluted with EtOAc (20 mL). The organic layer was washed with sat. NH4Cl (3 x 20 mL) and brine (2 x 30 mL). The combined aqueous layers were extracted using EtOAc (2 x 50 mL). The combined organic layers dried over Na2SO4 and evaporated to dryness under vacuum. The residue was purified by column chromatography (2:1 hexanes/EtOAc) yielding 289 mg (55%) of S3.

1H NMR (400 MHz, CDCl3) δ 7.92 (s, 1H), 7.35 (d, J = 8 Hz, 2H), 7.24 (dd, J = 3.7, 8 Hz, 7H), 6.78 (d, J = 8 Hz, 4H), 6.27 (t, J = 6.2 Hz, 1H), 4.49 (td, J = 2.4, 3.7 Hz, 1H), 4.05 (d, J = 3.7 Hz, 1H), 3.75 (s, 6H), 3.56 (d, J = 13.7 Hz, 1H), 3.52 (dd, J = 2.7, 10.7 Hz, 1H), 3.42 (d, J = 13.7 Hz, 1H), 3.33 (dd, J = 2.7, 10.7 Hz, 1H), 2.39 (dd, J = 3.7, 6.2 Hz, 1H), 2.28 (dd, J = 6.2, 13.7 Hz, 1H), 0.85 (s, 9H), 0.07 (s, 3H), 0.02 (s, 6H).

Preparation of 4

Compound S3 (153.9 mg, 0.261 mmol) dissolved in a solution of 60% MeOH, 20% tBuOH, 20% H2O (5.2 mL, 50 mM). Activated palladium on carbon (102.4 mg, 66 wt%) was added and the mixture was flushed with a hydrogen-filled balloon three times. The reaction was continuously sparged with H2 (1 atm). After 4 h, TLC (1:1 Hex/EtOAc, UV active and stained with PAA) confirmed that the starting material (Rf = 0.6) was converted to the product (Rf = 0.02). The crude mixture was filtered through Celite and evaporated to dryness under vacuum to yield 139.7 mg (93%) of S5.

1H NMR (400 MHz, CD3OD) δ 7.85 (s, 1H), 7.42 (d, J = 6.8 Hz, 2H), 7.36 – 7.21 (m, 7H), 6.97 – 6.71 (m, 4H), 6.24 (m, 1H), 4.52 (t, J = 4.0 Hz, 1H), 3.94 (dd, J = 4.0, 6.8 Hz, 1H), 3.82 (dd, J = 3.2, 10.7 Hz,
1H), 3.76 (s, 6H), 3.69 (d, J = 4.0 Hz, 1H), 3.49 (dd, J = 3.2, 10.7 Hz, 1H), 3.18 – 3.03 (m, 1H), 2.40 – 2.24 (m, 2H), 0.82 (s, 9H), 0.00 (s, 3H), -0.03 (s, 3H).

ESI-TOF m/z calculated for C_{37}H_{47}N_{3}O_{7}Si (M + H) – 675.3183 calculated, 675.3205 observed.

Crude S5 (83.8 mg, 0.12 mmol) was dissolved in THF (2.48 mL, 50 mM). Distilled triethylamine (0.26 mL, 1.86 mmol, 15 eq) and ethyl trifluoroacetate (0.15 mL, 1.24 mmol, 10 eq) were added to the flask. The reaction was stirred for 4 h at 25 °C. TLC (1:1 Hex/EtOAc, R_f = 0.5, UV active and stained orange then blue by PAA) confirmed conversion to product. The reaction evaporated to dryness under vacuum to yield 65.4 mg (70%) of 4. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 7.82 (s, 1H), 7.40 (d, J = 7.5 Hz, 2H), 7.35 – 7.09 (m, 7H), 6.81 (d, J = 7.5 Hz, 4H), 6.24 (t, J = 6.4 Hz, 1H), 4.40 (t, J = 2.8 Hz, 1H), 3.93 (d, J = 3.4 Hz, 1H), 3.74 (s, 6H), 3.69 (t, J = 6.4 Hz, 3H), 3.37 (dd, J = 2.8, 10.6 Hz, 1H), 3.29 (dd, J = 3.4, 10.6 Hz, 1H), 2.28 (m, 1H), 2.14 (m, 1H), 0.79 (s, 9H), 0.01 (s, 3H), -0.04 (s, 3H).

\textsuperscript{13}C NMR (400 MHz, CD\textsubscript{3}OD) δ 173.8, 172.4, 169.0, 163.8, 161.7, 155.8, 150.8, 138.1, 135.0, 133.4, 132.2, 131.2, 130.8, 129.8, 128.5, 127.1, 126.7, 125.7, 125.2, 122.4, 116.3, 113.7, 104.5, 47.7, 31.7, 30.1, 29.4, 29.1, 28.7, 24.9, 22.3, 13.0, 0.1.

ESI-TOF m/z calculated for C_{39}H_{48}F_{3}N_{3}O_{8}Si (M + H) – 770.3006 calculated, 770.2996 observed.

**Preparation of 5**

Crude 4 (116.9 mg, 0.15 mmol) was dissolved in 3% TCA in DCM (2.48 mL, 50 mM). The reaction stirred for 1.5 h until TLC (1:1 hex/EtOAc, R_f = 0.2, UV active and stained blue with PAA) showed starting material conversion to product. The reaction was quenched with sat. sodium bicarbonate (3 mL) and diluted with DCM (5 mL). The organic layer was washed with bicarbonate solution (1 x 8 mL). The aqueous layer was extracted with DCM (3 x 10 mL). The combined organic layers were
washed with brine (1 x 40 mL), dried with Na$_2$SO$_4$, and evaporated to dryness under vacuum. The crude mixture was purified by column chromatography (2:1 Hex/EtOAc) to yield 59 mg (64%) of S6. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.44 (s, 1H), 8.26 (s, 1H), 6.20 (t, $J$ = 6.4 Hz, 1H), 4.47 (q, $J$ = 3.2 Hz, 1H), 4.17 (d, $J$ = 6.4 Hz, 2H), 3.97 (s, 1H), 3.96 (dd, $J$ = 3.2, 9.8 Hz, 2H), 3.76 (d, $J$ = 9.8 Hz, 1H), 2.42 – 2.29 (m, 1H), 2.25 – 2.11 (m, 1H), 0.88 (m, 9H), 0.11 – 0.00 (m, 6H).

$^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 164.3, 158.5, 158.1, 150.4, 141.8, 117.2, 114.3, 109.1, 88.5, 86.7, 77.4, 77.1, 76.8, 71.8, 61.8, 41.9, 36.6, 25.7, 21.1, 18.0, -4.78, -4.9.

ESI-TOF m/z calculated for C$_{18}$H$_{28}$F$_3$N$_3$O$_6$Si (M + H) – 468.1699 calculated, 468.1766 observed.

Compound S6 (59 mg, 0.126) was azeotropically dried with pyridine (2 x 0.5 mL). The flask was cooled to 0 °C. DIPEA (65 mg, 90 μL, 0.504 mmol, 4 eq) was added to the cold starting material and the reactants dissolved in DCM (1.25 mL, 100 mM). 2-Cyanoethyl- $N$, $N$-diisopropylchlorophosphoramidite (35.7 mg, 34 μL, 0.151 mmol. 1.2 eq) was added and the reaction was monitored periodically by TLC (1:1 Hex/EtOAc, $R_f$ = 0.6, stained with PAA). After 2 h, TLC showed complete conversion to the phosphoramidite. The reaction was diluted with freshly distilled EtOAc (3 mL). The organic layer was washed with sat. bicarbonate solution (2 x 5 mL) and the combined aqueous layers were extracted with distilled EtOAc (2 x 10 mL). The combined organic layers were washed with brine (1 x 25 mL) and dried over Na$_2$SO$_4$. The organic layers were evaporated to dryness under vacuum and the crude material was purified by column chromatography (3:1 distilled hexanes/distilled EtOAc) yielding 44.3 mg (55%) of 5. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.02 (s, 1H), 6.31 (t, $J$ = 6.5 Hz, 1H), 4.44 (dd, $J$ = 3.4, 6.6 Hz, 1H), 4.19 (dd, $J$ = 3.4, 13.3 Hz, 1H), 4.03 (d, $J$ = 2.4 Hz, 2H), 3.93 (t, $J$ = 2.4 Hz, 2H), 3.82 (m, 2H), 3.59 (m, 2H), 2.67 (t, $J$ = 6.6 Hz, 2H), 2.40 – 2.22 (m, 2H), 2.11 (dt, $J$ = 6.5, 13.3 Hz, 1H), 1.26 – 1.14 (m, 12H), 0.88 (s, 9H), 0.13 – 0.03 (m, 6H).
\[^{13}\text{C}\] NMR (400 MHz, CDCl\textsubscript{3}) δ 171.1, 163.84, 163.81, 150.1, 150.0, 140.1, 117.1, 109.2, 109.1, 85.2, 77.3, 77.0, 76.6, 72.4, 71.7, 60.3, 58.5, 43.1, 43.0, 41.1, 34.6, 31.5, 29.0, 25.6, 25.2, 24.65, 24.57, 24.5, 22.6, 20.96, 20.4, 20.3, 18.7, 17.8, 14.1, 14.0, 11.3.

\[^{31}\text{P}\] NMR (400 MHz, CDCl\textsubscript{3}) δ 148.9, 148.7.

ESI-TOF m/z calculated for C\textsubscript{27}H\textsubscript{45}F\textsubscript{3}N\textsubscript{5}O\textsubscript{7}PSi (M + Na) − 690.2778 calculated, 690.2438 observed.

**Preparation of 7**

Compounds 3 (44.3 mg, 0.068 mmol, 1 eq) and 6\textsuperscript{8} (29.7 mg, 0.082 mmol, 1.2 eq) were azeotropically dried together with toluene (2 x 2 mL). S-Ethyl tetrazole/MeCN (250 mM, 330 μL, 0.082 mmol, 1.2 eq) was added to the flask. After 3.5 h, TLC (2.5:1 EtOAc/Hex, R\textsubscript{f} = 0.6, UV active and stained with PAA) indicated that the majority of 6 was consumed. tBuOOH (500 mM, 40.8 μL, 0.204 mmol, 3 eq) was added for 15-20 min. The reaction was evaporated to dryness under vacuum and purified by column chromatography (1:1 EtOAc/Hex -> 3:1 EtOAc/Hex) yielded 43 mg (75 %) of 7. \[^{1}\text{H}\] NMR (400 MHz, CDCl\textsubscript{3}) δ 9.61 (m, 1H), 8.15 (s, 1H), 7.76 (s, 1H), 6.28 (q, J = 6.8 Hz, 1H), 5.80 (m, 2H), 5.58 (m, 1H), 5.65 – 4.89 (m, 6H), 4.77 (d, J = 3.4 Hz, 1H), 4.40 (d, J = 3.4 Hz, 1H), 4.36 – 4.25 (m, 5H), 4.02 (s, 1H), 3.68 (dd, J = 3.2, 6.0 Hz, 2H), 3.54 (m, 1H), 2.77 (t, J = 6.0 Hz, 2H), 2.32 (m, 1H), 2.10 (d, J = 6.8 Hz, 5H), 1.84 (m, 1H), 1.73 – 1.61 (m, 6H), 0.87 (s, 9H), 0.08 (d, J = 1.9 Hz, 6H).

\[^{13}\text{C}\] NMR (400 MHz, CDCl\textsubscript{3}) δ 163.4, 150.1, 138.1, 114.83, 114.8, 110.8, 108.0, 85.1, 68.7, 67.3, 67.1, 67.0, 62.7, 31.6, 30.2, 29.0, 28.9, 25.6, 22.6, 21.1, 17.8, 14.2, 14.1, 11.4, -4.8, -5.0.
$^{31}$P NMR (400 MHz, CDCl$_3$) $\delta$ -1.8, -1.9.

ESI-TOF m/z calculated for C$_{36}$H$_{56}$F$_3$N$_4$O$_{12}$PSi (M - H) – 851.3354 calculated, 851.3229 observed.

**Preparation of 8**

Compound 7 (28.5 mg, 0.033 mmol) was dissolved in THF (330 μL, 100 mM). TEA•3HF (28 μL, 0.17 mmol, 5 eq) was added to the flask and the reaction was stirred at 25 °C overnight. After confirmed complete by TLC (3:1 EtOAc/Hex, R$_f$ = 0.1, UV active and stained with PAA), the reaction was diluted with DCM (2 mL) and washed with H$_2$O (1 x 3 mL), sat. sodium bicarbonate (1 x 3 mL), and brine (1 x 3 mL). The combined organic layers were dried over Na$_2$SO$_4$ and evaporated to dryness under vacuum. Column chromatography (4:1 EtOAc/Hex -> 100% EtOAc) provided 22 mg (92 %) of the desilylated intermediate (S8). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.75 (s, 1H), 8.09 (s, 1H), 7.76 (s, 1H), 6.25 (s, 1H), 5.80 (dd, $J$ = 6.2, 10.3 Hz, 2H), 5.60 (m, 1H), 5.10-4.90 (m, 6H), 4.77 (d, $J$ = 10.3 Hz, 1H), 4.49 (s, 1H), 4.32 (s, 4H), 4.21 (s, 2H), 4.09 (s, 2H), 3.68 (m, 2H), 3.60 – 3.46 (m, 1H), 3.40 (s, 1H), 2.80 (d, $J$ = 6.2 Hz, 2H), 2.41 (s, 1H), 2.16 (s, 2H), 2.09 (m, 4H), 1.75 – 1.56 (m, 4H).

$^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 137.9, 131.1, 114.7, 107.7, 105.2, 77.3, 77.0, 76.7, 53.4, 50.3, 46.0, 30.0, 28.8, 25.6, 8.4.

$^{31}$P NMR (400 MHz, CDCl$_3$) $\delta$ -1.81, -1.84, -1.9, -2.1.

ESI-TOF m/z calculated for C$_{30}$H$_{42}$F$_3$N$_4$O$_{12}$P (M - H) – 737.2489 calculated, 737.2353 observed.
**S8** (27.5 mg, 0.037 mmol) was dissolved in concentrated aqueous ammonia (560 μL). The reaction was capped and stirred at 0 °C. After 10 h, TLC (15% MeOH in DCM, 

\[ R_f = 0.2 \text{, UV active and stained with } \text{KMnO}_4 \] confirmed the disappearance of starting material. The reaction was evaporated to dryness under vacuum to yield 22.1 mg (100%) of **8**.

\[ ^1H \text{ NMR (400 MHz, CD}_3\text{OD)} \] δ 8.36 (s, 1H), 7.98 (s, 1H), 6.27 (m, 1H), 5.84 (m, 2H), 5.60 (m, 1H), 4.99 (m, 6H), 4.54 (m, 1H), 4.49 (m, 1H), 4.20 (m, 1H), 4.09 (s, 1H), 4.01 (m, 2H), 3.92 (d, \( J = 12 \) Hz, 1H), 3.86 (d, \( J = 12 \) Hz, 2H), 3.67 (m, 2H), 3.05 (q, \( J = 8 \) Hz, 6H), 2.38 (m, 1H), 2.28 (m, 1H), 2.12 (m, 4H), 1.65 (m, 4H), 1.32 (t, \( J = 8 \) Hz, 9H).

\[ ^{13}C \text{ NMR (400 MHz, CD}_3\text{OD)} \] δ 172.9, 159.1, 158.7, 151.9, 141.0, 118.7, 115.9, 110.4, 86.4, 86.3, 62.4, 61.7, 61.4, 38.4, 37.4, 20.8, 14.4.

\[ ^31P \text{ NMR (400 MHz, CD}_3\text{OD)} \] δ 8.2, 7.6, -2.2, -2.3.

ESI-TOF m/z calculated for C\(_{25}\)H\(_{40}\)N\(_3\)O\(_{11}\)P (M -H) = 588.2400 calculated, 588.2308 observed.

**Preparation of 9**

A stock solution of **S13** (20 mM) in 6% H\(_2\)O in MeCN was prepared and stored at -20 °C. An aliquot of the stock solution (5 μL, 100 nmol) was cooled to 0 °C. A solution of recrystallized NBS (5 μL, 30 mM, 300 nmol, 3 eq) was added and the reaction was stirred for 4 min (final: [**S13**] = 5 mM, 3% H\(_2\)O, [NBS] = 15 mM). After 4 min, saturated Na\(_2\)S\(_2\)O\(_3\) (10 μL) was added and the reaction was stirred for 10 min to quench it. The reaction was evaporated to dryness under vacuum, dissolved in 1:1 MeCN/H\(_2\)O, and analyzed by ESI. For characterization, **S13** (3 mg, 3.8 μmol) was deprotected using the above method and purified by high-performance liquid chromatography equipped with a UV-detector set.
at 260 nm. Chromatography was carried out using a Luna 5 µm C18 100 Å LC column (250 x 4.6 mm). The product was eluted using a gradient from 3% to 97% MeCN in H₂O (1.00 mL/min) over 36 min. Fractions containing 9 (11.6 min) were collected and evaporated to dryness by lyophilization.

¹H NMR (800 MHz, CD₃CN) δ 9.58 (m, 1H), 8.85 (m, 1H), 7.88 (m, 3H), 7.71 (s, 1H), 7.53 (s, 1H), 7.40 (m, 1H), 7.29 (m, 1H), 7.14 (m, 1H), 6.89 (m, 1H), 6.34 (m, 1H), 4.93 (s, 2H), 4.85 (m, 2H), 4.65 (m, 2H), 4.47 (s, 6H), 4.34 (m, 1H), 4.24 (m, 1H), 3.95 (m, 6H), 3.77 (m, 2H), 1.64 (m, 1H), 1.51 (m, 1H), 1.30 (m, 9H).

ESI-TOF m/z calculated for C₂₆H₃₀N₃O₁₄P (M - H) – 638.1393 calculated, 638.1529 observed.

ε₃₆₄ = 1.72 × 10³ M⁻¹cm⁻¹; ε₂₆₀ = 1.48 × 10⁴ M⁻¹cm⁻¹

Scheme S2. Independent synthesis of inhibitor hit precursors.

Preparation of S9

3,5-Dihydroxy naphthoic-2-carboxylic acid (485 mg, 2.37 mmol) was azeotropically dried with pyridine (3 x 1 mL). TBDMScI (1.09 g, 7.11 mmol, 3 eq) and imidazole (967 mg, 14.2 mmol, 6 eq) were added to the flask. The
contents were dissolved in DMF (9.5 mL, 250 mM) and reaction was heated at 60 °C and stirred overnight. The following morning, TLC (3% MeOH in DCM) suggested starting material was consumed so H₂O (0.2 mL) was added, and the reaction was stirred for 2 h. The reaction was evaporated to dryness under vacuum and purified by column chromatography (4:1 Hex/EtOAc -> 1:1 Hex/EtOAc) to yield 400 mg (53%) of S9. ¹H NMR (400 MHz, CDCl₃) δ 8.46 (s, 1H), 7.44 (s, 1H), 7.26 (d, J = 7.6 Hz, 1H), 6.96 (d, J = 7.6 Hz, 1H), 6.72 (d, J = 7.6 Hz, 1H), 1.00 (s, 9H), 0.19 (s, 6H).

Preparation of S10

Compound S9 (238 mg, 0.75 mmol) was azeotropically dried with pyridine (2 x 1 mL). NHS (128.8 mg, 1.12 mmol, 1.5 eq), and EDC (219 mg, 1.12 mmol, 1.5 eq) were added to the flask and the reagents were dissolved in DMF and stirred overnight at 25 °C. After 16 h, TLC (5% MeOH in DCM, Rf = 0.7) suggested the SM was completely converted so the reaction was evaporated to dryness under vacuum. The product was purified by column chromatography (3:1 Hex/EtOAc -> 1:1 Hex/EtOAc) to yield 62 mg (20%) of S10. ¹H NMR (400 MHz, CDCl₃) δ 8.66 (s, 1H), 7.68 (s, 1H), 7.44 (d, J = 8 Hz, 1H), 7.21 (t, J = 8 Hz, 1H), 6.94 (d, J = 8 Hz, 1H), 2.95 (s, 4H), 0.88 (s, 9H), 0.29 (s, 6H).

Preparation of S11

3,7-Dihydroxy naphthoic-2-carboxylic acid (121 mg, 0.41 mmol) was azeotropically dried with pyridine (3 x 1 mL). TBDMSCI (155 mg, 1.02 mmol, 2.5 eq) was added to the flask. The contents were dissolved in DMF (4.1 mL, 100 mM). DBU (187 mg, 183 μL, 1.23 mmol, 3 eq) was added and the reaction was heated to 60 °C. After 2 h, TLC (5% MeOH in DCM, Rf = 0.8) suggested complete conversion. The reaction was diluted with H₂O (5 mL) and the product was extracted with DCM (2 x 15 mL). The combined organic layers were washed with brine (1 x 30 mL), dried over Na₂SO₄,
evaporated to dryness under vacuum. Column chromatography (DCM -> 1% MeOH in DCM) yielded 967 mg (81%) S11. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.64 (d, $J = 8$ Hz, 1H), 7.24 (m, 4H), 1.11 (s, 18H), 0.47 (d, $J = 8$ Hz, 6H), 0.33 (s, 6H).

**Preparation of S12**

Compound S11 (86.5 mg, 0.2 mmol) was azeotropically dried with pyridine (1 x 0.8 mL). NHS (34.7 mg, 0.3 mmol, 1.5 eq) and EDC (57.8 mg, 0.3 mmol, 1.5 eq) were added to the flask and the reagents were dissolved in DMF and stirred overnight at 25 $^\circ$C. After 16 h, TLC (5% MeOH in DCM, R$_f$ = 0.7) suggested the SM was consumed, so the reaction was evaporated to dryness under vacuum. The product was purified by column chromatography (DCM -> 1% MeOH in DCM) to yield 62 mg (60%) of S12. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.93 (s, 1H), 8.55 (s, 1H), 7.59 (d, $J = 8.8$ Hz, 1H), 7.32 (s, 1H), 7.18 (d, $J = 8.8$ Hz, 1H), 2.94 (s, 4H), 1.01 (s, 9H), 0.24 (s, 6H).

**Preparation of S13**

NHS ester S10 (10 mg, 16 μmol, 1.2 eq), scaffold 8 (8 mg, 14 μmol), and DIPEA (3 μL, 16 μmol, 1.2 eq) were dissolved in DMF (270 μL, 50 mM). The reaction was heated at 60 $^\circ$C overnight. After 16 h, TLC (20% MeOH in DCM, R$_f$ = 0.3) suggested the scaffold was still present but a significant number of new spots had formed so the reaction was evaporated to dryness under vacuum and purified by column chromatography (15% MeOH in DCM -> 20% MeOH in DCM) to yield 11 mg of the amide intermediate (77%). The TBS group is labile and the NMR indicates these protons integrate to less than expected. The product(s) isolated is likely a mixture of silylated and desilylated compounds. This is acceptable because this intermediate is immediately desilylated (below). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.40 (m, 1H), 8.08 (s, 1H), 7.53
(d, J = 14 Hz, 1H), 7.13 (m, 1H), 6.84 (dd, J = 8, 14 Hz, 1H), 6.29 (m, 1H), 5.80 (m, 4H), 5.55 (m, 1H), 4.96 (m, 6H), 4.56 (m, 1H), 4.48 (m, 2H), 4.35 (m, 1H), 4.11 (m, 2H), 4.03 (m, 2H), 3.92 (m, 1H), 3.56 (m, 2H), 3.47 (m, 1H), 3.05 (q, J = 8 Hz, 6H), 2.92 (m, 1H), 2.29 (m, 2H), 2.07 (m, 6H), 2.63 (m, 6H), 1.32 (t, J = 8 Hz, 9H), 1.10 (s, 4H), 0.28 (s, 3H).

ESI-TOF m/z calculated for C₄₂H₅₉N₃O₁₄P (M - H) – 888.3509 calculated, 888.3554 observed.

The amide intermediate (11 mg, 0.012 mmol) was dissolved in THF (120 μL, 100 mM). TEA•3HF (10 μL, 0.06 mmol, 5 eq) was added to the flask and the reaction was stirred at 25 °C overnight. After confirmed complete by TLC (15% MeOH in DCM, R_f = 0.4, UV active and stained with PAA), the reaction was evaporated to dryness under vacuum and purified by column chromatography (10% MeOH in DCM -> 20% MeOH in DCM) provided 10 mg (99 %) of S13. ¹H NMR (800 MHz, CDCl₃) δ 8.44 (s, 1H), 8.02 (d, J = 8 Hz, 1H), 7.55 (s, 1H), 7.36 (d, J = 8 Hz, 1H), 7.10 (t, J = 8 Hz, 1H), 6.80 (d, J = 8 Hz, 1H), 6.29 (m, 1H), 5.79 (m, 4H), 4.94 (m, 6H), 4.49 (m, 5H), 4.30 (m, 1H), 4.08 (m, 4H), 3.57 (m, 2H), 3.05 (q, J = 8 Hz, 6H), 2.30 (m, 2H), 2.11 (m, 3H), 2.08 (m, 4H), 1.60 (m, 4H), 1.32 (t, J = 8 Hz, 9H).

¹³C NMR (800 MHz, CD₃OD) δ 141.7, 138.0, 125.6, 119.9, 113.8, 109.2, 107.6, 106.6, 106.1, 105.8, 105.5, 86.8, 86.1, 85.2, 71.2, 70.8, 66.4, 65.0, 60.5, 54.4, 48.0, 47.9, 47.8, 40.6, 30.0, 28.9.

³¹P NMR (400 MHz, CD₃OD) δ -1.8, -1.9, -2.0, -2.1.

ESI-TOF m/z calculated for C₃₆H₄₅N₃O₁₄P (M + H) – 777.2717 calculated, 777.2330 observed.

\[ \varepsilon_{364} = 1.72 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}; \varepsilon_{260} = 1.48 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \]
Preparation of S14

NHS ester S12 (10 mg, 16 μmol, 1.2 eq), scaffold 8 (8 mg, 14 μmol), and DIPEA (3 μL, 16 μmol, 1.2 eq) were dissolved in DMF (270 μL, 50 mM). The reaction was heated at 60 °C overnight. After 16 h, TLC (20% MeOH in DCM, Rf = 0.3) suggested the scaffold was still present but a significant number of new spots had formed so the reaction was evaporated to dryness under vacuum and purified by column chromatography (15% MeOH in DCM -> 20% MeOH in DCM) to yield 11 mg of the precursor to S14 (77%). $^1$H NMR (400 MHz, CDCl₃) δ 9.67 (s, 2H), 7.96 (s, 1H), 7.86 (s, 1H), 7.55 (d, J = 9.6 Hz, 1H), 7.37 (d, J = 9.6 Hz, 1H), 7.25 (dd, J = 2.4, 8.8 Hz, 2H), 7.06 (m, 1H), 6.16 (t, J = 6.6 Hz, 1H), 5.80 (m, 2H), 4.94 (m, 4H), 4.45 (q, J = 2.4 Hz, 2H), 4.41 (s, 1H), 4.10 (d, J = 12 Hz, 2H), 4.05 (m, 1H), 3.89 (m, 1H), 3.85 (q, J = 3.3 Hz, 1H), 3.71 (dd, J = 3.3, 12 Hz, 1H), 3.65 (dd, J = 3.3 Hz, 4H), 3.05 (q, J = 8 Hz, 6H), 2.23 (m, 4H), 2.18 (m, 3H), 1.56 (m, 4H), 1.32 (t, J = 8 Hz, 9H), 0.99 (m, 5H), 0.88 (s, 7H), 0.08 (s, 4H).

The amide intermediate (11 mg, 0.012 mmol) was dissolved in THF (120 μL, 100 mM). TEA•3H F (10 μL, 0.06 mmol, 5 eq) was added to the flask and the reaction was stirred at 25 °C overnight. After confirmed complete by TLC (15% MeOH in DCM, Rf = 0.4, UV active and stained with PAA), the reaction was evaporated to dryness under vacuum and purified by column chromatography (10% MeOH in DCM -> 20% MeOH in DCM) provided 10 mg (99%) of S14. $^1$H NMR (400 MHz, CD₃OD) δ 10.00 (s, 1H), 8.60 (s, 1H), 7.95 (s, 1H), 7.53 (d, J = 6 Hz, 1H), 7.34 (s, 1H), 7.16 (s, 1H), 7.09 (d, J = 6 Hz, 1H), 6.22 (t, J = 3.8 Hz, 1H), 5.84 (m, 4H), 4.99 (m, 5H), 4.46 (m, 4H), 4.07 (m, 4H), 3.92 (s, 1H), 3.77 (d, J = 11 Hz, 1H), 3.48 (m,
2H), 3.05 (q, J = 8 Hz, 6H), 2.25 (m, 4H), 2.16 (m, 2H), 2.04 (m, 4H), 1.56 (m, 2H), 1.32 (t, J = 8 Hz, 9H).

**Preparation of S15**

A stock solution of S14 (20 mM) in 6% H$_2$O in MeCN was prepared and stored at -20 °C. An aliquot of the stock solution (5 μL, 100 nmol) was cooled to 0 °C. A solution of recrystallized NBS (5 μL, 30 mM, 300 nmol, 3 eq) was added and the reaction stirred for 4 min (final: [S14] = 5 mM, 3% H$_2$O, [NBS] = 15 mM). After 4 min, saturated Na$_2$S$_2$O$_3$ (10 μL) was added and the reaction was stirred for 10 min to quench it. The reaction was evaporated to dryness under vacuum, dissolved in 1:1 MeCN/H$_2$O, and analyzed by ESI.

ESI-MS m/z calculated for C$_{26}$H$_{28}$N$_3$O$_{13}$P (M - H$_2$O - H) − 621.14 calculated, 621.16 observed.

$\varepsilon_{263} = 2.22 \times 10^4$ M$^{-1}$cm$^{-1}$

**Radiolabeling and Preparation of Oligonucleotide Complexes**

The 5’-dRP oligonucleotide was 3’-$^{32}$P labeled using $\alpha$-$^{32}$P Cordycepin triphosphate and terminal deoxynucleotidyl transferase. 3’-$^{32}$P labeling was also completed using Klenow exo$^-$ and $\alpha$-$^{32}$P-ATP, within a duplex containing a single 5’-dT overhang. In this case, the 3’-terminus of the 5’-dRP strand was $^{32}$P-labeled, denatured from the duplex, and purified by 20% denaturing PAGE. 5’-$^{32}$P labeling was completed by T4 polynucleotide kinase and $\gamma$-$^{32}$P-ATP. Ternary complexes were hybridized by mixing $^{32}$P-labeled oligonucleotides with the appropriate template and flanking strand in a 1:2.5:5 ratio in phosphate buffered saline (10 mM sodium phosphate, 100 mM NaCl, pH 7.3), heating to 95 °C, and slowly cooling to 25 °C.
Ternary complexes containing fluorophore labeled oligonucleotides were prepared by annealing the fluorophore-labeled strand with the appropriate quencher-labeled template and flanking strand in a 1:2:3 ratio. All oligonucleotides used to prepare ternary complexes are described in Table S1. Oligonucleotides containing a photochemical precursor (TC1) were generated fresh by photolysis (350 nm, 20 min) using a Rayonet.\textsuperscript{10}

**Table S1.** DNA substrates for assays.

| Pol β\textsuperscript{1} (TC1) | 5′-d(TAA TGG CTA ACG CAA XAC GTA ATG CAG TCT) -3′  
3′-d(ATT ACC GAT TGC GTT__ATG CAT TAC GTG AGA) -5′ |
|---|---|
| Pol β\textsuperscript{2} (TC2) | 5′-d(TCA CCC TCG TAC GAC TC TTT TTT TTT TGC F) -3′  
3′-d(AGT GGG AGC ATG CTG AG__AAA AAA AAA ACG Q) -5′ |
| Fluorescence Anisotropy (TC3) | 5′-d(TAA TGG CTA ACG CTT pFCC GTA ATG CAG TCT) -3′  
3′-d(ATT ACC GAT TGC GAA__AGG CAT TAC GTG AGA F) -5′ |

\textsuperscript{1} = lyase assay, \textsuperscript{2} = fluorescence assay  
X= 5′-dRP, F= TAMRA, Q= BHQ,  
Fl= dichloro-diphenyl-fluorescein (SIMA-HEX), pF=  

**Strand Displacement Assay with 31 kDa Pol β**

A 1 mM stock solution of each inhibitor is prepared using 50% MeCN in H\textsubscript{2}O. A solution of 31 kDa Pol β (500 nM) was preincubated with 1 or 9 (30 \textmu M) in 1X reaction buffer (total volume: 50 \textmu L; 50 mM HEPES buffer pH = 7.4, 5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol by volume) in a 384-well plate at 25 °C for 30 min. In control experiments, an equal volume of a control solution (containing all coupling and deprotection reagents but lacking inhibitor) was added to keep the percentage of solvents and reagents consistent. An aliquot (15 \textmu L) was diluted with a 2X solution (15 \textmu L) containing TC2 (100 nM, Table S1) and dTTP (200 \textmu M) in 1X reaction buffer (total volume: 30 \textmu L) in a different 384-well plate. The final reaction mixture contained 250 nM 31 kDa Pol β, 15 \textmu M inhibitor, 50 nM DNA,
100 µM dTTP, 1X reaction buffer, and 0.25% MeCN. The solution in each well was mixed thoroughly, and the fluorescence measurements were collected immediately.

**Gel-based Lyase Assay**

A solution of Pol β (20 nM) was preincubated with inhibitor at various concentrations (e.g. 5 µM) in 1X reaction buffer (total volume: 50 µL; 50 mM HEPES buffer pH = 7.4, 5 mM MgCl₂, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol by volume) at 25 °C for 30 min. In control experiments, an equal volume of a control solution (containing all coupling and deprotection reagents but lacking inhibitor) was added to keep the percentage of solvents and reagents consistent. The volume of inhibitor added was unchanged across experiments that used various inhibitor concentrations. To achieve various inhibitor concentrations, the stock solution of inhibitor (1 mM in 50% MeCN in H₂O) was diluted appropriately for the desired conditions.

An aliquot (3 µL) was mixed with freshly prepared TC1 (150 nM, Table S1) in 1X reaction buffer (total volume: 30 µL). The final mixture in the reaction well contained 2 nM Pol β, 0.5 µM inhibitor, 150 nM DNA, and 1X reaction buffer. Aliquots (4 µL) were removed at various times (0, 2, 5, 10, 15, 20 min) and flash frozen in dry ice. Afterwards, the thawed mixtures were immediately stabilized by NaBH₄ (4 µL, 300 mM) for 2 h at 4 °C. The samples were mixed with formamide loading buffer (8 µL, 90%, 10 mM EDTA). An aliquot (6 µL) was loaded onto a 20% denaturing polyacrylamide gel. The gel was exposed in a radiography cassette and the product was analyzed using a Phosphorimager.

This procedure was also used to measure lyase activity of 8 kDa Pol β with minor changes: (1) The concentration of 8 kDa Pol β during preincubation was 100 nM (2) After preincubation, an aliquot (3 µL) was mixed with of freshly prepared TC1 (20 nM, Table S1) in 1X reaction buffer
(total volume: 30 µL). Therefore, the reaction mixture contained 10 nM 8 kDa Pol β and 20 nM TC1, along with the other components.

**General Procedure for Dialysis**

Pol β (100 nM, total volume 200 µL) was preincubated in the absence or presence of 9 (e.g. 80 µM) in 1X reaction buffer (50 mM HEPES buffer pH = 7.5, 5 mM MgCl2, 0.2 mM EDTA, 50 mM KCl, 0.01 % Tween 20, 0.01 mg/mL BSA, and 4% glycerol) at 25 °C for 30 min. The lyase activity of each sample was immediately measured by mixing an aliquot of each sample (3 µL, 100 nM Pol β ± 80 µM 9) with 2X solution (15 µL) containing TC1 (150 nM) in 1X reaction buffer (total volume: 30 µL) (Table 21). The final concentrations during lyase kinetics were 10 nM Pol β, 80 µM 9, and 75 nM DNA.

The lyase kinetics were carried out as previously described. Briefly, aliquots (4 µL) were removed (0, 2, 5, 10, 15, and 20 min) from the reaction mixture. The reaction was halted by flash freezing the aliquot in dry ice. After 25 min, all aliquots were stabilized by NaBH₄ (4 µL, 300 mM) for 2 h at 4 °C. The samples were loaded onto a 20% denaturing PAGE gel and run for 4 h at 55 watts. The gel was exposed in a radiography cassette, which was scanned using a Phosphorimager.

The remaining sample (197 µL) was dialyzed in a 3.50K MW cassette in reaction buffer (1 L, buffer exchanged after 12 h) containing 50 mM HEPES buffer (pH = 7.5, 5 mM MgCl2, 4 mM DTT) for 24 h. The volume of the solution in the cassette was marked and no considerable volume change was observed after dialysis. The remaining lyase activity of the enzyme was measured as previously described. Aliquots (4 µL) were removed (0, 2, 5, 10, 15, 20 min) and stabilized with NaBH₄ (4 µL, 300 mM) for 1-2 h at 4 °C. The time points were analyzed by 20% denaturing PAGE (described above).
UPLC MS/MS Analysis of Pol β Modification by Covalent Inhibitors

A solution of Pol β (25 µL, 20 µM, 500 pmol) was mixed with 1 or 9 (5 µL, 50 µM, 100X), H₂O (420 µL) and 10X reaction buffer (50 µL, 500 mM HEPES buffer, pH = 7.4, 50 mM MgCl₂, 20 mM DTT) and incubated at 25 °C for 30 min. The reaction mixture was concentrated by centrifugation using an Amicon 3K centrifugal filter. To prevent the loss of protein, the centrifugal was blocked with Pol β prior to addition of the sample. Blocking was conducted by adding Pol β (500 µL, 0.5 µM), followed by centrifugation (13,000 g, 25 min, 4 °C) and removal of the supernatant. Following blocking of the membrane filter, the sample (500 µL, 1 µM Pol β ± 5 µM 1 or 9) was added to the Amicon centrifugal filter, and centrifugation was carried out (13,000 g, 25 min, 4 °C). The sample was then washed twice with 500 µL of 1X reaction buffer can concentrated by centrifugation in the Amicon filter to 50 µL (10 µM Pol β). Digestion buffer (25 µL, 500 mM Tris•HCl pH 8.0), 10X trypsin (25 µL, 400 µM), and H₂O (150 µL) were added to yield a final mixture of 2 µM Pol β and 40 µM trypsin (1:20 ratio) in 1X digestion buffer (total volume, 250 µL, 50 mM Tris•HCl, pH 8.0).

The digestion sample was incubated at 37 °C overnight. A portion (100 µL) of the digestion mixture was spun down (16,000 g, 10 min, 4 °C). The sample (10 µL) was injected onto and analyzed by UPLC-MS/MS using an ACQUITY UPLC HSS T3 Column (100 Å, 1.8 µm, 2.1 mm x 100 mm). The flow rate was 0.3 mL/min running a gradient from 85:5:10 water: acetonitrile: 1% formic acid to 50:40:10 water: acetonitrile: 1% formic acid over 35 min. Analysis was conducted using BioPharmaLynx with tolerance set to 30 ppm and allowing for 4 missed cleavages.

Fluorescence Anisotropy

Anisotropy measurements were conducted using a solution of dichloro-diphenyl-fluorescein-labeled TC3 (2.5 nM, Table S1) and Pol β (varying concentrations) in reaction buffer (50 mM HEPES, pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol). Samples also
contained 10% storage buffer (20 mM Tris•HCl, pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) by volume.

In a typical experiment, a sample (300 µL) was prepared by mixing Pol β (30 µL, 1 µM) in storage buffer with 10X reaction buffer (30 µL), TC3 (30 µL), a solution 50% MeCN in H2O containing or lacking inhibitor ([1] = 3, 6 mM, [9] = 5 mM; 3 µL) and H2O (207 µL). These samples, termed solutions A and A’ (A did not contain inhibitor and A’ contained a fixed concentration of inhibitor), contained 250 pM TC3, 100 nM Pol β, ± 50 µM 9. Samples containing various concentrations of Pol β were prepared by serial dilution with solution B and B’. Solution B (10 mL) was prepared by mixing H2O (7.85 mL), with 10X reaction buffer (1 mL), 10X storage buffer (1 mL), TC3 (50 nM, 50 µL), and a solution of 50% MeCN in H2O containing or lacking inhibitor (100 µL). Similarly, solution B did not contain inhibitor and was used exclusively to dilute solution A, whereas solution B’ contained a fixed concentration of inhibitor (e.g. 50 µM 9) and was used to dilute solution A’. By mixing equal volumes of A or A’ (150 µL) with B or B’ (150 µL) respectively, the concentration of Pol β decreased to 50 nM, while the concentration of DNA and inhibitor remain unchanged. An aliquot (150 µL) of this new solution was then mixed with solution B or B’ (150 µL) to prepare a new solution containing 25 nM Pol β. Serial dilutions were repeated such that samples contained Pol β concentrations of 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.13 nM, 1.56 nM, 0.78 nM, 0.39 nM, and 0.2 nM.

Samples were incubated at 25 °C for 1 h and fluorescence anisotropy (A) was measured using a portion (125 µL) of each sample with a PMT voltage of 800 mV, 8 nm slit width, 535 nm excitation and 556 nm emission. Fluorescence anisotropy was measured for TC3 in the absence of enzyme (A0), and the change in anisotropy (A-A0) was calculated for each sample and plotted against the concentration of Pol β. Each fluorescence anisotropy measurement was collected in triplicate.
This method was also used for the 31 kDa domain of Pol β and 8 kDa domain of Pol β.

This method was adapted to analyze inhibitors at various concentrations. The following concentrations were used: \([1] = 30 \text{ µM}, [9] = 50 \text{ µM}.\)
Chart S1. Carboxylic acids used to prepare inhibitor candidates.
Figure S1. Fluorescence-based strand displacement assay.
Figure S2. Biochemical characterization of inhibitor candidates. (a) Two candidates identified from screen (b) Effect of inhibitor(s) on dRP lyase activity in 8 kDa Pol β.
**Figure S3.** The effect of dialysis on Pol β lyase inactivation by 9.

**Figure S4.** Effect of (A) 1 and (B) 9 on strand displacement synthesis by 31 kDa fragment of Pol β.
| Res | B | z | Calculated | Observed | Y | z | Calculated | Observed |
|-----|---|---|------------|----------|---|---|------------|----------|
| K*  | 1 |   |            |          | 15|   |            |          |
| F   | 2 |   |            |          | 14| 1 | 1662.9062  | 1662.9055|
| V   | 3 |   |            |          | 13|   |            |          |
| D   | 4 |   |            |          | 12|   |            |          |
| E   | 5 | 2 | 620.1937   | 620.1929 | 11|   |            |          |
| G   | 6 |   |            |          | 10| 1 | 1172.6998  | 1172.6986|
| I   | 7 |   |            |          | 9 |   |            |          |
| K   | 8 | 1 | 1538.5879  | 1538.5890| 8 |   |            |          |
| T   | 9 |   |            |          | 7 | 1 | 874.4993   | 874.5012 |
| L   | 10|   |            |          | 6 |   |            |          |
| E   | 11| 2 | 949.8811   | 949.8806 | 5 |   |            |          |
| D   | 12|   |            |          | 4 | 1 | 531.3249   | 531.3256 |
| L   | 13| 2 | 1062.9366  | 1062.9375| 3 | 1 | 416.2980   | 416.2977 |
| R   | 14|   |            |          | 2 |   |            |          |
| K   | 15|   |            |          | 1 |   |            |          |
Figure S5. Annotated MS/MS of modified peptides observed in trypsin digest of Pol β preincubated with 1 (A) peptide containing K113 and (B) peptide containing K230.
| Res | B | z | Calculated  | Observed  | Y | z | Calculated  | Observed  |
|-----|---|---|-------------|-----------|---|---|-------------|-----------|
| I   | 1 | 13| 185.1285    | 185.1274  |   |   |             |           |
| A   | 2 | 12| 314.1711    | 314.1720  | 11| 2 | 935.9994    | 935.9003  |
| K*  | 4 | 10| 1063.4021   | 1063.4018 |   |   |             |           |
| D   | 5 |  9|  993.5252   |  993.5268 |   |   |             |           |
| E   | 6 |  8|  880.4411   |  880.4102 |   |   |             |           |
|    | 7 |  7| 1420.5565   | 1420.5534 |   |   |             |           |
| F   | 8 |   |            |           |   |   |             |           |
| L   | 9 |  5|  489.3032   |  489.3017 |   |   |             |           |
| A   |10 |  4|  376.2191   |  376.2184 |   |   |             |           |
| T   |11 |   |            |           |   |   |             |           |
| G   |12 |  2|  204.1343   |  204.1350 |   |   |             |           |
| K   |13 |   |            |           |   |   |             |           |
Figure S6. Annotated MS/MS of modified peptides observed in trypsin digest of Pol β preincubated with 9. (A) peptide containing K72 and (B) peptide containing K81.
Figure S7. $^1$H NMR Spectrum of S1

Figure S8. $^1$H NMR Spectrum of S2
Figure S9. $^1$H NMR Spectrum of S3

Figure S10. $^1$H NMR Spectrum of S4
Figure S11. $^1$H NMR Spectrum of 3

Figure S12. $^1$H NMR Spectrum of S5
Figure S13. $^1$H and $^{13}$C NMR Spectra of 4
Figure S14. $^1$H and $^{13}$C NMR Spectra of S6
Figure S15. $^1$H and $^{13}$C NMR Spectra of 5
Figure S16. $^{31}$P NMR Spectrum of 5

Figure S17. $^{31}$P NMR Spectrum of 7
Figure S18. $^1$H and $^{13}$C NMR Spectra of 7
Figure S19. $^1$H and $^{13}$C NMR Spectra of S8
Figure S20. $^{31}$P NMR Spectrum of S8

Figure S21. $^{31}$P NMR Spectrum of 8
Figure S22. $^1$H and $^{13}$C NMR Spectra of 8
Figure S23. $^1$H NMR Spectrum of S9

Figure S24. $^1$H NMR Spectrum of S10
Figure S25. $^1$H NMR Spectrum of S11

Figure S26. $^1$H NMR Spectrum of S12
Figure S27. $^1$H NMR Spectrum of Precursor to S13

Figure S28. $^{31}$P NMR Spectrum of S13
Figure S29. $^1$H and $^{13}$C NMR Spectra of S13
Figure S30. $^1$H NMR Spectrum of 9

^ - CD$_3$CN, # - H$_2$O, * triethyl ammonium salt, ** impurity from HPLC
Figure S31. $^1$H NMR Spectrum of Precursor to S14

Figure S32. $^1$H NMR Spectrum of S14
**Figure S33.** UPLC-MS analysis of precursor to 1. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (m/z = 790.1916 ± 5.00 ppm).
Figure S34. UPLC-MS analysis of 1. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (m/z = 654.0664 ± 5.00 ppm).
Figure S35. UPLC-MS analysis of S13. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion ($m/z = 774.2645 \pm 5.00$ ppm).
Figure S36. UPLC-MS analysis of 9. A) Total ion chromatogram. B) Extracted ion chromatogram of the molecular ion (m/z = 638.1393 ± 5.00 ppm).
Figure S37. Primary fluorescence data of screened first-generation library (30 μM inhibitor).
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