SUPPLEMENTAL MATERIALS

RecO and RecR are necessary for RecA loading in response to DNA damage and replication fork stress

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Running Title: RecO and RecR load RecA

Keywords: RecO, RecR, RecA, DNA replication, recombination

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CHEMISTRY EXPERIMENTAL SECTION

Synthesis of HPUra and congeners

The scheme shown in **Figure 2A** in the main text was utilized for the synthesis of HPUra and analogs. Condensation of 5-aminouracil (**1**) with hydrazine (1) or phenylhydrazine (2) led to the adducts **2** and **6**, respectively, in good yield. Reaction of **2** with *p*-benzoquinone proceeded cleanly to yield HPUra (**3**) as a brilliant orange solid. It was identical analytically and spectroscopically to a reference sample obtained from external sources. Compound **3** was further characterized as a *tert*-butyldimethylsilyl (TBS) ether derivative **5** (~ 87:13 mixture of isomers with the major one as assigned). The attempted synthesis of the hydrazino congener **4** (H_2-HPUra) by reduction of **3** was conducted by procedures described previously (2-4). Numerous attempts at sodium dithionite, following the literature procedure (3) or slight variations thereof, did not provide **4** but only recovered **3**. Any quenching of solution color was always transient, with a return to orange or pale orange upon acidification on workup. Other known methods of reducing diazenes were investigated including zinc in refluxing acetic acid (5), hydrazine hydrate in ethanol (6), and stannous chloride. Neither a color discharge nor reaction was observed for any of these except stannous chloride, which led to degradation of **3**. Since we were unable to produce the reduced form, we tested the oxidized form and found that addition of HPUra (compound **3**) to cells caused a rapid arrest to DNA synthesis *in vivo*. We found that compound 3 indeed blocked replication fork progression **(Figure 2B)**. Therefore, we conclude that compound **3** (HPUra) cannot be reduced to compound **4** (H_2-HPURra).

General chemical methods. All starting materials were obtained from commercial suppliers and were used without further purification. Reactions were performed under a blanket of nitrogen unless specified otherwise. Melting points were determined in open capillary tubes on a Laboratory Devices Mel-Temp apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz or 400 MHz instrument utilizing d_6-DMSO or D_2O/NaOD as solvent. Chemical shifts are reported relative to the residual solvent peak in δ (ppm). Mass spectra were recorded on a Micromass LCT time-of-flight

instrument utilizing the electrospray ionization mode. Thin-layer chromatography (TLC) was performed on silica gel GHLF plates (250 microns) purchased from Analtech.

6-Hydrazinylpyrimidine-2,4(1*H*,3*H*)-dione (2). A stirred suspension of 6-aminouracil (**1**; 5 g, 39.3 mmol), 62% hydrazine monohydrate (9.85 mL, 197 mmol), acetic acid (5 mL), and water (30 mL) was heated at reflux for 6 h and then cooled. The precipitate was collected by filtration, washed successively with water, ethanol, ether, and then dried to leave 4.58 g (82%) of **2** as an off-white solid, mp 279 °C (dec); TLC (4:1 chloroform / methanol) showed complete absence of starting material. See **Figure S2** for ^{1}H NMR spectrum.

6-((4-Hydroxyphenyl)diazenyl)pyrimidine-2,4(1*H*,3*H*)-dione (3). A suspension of 6-hydrazinylpyrimidine-2,4(1*H*,3*H*)-dione (**2**; 12.7 g, 89 mmol), benzoquinone (9.66g, 89 mmol), and formic acid (68.5 mL) was maintained at room temperature for 5 min, and then heated at 50 °C for 5-8 min. After cooling the orange solids were collected by filtration, and then washed successively with formic acid, water, ethanol, ether, and vacuum dried to leave 17.4 g (84%) of **3**, mp >260 °C (dec);TLC R_f 0.67 (4:1 chloroform / methanol). See **Figures S3 and S4** for ^{1}H NMR spectra, **Figure S5** for ^{13}C NMR spectrum and **Figure S6** for mass spectrum.

2-((*tert*-Butyldimethylsilyl)oxy)-6-((4-hydroxyphenyl)diazenyl)pyrimidin-4(3*H*)-one (5). A mixture of **3** (46 mg, 0.2 mmol), *tert*-butyldimethylsilyl chloride (179 mg, 1.19 mmol), diisopropylethylamine (DIPEA; 0.31 mL, 1.78 mmol), and DMF (0.8 mL) under nitrogen was heated at 50 °C for 20 h. A deep-red solution formed within a few minutes. After cooling, the mixture was diluted with 0.5 mL 2-propanol and placed in the refrigerator to initiate precipitation. The precipitated solids were collected by filtration, rinsed with 2-propanol, and dried to leave 40 mg (58%) of **5** as an orange solid, mp 260 °C;TLC R_f 0.24 (1:1 ethyl acetate / hexanes). See **Figure S7** for ^{1}H NMR spectrum, **Figure S8** for ^{13}C NMR spectrum and **Figure S9** for mass spectrum.

6-(2-Phenylhydrazinyl)pyrimidine-2,4(1*H*,3*H*)-dione (6). A mixture of 6-aminouracil (**1**; 0.5 g, 3.9

mmol), phenylhydrazine (0.78 mL, 0.851g, 7.87 mmol), acetic acid (0.45 mL, 7.91 mmol), and water (15 mL) was heated at reflux for 3 h. After cooling, the precipitated solids were collected by filtration, washed well with water and then ethanol, and dried to give 0.72 g (84%) of product as cream-colored solid; TLC R_f 0.68 (3:1 acetonitrile / 0.2M aq. ammonium chloride). See **Figure S10** for ^{1}H NMR spectrum, **Figure S11** for ^{13}C NMR spectrum and **Figure S12** for mass spectrum.

BIOLOGY METHODS SECTION

Plasmids used in this study. Unless otherwise indicated, all plasmids used in this study were constructed using standard cloning procedures (7). All primers used for plasmid construction are available upon request.

pEB1 was constructed for integration and expression of *recO* from a P_{spac} promoter at the *amyE* locus. The 765 nucleotide *recO* coding sequence was PCR amplified using primers oEB1 and oEB2. The PCR product was digested with SphI and HindIII, the same enzymes used to digest pDR66 (8). Plasmid pEB1 was then constructed by ligation of the *recO* coding region with double digested pDR66 using the same enzymes.

pEB20 was constructed by SLIC (9) of a partial fragment of *recR* into pBGSC6 for the disruption of the *recR* gene. The fragmented *recR* coding region was PCR amplified using primers oEB78 and oEB79.

pEB21 was constructed by SLIC of the entire *recR* coding region into pJS101 for integration at the *amyE* locus under a xylose inducible promoter. The *recR* coding region was PCR amplified using primers oEB83 and oEB84. The pJS101 vector was amplified using primers oJS431 and oJS432.

pJSL112 was constructed by Gibson assembly. A ~1 kB region corresponding to the immediate upstream and downstream regions of *recF* was amplified by PCR with primers oJSL304/oJSL305 and oJSL306/oJSL307, respectively. The chloramphenicol cassette was amplified from pGEM with primers

oJSL294 and oJSL295. A basic plasmid backbone containing the *ori* and the ampicillin cassette was amplified from pDR111 using oJSL302 and oJSL303. All four DNA fragments were assembled in a single Gibson Assembly reaction at 50˚ C for 1 hour using standard Gibson protocol.

All constructed clones were sequenced prior to use by the University of Michigan core sequencing facility (http://seqcore.brcf.med.umich.edu/).

Immunoblot analysis. Immunoblot analysis was done essentially as described (11). Briefly, strains were grown in 10 mL defined $S7_{50}$ minimal media containing 2% glucose, with appropriate antibiotics to mid exponential phase, and concentrated by centrifugation. Cells were resuspended using 300 µL lysis buffer [10 mM Tris-HCL (pH 7.0), 1X Protease Inhibitor, 0.5 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 1 mg/mL lysozyme, 1 U/mL DNase I in ddH_2O] as previously described (12) and lysed via sonication for 45 seconds three times with a 1 minute pause in-between, followed by the addition of SDS to 1%. Total soluble protein was quantified using a Pierce BCA Protein Assay kit according to manufacturer's instructions. Proteins were separated on a 4-15% gradient gel (BioRad) by SDS-PAGE followed by transfer to a nitrocellulose membrane (Whatman) using 1X transfer buffer (24 mM Tris, 192 mM glycine, pH 8.2, 15% methanol) overnight at 15 volts as described (13, 14). The membrane was blocked with 5% non-fat milk in TBS+0.02% Tween 20. 1:1000 primary antibody was added to fresh blocking solution, and incubated with the membrane overnight on an orbital shaker at 4 °C. The nitrocellulose membrane was washed 3X in 1X TBS-Tween 20 (0.02%) followed by 1:1000 dilution of goat-anti-rabbit-HRP conjugated secondary antibody in 5% milk/TBS-Tween on an orbital shaker at room temperature for 2 hours. The nitrocellulose membrane was washed again 3X with 1X TBS-tween (0.02%) followed by incubation with 2 mL SuperSignal West Pico Luminol/ Enhancer Solution and 2 mL SuperSignal West Pico Stable Peroxide Solution (Thermo Scientific). Blots were exposed to film (BioExpress) for 1 minute prior to developing.

BIOLOGY RESULTS

We also established the time dependency of formation of RecA foci before performing the experiments below. We found that the DNA break inducing peptide phleomycin (30) caused a linear increase in the percentage of cells with foci beginning to form 7.5 minutes after chemical treatment, with 1.2% of cells gaining RecA-GFP foci/minute (R^2=0.983) over a 60 minute time course **(Figure S1B)**. Mitomycin C (MMC), which upon being imported into the cell is reduced to produce its toxic form, begins producing a linear increase in the percentage of cells with RecA-GFP foci at a similar rate (1.2% of cells gaining foci/minute) starting at 20 minutes past treatment (R^2=0.998). At 30 minutes past treatment, both MMC and phleomycin cause production of RecA filaments.

SUPPLEMENTAL TABLES

Table S1. List of plasmids

Plasmids	Characteristics	Source
pEB1	*recO* in pDR66	This work
pEB21	*recR* in pJS101	This work
pEB22	*recO* in pJS102	This work
pCm::Tet	*pCM::Tet*	BGCS
pCM::Er	*pCM::Er*	BGCS
pJSL112	*recF::cm*	This work

Table S2. List of Strains

Strains	Genotype	Source
LAS508	PY79 (wild type)	(15)
LAS40	*recA-23mgfpA206K(spc^r)* (referenced below as *recA-gfp*)	(16)
ERB1	*amyE::P$_{spac}$-recO(cm^r)*	This work
ERB2	*amyE::P$_{spac}$-recO(tet^r)*	This work
ERB5	*amyE::P$_{spac}$-recO (tet^r); recO::cat (cm^r)*	This work
ERB5	*amyE::P$_{spac}$-recO(tet^r) ; recO::cat (cm^r); recA-gfp (spc^r)*	This work
ERB63	*recR::cat(cm^r)*	This work
ERB64	*recR::cat(cm^r), recA-gfp(spc^r)*	This work
ERB69	*amyE::P$_{xyl}$-recR(mls^r)*	This work
ERB70	*amyE::P$_{xyl}$-recR(mls^r); recR::cat(cm^r)*	This work
ERB76	*amyE::P$_{xyl}$-recR(mls^r); recR::cat; recA-gfp(spc^r)*	This work
ERB77	*dnaB134(ts) (tet^r); recO::cat(cm^r); recA-gfp(spc^r)*	This work
ERB78	*recO::cat (cm^r)*	This work
ERB84	*amyE::P$_{xyl}$-recOΔC24-mCitrine(mls^r)*; *recO::cat(cm^r)*	This work
ERB85	*recO::cat(cm^r); recA-gfp(spc^r)*	This work
LAS197	*recO::cat(cm^r); recA-gfp(spc^r)*	Lab Stock
JSL496	*recF::cat(cm^r); recA-gfp(spc^r)*	This work
JSL532	*SceI CS yyaC, amyE::Pxyl I SceI (mls^r),*	This work

	recA-gfp (spc^r)	
JSL533	*SceI CS yyaC, amyE::Pxyl I SceI (mls^r), recA-gfp (spc^r), recF::cat(cm^r)*	This work
JWS159	*ssb3$^+$ (mls^r) PspacrpsR, recA-gfp(spc^r)*	This work
JWS160	*ssbΔ35 (mls^r) Pspac-rpsR, recA-gfp(spc^r)*	This work
JSL656	*ssbΔ35(mls^r) PspacrpsR, recA-gfp(spc^r), recO::cat (cm^r)*	This work
JSL658	*ssbΔ35(mls^r) Pspac-rpsR, recA-gfp(spc^r), recR::cat (cm^r)*	This work
JSL660	*ssb3$^+$(mls^r) Pspac-rpsR, , recA-gfp(spc^r), recR::cat (cm^r)*	This work
JSL661	*ssb3$^+$(mls^r) Pspac-rpsR, , recA-gfp(spc^r), recO::cat (cm^r)*	This work
JSL662	*ssb3$^+$(mls^r) Pspac-rpsR, recR::cat (cm^r)*	This work
JSL663	*ssb3$^+$(mls^r) Pspac-rpsR, recO::cat (cm^r)*	This work
JSL665	*ssbΔ35(mls^r) PspacrpsR, recO::cat (cm^r)*	This work
JSL667	*ssbΔ35(mls^r) PspacrpsR, recR::cat (cm^r)*	This work
JSL669	*SceI CS yyaC, amyE::Pxyl I SceI (mls^r), recA-gfp (spc^r), recO::cat(cm^r)*	This work
JSL673	*SceI CS yyaC, amyE::Pxyl I SceI (mls^r), recA-gfp (spc^r), recR::cat(cm^r)*	This work
JSL677	*ssb3$^+$(mls^r) Pspac-rpsR, recA::spc (spc^r)*	This work

SUPPLEMENTAL FIGURES

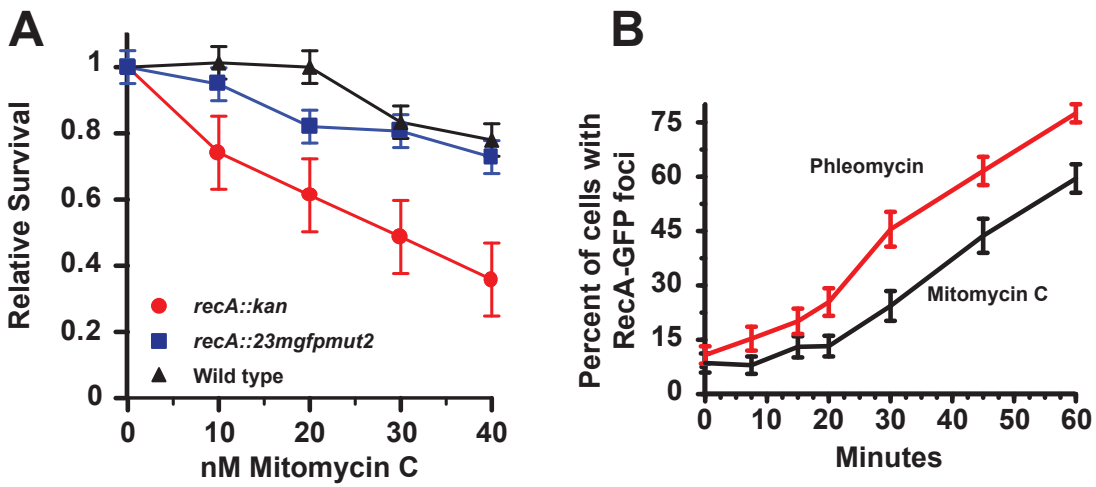
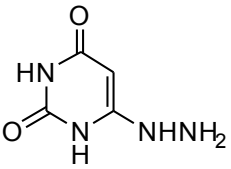


Figure S1. RecA-GFP focus formation in response to DNA damage. (A) Shown is a survival curve of cells challenged with concentrations of mitomycin C ranging from 0-40 nM for 30 minutes. The error bars represent the standard error of the mean. **(B)** Shown is a time course experiment representing the percentage of cells with RecA-GFP foci at 40 nM mitomycin C (black) or 400 nM phleomycin (red) over 60 minutes. Each treatment was scored in triplicate at the time points indicated. Error bars reflect the 95% confidence interval.



2 $C_4H_6N_4O_2$
MW 142.12

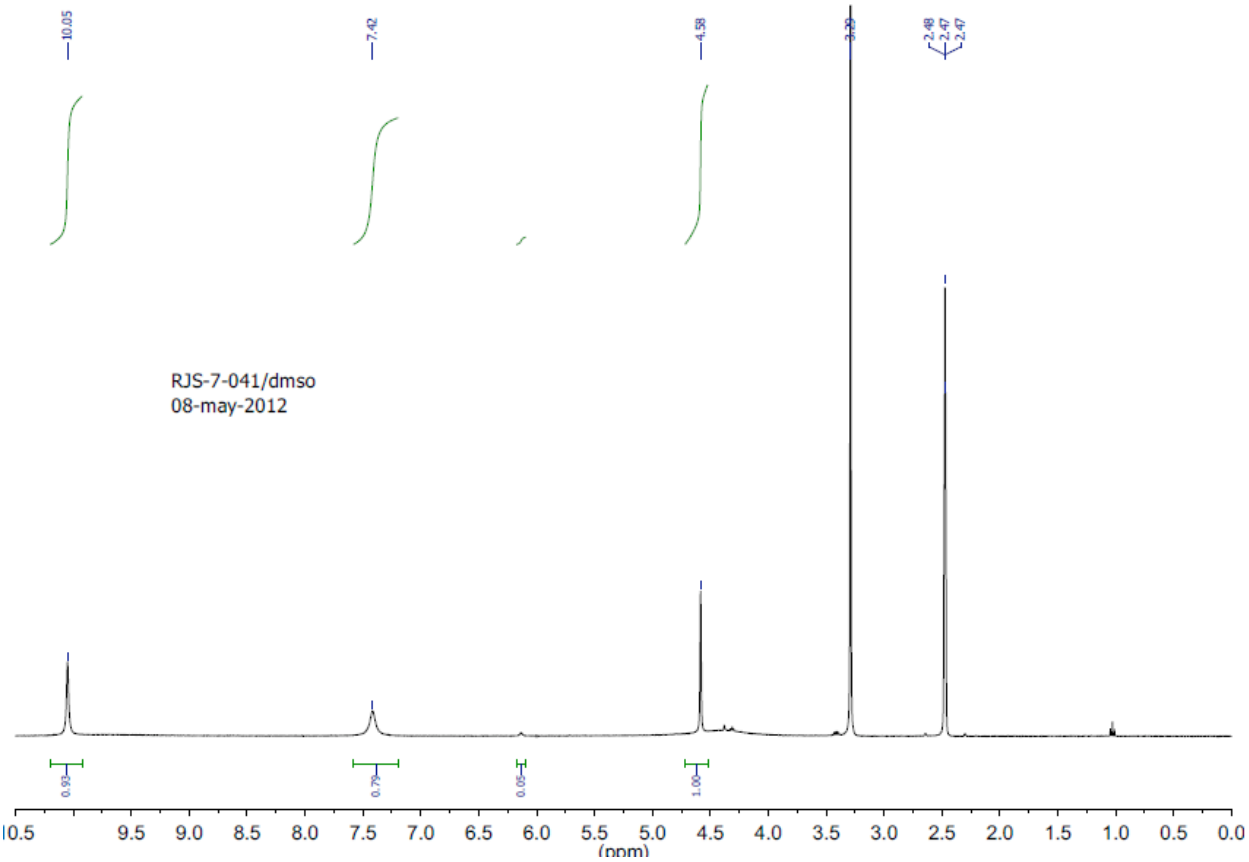
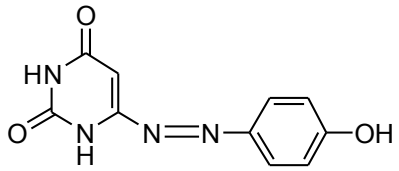


Figure S2. ^{1}H NMR spectrum of 2 in DMSO-d_6.



3 $C_{10}H_8N_4O_3$
MW 232.20

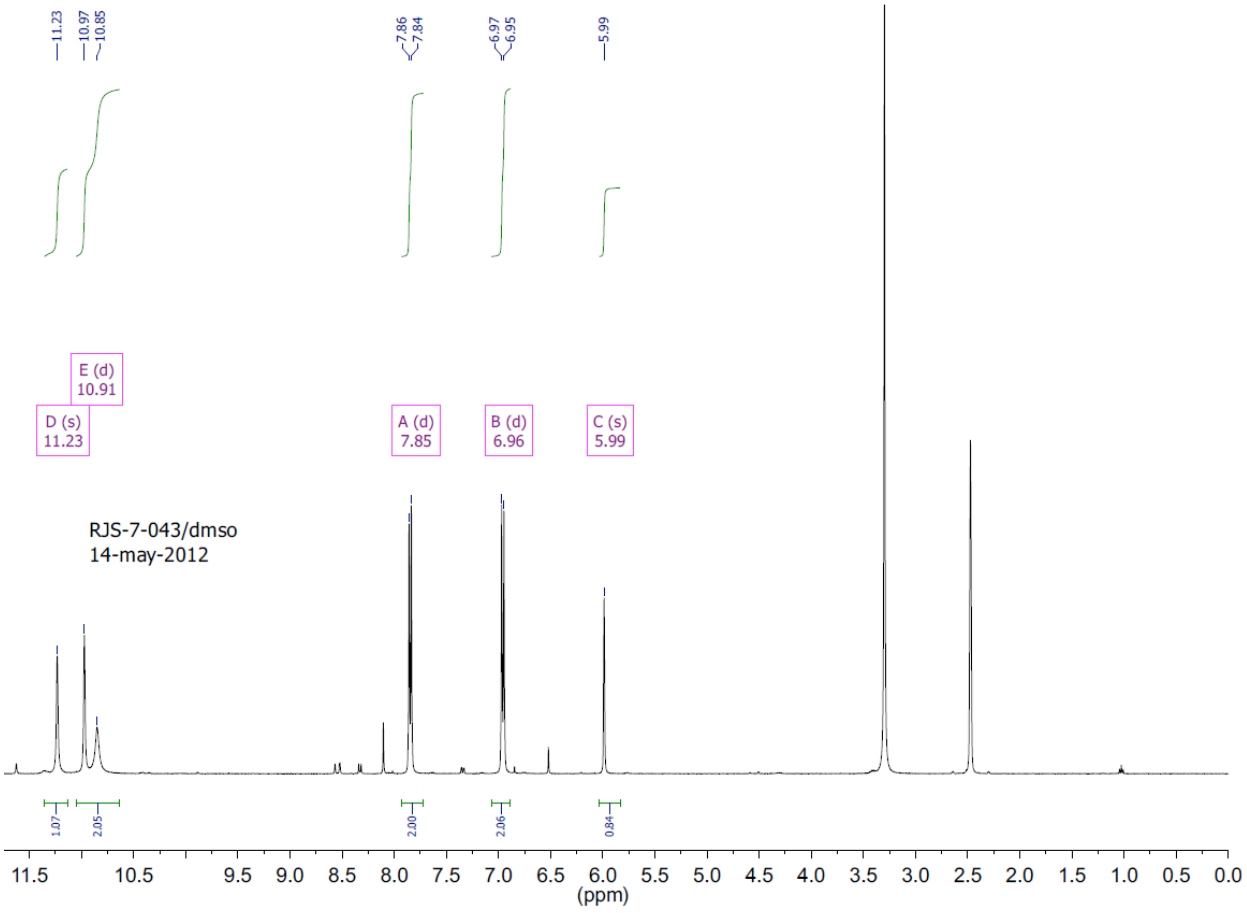


Figure S3. ^{1}H NMR spectrum of 3 (HPUra) in DMSO-d_6.

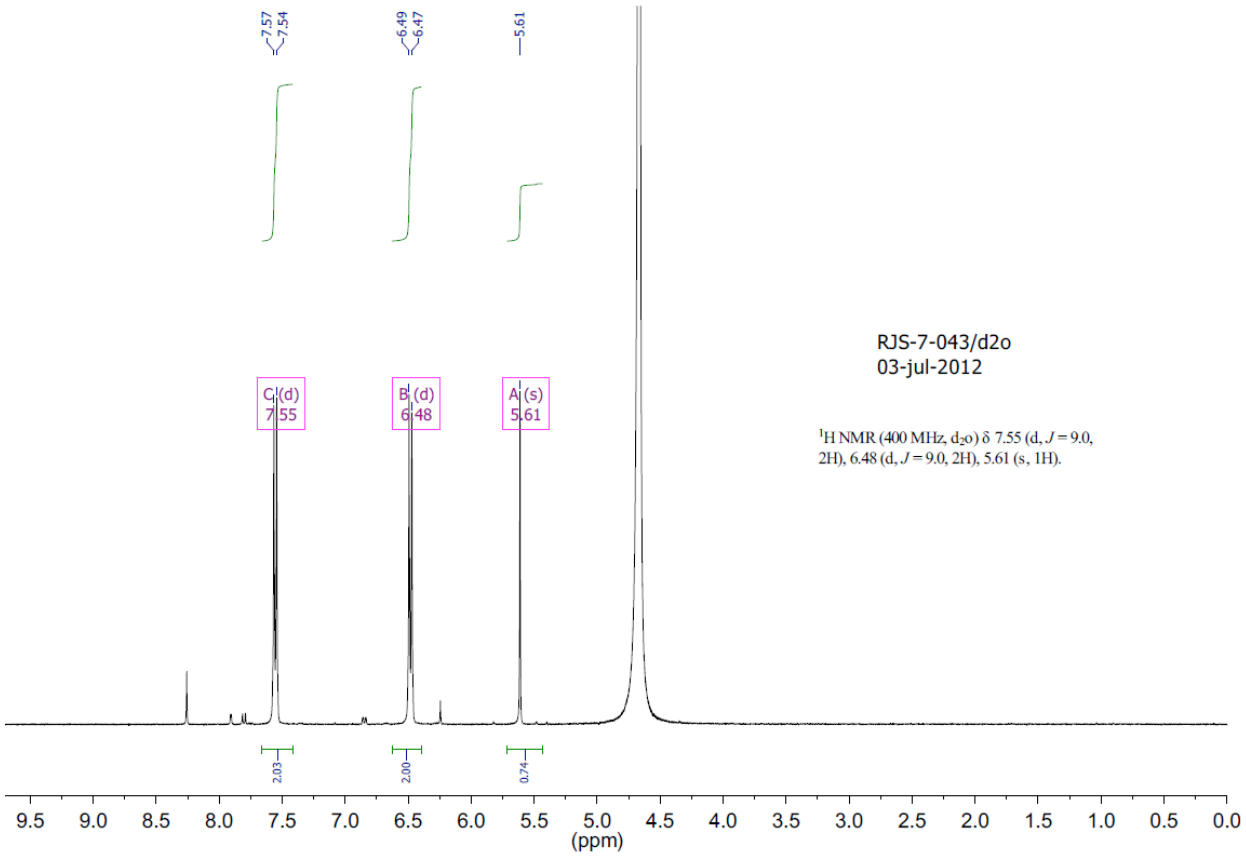


Figure S4. ^{1}H NMR spectrum of 3 (HPUra) in D_2O

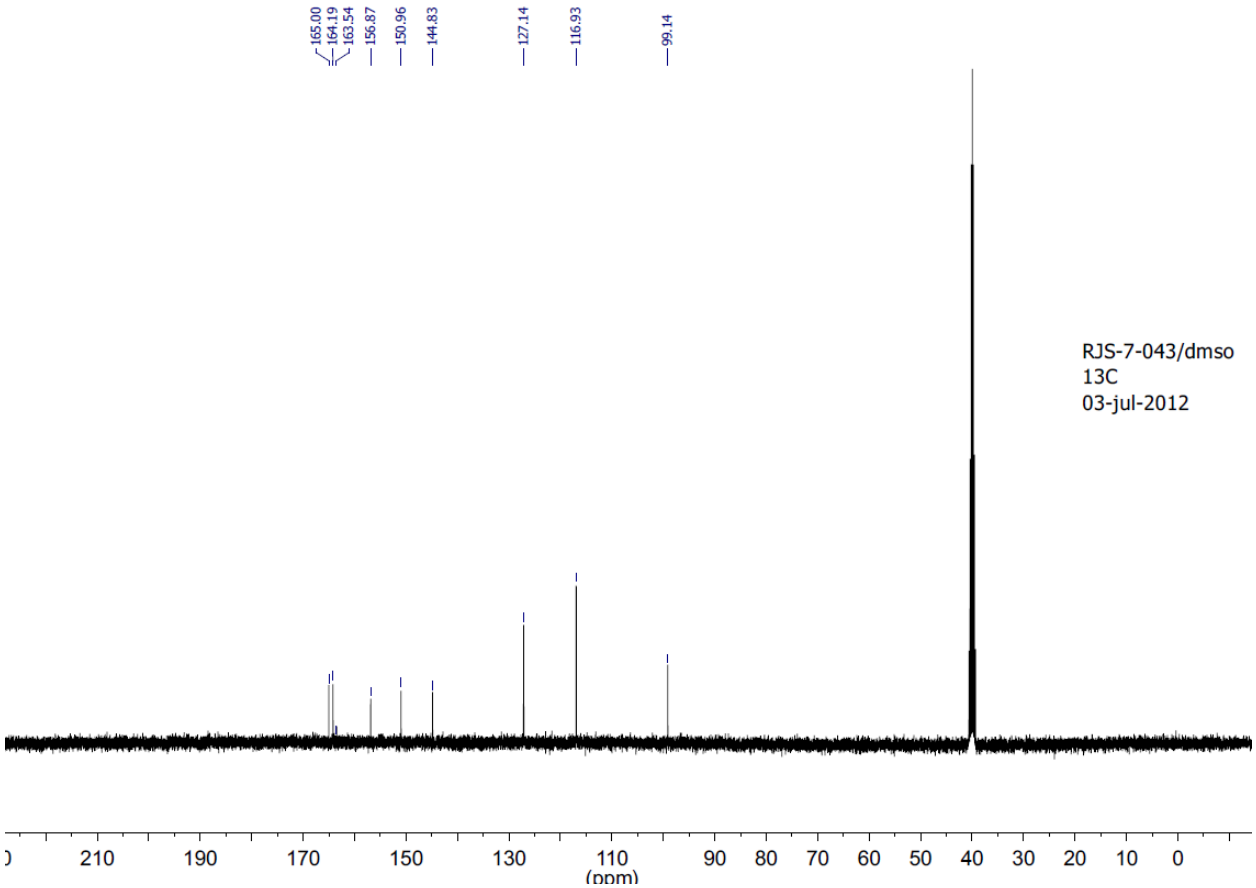


Figure S5 . ^{13}**C NMR spectrum of 3 (HPUra) in DMSO-*d*$_6$.**

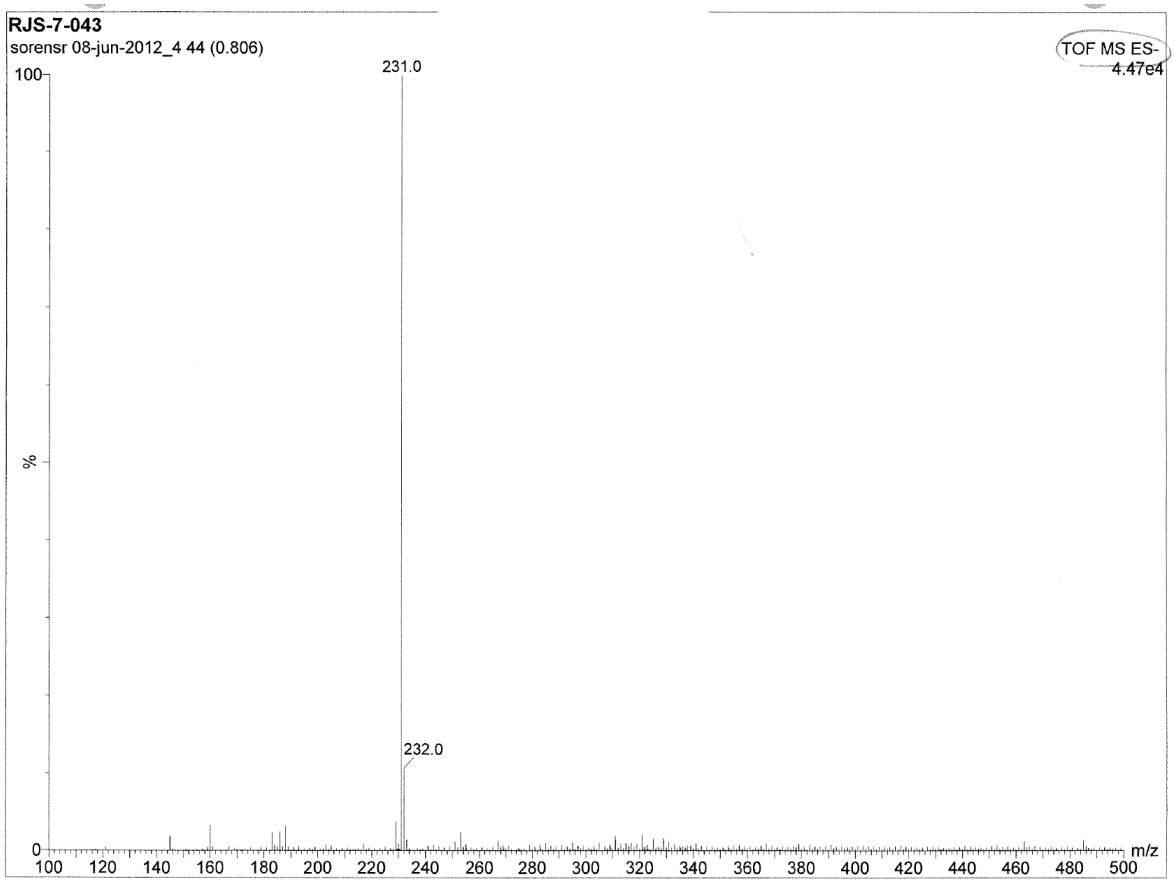
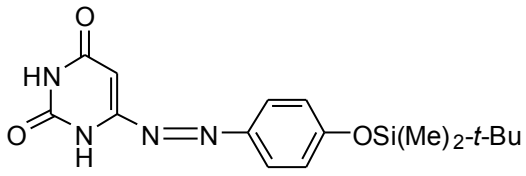


Figure S6. Electrospray (negative mode) mass spectrum of 3 (HPUra).



5 $C_{16}H_{22}N_4O_3Si$
MW 346.46

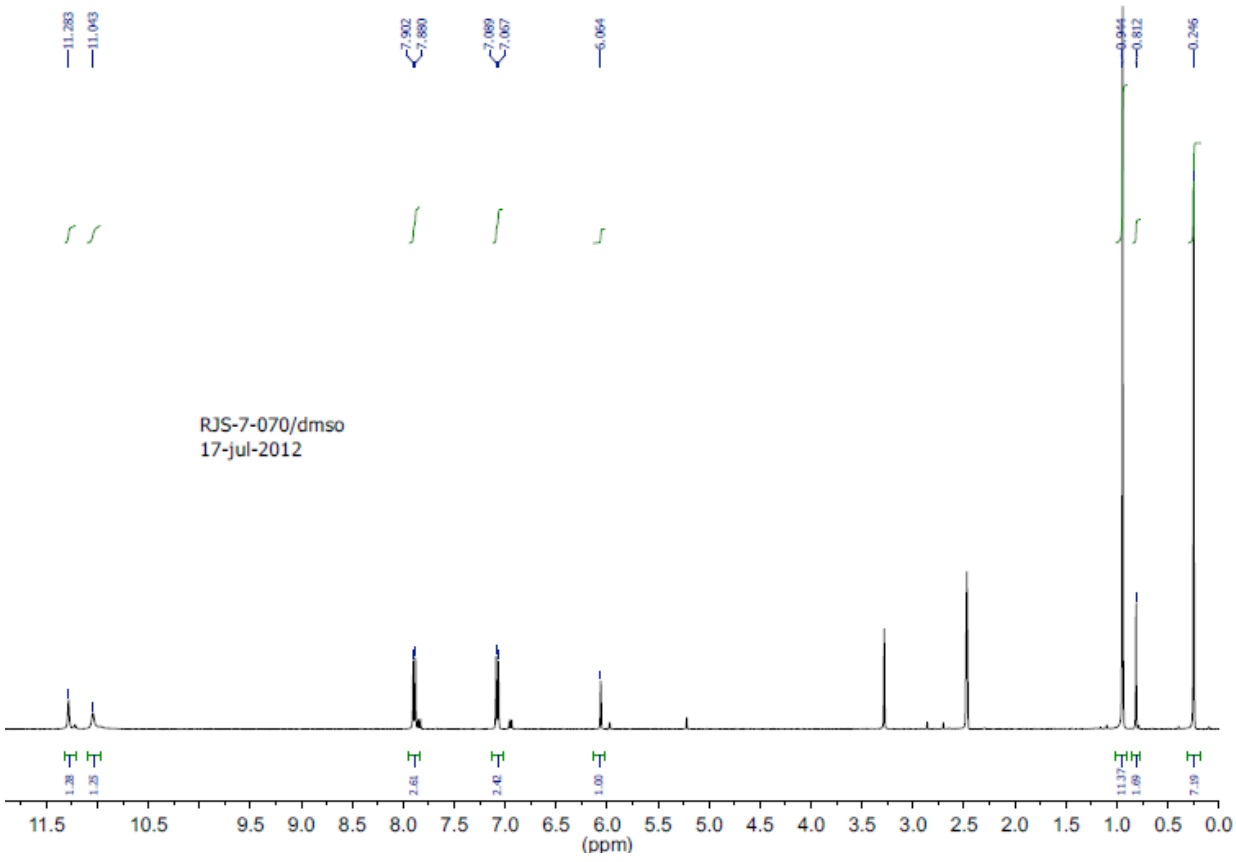


Figure S7. ^{1}H NMR spectrum of silylated HPUra (5) in DMSO-d_6.

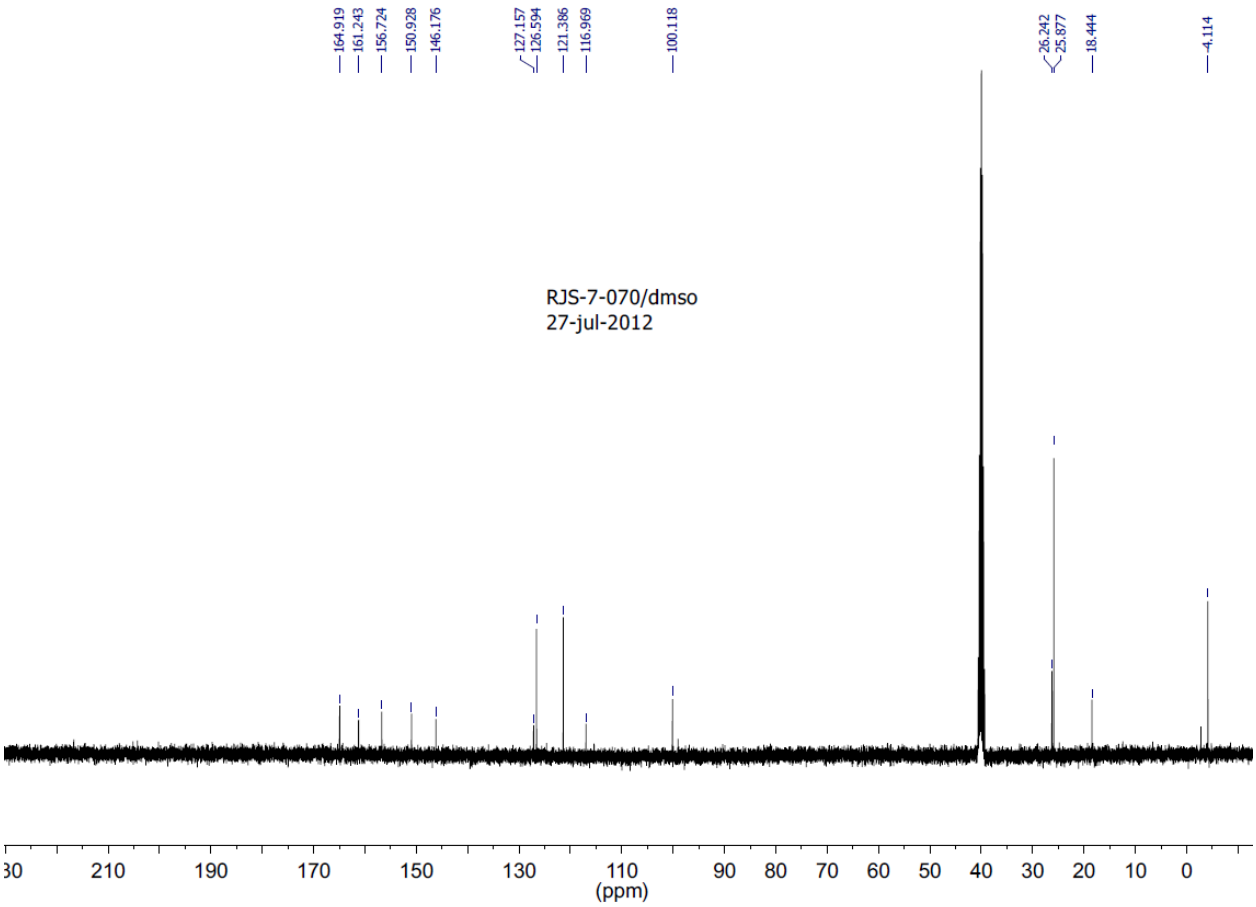


Figure S8. ^{13}C NMR spectrum of silylated HPUra (5) in DMSO-d_6.

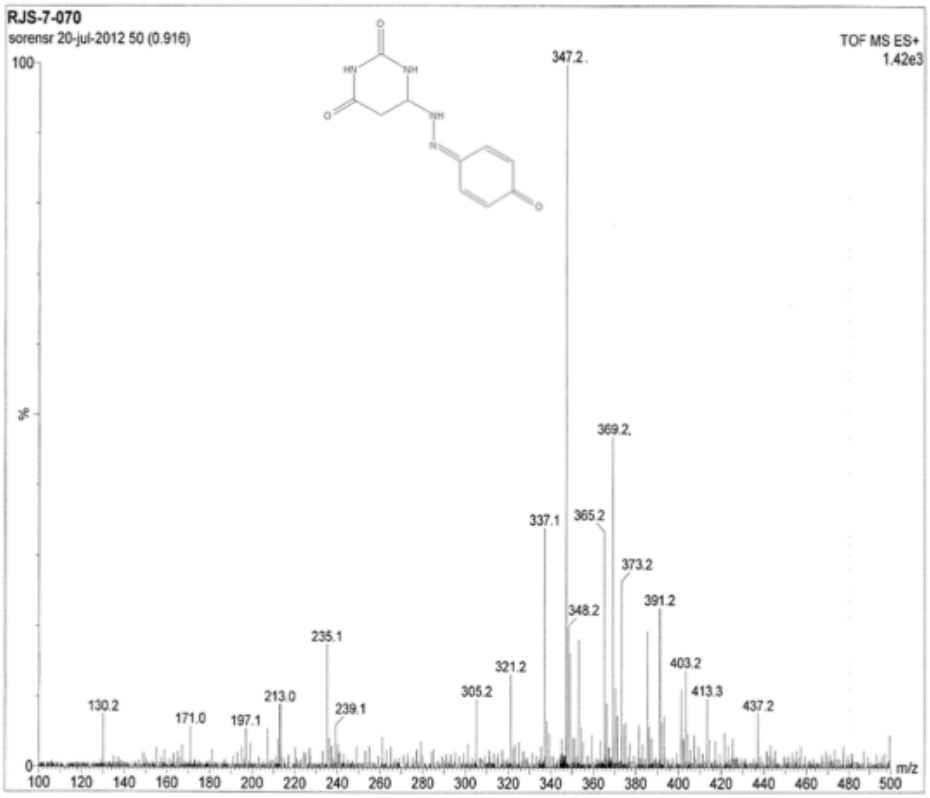
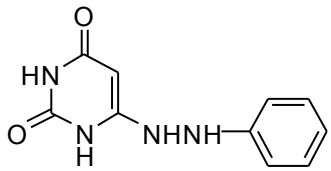


Figure S9. Electrospray (positive mode) mass spectrum of silylated HPUra (5).



6 $C_{10}H_{10}N_4O_2$
MW 218.22

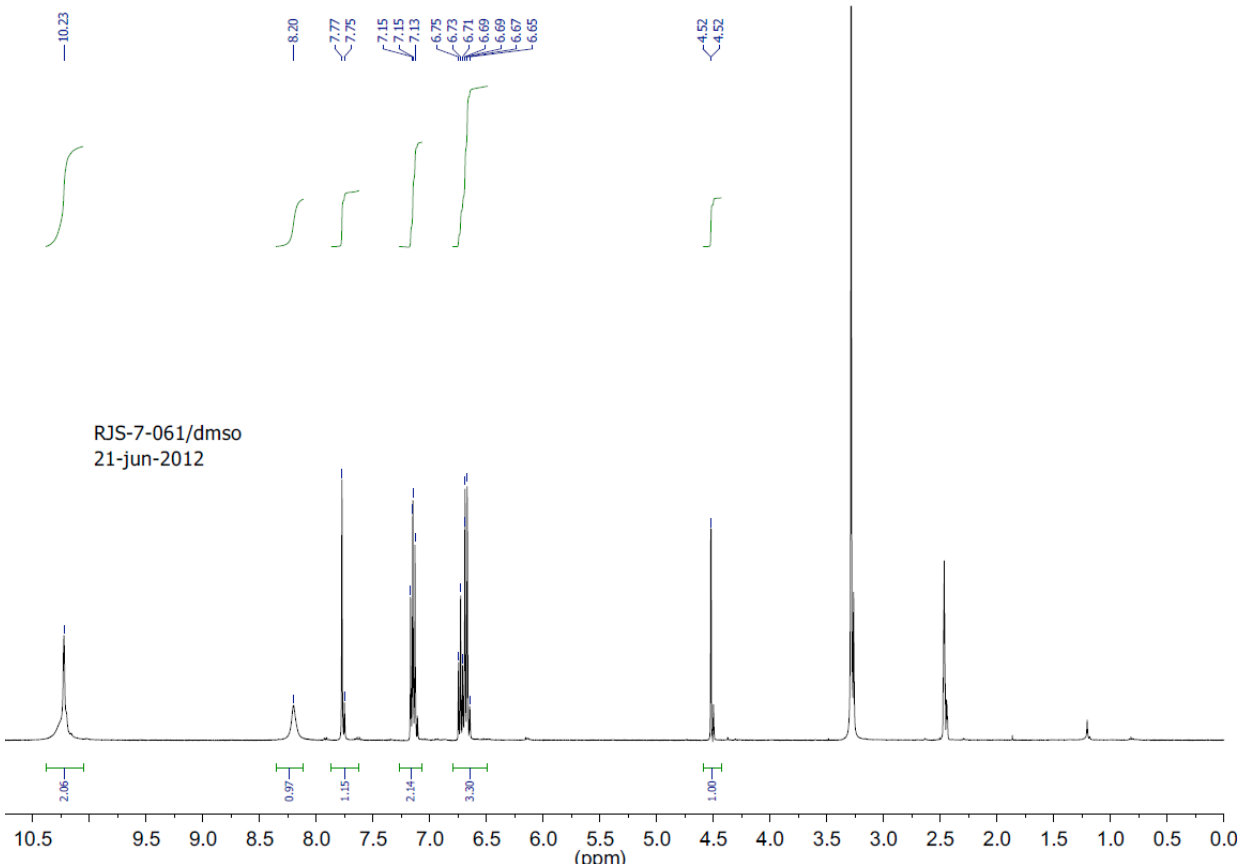


Figure S10. ^{1}H NMR spectrum of 6 in DMSO-d_6.

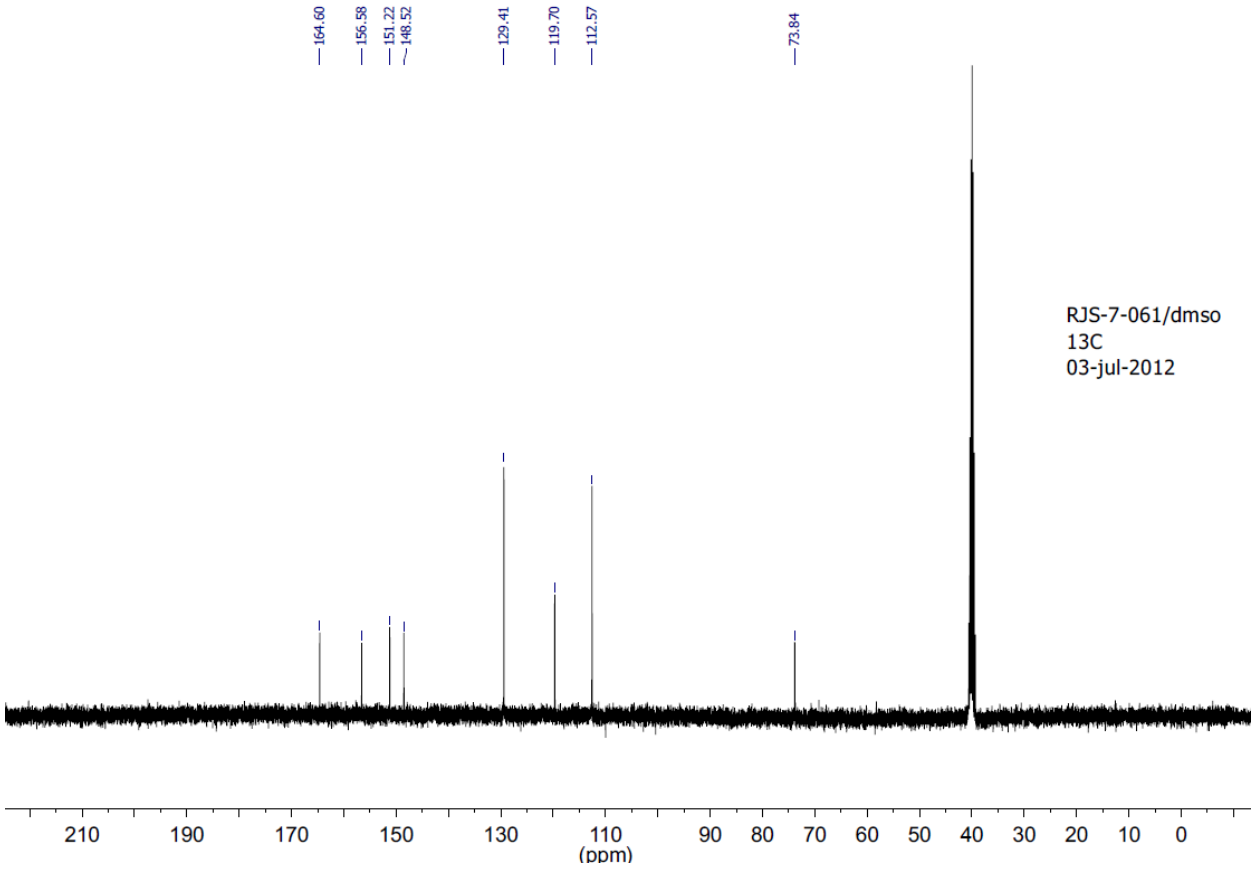


Figure S11. ^{13}C NMR spectrum 6 in DMSO-d_6.

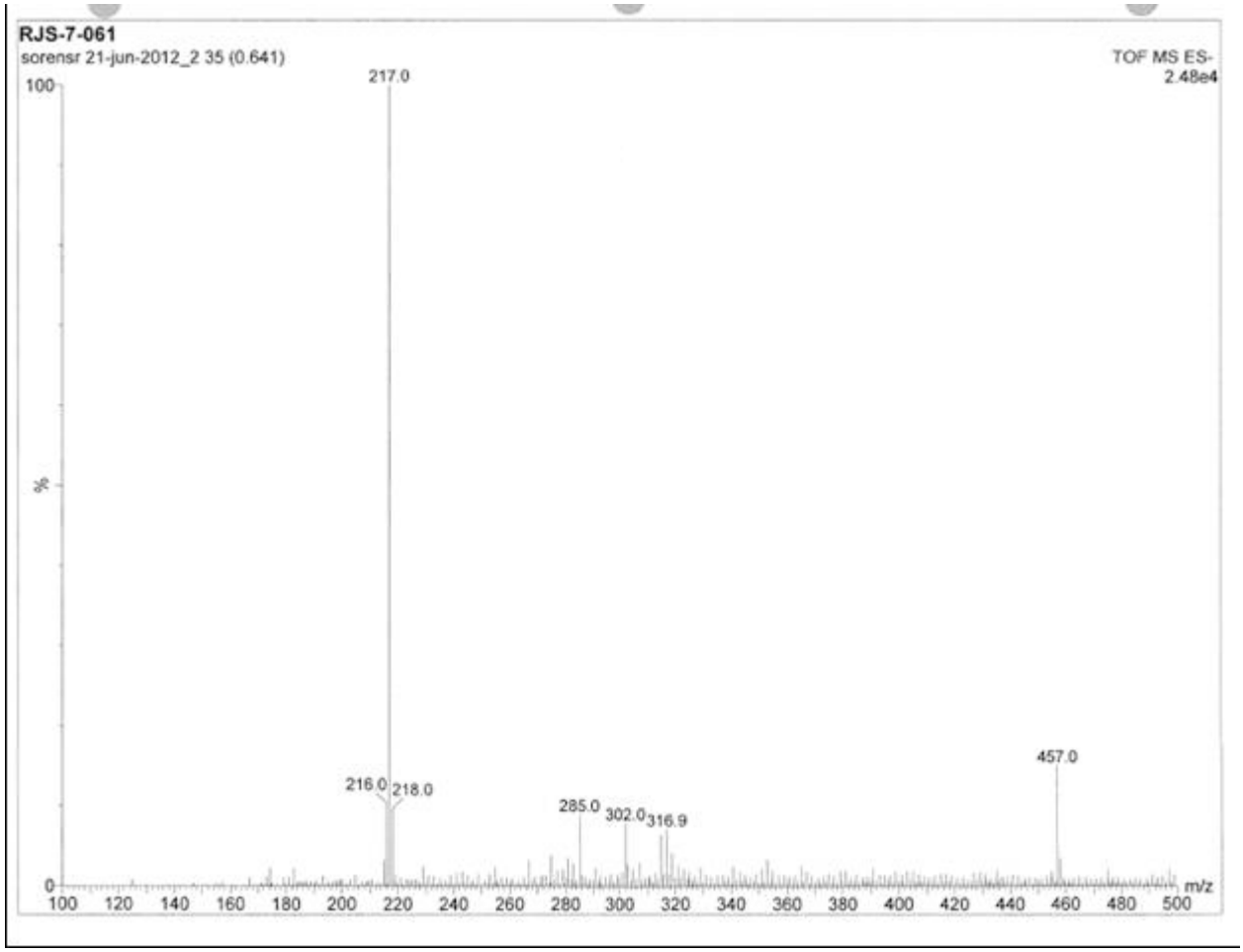


Figure S12. Electrospray (negative mode) mass spectrum of 6.

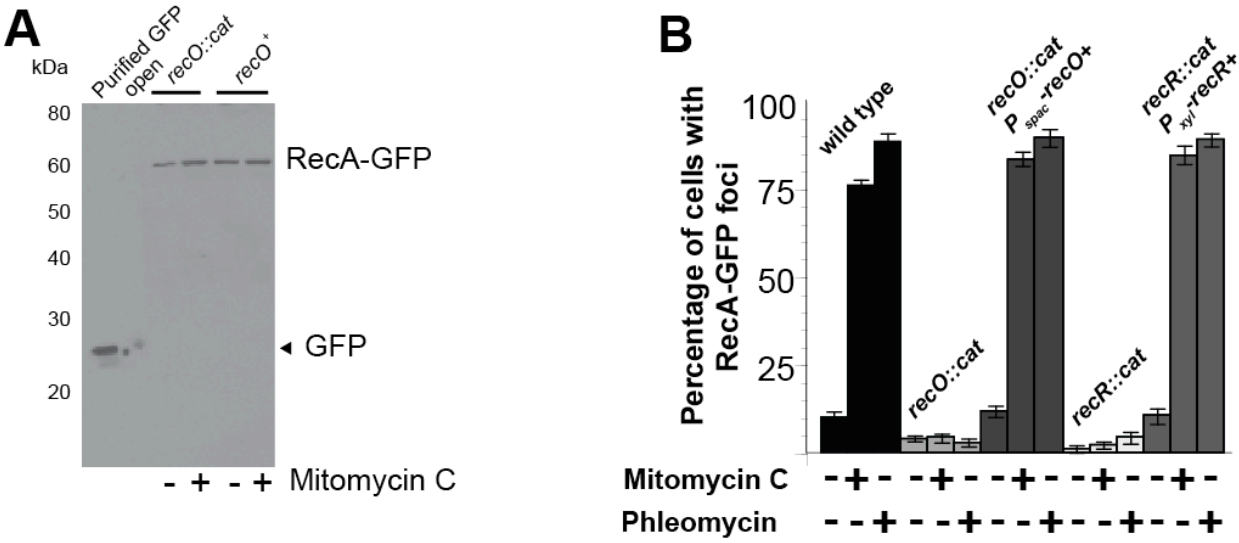


Figure S13 (A) Immunoblot of RecA-GFP in cells that were wild type or contained the *recO::cat* allele. Samples treated with mitomycin C (100 ng/ml) are indicated. **(B)** A bar graph quantifying the percentage of cells with RecA-GFP foci under the condition of mitomycin C (100 ng/ml) or phleomycin (3 μM). The error bars represent the 95% confidence interval. For each condition, at least 850 cells were scored from at least two independent experiments.

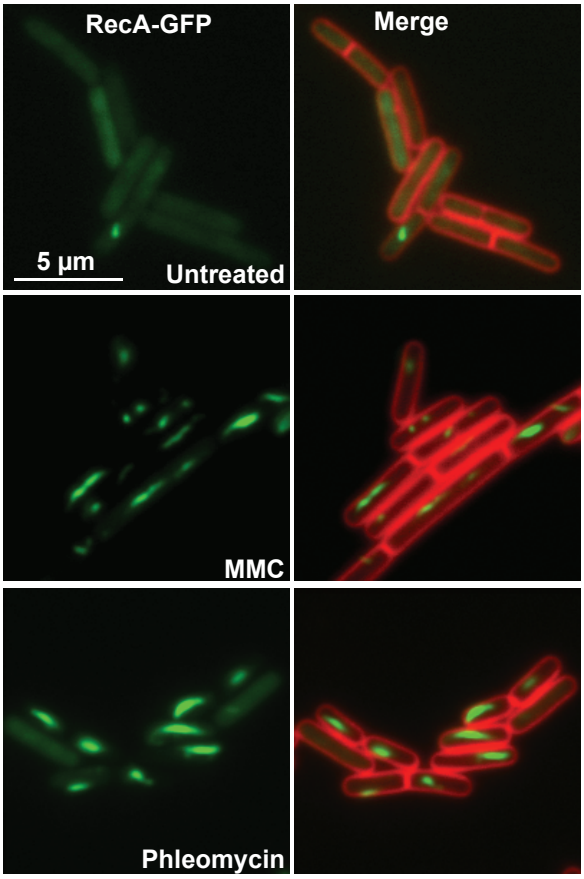


Figure S14. RecA-GFP focus formation in response to DNA damage. (A) Shown are representative images of cells with the *recA-gfp* allele that were untreated, or challenged with 100 ng/ml MMC or 3 µM phleomycin. The membrane is stained with TMA-DPH and is pseudocolored red.

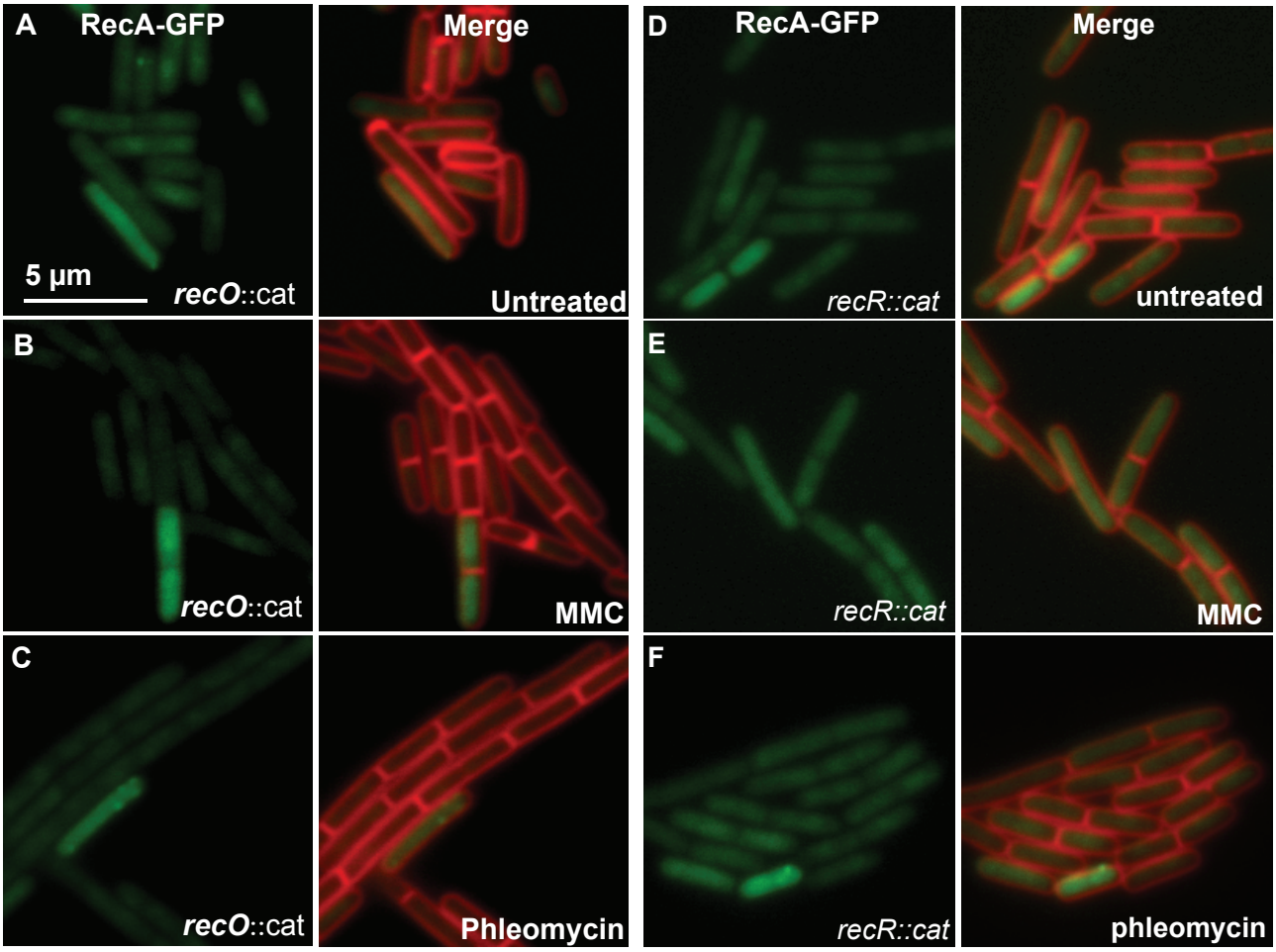


Figure S15. The *recO* and *recR* genes are necessary for RecA-GFP focus formation. Shown are representative images of a *recA-gfp* allele in a *recO::cat* **(A-C)** or *recR::cat* background **(D-F)**. Each strain was untreated, or challenged with mitomycin C (100 ng/ml) or phleomycin (3 μM) as indicated. Membranes were stained with TMA-DPH and pseudocolored red.

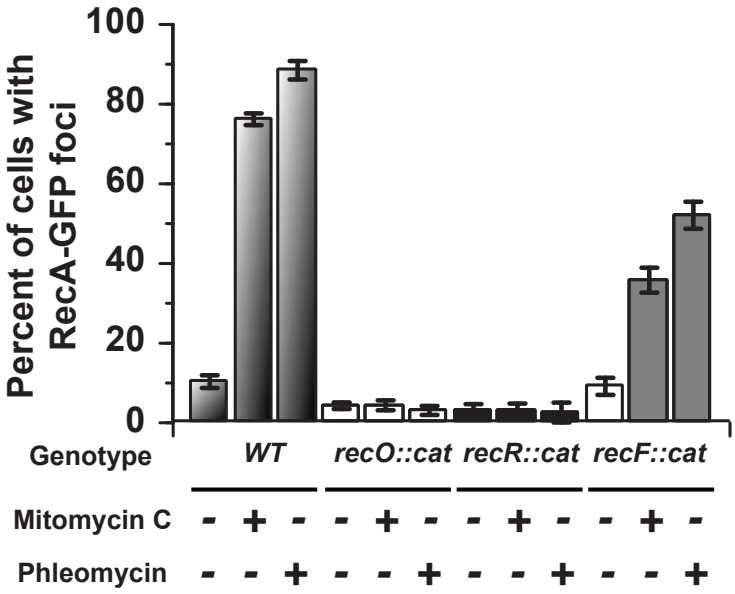


Figure S16. **RecA-GFP is unable to organize into repair centers in *recO* and *recR* deficient cells**. Shown is a bar graph for the percentage of cells with RecA-GFP foci in the indicated backgrounds untreated or following challenge with 100 ng/mL mitomycin C or 3 μM phleomycin. The error bars represent the 95% confidence interval.

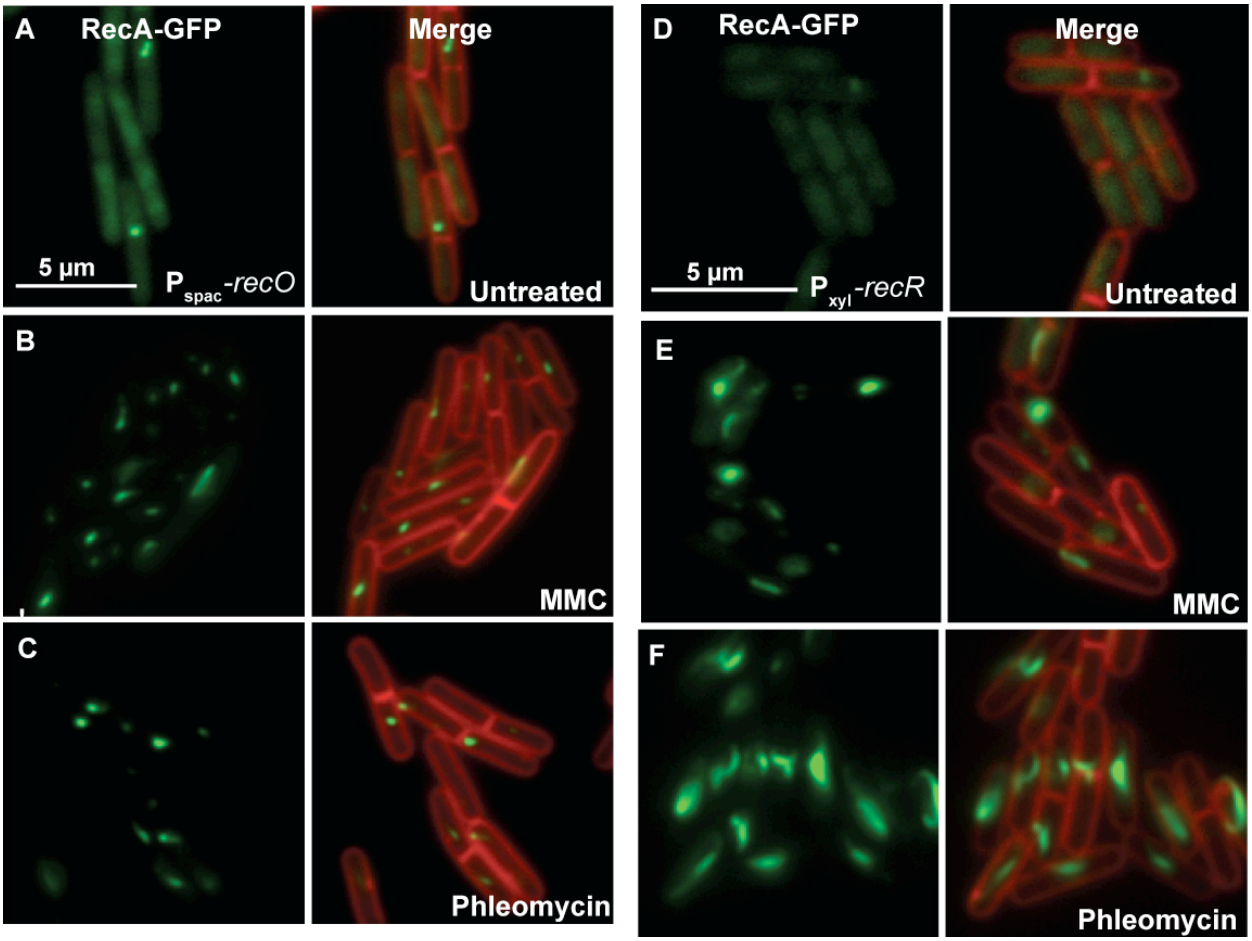


Figure S17. Ectopic expression of *recO* and *recR* restores RecA-GFP focus formation. (A-C) Shown are representative images of cells with *recA-gfp*; *recO::cat* with P_{spac}-*recO*$^{+}$ expressed from *amyE*. (A) Untreated; challenged with (B) 100 ng/ml MMC or (C) 3 µM phleomycin. **(D-F)** Shown are representative images of cells with *recA-gfp*; *recR::cat* with P_{xyl}-*recR*$^{+}$ expressed from *amyE*. (D) Untreated; challenged with (E) 100 ng/mL MMC or (F) 3 µM phleomycin. The membrane was stained with the vital membrane dye TMA-DPH and pseudocolored red.

Figure S18. Sequence alignment of the C-terminal residues of *B. subtilis* and *E. coli* SSB. Shown is the primary structure of the C-terminal 57 amino acids of *B. subtilis* SSB aligned with *E. coli*. The red residues correspond to the PF motif and the underlined region denotes the portion missing in the *ssbΔ35* allele (17).

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