Supporting Information to:

Probing the Active Site of a Diels-Alderase Ribozyme by Photoaffinity Crosslinking
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Materials and methods
Oligonucleotide synthesis and labeling
RNA oligonucleotides were synthesized by phosphoramidite chemistry (Expedite 8900 synthesizer, standard protecting groups, standard deprotection). Incorporation of oligo-(ethylene glycol)-tethered anthracene was performed chemically by using the respective phosphoramidite.1 Oligonucleotides were purified by HPLC on a Agilent 1100 series system equipped with a diode array detector using a Phenomenex® Luna 5µm C18 column (4.6×250 mm), eluting with a gradient of 100 mM triethylammonium acetate (TEAA) pH 7.0 (buffer A) and 80:20 acetonitrile/TEAA (buffer B) at 1 mL/min. Oligonucleotides and conjugates were 32P-labeled at their 5'ends with polynucleotide kinase or at the 3'ends with T4 RNA ligase according to standard procedures.

Chemical synthesis
All reagents used were purchased from Aldrich, Fluka, or Acros Organics and used without further purification. Solvents were purchased from Fluka (dry solvents over molecular sieves). TLC analyses were carried out using silica gel plates Polygram® Sil G/UV 254 (40×80 mm) from Macherey-Nagel. All synthesis and purification steps were performed under exclusion of light. Flash chromatography was carried out on silica gel 40µm from J.T. Baker ready-to-use. NMR spectra were recorded on Bruker an AM-360 spectrometer. 1H and 13C NMR spectra were calibrated to TMS on the basis of the relative chemical shift of the solvent as an internal standard. EI mass spectra were recorded on a JEOL JMS-700 sector field mass spectrometer.

p-Azidobenzylamine was synthesized starting from p-toluidine according to literature.2 (Supporting Figure S1a).

1-(4-azidobenzyl)-1H-pyrrole-2,5-dione (1)
500 mg (3.38 mmol) 4-azidobenzyl amine and maleic anhydride (3.38 mmol) were dissolved in dry CH2Cl2 and cooled to 0°C in an ice bath, followed by addition of 517 mg (3.38 mmol) 1-hydroxy benzotriazole and N,N-dicyclohexyl carbodiimide.3 The reaction mixture was stirred for 12 h at 0°C and subsequently filtered to remove dicyclohexylurea. After evaporating the solvent, purification of the crude product by flash chromatography (elution with 100% ethyl acetate) gave 516 mg (2.26 mmol, 67 %) of an amber solid which was stored under exclusion of light. 1H-NMR (360 MHz, CDCl3): δ 7.35 (d, 2H, J = 8.5 Hz), 6.97 (d, 2H, J = 8.6 Hz), 6.71 (s, 2H), 4.64 (s, 2H) ppm. 13C-NMR (90 MHz, CDC13): δ 166.8, 139.55, 134.05, 132.72, 129.88, 119.08, 40.64 ppm. MS-EI: (m/z) [M]+ 228 (calculated for [C11H8N4O2]+ 228.21), [M-N2]+ 200 (calculated for [C11H8N2O2]+ 200.06).

Photoreactivity of (1)
The photosensitivity of 1 was investigated by comparing absorbance spectra of a 100 µM ethanolic solution before and after irradiation with UV light of 254-nm (Benda NU-8 KL hand lamp, Supp. Figure S1b), or 302 nm (AIML-M26 Alpha innotech lamp, Supp. Figure S1c) wavelength, either with or without polystyrene filter (cutoff ~ 290 nm), for 200 sec from a 1.5 cm distance. A 100 µM solution of 1 was irradiated repeatedly for 20 sec time periods with 254 nm UV light from a distance of 1.5 cm, followed by recording full UV-spectra from 200 to 450 nm. UV-spectra were recorded on a Cary 50 Varian UV-spectrometer. Absorbance in the 230 – 270 nm wavelength range decreased continuously, while it increased above the isosbestic point at 270 nm, indicative of the formation of reactive keteneimine species (Supp. Figure S1b).

Synthesis of 11-mer Diels-Alder product photoaffinity probe (11-mer-DAN3) and photocross-linking
Synthesis: Compound 1 was converted to the 11-mer-Diels-Alder product conjugate 11-mer-DAN3 using the bipartite
A ribozyme construct consisting of a 38-mer and a 11-mer covalently linked to anthracene via a hexa(ethylene glycol) tether (11-mer-A). To a solution of 0.2 mM 38-mer and 0.1 mM 11-mer-A in buffer (30 mM Tris-HCl pH 7.4, 300 mM NaCl, 80 mM MgCl₂), a 5 mM solution of 1 in ethanol was added to a final concentration of 0.5 mM in a total volume of 10µl. After incubation for 10 min at room temperature, excess of 1 was removed by gel filtration (Dye Ex®, Qiagen). Product peak fractions from gel filtration were collected and lyophilized. All experiments were performed in amber vials. E.e. determinations were done as described previously.5

Cross-linking: The RNA strands to be cross-linked (11-DAN₃ and 38-mer) were dissolved in 10 µl buffer (30 mM Tris-HCl pH 7.4, 300 mM NaCl, 80 mM MgCl₂) and irradiated with polystyrene-filtered light of 302 nm wavelength for 20 min from a 1.5 cm distance. After irradiation, 1 µl of an aqueous solution of 0.1 mM dithiothreitol was added to quench contaminating 1. Cross-linked product was separated by electrophoresis on 15% denaturing PAGE gels and analyzed by phosphorimaging (Typhoon 9400, Molecular Dynamics).

Variations of the procedure (See captions to Supporting figures): variant 1. One labeled and one unlabeled strand (38-mer and 11-A) were reacted as described above. The pellet obtained after gel filtration was used without further purification, dissolved in reaction buffer, and subjected to irradiation. Cross-linked fractions were isolated by denaturing PAGE (15% gels) using phosphorimaging detection.

Variant 2. Both 11-mer-A and 38-mer strands were used unlabeled. After reaction and cross-linking as in variant 1, the cross-linked fractions was isolated by HPLC (UV detection at 254 nm), lyophilized, and 32P-labeled by polynucleotide kinase. This procedure afforded higher specific radioactivities of the cross-linked fractions and facilitated analysis of cross-link positions.

Variant 3. 11-mer-A and 38-mer strands were reacted (but not cross-linked) as described above (see paragraph Synthesis). After reaction, the reaction product photoaffinity label 11-DAN₃ was isolated by HPLC (Supp. Figure S2) and lyophilized. In a second step, 11-DAN₃ was then mixed stoichiometrically with fresh 38-mer, and either 11-DAN₃ or the fresh 38-mer could now be used 32P-labeled. Cross-linked fractions were isolated by denaturing PAGE (15% gels) using phosphorimaging detection. This variant combined a variable labeling position with high specific radioactivity. Variant 3 was used for the experiments in Fig. 2a,b (main text).

Mapping the cross-link sites
The cross-link bands were excised and eluted with buffer (0.5 M NH₄OAc; pH 5.5; 10 mM Mg(OAc)_2, 0.1 mM EDTA, 0.1% SDS) for 6 h at 37°C. After ethanol precipitation, the pellet was subjected to limited alkaline hydrolysis by addition of 11 µg carrier tRNA and subsequent treatment with 50 mM NaHCO₃ pH 9 for 10 min at 90°C. The cross-link position was assigned by running parallel alkaline RNA hydrolysis and limited ribonuclease T1 digestion of the untreated 38-mer or 11-mer strands. Alkaline hydrolysis ladders were generated as described above, and partial T1 nuclease digestion was performed under denaturing conditions (12.5 mM sodium citrate [pH 4.5], 0.5 mM EDTA, 3.5 M urea) using 0.08 units of the enzyme at 55°C for 10 min (8 min) (supplemented with tRNA to a final RNA concentration of 1.1 mM). The reactions were quenched on ice by the addition of formamide. The samples were loaded on a 15% denaturing polyacrylamide sequencing gel and analyzed by phosphorimaging.

Mapping of the cross-link sites was performed by placing the 32P label at different positions, followed by limited alkaline hydrolysis (see Supporting Figures S3-S5).

Supporting Figure S2. Preparative HPLC separation of a typical reaction mixture for the synthesis of 11-DAN₃. Column: C18 (Luna, Phenomenex, 4.6 x 250 mm), radio flow detector (Berthold LB 509). Flow rate: 3 ml/min; eluent A: 0.1 M TEAA, in H₂O (pH 7.0); eluent B: 0.1 M TEAA in 80% aq. acetonitrile (pH 7.0); 1-50% B in A within 15 min, followed by 50 - 60% B in A within 5 min and 60-74% B in A within 2 min. From the peak areas, conversion is determined to be 65%. For the uncatalyzed reaction (without 38-mer), yields are < 0.5% under these conditions.

Supporting Figure S3. Mapping the photoaffinity cross-link XlnA. Left panel: lane 1) alkaline hydrolysis of cross-link XlnA of 5'-32P-labeled 38-mer (position of radiolabel indicated by asterisk; lanes 2, 3, 5, 6) alkaline hydrolysis of 5'-32P-labeled 38-mer (different intensities); lanes 4, 7) T1 ribonuclease digest of 38-mer. Right panel: enlargement of lanes 1 and 2 of the left panel. Cross-links generated by using variant 1. The right panel is identical to Figure 2c in the main text.
Supporting Figure S4. Further analysis of the photoaffinity cross-link XlnA. (a) Analysis of XlnA containing 3'-32P-radiolabeled 38-mer (position of radiolabel indicated by asterisk). 49-mer (lane 1), 38-mer (lane 2), T1 ribonuclease digest of 38-mer (lane 3), alkaline hydrolysis of 38-mer (lane 4), alkaline hydrolysis of XlnA (lane 5) and XlnA (lane 6). (b) Analysis of XlnA containing 3'-32P-radiolabeled 11-mer-DAN3. 11-mer-DAN3 (lane 1), T1 ribonuclease digest of 11-mer-DAN3 (lane 2), alkaline hydrolysis of 11-mer-DAN3 (lane 3), alkaline hydrolysis of XlnA (lane 4) XlnA (lane 5), 11-mer-A, the T1 ribonuclease digest and the alkaline hydrolysis of 11-mer-A (lane 6, 7 and 8); The dashed box indicates the pattern identity of 11-mer-DAN3 and its cross-link product XlnA. Cross-links generated by variant 2. (c) Normalized densitometric scan of lanes 4 and 5 in panel (a), showing that the bands from A18 to G21.3 have comparable intensities, while those from U17 up are significantly reduced in lane 5.

Supporting Figure S5. Mapping the photoaffinity cross-link XlnC generated by using variant 2 with 8 mM MgCl2 in the irradiation step and 5'-end-labeled 38-mer. 38-mer (lane 1), T1 ribonuclease digest of 38-mer using 100 and 1000 times diluted enzyme stock solution of 8 units (lane 2 and 3), alkaline hydrolysis of 38-mer (lane 4 and 6), alkaline hydrolysis of XlnA (lane 5) and alkaline hydrolysis of XlnC (lane 7). XlnA (U17) is shown as a reference.
Irrespective of the applied labeling procedure and label position, digests of diffuse cross-link band XlnB (Figure 2b, main text) failed to yield recognizable gaps in the hydrolysis band patterns (data not shown), suggestive of a larger collection of cross-link products with various attachment sites.

References to Supporting Information