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Supporting Information

Catalytic carbene transfer allows the direct customization of cyclic dinucleotides

Na Fei,^{*a*} Daniel Häussinger,^{*a*} Seraina Blümli,^{*a*} Lorenzo D. Bizzini^{*a*}, Benoît-Joseph Laventie,^{*b*} Urs Jenal,^{*b*} Dennis Gillingham^{*a*}

^{*a*} Department of Chemistry, University of Basel, St. Johanns-Ring 19, Basel, Switzerland. ^{*b*} Biozentrum, University of Basel Klingelbergstrasse 50, Basel, Switzerland.

- 1. Material, instruments and general methods
- 2. Modification of c-di-nucleotide
- 3. Synthesis of fluorescent tag of c-di-GMP
- 4. Modification of DgrA

General

All reagents and solvents used were of analytical grade. Buffers were prepared with nanopure water. All chemicals were purchased from Sigma or Acros and used as received. Analytical TLC was performed on Silica gel 60 F₂₅₄ pre-coated aluminium sheets. Flash chromatography was performed on Silica gel 60 40-63 µm (230-400 mesh) (SiliCycle, Quebec). ¹H and ¹³C NMR and 2D spectra of modified CDN were acquired on a Bruker AvanceIII+ 600 MHz. Other compounds were recorded on 400 MHz proton frequency spectrometer at 298 K. Chemical shifts relative to TMS were referenced to the solvent's residual peak and are reported in ppm. ESI MS-MS spectra were obtained on a Bruker Esquire3000plus spectrometer by direct injection in positive polarity of the ion trap detector. High resolution mass spectra were acquired on a Bruker maXis 4G QTOF ESI mass-spectrometer. MALDI TOF analyses were carried out on a Bruker Microflex mass-spectrometer in linear positive mode using sinapic acid as matrice. HPLC procedures were carried out on an Agilent 1100 LC system equipped with Eclipse XDB-C8 5µm 4.6 x 100 mm column (Agilent) for analytic analysis. Shimadzu preparative HPLC (LC-20AP) equipped with phenomenex column (Gemini® 10 µm C18 110 Å, LC Column 250 x 21.2 mm, AXIA[™] Packed) was employed for preparative purification. 100 mM triethylammonium acetate (pH 7.2-7.3) and acetonitrile was used as a mobile phase. Method for analytical measurement: 1 ml/min: 0-35 % acetonitrile in 12 min, 35-80 % acetonitrile in 3 min, 80 % acetonitrile in 2 min. For preparative separation: flow rate: 20 ml/min, 0% acetonitrile in 2 min, 0-60 % acetonitrile in 27 min. Detection was carried out by monitoring the absorbance of the column effluent at 254 nm. The UV cross-linking reaction was carried out with CAMAG TLC UV lamp at 366nm. The sample was placed 2 cm away from the UV lamp.

Chemical synthesis

Diazo substrates were synthesized according to literature methods.¹

All cyclic dinucleotides were synthesized according to literature methods.²

General procedure for $Rh_2(OAc)_4$ -catalyzed CDN modification with diazocarbonyl compounds. For analytical reactions, typically 20 µl reaction mixtures containing 2 mM oligonucleotide, 200 µM $Rh_2(OAc)_4$ and 20 mM α -diazocarbonyl compound in 100 mM MES buffer, pH 6.0 were reacted at room temperature. Only aryl azide diazo modification of CDN was carried out in 50% water and 50% t-BuOH due to the solubility of aryl azide diazo compound. The reaction was traced by analytic HPLC and the identity of the product in each fraction was confirmed by HR-ESI.

To obtain enough products for NMR characterization preparative reactions were run on a 12 ml reaction scale. For example 2.4 ml 10 mM c-di-GMP, 1.2 ml 2mM Rh₂(OAc)₄ and 6 ml 40mM aryl azide diazo in *tert*-butanol and additional 2.4 ml water were mixed together. The reaction finished in 30 minutes. 5 ml Ethyl acetate was added to the reaction mixture to extract the organic side product, and aqueous layer was freezed dried and applied to preparative HPLC for purification. The modified compounds were freeze dried and confirmed by HRMS (ESI) and NMR. In some cases, pure diastereomers could be separated. c-di-GMP-N₃ (entry 4 in Table 1), c-GAMP-N₃ (entry 5 in Table 1) and c-di-GMP-ArNMe₂ (entry 3 in Table 1) were fully characterized by 1D NMR, 2D NMR, and MS analysis. The remaining compounds were characterized by 1H NMR and/or high resolution ESI-MS. The site of modification in the unsymmetrical compounds was determined by NMR through a combination of HMBC correlations and ROESY contacts, and also confirmed by analysis of 2D NMR data.



12 ml reaction mixtures containing 2 mM c-di-GMP, 200 μM Rh₂(OAc)₄ and 20 mM aryl azide diazocarbonyl compound in 50% water and 50% *t*-BuOH were kept at room temperature for 30 min. The reaction was monitored by analytical HPLC (conversion: 58%) and isolated by preparative HPLC (yield: 31%). ¹H NMR (600 MHz, DMSO) δ 10.54 (s, 2H), 7.99 (s, 1H), 7.93 (d, J = 2.7 Hz, 1H), 7.80 (s, 2H), 7.52 (d, J = 8.5 Hz, 2H), 7.30 (d, J = 6.4 Hz, 1H), 7.20 (d, J = 8.5 Hz, 2H), 6.52 (s, 3H), 6.42 (s, 1H), 5.76 (d, J = 7.6 Hz, 1H), 5.69 (d, J = 7.9 Hz, 1H), 5.48 (d, J = 6.5 Hz, 1H), 4.83 (dd, J = 7.9, 4.3 Hz, 1H), 4.65 (d, J = 7.0 Hz, 11H), 1.17 (t, J = 7.2 Hz, 16H). ¹³C NMR (151 MHz, DMSO) δ 171.00, 156.60, 156.08, 153.70, 151.68, 151.18, 150.14, 139.76, 136.97, 135.33, 132.42, 129.13, 125.19, 119.63, 117.87, 116.59, 86.95, 85.60, 80.85, 73.64, 71.57, 71.01, 70.75, 64.10, 57.15, 52.37, 45.71, 10.84, 10.76, 8.66. ³¹P NMR (243 MHz, DMSO) δ 0.59, 0.55. HRMS (ESI): calc'd for [C₂₉H₃₁N₁₃O₁₆P₂+2Et₃N⁺] = 1082.3896; found 1082.3973.







Fig S2 NMR assignment (HMQC and COSY analysis see in Fig S6 and Fig. S7)



Fig S3 ¹HNMR in DMSO, 313K, 600MHz



Fig. S4 ¹³CNMR in DMSO, 313K, 600MHz



Fig. S6 HMQC spectrum in DMSO, 313K, 600MHz

 \gtrsim 0.59 0.55



Fig. S7 COSY spectrum in DMSO, 313K, 600MHz



12 ml reaction mixtures containing 2 mM c-GAMP, 200 μM Rh₂(OAc)₄ and 20 mM aryl azide diazocarbonyl compound in 50% water and 50% *t*-BuOH were kept at room temperature for 30 min. The reaction was traced by analytic HPLC (conv. 67%) and isolated by preparative HPLC (iso. 22%). The modified site is consistent with MS-MS and Roesy spectrum of NMR. ¹H NMR (600 MHz, MeOD) δ 8.44 (s, 1H), 8.15 (s, 1H), 8.04 (s, 1H), 7.55 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.5 Hz, 2H), 6.08 (s, 1H), 5.82 (s, 1H), 5.61 (s, 1H), 5.00 (s, 1H), 4.95 (d, J = 4.7 Hz, 1H), 4.41 – 4.29 (m, 5H), 4.13 – 4.09 (m, 2H), 3.78 (s, 3H), 3.20 (q, J = 7.3 Hz, 3H), 1.30 (t, J = 7.3 Hz, 5H). ¹³C NMR (151 MHz, MeOD) δ 173.10, 159.23, 156.91, 153.10, 150.92, 150.00, 142.03, 141.10, 138.05, 135.32, 131.05, 120.67, 91.77, 91.43, 82.30, 82.06, 75.96, 75.77, 72.91, 72.70, 64.10, 59.62, 53.77, 49.44, 49.30, 49.15, 9.51. ³¹P NMR (243 MHz, MeOD) δ -0.67, -0.84. HRMS (ESI): calc'd for [C₂₉H₃₂N₁₃O₁₅P₂⁺] = 864.5821; found 864.1605.



Fig. S10 NMR assignment of product



Fig. S12 ¹³CNMR in CD₃OD, 318K, 600MHz



12 ml reaction mixtures containing 2 mM c-GAMP, 200 μ M Rh₂(OAc)₄ and 20 mM α -diazocarbonyl compound in 100 mM MES buffer, pH 6.0 were kept at room temperature for 2h. The reaction was traced by analytic HPLC (73%) and the mixture of diasteromer was isolated by preparative HPLC (29%). The epimerized mixture (ratio 2:1 in MeOD) was applied directly to NMR study. The modified site was confirmed by HMBC and consistent with MS-MS data. ¹H NMR (600 MHz, MeOD) δ 8.68 (s, 1H), 8.39 (s, 2H), 8.17 (s, 1H), 8.11 (s, 2H), 7.99 (s, 2H), 7.76 (s, 1H), 7.66 (d, J = 8.2 Hz, 4H), 7.64 (d, J = 8.1 Hz, 2H), 7.59 (d, J = 8.2 Hz, 4H), 7.52 - 7.50 (m, 2H), 6.14 (d, J = 1.3 Hz, 1H), 6.09 (s, J =2H), 5.80 (d, J = 2.1 Hz, 2H), 5.60 (s, 2H), 5.50 (d, J = 6.5 Hz, 2H), 4.90 (s, 1H), 4.87 (d, J = 4.6 Hz, 2H), 4.83 – 4.81 (m, 1H), 4.63 (d, J = 3.7 Hz, 2H), 4.47 (s, 2H), 4.43 (s, 1H), 4.40 (s, 1H), 4.39 (s, 2H), 4.36 (d, J = 2.5 Hz, 4H), 4.29 – 4.23 (m, 4H), 4.18 (s, 4H), 4.17 (d, J = 4.2 Hz, 1H), 4.11 (s, 3H), 4.10 (d, J = 4.1 Hz, 2H), 4.07 (s, 2H), 4.02 - 3.99 (m, 2H), 3.97 (d, J = 13.0 Hz, 1H), 3.93 (d, J = 12.8 Hz, 10.0 Hz)2H), 3.75 (s, 6H), 3.65 (s, 3H), 2.84 (s, 6H), 2.77 (s, 12H). ¹³C NMR (151 MHz, MeOD) δ 172.18, 153.86, 152.16, 150.31, 141.43, 140.91, 140.74, 137.63, 132.96, 132.86, 131.94, 131.62, 130.09, 129.14, 128.83, 128.70, 120.56, 91.55, 91.30, 91.14, 75.81, 75.73, 75.22, 74.08, 73.50, 73.47, 72.78, 72.74, 64.23, 64.19, 64.10, 64.07, 62.67, 62.51, 62.23, 62.15, 59.91, 53.70, 53.64, 52.92, 43.71, 43.25. ³¹P NMR (243 MHz, MeOD) δ -0.15, -1.19, -1.28, -1.33. HRMS (ESI): calc'd for $[C_{32}H_{40}N_{11}O_{15}P_2^+Et_3N] = 980.2102$; found 980.2210.



Fig. S14 HPLC trace of reaction mixture and pure product



Fig. S15 MS/MS fragment of main product



Fig. S16 MS/MS fragment of side product



Fig S17 NMR assignment of main product(important HMBC analysis see in Fig S22 and Fig. S23)



Fig. S18 NMR assignment of main product: minor diasteromer;







Fig. S20 ¹³CNMR in CD3OD, 318K, 600MHz





Fig. S21 ³¹PNMR in CD₃OD, 318K, 600MHz



Fig. S22 HMBC in CD₃OD, 318K, 600MHz







12 ml reaction mixtures containing 2 mM c-di-GMP, 200 μM Rh₂(OAc)₄ and 20 mM α-diazocarbonyl compound in 100 mM MES buffer, pH 6.0 were kept at room temperature for 3h. The reaction was traced by analytical HPLC (conv. 51%) and the mixture of diasteromers was isolated by preparative HPLC (39%). ¹H NMR (600 MHz, DMSO) δ 8.01 (d, J = 7.4 Hz, 1H), 7.99 – 7.93 (m, 2H), 7.43 (t, J = 8.2 Hz, 2H), 7.35 (dd, J = 13.6, 8.0 Hz, 3H), 7.25 (d, J = 7.9 Hz, 1H), 6.62 (s, 2H), 6.29 (s, 1H), 5.77 (t, J = 8.0 Hz, 1H), 5.67 (d, J = 8.2 Hz, 1H), 5.41 (dd, J = 19.0, 6.2 Hz, 1H), 4.89 (s, 1H), 4.65 (dd, J = 38.2, 17.6 Hz, 4H), 4.18 (dd, J = 10.5, 5.8 Hz, 2H), 4.00 (s, 2H), 3.79 (dd, J = 21.2, 11.1 Hz, 3H), 3.69 (d, J = 14.0 Hz, 3H), 2.45 (q, J = 7.1 Hz, 6H), 1.87 (s, 6H), 0.94 (t, J = 7.1 Hz, 9H).HRMS (ESI): calc'd for [C₃₂H₄₀N₁₁O₁₆P₂⁺] = 896.2051; found 896.2121.



Fig. S24 HPLC trace of reaction mixture and purified product



20 µl reaction mixtures containing 2 mM c-di-GMP, 200 µM Rh₂(OAc)₄ and 20 mM α -diazocarbonyl compound in 100 mM MES buffer, pH 6.0 were kept at room temperature for 2h. The reaction was monitored by analytical HPLC (conv. 33%). HRMS (ESI): calc'd for [C₃₂H₄₀N₁₁O₁₄P₂⁺Et₃N] = 965.3358; found 965.3430.

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Fig. S26 HPLC trace of reaction mixture and purified product



2.4 ml reaction mixtures containing 2 mM c-di-GMPS, 200 μ M Rh₂(OAc)₄ and 20 mM aryl azide diazocarbonyl compound in 50% water and 50% *t*-BuOH were kept at room temperature for 50 min. The reaction was monitored by analytical HPLC (80%) and the product isolated by semi-preparative HPLC (41% yield). The pure diastereomer was separated by HPLC, however it epimerized after adding DMSO-d₆. ¹H NMR (600 MHz, DMSO) δ 7.98 (d, *J* = 3.1 Hz, 1H), 7.81 (dd, *J* = 33.5, 7.7 Hz, 1H), 7.65 – 7.57 (m, 1H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.34 (s, 1H), 7.19 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.83 (s, 1H), 6.71 (d, *J* = 22.8 Hz, 2H), 5.75 (d, *J* = 6.1 Hz, 1H), 5.65 (s, 1H), 5.47 (s, 1H), 5.40 (s, 1H), 4.80 (s, 1H), 4.72 (s, 1H), 4.29 (s, 2H), 4.10 (s, 2H), 3.94 (dd, *J* = 24.2, 12.7 Hz, 2H), 3.69 (d, *J* = 18.7 Hz, 3H). HRMS (ESI): calc'd for [C₂₉H₃₁N₁₃O₁₆P₂+2Et₃N⁺] = 1082.3896; found 1082.3973.



Fig. S27 HPLC trace of reaction mixture and pure product



20 µl reaction mixtures containing 2 mM c-di-GMP, 200 µM Rh₂(OAc)₄ and 20 mM α -diazocarbonyl compound in 100 mM MES buffer, pH 6.0 were kept at room temperature for 2h. The reaction was traced by analytic HPLC (conv. 30%). HRMS (ESI): calc'd for [C₃₇H₄₅N₁₂O₁₆P₂⁺] = 975.2473; found 975.2488.



Fig. S29 HPLC trace of reaction mixture

Click reaction to synthesize fluorescent tag on c-di-GMP



Fig. S30 preparation of fluorescent tag

Rhodamine B (0.600 g, 1.35 mmol), 2,3,4,5,6-pentafluorophenol (0.250 g, 1.36 mmol) and 4-(N,Ndimethylamino)pyridine (0.021 g, 0.17 mmol) were dissolved in 20 ml of dry DCM under nitrogen and N,N'-dicyclohexylcarbodiimide (0.327 g, 1.59 mmol) as a solution in 2 ml of DCM was added with continuous stirring. The mixture was stirred at room temperature until complete as judged by TLC (2 h). It was then evaporated and the residue purified by column chromatography on Si60 in DCM/methanol to afford 0.46 g target product as a dark purple solid (55%). The obtained rhodaminepentafluoroester (0.092 g, 0.14 mmol) was dissolved in 11 ml of dry acetonitrile and N-propargyl piperazine (0.018 g, 0.17 mmol) as a solution in 2.5ml of acetonitrile was added with continuous stirring under nitrogen. The mixture was stirred for 24 h at room temperature, then evaporated under vacuum and the residue purified by column chromatography on Si60 in DCM/methanol (20:1 to 10:1) to afford a deep purplish-violet solid 0.4 g (40%). ¹H NMR (400 MHz, CDCl₃) δ 7.69 – 7.60 (m, 2H), 7.53 (dd, J = 6.7, 2.1 Hz, 1H), 7.34 – 7.29 (m, 1H), 7.21 (d, J = 9.5 Hz, 2H), 6.91 (dd, J = 9.5, 2.2 Hz, 2H), 6.77 (d, J = 2.4 Hz, 2H), 3.60 (dt, J = 11.9, 7.3 Hz, 8H), 3.37 (d, J = 37.7 Hz, 4H), 3.25 (d, J = 2.3 Hz, 2H), 2.68 (s, 2H), 2.36 (s, 2H), 2.23 (s, 1H), 1.29 (t, J = 7.1 Hz, 13H). ¹³C NMR (101 MHz, CDCl₃) & 167.33, 157.65, 155.60, 155.54, 135.30, 131.99, 130.35, 130.10, 129.82, 127.56, 113.97, 113.61, 96.29, 77.32, 77.00, 76.68, 73.96, 51.42, 50.87, 47.16, 46.46, 46.01, 41.36, 12.52. HRMS (ESI): calc'd for $[C_{35}H_{41}N_4O_2^+] = 549.3224$; found 549.3225.





A reaction mixture of 0.4 mM c-di-GMP-N₃, 4 mM Rhodamine alkyne, 0.2 mM CuSO₄ and 0.16 mM sodium ascorbate was left over night and check by HPLC and the product confirmed by HRMS (ESI): Calc'd for $[C_{64}H_{72}N_{17}O_{18}P_2^+] = 1428.4711$; found 1428.4726.



Fig. S33 Click reaction of fluorescent c-di-GMP



Fig. S34 HPLC trace of click reaction of fluorescent c-di-GMP

Protein modification

The production of DgrA and its mutant have been reported previously³. DgrA was subcloned into pET42b vector to include an N-terminal His₆ tag for purification of the protein. The construct was then transformed into *Escherichia coli* BL21 (DE3). Cells were grown in LB medium at 37 °C to an OD600 of approximately 0.6–0.9. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM and left to grow for another 2-3 hours. Cells were collected by centrifugation at 6,500 r.p.m. and resuspended in 15 ml of lysis buffer (20 mM PBS, pH 7.0) per liter of culture. Resuspended cells were then lysed by sonication, and cell debris was removed by centrifugation at 22,000 r.p.m. 0.7 ml NTA solution was added to the clear lysis solution and stirred for half an hour then loading onto a 5-mL HisTrap column (GE Healthcare). Impurities were washed away with 20 mM imidazole in lysis buffer, and target protein was eluted with 250 mM imidazole. The peak fractions were checked by SDS-PAGE, and fractions containing the target protein were pooled together and dialysis in 10 mM PBS buffer overnight.

Sequence of DgrA

WT:

MVMVETSGAERRAHPRMPAARKIYIVDDPRSWKASLLDVAEKGGRISIAGIASPPDTFVFVDA GGRRVHLANVVWRSGTEVGVQFAATQRIGPRAGGAAGALEIARRFLATLPAEDDALEHHHH HHHH

W75A:

MVMVETSGAERRAHPRMPAARKIYIVDDPRSWKASLLDVAEKGGRISIAGIASPPDTFVFVDA GGRRVHLANVVARSGTEVGVQFAATQRIGPRAGGAAGALEIARRFLATLPAEDDALEHHHH HHHH

R11A/R12A:

MVMVETSGAEAAAHPRMPAARKIYIVDDPRSWKASLLDVAEKGGRISIAGIASPPDTFVFVD AGGRRVHLANVVWRSGTEVGVQFAATQRIGPRAGGAAGALEIARRFLATLPAEDDAHHHHH H

DgrA modification

The reaction was carried out by mixing protein and c-diGMP-N₃ together in following step and the protein concentration was kept at 115 μ M. The reaction was put under UV light 366 nm for 15 hours and later the sample was applied to 18% SDS gel and HR-ESI and MALDI-TOF analysis.

1: 2 µl 691 µM DgrA-wt and 10µl water

2: 1.5 μ l 1 mM c-diGMP-N₃ added 2 μ l 691 μ M DgrA-wt and 8.5 μ l water protein

3: 1.2 µl 1 mM c-diGMP-N3 added 8 µl 148 µM DgrA-R11/R12A protein and 1µl water

4: 8 µl 148 µM DgrA-R11A/R12A protein and 2.2 µl water

5: 1.6 µl 1 mM c-diGMP-N₃ added 6 µl 270 µM DgrA-W75A protein and 6.4µl water

6: 6 µl 270 µM DgrA-W75A protein and 7.6µl water

7: 1.5 µl 10 mM c-diGMP-N₃ added 2 µl 691 µM DgrA-wt protein 8.5 µl water

Modification of DgrA-wt







Fig. S38 HR-ESI of modification DgrA-wt with 10 equivalents of c-di-GMP-N₃(reaction 7)

Modified MS of mutant dgrA-W75 and DgrA-R11A/R12A was not observed in HR-ESI or in MALDI-TOF when treated with 1 equivalent of c-di-GMP-N₃. Even when c-di-GMP-N₃ was increased to 10 equivalents the MS spectrum showed only broad indistinct peaks due to the high concentration of c-di-GMP-N₃. These observations are consistent with the gel data and microscale thermophoresis measurement suggesting low or no modification.

Trypsin digestion for DgrA-wt modification (digestion of reaction 2)

	final concentration	stock solution	volume
protein	107µM	115µM	15µl
Trypsin	2.1µM	85μΜ	0.4µl
CaCl ₂	4.2µM	100µM	0.7µl

The trypsin digestion reaction mixture was left at 37 °C for 3-4 h and quenched by 10% formic acid and then injected to UPLC-MS to analyze the digested fractions. The mass of the fragment shown corresponds to the GGR peptide fragment plus the azide cross-linker. This fragment is the only species not seen in the control digestion of pure DgrA. The peak at 1118.4 Da is a dehydration product of the initial fragment, which likely forms from the acidic formic acid quench.



Fig. S39 UPLC-MS of trypsin gigestion of modified DgrA-c-d-GMP-N₃

Microscale thermophoresis

MST measurements were performed as previously described.⁴ DgrA and DgrA W75A were labeled with the fluorescent dye DyLight488 NHS Ester according to the manufacturer's protocol (ref. 46402, ThermoScientific, Rockford, IL-USA), with a degree of labeling of 80% and 33%, respectively. The excess of free dye was removed by dialysis. DyLight488-labelled protein concentration was kept constant at 50 nM for DgrA and 100 nM for DgrA W75A, while c-di-GMP and c-di-GMP-N₃ were titrated in PBS-T (PBS, 0.1% v/v Tween-20, pH 7.4). The samples were loaded into MonolithTM standard-treated capillaries and thermophoresis was measured at 25°C after 15 and 30 min incubation using a Monolith NT.115 instrument (NanoTemper Technologies, München, Germany). Laser power was set to 100% using 30 seconds on-time. The LED power was set to 100%. The K_d values were fitted from triplicates with Prism 6.00 (GraphPad Software) and expressed as mean \pm SD.



Fig. S40 MST spectrum of modified c-di-GMP-N3 and c-di-GMP

Microscale thermophoresis of the DyLight488-labelled DgrA (A) and DgrA W75A (B) shows binding to c-di-GMP (black circles) and c-di-GMP-N₃ (grey squares). The thermophoresis change upon addition of c-di-GMP is plotted as normalized fluorescence. DgrA and DgrA W75A showed a decreased fluorescence in the bound state. The K_d of DgrA was fitted to 79 ± 12 nM for c-di-GMP and 231 ± 48 nM for c-di-GMP-N3 (n = 3). The K_d of DgrA W75A was fitted to 372 ± 190 µM for c-di-GMP and 397 ± 202 µM for c-di-GMP-N₃ (n = 3). The error bars represent SD (n = 3). The Kd of DgrA was fitted to 372μ M for c-di-GMP and 231 nM for c-di-GMP-N₃, and the K_d of DgrA W75A was fitted to 372μ M for c-di-GMP and 397μ M for c-di-GMP-N3 (n = 3). The aryl azide modification of c-di-GMP has a very minor effect on the binding affinity for DgrA and DgrA W75A (2.9 and 1.1 fold K_d increase respectively).

References

- (a) K. Tishinov, K. Schmidt, D. Haussinger and D. G. Gillingham, *Angew. Chem. Int. Ed.*, 2012, 51, 12000-12004; (b) K. Tishinov, N. Fei and D. Gillingham, *Chem. Sci.*, 2013, 4, 4401-4406.
- 2. B. L. Gaffney, E. Veliath, J. W. Zhao and R. A. Jones, *Org. Lett.*, 2010, **12**, 3269-3271.
- 3. M. Christen, B. Christen, M. G. Allan, M. Folcher, P. Jeno, S. Grzesiek and U. Jenal, *P Natl Acad Sci USA*, 2007, **104**, 4112-4117.
- 4. M. Jerabek-Willemsen, C. J. Wienken, D. Braun, P. Baaske and S. Duhr, *Assay and drug development technologies*, 2011, **9**, 342-353.