Structural-based engineering expands the substrate scope of a cyclodipeptide synthase

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Supplementary Information

Methods
- Cloning, expression, and purification
- Crystallography of Parcu-CDPS
- tRNA Pool Extraction
- S30 Extract Preparation
- Cyclodipeptide Synthase Activity Assay
- Activity Assay using DBE Substrates
- Cyclic Dipeptide Identification via LC-MS
- Synthesis of Amino-DBE Compounds – His-DBE and Phe-DBE
- Trapped acyl-enzyme intermediate

Mass Spectrometry of Unnatural CDP Library

HPLC Analysis of cHE and cHP

Parcu-CDPS Mutant Crystal Structures

Crystallographic Data Table

Intact Mass Spectrometry of Parcu-CDPS Mutants

References
Methods

Cloning, expression, and purification

The CDPS genes – Para-CDPS from *Parabacteroides* sp. 20_3 and Parcu-CDPS from *Paracubacteria bacterium RAAC4_OD1_1* – were synthesised as a codon optimized for *E. coli* expression gBlock from IDT. The synthesised genes were inserted into a pJ411 expression plasmid with a C-terminal 6xHis protein tag by Gibson assembly cloning technique. Mutants of Parcu-CDPS were created by site-directed mutagenesis based on the NEB Q5 site-directed mutagenesis kit. Presence of both the WT and mutant were confirmed via sequencing before cloning into the commercially available *E. coli* BL21(DE3) expression strain (NEB). Cells were grown at 37 °C until the OD₆₀₀ had reached 0.6 and protein expression was induced using IPTG (1mM). The cells were then grown at 16 °C overnight.

The resultant protein pellet was purified, first by resuspending in the appropriate volume of lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 20 mM imidazole, 5% glycerol). The solution was lysed using a high-pressure cell disruptor (Constant Systems) before being centrifuged at 20000RPM for 30 minutes, 4 °C. The cell lysate was filtered through an 80 µm membrane and loaded onto a 5 mL HisTrap HP column (GE Healthcare), pre-equilibrated with lysis buffer. The column was washed with 20 column volumes (CV) of lysis buffer and the adsorbed proteins were eluted using elution buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 300 mM imidazole, 5% glycerol) in 3 steps: 10%, 20% and 100%. Proteins of interest were dialysed into dialysis buffer (20 mM HEPES pH 7.0, 250 mM NaCl, 5 mM 2-mercaptoethanol) overnight at 4 °C. The wild-type proteins were purified further using size exclusion chromatography in the dialysis buffer mentioned previously. (Superdex 200 Increase 16/60).

Crystallography of Parcu-CDPS

Crystals of Parcu-CDPS and mutants were grown at 4 °C using the sitting drop vapour diffusion technique. Equal volumes of protein and reservoir solution (0.15 µL) were used. Initial attempts to crystallise Parcu-CDPS in its original storage buffer were unsuccessful which lead to a repeat purification to change the storage buffer to 50mM citrate pH 6.5, 150mM NaCl, 2mM DTT and 2mM EDTA. From this new prep, crystals of Parcu-CDPS were grown in 2.27 M ammonium sulphate and 0.1 M sodium acetate pH 5.13. Crystals were cryoprotected in mother liquor supplemented with 10% (v/v) ethylene glycol and stored in liquid nitrogen. Initial phasing of wild-type Parcu-CDPS was achieved by soaking crystals in mother liquor supplemented with 0.5M NaI for 5 minutes before being fished - the structure was consequently solved using an iodine-SAD data set. The subsequent mutants of Parcu-CDPS were solved using the wild-type as the molecular replacement model in Phaser. X-Ray Diffraction data was collected at the Diamond Light Source in Oxford, UK. Protein structures were built and refined using COOT, optimised using PDB-redo and this refinement was assessed in PHENIX.

tRNA Pool Extraction

To extract the pool of all tRNA produced by *E. coli*, a method was adapted from a protocol described by Mechulam *et al*. Firstly, an overnight culture was prepared from commercially available BL21 DE3 cells in lysogeny broth (LB). Terrific broth (TB, 1L) was inoculated from this and split into two separate flasks before being incubated at 37 °C until the OD₆₀₀ had reached 4. The cells were pelleted via centrifugation (6000 RPM, 15 minutes, 4 °C) and resuspended in a buffer containing 1 mM HEPES-KOH, pH 7.5 and 10 mM magnesium acetate (8.6 mL). Phenol:chloroform (5:1) was added to the suspension and the mixture was centrifuged (15000 RCF, 30 minutes, 20°C). The top layer containing RNA was moved to a new falcon tube where 0.1 volumes of 5M NaCl and 2.2 volumes of cold ethanol were added. The mixture was centrifuged (15000 RCF, 30 minutes, 4 °C) and the supernatant was discarded. The pellet was resuspended in 1 M NaCl (5 mL) and the solution was centrifuged (15000
RCF, 30 minutes, 4 °C). The supernatant was transferred to a new falcon tube and the tRNA was precipitated using 2 volumes of cold ethanol. The solution was centrifuged (15000 RCF, 30 minutes, 4 °C) and the pellet was dissolved in 100 mM HEPES-KOH, pH 8 (2 mL). Deacylation of tRNA was performed by incubating at 37 °C for one hour followed by precipitation using 0.1 volume of 5 M NaCl and 2.2 volumes of cold ethanol. The solution was centrifuged (4000 RPM, 10 minutes, 4 °C) and the pellet was dissolved in 100 mM HEPES-KOH, pH 8 (2 mL).

Deacylation of tRNA was performed by incubating at 37 °C for one hour followed by precipitation using 0.1 volume of 5 M NaCl and 2.2 volumes of cold ethanol. The solution was centrifuged (4000 RPM, 10 minutes, 4 °C) and the pellet was resuspended in 70% cold ethanol (2 mL) to perform an overnight precipitation at -80 °C. Following incubation, the tRNA was pelleted via centrifugation (4000 RPM, 10 minutes, 4 °C) and resuspended in 70% cold ethanol (2 mL) to perform an overnight precipitation at -80 °C. Following incubation, the tRNA was pelleted via centrifugation (4000 RPM, 10 minutes, 4 °C) and resuspended in DEPC-treated water (4 mL). The concentration of this tRNA pool was calculated using the Beer-Lambert where the extinction coefficient is a sum of the extinction coefficients for the four RNA nucleobases present.

**S30 Extract Preparation**

The synthesis of a cell-free bacterial lysate was first published by Krinsky et al. and this method follows theirs closely. TB (1 L) was inoculated with an overnight culture prepared from BL21-DE3 cells grown in LB at a 1:50 (cells:media) ratio. The cells were grown at 37 °C until OD$_{600}$ reached 4 whereupon the cells were pelleted via centrifugation (7000 RCF, 10 minutes, 4 °C). The pellet was resuspended in 1 litre of S30 buffer containing 10 mM tris acetate pH 7.4, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT and 0.5 ml/L 2-mercapoethanol. The solution was centrifuged again (7000 RCF, 10 minutes, 4 °C) and the pellet was resuspended in S30 buffer (15 mL). The cells were lysed using a high-pressure cell disruptor at 15 kpsi and 0.1 M DTT was added to the lysate (100 µL for every 10 mL of suspension). The lysate was then centrifuged (24700 g, 30 minutes and 4 °C) to yield the S30 extract in the supernatant. The concentration of this was measured using a spectrophotometer.

**Cyclodipeptide Synthase Activity Assay**

To investigate the activity of the CDPS enzymes in vitro, the following reactions were performed as detailed. The assay contained a buffer composed of 100mM HEPES pH 7.0, 100 mM KCl, 20mM MgCl$_2$, 5mM ATP, and 10mM DTT. Respective amino acids were added to a final concentration of 500 µM followed by 50 µM of the tRNA pool purified as described above. Before the enzymes were added to the mixture, the pH of the solution was checked to ensure it was 7. Following any necessary pH correction, DEPC-treated water was added to achieve the desired final volume followed by the addition of tRNA synthetases and CDPS enzyme – both at 5 µM final concentration. The reaction was left to proceed overnight at room temperature.

Using the S30 extract required a different buffer system composed of 55mM HEPES-KOH pH 8.0, 14 mM MgOAc, 50mM KOAc, 155mM NH$_4$OAc supplemented with 5mM ATP and the necessary amino acids at 2.5 mM final. 22mg/mL of purified S30 extract was used in conjunction with 5 µM CDPS to yield cyclodipeptide products.

To quench the assays, cold methanol was added so the total final volume contained 80% methanol. The samples were incubated at -80 °C for 15 minutes and centrifuged for 10 minutes afterwards. The supernatant from this was moved to a new eppendorf and dried under nitrogen. The resultant residue was reconstituted in LC-MS grade water for analysis.

To monitor the production of the cyclic dipeptide formed by the enzyme, a discontinuous time course of the activity assay was performed in duplicate. The reaction was initiated by adding enzyme and 10 time points were taken (20 µL x 10) ranging from 0 minutes to 300 minutes. For each sample, the methanol extraction protocol was followed and analysed via LC-MS.
Activity Assay using DBE Substrates

For reactions using two DBE substrates, a simplified version of the previously described CDPS activity assay was employed. The reaction solution included 20 mM HEPES, 500 mM NaCl, 5 mM DTT and the DBE substrates both at 500 µM. For experiments using one DBE substrate with one aminoacylated tRNA substrate, the original activity assay procedure was followed with the substitution of one amino acid and its corresponding synthetase with the aa-DBE substrate. The assay was quenched using the same protein precipitation mentioned above.

Cyclic Dipeptide Identification via LC-MS

The activity assays were analysed using a Waters ACQUITY UPLC liquid chromatography system coupled to a Xevo G2-XS QTof mass spectrometer equipped with an electrospray ionization (ESI) source. The samples (10 µL) were loaded onto an HSS-T3 column (2.1 x 100 mm, 1.8 µm, Waters Acquity) and ran at 40 °C. The analytes were separated using a gradient mobile phase from 1%B to 50%B where the two mobile phases consisted of A - 0.1% formic acid in water and B – 0.1% formic acid (F.A) in acetonitrile at a flow rate of 400 µL min⁻¹. The capillary voltage was set at 2.5 kV in positive ion mode. The source and desolvation gas temperatures of the mass spectrometer were set at 120 °C and 500 °C, respectively. The cone gas flow was set to 50 L/hr whilst the desolvation gas flow was set at 1000 L/hr. An MS³ scan was performed between 50 – 700 m/z where function 1 employed MS analysis whilst function 2 applied a collision energy ramp from 15 to 30 V to perform MS/MS fragmentation. In addition, a lockspray signal was measured and a mass correction was applied by collecting every 10s, averaging 3 scans of 1s each using Leucine Enkephalin as a standard (556.2771 m/z).

Synthesis of Amino-DBE Compounds – His-DBE and Phe-DBE

The synthesis of the amino acid-DBE substrates was followed using the method described by Harding et al. 7, 8

1. Synthesis of Boc-aa-DBE

N-Boc-L-amino acid was added to a 25 mL flask (1.97 mmol) with 3,5-dinitrobenzyl chloride (1.65 mmol). The reaction was placed under argon and DMF (0.4 mL) was added, forming a yellow solution. Et₃N (3.98 mmol) was added last, and the reaction was stirred overnight at room temperature. The mixture was diluted in diethyl ether (25mL) and an extraction was performed using 1M HCl (2 x 25mL) followed by sat. NaHCO₃ (2 x 25mL). A brine wash was performed, and the resultant organic layer was dried over MgSO₄. The filtered solution was concentrated in vacuo to yield an orange oil.
2. Boc deprotection

Crude N-Boc-amino acid-3,5-dinitrobenzyl ester (aa-DBE) was added to a 25 mL flask whereupon 4 N HCl in 1,4-dioxane (2.5 mL) were added. The resulting solution was stirred at room temperature for 1 hour and the solvent removed in vacuo. The resulting oil was triturated with diethyl ether to yield the final compound.

His-DBE: $^1$H NMR (400 MHz, Methanol-d$_4$) δ 8.93 (t, $J = 2.0$ Hz, 1H), 8.85 (s, 1H), 8.59 (d, $J = 2.0$ Hz, 2H), 7.49 (s, 1H), 5.48 (d, $J = 6.4$ Hz, 2H), 4.57 (t, $J = 6.8$ Hz, 1H), 3.54 – 3.35 (m, 2H).

Figure S1. NMR spectra of His-DBE

Phe-DBE: $^1$H NMR (400 MHz, Methanol-d$_4$) δ 8.96 (t, $J = 2.1$ Hz, 1H), 8.51 (d, 2H), 7.30 – 7.19 (m, 5H), 5.41 (s, 2H), 4.45 (t, $J = 7.2$ Hz, 1H), 3.23 (dd, $J = 7.2, 2.5$ Hz, 2H).

Figure S2. NMR spectra of Phe-DBE
**Trapped acyl-enzyme intermediate**

Acyl-intermediate enzyme preparation was performed by incubating 20 mM enzyme with excess aa-DBE (5 mM) overnight. The samples were then sent for analysis at the University of St Andrews mass spectrometry and proteomics facility. The sample was diluted to 1 µM and 10 µL was injected onto a Waters MassPrep column cartridge on a Waters G2TOF LC-MS system. The system used a two solvent system composed of solvent A - Water + 1% F.A.:Acetonitrile (95:5) and solvent B - Water + 1% F.A.:Acetonitrile (5:95). A gradient was employed from 2% B to 98% B over 4 minutes, held for 0.5 minutes then returned to 2% B. MS data was collected in ESI+ mode and scanned from 500–2500 m/z. The raw data was combined and processed to mass using Waters MaxEnt algorithm at 0.1 resolution using peak width of half height of 0.4 Da. The instrument was calibrated externally against a solution of Horse hear myoglobin, and an internal lock mass of LeuEnk was additionally used.

![Figure S3](image)

**Figure S3.** Intact protein mass spectrometry of mutants incubated with different aa-DBE substrates: histidine, phenylalanine and leucine. None of the mutants are able to react with his-DBE and instead accept phenylalanine and leucine (except Y55V+Y189L which accepts phenylalanine exclusively).
### Table S1. All non-canonical amino acids tested with Para-CDPS are listed here and rows in green highlight the accepted substrates.
| 4-Amino-cis-L-proline Dihydrochloride | No                  |
|--------------------------------------|---------------------|
| Histidine                           | (2S,4S)-4-Hydroxypyrrolidine-2-carboxylic acid No |
| Histidine                           | (2R,4S)-4-Hydroxypyrrolidine-2-carboxylic acid No |
| Histidine                           | H-Hyp-OH (trans-hydroxy-Pro) No |
| Histidine                           | cis-4-hydroxy-D-proline No |
| Histidine                           | Glutamate Yes |
| Histidine                           | Quisqualic acid No |
| Histidine                           | Ibotenic acid No |
| Histidine                           | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid No |

**Table S2.** All non-canonical amino acids tested with Parcu-CDPS are listed here and rows in green highlight the accepted substrates.
Figure S4. Mass spectrometry of the products formed by Para-CDPS using a combination of canonical and non-canonical amino acids.
Figure S5. Mass spectrometry of the products formed by Parcu-CDPS using a combination of canonical and non-canonical amino acids.
**HPLC Analysis of cHE and cHP**

A time course experiment was performed using a reaction mixture composed of equal concentrations of proline and glutamate with Parcu-CDPS. Reaction was quenched at different time points – 5 minutes, 30 minutes, 1 hour, 3 hour and overnight – using the methanol method previously described. High performance liquid chromatography (HPLC) of each sample was performed using a Shimadzu HPLC system coupled to a UV-vis detector equipped with a Waters XSelect Premier HSS T3 column (2.5 µm, 4.6mm x 50mm). The column was heated to 40 °C for the run and 25 µL of sample was injected per run. Mobile phases were A - water + 0.1% trifluoroacetic acid, and B – 100% acetonitrile. The analytes were separated using a gradient mobile phase from 1% B to 50% B over 5 minutes using a flow rate of 1 mL min⁻¹ and the absorbance at 214 nm and 254 nm was monitored.

![HPLC chromatogram](image)

**Figure S6.** HPLC chromatogram of reaction mixture at 214 nm.

**Parcu-CDPS Mutant Crystal Structures**

**Figure S7.** Superimposition of Parcu-CDPS WT and the 6 crystallised mutant structures: WT (blue); Y55F (green); D58N (pink); E171Q (orange); E174A (yellow); E174L (beige) and Y189F in lilac.
| Accession code | Parcu-CDPS WT | Parcu-CDPS Y55F | Parcu-CDPS D58N | Parcu-CDPS E171Q | Parcu-CDPS E174A | Parcu-CDPS E174L | Parcu-CDPS Y189F |
|----------------|--------------|----------------|----------------|-----------------|----------------|----------------|-----------------|
| Resolution (Å) | 51.31 - 1.90 | 45.28 - 2.09 | 46.26 - 2.30 | 46.05 - 2.09 | 44.85 - 2.40 | 41.24 - 2.11 | 45.26 - 2.29 |
| Space group    | P 42 21 2    | P 42 21 2    | P 42 21 2    | P 42 21 2    | P 42 21 2    | P 42 21 2    | P 42 21 2    |
| Cell Dimensions a, b, c (Å) | 102.61, 102.61, 96.76 | 101.26, 101.26, 95.90 | 103.44, 103.44, 95.95 | 102.97, 102.97, 95.91 | 102.60, 102.60, 95.90 | 103.79, 103.79, 95.90 | 101.20, 101.20, 95.90 |
| α, β, γ (°)    | 90, 90, 90   | 90, 90, 90   | 90, 90, 90   | 90, 90, 90   | 90, 90, 90   | 90, 90, 90   | 90, 90, 90   |
| Total reflections | 1128270 (101638) | 192351 (6030) | 311233 (30217) | 222211 (4134) | 260149 (25838) | 323801 (11158) | 187130 (8625) |
| Unique reflections | 21493 (2107) | 14927 (1536) | 12408 (1189) | 15221 (1565) | 10887 (1064) | 14543 (1555) | 12240 (1215) |
| Multiplicity    | 52.5 (48.2)  | 12.9 (7.9)   | 25.1 (25.4)  | 14.5 (5.5)   | 23.9 (24.3)  | 22.6 (15.6)  | 15.3 (14.5)  |
| Completeness (%)| 99.86 (99.86)| 94.1 (100.0)| 98.92 (97.62)| 92.57 (96.84)| 99.94 (99.91)| 89.55 (98.17)| 99.12 (99.92)|
| Mean I/σ(I)     | 31.45 (1.95) | 94.02 (99.81)| 27.09 (4.21) | 15.80 (0.90) | 26.17 (7.24) | 28.90 (1.30) | 18.80 (1.30) |
| R-merge         | 0.073 (1.452)| 0.069 (1.452)| 0.088 (1.435)| 0.090 (1.637)| 0.155 (0.9867)| 0.049 (1.992)| 0.072 (2.230)|
| R-pim           | 0.010 (0.207)| 0.027 (0.804)| 0.018 (0.287)| 0.023 (0.807)| 0.033 (0.202)| 0.010 (0.520)| 0.019 (0.604)|
| CC1/2           | 0.999 (0.926)| 1.000 (0.490)| 1.000 (0.892)| 0.999 (0.331)| 0.999 (0.963)| 0.999 (0.434)| 1.000 (0.511)|
| R-work          | 0.2240 (0.3893)| 0.2073 (0.3010)| 0.2230 (0.3321)| 0.2179 (0.3297)| 0.2048 (0.2517)| 0.2247 (0.3501)| 0.2195 (0.4107)|
| R-free          | 0.2431 (0.3568)| 0.2698 (0.3207)| 0.2573 (0.4003)| 0.2620 (0.3456)| 0.2541 (0.4073)| 0.2890 (0.3441)| 0.2548 (0.4193)|
| Total non-hydrogen atoms | 1903 | 1819 | 1817 | 1863 | 1827 | 1811 | 1825 |
| Total macromolecules | 1862 | 1809 | 1805 | 1855 | 1803 | 1809 | 1820 |
| Total ligands | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total solvent | 41 | 10 | 12 | 8 | 24 | 2 | 5 |
| Total protein residues | 221 | 215 | 214 | 221 | 215 | 215 | 216 |
| RMS(bonds) (Å) | 0.009 | 0.008 | 0.007 | 0.008 | 0.008 | 0.008 | 0.009 |
| RMS(angles) (°) | 1.26 | 1.15 | 1.14 | 1.21 | 1.13 | 1.28 | 1.3 |
| Ramachandran favored (%) | 98.16 | 96.65 | 97.6 | 96.77 | 97.61 | 96.65 | 96.67 |
| Ramachandran allowed (%) | 1.38 | 2.87 | 1.92 | 2.76 | 1.91 | 2.87 | 2.86 |
| Ramachandran outliers (%) | 0.46 | 0.48 | 0.48 | 0.46 | 0.48 | 0.48 | 0.48 |
| Average B-factor (Å²) | 62.31 | 71.85 | 67.24 | 69.19 | 53.79 | 85.04 | 84.32 |

Table S3. Crystallographic data table for Parcu-CDPS and its corresponding variants.
Intact Mass Spectrometry of *Parcu*-CDPS Mutants

**Figure S8.** Intact protein mass spectrometry analysis of *Parcu*-CDPS variants in order to confirm the desired mutation is present.
| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 6.32  | 3.8       |
| E171    | 10.51 | 4.5       |
| E174    | 4.96  | 4.5       |
| Y55     | 18.05 | 10        |
| Y167    | 9.89  | 10        |
| Y189    | 14.12 | 10        |
| Y191    | 13.19 | 10        |

| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 6.18  | 3.8       |
| E171    | 11.36 | 4.5       |
| E174    | 8.89  | 4.5       |
| Y55     | 9.75  | 10        |
| Y167    | 9.13  | 10        |
| Y189    | 12.84 | 10        |

| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 5.59  | 3.8       |
| E171    | 9.95  | 4.5       |
| E174    | 14.49 | 10        |
| Y55     | 9.54  | 10        |
| Y167    | 13.16 | 10        |
| Y189    | 12.72 | 10        |
| Y191    | 12.72 | 10        |

| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 6.03  | 3.8       |
| E171    | 10.09 | 4.5       |
| E174    | 14.08 | 10        |
| Y55     | 9.57  | 10        |
| Y167    | 13.25 | 10        |
| Y189    | 12.68 | 10        |

| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 6.11  | 3.8       |
| E171    | 5.78  | 4.5       |
| E174    | 18.28 | 10        |
| Y55     | 9.85  | 10        |
| Y167    | 14.21 | 10        |
| Y191    | 13.13 | 10        |

| Residue | pKa   | model-pKa |
|---------|-------|-----------|
| D58     | 5.61  | 3.8       |
| E171    | 10.83 | 4.5       |
| E174    | 7.73  | 4.5       |
| Y55     | 16.49 | 10        |
| Y167    | 9.41  | 10        |
| Y189    | 11.72 | 10        |

Table S4. pKa values calculated using PROPKA. Model pKa refers to expected pKas for the side chain of each amino acid free in solution, while pKa is the calculated number for each residue using the pdb file for each variant.
Figure S8. Differential scanning fluorimetry of Parcu-CDPS and mutants of interest. Each line represents a sigmoidal fit of the normalised mean of 3 replicates for each protein. The melting temperatures were recorded: 32.5 °C (WT); 30 °C (Y55V); 40 °C (E174L); 35 °C (Y189L); 41 °C (Y55V+E174L); 34 °C (Y55V+Y189L) and 49 °C (E174L+Y189L).

References

1. D. G. Gibson, L. Young, R.-Y. Chuang, J. C. Venter, C. A. Hutchison and H. O. Smith, *Nature Methods*, 2009, 6, 343-345.
2. D. Liebschner, P. V. Afonine, M. L. Baker, G. Bunkoczi, V. B. Chen, T. I. Croll, B. Hintze, L.-W. Hung, S. Jain, A. J. McCoy, N. W. Moriarty, R. D. Oeffner, B. K. Poon, M. G. Prisant, R. J. Read, J. S. Richardson, D. C. Richardson, M. D. Sammito, O. V. Sobolev, D. H. Stockwell, T. C. Terwilliger, A. G. Urzhumtsev, L. L. Videau, C. J. Williams and P. D. Adams, *Acta Crystallographica Section D*, 2019, 75, 861-877.
3. P. Emsley, B. Lohkamp, W. G. Scott and K. Cowtan, *Acta Crystallogr D Biol Crystallogr*, 2010, 66, 486-501.
4. R. P. Joosten, J. Salzemann, V. Bloch, H. Stockinger, A.-C. Berglund, C. Blanchet, E. Bongcam-Rudloff, C. Combet, A. L. Da Costa, G. Deleage, M. Diarena, R. Fabbretti, G. Fettahi, V. Flegel, A. Gisel, V. Kasam, T. Kervinen, E. Korpelainen, K. Mattila, M. Pagni, M. Reichstadt, V. Breton, I. J. Tickle and G. Vriend, *J Appl Crystallogr*, 2009, 42, 376-384.
5. Y. Mechulam, L. Guillon, L. Yatime, S. Blanquet and E. Schmitt, in *Methods in Enzymology*, ed. J. Lorsch, Academic Press, 2007, vol. 430, pp. 265-281.
6. N. Krinsky, M. Kaduri, J. Shainsky-Roitman, M. Goldfeder, E. Ivanir, I. Benhar, Y. Shoham and A. Schroeder, *PLOS ONE*, 2016, 11, e0165137.
7. C. J. Harding, E. Sutherland, J. G. Hanna, D. R. Houston and C. M. Czekster, *RSC Chem. Bio.*, 2021, 2, 230-240.
8. J. R. Peacock, R. R. Walvoord, A. Y. Chang, M. C. Kozlowski, H. Gamper and Y.-M. Hou, *RNA*, 2014, 20, 758-764.
9. E. Jurrus, D. Engel, K. Star, K. Monson, J. Brandi, L. E. Felberg, D. H. Brookes, L. Wilson, J. Chen, K. Liles, M. Chun, P. Li, D. W. Gohara, T. Dolinsky, R. Koncny, D. R. Koes, J. E. Nielsen, T. Head-
Gordon, W. Geng, R. Krasny, G. W. Wei, M. J. Holst, J. A. McCammon and N. A. Baker, *Protein Sci*, 2018, **27**, 112-128.