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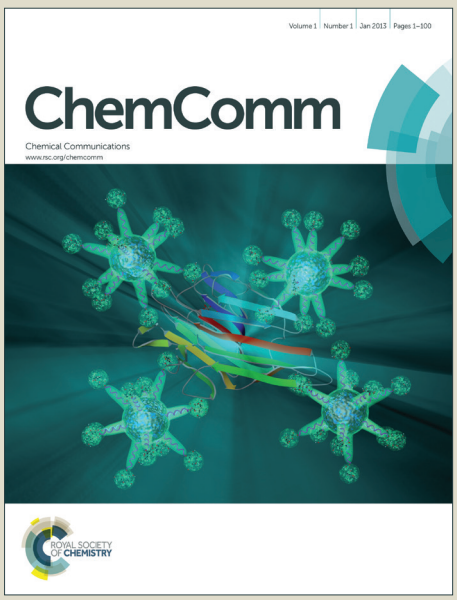

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ARTICLE TYPE

Expanding the chemical diversity of lasso peptide MccJ25 with genetically encoded noncanonical amino acids †

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Using the amber suppression approach, four noncanonical amino acids (ncAAs) were used to replace existing amino acids at four positions in lasso peptide microcin J25 (MccJ25). The lasso peptide biosynthesis enzymes tolerated all four ncAAs and produced antibiotics with efficacy equivalent to wild-type in some cases. Given the rapid expansion of the genetically encoded ncAA pool, this study is the first to demonstrate an expedient method to significantly increase the chemical diversity of lasso peptides.

Lasso peptides are a class of topologically constrained peptides typified by their pseudoknot structure resembling a lasso.[1] Such peptides belong to the superclass of ribosomally-derived natural products referred to as ribosomally-synthesized and posttranslationally-modified peptide (RiPPs).[2] RiPPs commonly contain posttranslationally-installed chemical moieties that differentiate them from conventional peptides.[3] While some RiPPs can be heavily modified,[4-5] lasso peptides have only a single posttranslational modification (PTM). The PTM in lasso peptides is an isopeptide bond that forms a ring of 7-9 amino acids[6-7] in which the C-terminal tail of the peptide is trapped (Figure 1A). Since lasso peptides to date have been confined to the chemistry of the twenty common proteinogenic amino acids, here we expand the chemical functionality of the antimicrobial lasso peptide microcin J25 (MccJ25)[8] via the incorporation of noncanonical amino acids (ncAAs). Since lasso peptides are a promising, protease-resistant scaffold for peptide drug design,[9] ncAAs will broaden the set of building blocks for such efforts and provide further insights into the tolerance of the lasso peptide scaffold for such building blocks.

Several examples of ncAA incorporation into RiPPs have been described in the past 2-3 years using different methodologies.[10] ncAAs have been introduced into the lanthipeptide lacticin 481 via solid-phase peptide synthesis (SPPS) of the linear core peptide followed by maturation *in vitro* with a purified enzyme.[11] Another lanthipeptide, the two-component lichenicidin, has been engineered using residue-specific ncAA incorporation[12-13] in a heterologous expression system in *E. coli*.[14] Structural analogs of methionine, proline, and tryptophan that are accepted by the native translational apparatus were incorporated into the two different precursor peptides of lichenicidin by utilizing auxotrophic strains of *E. coli* and media supplementation with the ncAAs. Site-specific ncAA incorporation, which relies on the addition of a 21st aminoacyl-tRNA synthetase (aaRS)/tRNA pair to an organism,[15] has also been used to engineer RiPPs with ncAAs.[16-17] Both lanthipeptides and cyanobactins were engineered with *para*-substituted phenylalanine derivatives using heterologous expression systems in *E. coli*.

We considered each of the three different methodologies used for ncAA incorporation into RiPPs thus far (SPPS followed by *in vitro* reaction, residue-specific incorporation, and site-specific incorporation) for incorporation of ncAAs into MccJ25. MccJ25 expression experiments typically proceed for 16 h or more[18] in order to maximize yields and such long expression times can be problematic for residue-specific incorporation experiments that rely on auxotrophic strains due to protein turnover providing new pools of canonical amino acids. While an *in vitro* system for the production of MccJ25 has been described,[19-20] the product yields of this system were not reported and solid-phase synthesis of the 58 aa precursor is challenging. Thus we focused on site-specific methodologies, specifically the pyrrolysyl-tRNA synthetase (PylRS) system that can incorporate a wide variety of bulky amino acids in response to the amber stop codon.[21] A specific variant of *Methanosarcina mazei* PylRS that includes two amino acid substitutions, N346A and C348A, has extensive substrate polyspecificity and can incorporate a growing list of aromatic

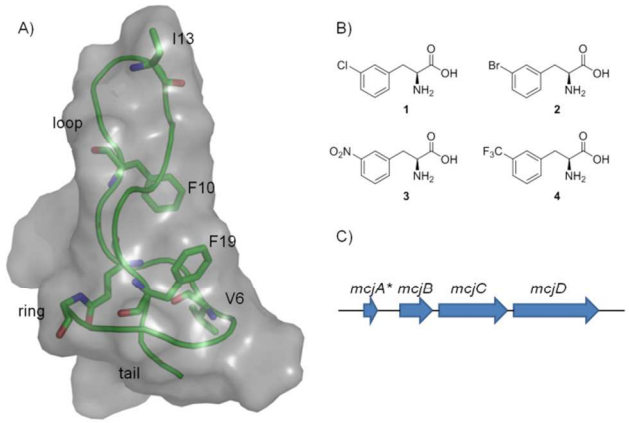

Fig. 1 Overview of ncAA incorporation into MccJ25. A) Structure of MccJ25 (PDB file: 1Q71). The loop, ring, and tail regions of the lasso are indicated, as are the residues being replaced by ncAAs. B) Structures of the ncAAs used in this study. C) Engineered gene cluster for the expression of MccJ25. The amber stop codon mutation (denoted by the asterisk) is present in the *mcjA* gene.

amino acids into recombinant proteins in *E. coli*.[22-26] For this study, we chose a panel of four *meta*-substituted Phe derivatives, *m*-ClPhe, *m*-BrPhe, *m*-NO_2Phe, and *m*-CF_3Phe (Figure 1B). This panel of Phe residues includes both polar (nitro) and non-polar (trifluoromethyl) substituents as well as halides, which introduce steric bulk to the *meta*-position

MccJ25 is derived from its 58 aa precursor protein McjA, and two enzymes, McjB and McjC, convert this precursor into the 21 aa MccJ25 product.[19-20, 27] A dedicated ABC transporter, McjD, pumps the lassoed MccJ25 product out of producing cells.[28] The structure of MccJ25 can be thought of as having three distinct components.[1] The "ring" portion of the peptide is composed of the first 8 aa with the N-terminal Gly residue covalently linked to the Glu-8 side chain (Figure 1A). The "loop" of MccJ25 includes residues 9-19 and is pictured above the ring in Figure 1A, while the "tail" portion of MccJ25 is comprised of only two aa. One of the most remarkable aspects of the MccJ25 structure is that the loop and tail portions of the peptide are held firmly in place by Phe-19 and Tyr-20.[29-31] These residues are referred to as the steric locks of the lasso peptide. We chose positions Val-6 and Ile-13 within the ring and loop of MccJ25, respectively, for substitution with ncAAs (Figure 1A). In addition, we chose the two existing Phe residues in MccJ25, Phe-10 and Phe-19, for substitution because of the structural similarity of Phe to the ncAAs discussed above. The Phe-10 residue is part of the loop region of the peptide, two amino acids away from the isopeptide bond while Phe-19 serves as a non-covalent steric lock as mentioned above. Therefore both of these Phe residues reside in sterically congested parts of the lasso structure (Figure 1A). For each of these four positions within MccJ25, we tested each of the four Phe analogs mentioned above for a total of 16 possible variants.

To enable the production of these 16 variants, we used a derivative of a plasmid-borne MccJ25 engineered gene cluster in which the *mcjA* gene is under the control of an IPTG-inducible T5 promoter while the *mcjBCD* operon is under its native constitutive promoter (Figure 1C).[18] Mutant *mcjA* genes harboring the amber codon (TAG) at the desired position were introduced to this plasmid. To enable ncAA incorporation, a pEVOL plasmid[32] harboring the gene for PylRS(N346A/C348A) and the *pylT* tRNA gene was used. Both of these plasmids were introduced into the BL21(DE3) *E. coli* host. The cells were grown in LB medium to an OD_{600} of 0.5 at which time the ncAA was added to the culture and both the *mcjA* gene and the gene for PylRS(N346A/C348A) were induced. As with previous MccJ25 expression experiments,[18] the culture was grown for 20 h. The culture supernatants were concentrated using solid-phase extraction and analysed via MALDI mass spectrometry and HPLC. Production of all 16 variants was observed via mass spectrometry on the extracted supernatant samples (Table 1, Figure S1). Unambiguous assignments of the retention time of 15 of the 16 variants could be made using HPLC (Table 1, Figure S1). Peaks corresponding to ncAA-substituted MccJ25 were collected and confirmed using MALDI (Figure S2).

Yields of ncAA-substituted MccJ25 variants depended both on the position of the substitution and the chemical nature of the substitution. In general, ncAA substitutions were best-tolerated at the I13 position, with yields of these peptides ranging from

Table 1 Characterization of engineered ncAA substituted MccJ25 variants

MccJ25 Variant	Retention time (min)	Expected/observed molecular weight (Da)	Relative Production[a]
V6TAG			
m-trifluoromethylPhe	17.99	2245.36/2246.06	0.07
m-bromoPhe	17.49	2256.26/2257.06	0.17
m-chloroPhe	17.33	2211.81/2212.06	0.16
m-nitroPhe	ND	2222.36/2222.08	ND
F10TAG			
m-trifluoromethylPhe	17.71	2197.32/2198.02	0.15
m-bromoPhe	16.92	2208.22/2208.97	0.17
m-chloroPhe	16.79	2163.77/2164.03	0.12
m-nitroPhe	16.19	2174.32/2175.04	0.05
I13TAG			
m-trifluoromethylPhe	18.3	2231.34/2231.87	0.17
m-bromoPhe	17.82	2242.24/2242.75	0.29
m-chloroPhe	17.56	2197.79/2197.83	0.29
m-nitroPhe	16.82	2208.34/2208.02	0.10
F19TAG			
m-trifluoromethylPhe	16.92	2197.32/2198.03	0.10
m-bromoPhe	16.39	2208.22/2208.94	0.14
m-chloroPhe	16.36	2163.77/2164.04	0.12
m-nitroPhe	16.28	2174.32/2175.06	0.02

[a] Relative production was calculated as the ratio of the HPLC peak area to that of wild-type MccJ25

~10-30% of wild-type MccJ25 production as estimated using HPLC peak area. In comparison, production of the variants substituted at F19 ranged from nearly undetectable on the HPLC to only 14% of wild-type MccJ25. The tolerance at the I13 position was expected given that many of the canonical aas can be substituted for I13[33] without a major impact on peptide yield.[34] Likewise, Pavlova *et al*. found that the steric lock residues F19 and Y20 were the most recalcitrant to substitutions with other canonical aas.[33] Trends could also be observed with regards to the ncAAs; variants with the nitro substitution were consistently the most poorly produced (Table 1, Figure S1). Of the four amino acids examined here, *m*-NO_2Phe exhibited the lowest protein yields in previous experiments on ncAA incorporation into GFP,[22] potentially indicating that *m*-NO_2Phe is simply a poorer substrate for the PylRS than the other ncAAs tested here.

We next examined the antimicrobial function of the ncAA-substituted MccJ25 variants. MccJ25 has narrow-spectrum antimicrobial activity against enteric bacteria including non-producing strains of *E. coli*, *Salmonella sp.*, and *Shigella sp.*[8] Its cytoplasmic target is RNA polymerase (RNAP),[35-37] and it enters susceptible cells through the outer membrane transporter FhuA.[38-40] Genetic evidence points to a role for SbmA in enabling transport of MccJ25 across the inner membrane.[41] Despite this requirement for productive interactions with multiple proteins, we detected measurable antimicrobial activity in solid-phase extracted culture supernatants for each of the 16 MccJ25 variants using a spot-on-lawn assay[42] (Figure S3). The relative antimicrobial activity in the supernatants was quantified using a

Table 2 Last active dilutions against *Salmonella newport* for ncAA substituted MccJ25 variants in antimicrobial assay[a]

	m-CF_3Phe	*m*-BrPhe	*m*-ClPhe	*m*-NO_2Phe
V6TAG	1 x	1/32 x	1/8 x	1/2 x
F10TAG	1/2 x	1/4096 x	1/1024 x	1/128 x
I13TAG	1/32 x	1/64 x	1/512 x	1/32 x
F19TAG	1/32 x	1/64 x	1/2048 x	1/32 x

[a]Wild-type MccJ25 has a last active dilution of 1/2048 x

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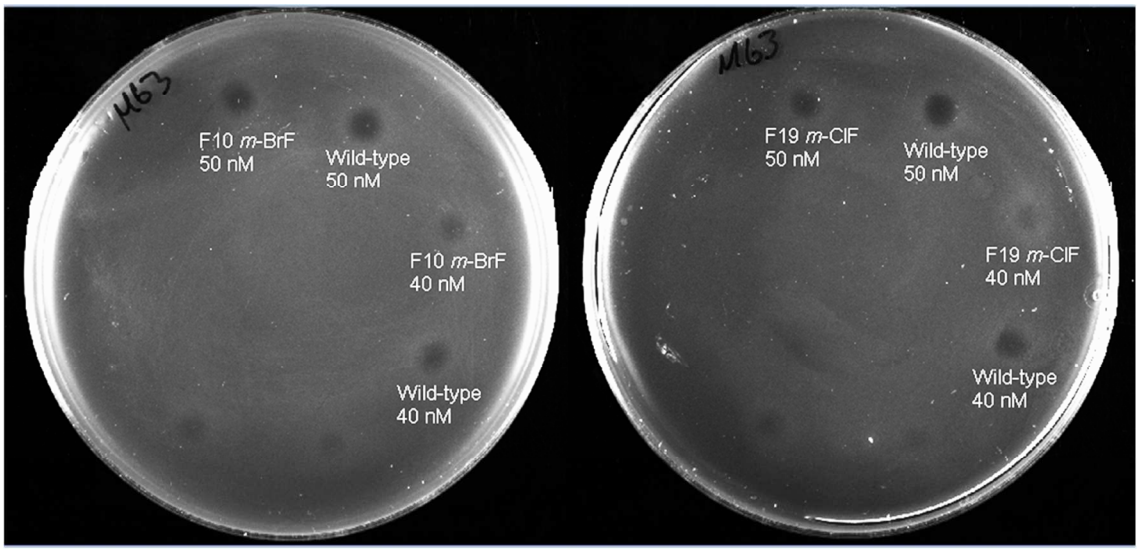

Fig. 2 Spot-on-lawn assays for the determination of minimal inhibitory concentrations against *Salmonella newport*. Left: F10 *m*-bromophenylalanine substituted MccJ25 compared to wild-type. Right: F19 *m*-chlorophenylalanine substituted MccJ25 compared to wild-type.

serial dilution method in which the last active dilution against *Salmonella newport* was determined[34] (Table 2, Figure S4). There was a large range in antimicrobial activities observed among the variants: the majority of them exhibited a decrease in potency, but several variants exhibited activities near or even above wild-type activity (Table 2).

In all four positions within MccJ25, the *m*-CF_3Phe substitution was highly deleterious with regards to antimicrobial activity. This loss in activity cannot be accounted for by decreased production because the *m*-CF_3Phe variants exhibited reasonable titers as judged by HPLC (Table 1). This indicates that while the trifluoromethyl moiety may be tolerated by the lasso peptide biosynthesis enzymes, it retards either the uptake of MccJ25 or its binding to RNAP. In contrast, 3 of the 4 *m*-ClPhe substituted variants exhibited last active dilutions near the wild-type value (Table 2). The exception was the V6 *m*-ClPhe variant, but all ncAA substitutions at this ring position led to severely decreased antimicrobial activity (Table 2). Given prior observations that substitutions with canonical aas at the F10 and F19 positions led to a total loss of antimicrobial activity,[33] we were pleased to see that all four ncAA variants at both of these positions exhibited antimicrobial activity (Table 2). It should be noted that a recent report showed weak antimicrobial activity for an F19S variant of MccJ25,[43] but the production of this variant was estimated as being less than 1% of wild-type. In contrast, several MccJ25 variants with ncAAs at the F10 and F19 positions had potent antimicrobial activity at or near wild-type levels as judged by the spot assays (Table 2).

Based on the spot assay results, the variants harboring *m*-BrPhe at the F10 position and *m*-ClPhe at the F19 position are the most potent of the 16 new variants, and were expected to have potency similar to wild-type MccJ25 (Table 2). We performed a large-scale expression of these peptides and purified them to homogeneity (Figure S5). The spot-on-lawn assay was used with defined concentrations of wild-type MccJ25 and these two variants in order to determine minimal inhibitory concentrations (MIC) against *Salmonella newport*. The wild-type and F10 *m*-BrPhe variant both exhibited a MIC of 40 nM, while the F19 *m*-ClPhe variant had a MIC of 50 nM (Figure 2). These data further demonstrate that large substituents at different positions within the lasso can be tolerated with retention of nanomolar potency. In the previous study mentioned above,[33] the F10 and F19 positions could not be substituted by any canonical amino acid, even tyrosine, with retention of antimicrobial activity. Our data indicate that the F10 and F19 positions not only can accept ncAAs but that these variants can exhibit wild-type or near wild-type activity.

Here we have demonstrated the use of a polyspecific PylRS variant to introduce ncAAs in a site-specific fashion into MccJ25, a lasso peptide. This expands the palette of RiPPs into which non-proteinogenic amino acids can be introduced. The MccJ25 scaffold is remarkably tolerant of substitutions by ncAAs with four different positions in the peptide each accepting four different *meta*-substituted Phe derivatives. Moreover, each of these sixteen new variants of MccJ25 retained measureable antimicrobial activity. We noted that substitutions at V6 were universally deleterious to MccJ25 activity. A recent report on the structure of MccJ25 bound to the outer membrane receptor FhuA[40] revealed that H5 is critical for binding of MccJ25 to the FhuA pocket. We suspect that substitutions at V6, especially with larger amino acids such as the ones used in this study, may disrupt the interaction of the MccJ25 ring with FhuA. This may mean that the decreased antimicrobial activity we observe is due in part to reduced uptake of the variants by FhuA. To date, the

only crystal structures associated with MccJ25 biosynthesis, uptake, or antimicrobial activity are the aforementioned structure of FhuA and the structure of the MccJ25 export protein McjD.[44] Of these, only the FhuA structure is liganded with MccJ25. Bromine atoms can be used in determining crystallographic phases via multiwavelength anomalous diffraction.[45] Thus we propose that the bromo-substituted variants described here could be useful in future crystallographic analyses of MccJ25-liganded biosynthetic enzymes, transporters, and targets.

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Notes and references

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1 M. O. Maksimov, S. J. Pan and A. J. Link, *Nat. Prod. Rep.*, 2012, **29**, 996.
2 P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, P. D. Cotter, D. J. Craik, M. Dawson, E. Dittmann, S. Donadio, P. C. Dorrestein, K.-D. Entian, M. A. Fischbach, J. S. Garavelli, U. Goransson, C. W. Gruber, D. H. Haft, T. K. Hemscheidt, C. Hertweck, C. Hill, A. R. Horswill, M. Jaspars, W. L. Kelly, J. P. Klinman, O. P. Kuipers, A. J. Link, W. Liu, M. A. Marahiel, D. A. Mitchell, G. N. Moll, B. S. Moore, R. Muller, S. K. Nair, I. F. Nes, G. E. Norris, B. M. Olivera, H. Onaka, M. L. Patchett, J. Piel, M. J. T. Reaney, S. Rebuffat, R. P. Ross, H.-G. Sahl, E. W. Schmidt, M. E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R. D. Sussmuth, J. R. Tagg, G.-L. Tang, A. W. Truman, J. C. Vederas, C. T. Walsh, J. D. Walton, S. C. Wenzel, J. M. Willey and W. A. van der Donk, *Nat. Prod. Rep.*, 2013, **30**, 108.
3 K. L. Dunbar and D. A. Mitchell, *ACS Chem. Biol.*, 2013, **8**, 473.
4 P. Yorgey, J. Lee, J. Kordel, E. Vivas, P. Warner, D. Jebaratnam and R. Kolter, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 4519.
5 H. E. Hallen, H. Luo, J. S. Scott-Craig and J. D. Walton, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 19097.
6 J. D. Hegemann, M. Zimmermann, S. Z. Zhu, H. Steuber, K. Harms, X. L. Xie and M. A. Marahiel, *Angew. Chem. Int. Edit.*, 2014, **53**, 2230.
7 M. O. Maksimov and A. J. Link, *J. Ind. Microbiol. Biotechnol.*, 2014, **41**, 333.
8 R. A. Salomon and R. N. Farias, *J. Bacteriol.*, 1992, **174**, 7428.
9 T. A. Knappe, F. Manzenrieder, C. Mas-Moruno, U. Linne, F. Sasse, H. Kessler, X. L. Xie and M. A. Marahiel, *Angew. Chem. Int. Edit.*, 2011, **50**, 8714.
10 N. Budisa, *Curr. Opin. Biotech.*, 2013, **24**, 591.
11 P. J. Knerr, T. J. Oman, C. V. G. De Gonzalo, T. J. Lupoli, S. Walker and W. A. van der Donk, *ACS Chem. Biol.*, 2012, **7**, 1791.
12 A. J. Link and D. A. Tirrell, *Methods*, 2005, **36**, 291.
13 N. Budisa, *Angew. Chem Int. Edit.*, 2004, **43**, 6426.
14 F. Oldach, R. Al Toma, A. Kuthning, T. Caetano, S. Mendo, N. Budisa and R. D. Sussmuth, *Angew. Chem. Int. Edit.*, 2012, **51**, 415.
15 L. Wang and P. G. Schultz, *Angew. Chem. Int. Edit.*, 2005, **44**, 34.
16 Y. X. Shi, X. A. Yang, N. Garg and W. A. van der Donk, *J. Am. Chem. Soc.*, 2011, **133**, 2338.
17 M. D. B. Tianero, M. S. Donia, T. S. Young, P. G. Schultz and E. W. Schmidt, *J. Am. Chem. Soc.*, 2012, **134**, 418.
18 S. J. Pan, W. L. Cheung and A. J. Link, *Protein Expres.Purif.*, 2010, **71**, 200.
19 S. Duquesne, D. Destoumieux-Garzón, S. Zirah, C. Goulard, J. Peduzzi and S. Rebuffat, *Chem. Biol.*, 2007, **14**, 793.
20 K. P. Yan, Y. Y. Li, S. Zirah, C. Goulard, T. A. Knappe, M. A. Marahiel and S. Rebuffat, *Chembiochem*, 2012, **13**, 1046.
21 W. Wan, J. M. Tharp and W. R. Liu, *BBA-Proteins Proteom.*, 2014, **1844**, 1059.
22 Y. S. Wang, X. Q. Fang, H. Y. Chen, B. Wu, Z. Y. U. Wang, C. Hilty and W. S. R. Liu, *ACS Chem. Biol.*, 2013, **8**, 405.
23 J. M. Tharp, Y.-S. Wang, Y.-J. Lee, Y. Yang and W. R. Liu, *ACS Chem. Biol.*, 2014.
24 A. Tuley, Y. S. Wang, X. Q. Fang, Y. Kurra, Y. H. Rezenom and W. R. Liu, *Chem. Comm.*, 2014, **50**, 2673.
25 Y. S. Wang, X. Q. Fang, A. L. Wallace, B. Wu and W. S. R. Liu, *J. Am. Chem. Soc.*, 2012, **134**, 2950.
26 A. Tuley, Y. J. Lee, B. Wu, Z. U. Wang and W. R. Liu, *Chem. Comm.*, 2014, **50**, 7424.
27 S. J. Pan, J. Rajniak, W. L. Cheung and A. J. Link, *Chembiochem*, 2012, **13**, 367.
28 J. O. Solbiati, M. Ciaccio, R. N. Farias, J. E. Gonzalez-Pastor, F. Moreno and R. A. Salomon, *J. Bacteriol.*, 1999, **181**, 2659.
29 K. A. Wilson, M. Kalkum, J. Ottesen, J. Yuzenkova, B. T. Chait, R. Landick, T. Muir, K. Severinov and S. A. Darst, *J. Am. Chem. Soc.*, 2003, **125**, 12475.
30 K. J. Rosengren, R. J. Clark, N. L. Daly, U. Goransson, A. Jones and D. J. Craik, *J. Am. Chem. Soc.*, 2003, **125**, 12464.
31 M. J. Bayro, J. Mukhopadhyay, G. V. T. Swapna, J. Y. Huang, L. C. Ma, E. Sineva, P. E. Dawson, G. T. Montelione and R. H. Ebright, *J. Am. Chem. Soc.*, 2003, **125**, 12382.
32 T. S. Young, I. Ahmad, J. A. Yin and P. G. Schultz, *J. Mol. Biol.*, 2010, **395**, 361.
33 O. Pavlova, J. Mukhopadhyay, E. Sineva, R. H. Ebright and K. Severinov, *J. Biol. Chem.*, 2008, **283**, 25589.
34 S. J. Pan and A. J. Link, *J. Am. Chem. Soc.*, 2011, **133**, 5016.
35 K. Adelman, J. Yuzenkova, A. La Porta, N. Zenkin, J. Lee, J. T. Lis, S. Borukhov, M. D. Wang and K. Severinov, *Mol. Cell*, 2004, **14**, 753.
36 J. Mukhopadhyay, E. Sineva, J. Knight, R. M. Levy and R. H. Ebright, *Mol. Cell*, 2004, **14**, 739.
37 J. Yuzenkova, M. Delgado, S. Nechaev, D. Savalia, V. Epshtein, I. Artsimovitch, R. A. Mooney, R. Landick, R. N. Farias, R. Salomon and K. Severinov, *J. Biol. Chem.*, 2002, **277**, 50867.
38 D. Destoumieux-Garzon, S. Duquesne, J. Peduzzi, C. Goulard, M. Desmadril, L. Letellier, S. Rebuffat and P. Boulanger, *Biochem. J.*, 2005, **389**, 869.
39 R. A. Salomon and R. N. Farias, *J. Bacteriol.*, 1993, **175**, 7741.
40 I. Mathavan, S. Zirah, S. Mehmood, H. G. Choudhury, C. Goulard, Y. Li, C. V. Robinson, S. Rebuffat and K. Beis, *Nat. Chem. Biol.*, 2014, **10**, 340.
41 R. A. Salomon and R. N. Farias, *J. Bacteriol.*, 1995, **177**, 3323.
42 W. L. Cheung, S. J. Pan and A. J. Link, *J. Am. Chem. Soc.*, 2010, **132**, 2514.
43 R. Ducasse, K. P. Yan, C. Goulard, A. Blond, Y. Y. Li, E. Lescop, E. Guittet, S. Rebuffat and S. Zirah, *Chembiochem*, 2012, **13**, 371.
44 H. G. Choudhury, Z. Tong, I. Mathavan, Y. Y. Li, S. Iwata, S. Zirah, S. Rebuffat, H. W. van Veen and K. Beis, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 9145.
45 Z. Dauter, M. Dauter and K. R. Rajashankar, *Acta Crystallogr. D*, 2000, **56**, 232.