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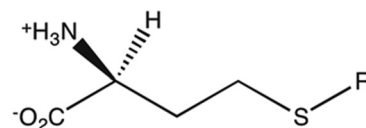
Intriguing cellular processing of a fluorinated amino acid during protein biosynthesis in *Escherichia coli*†

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Bioincorporation of the methionine analogue *S*-(2-fluoroethyl)-L-homocysteine (L-MFE) into bacteriophage lysozyme overproduced in *Escherichia coli* results not only in the expected L-MFE incorporation but surprisingly substantial L-vinthionine incorporation into the labeled lysozymes. Synthetic L-vinthionine itself however is not activated by purified *Escherichia coli* methionyl-tRNA synthetase. The indirect preparation of vinthionine-containing proteins has the potential to be an alternate strategy to prepare vinyl thioether moieties for click chemistry applications on proteins.

The incorporation of unnatural amino acids into proteins is a powerful tool in the armamentarium of chemical biologists.^{1–7} Modifiable unnatural amino acids, such as azidohomoalanine and various ketone- and acetylene-containing amino acid analogues, have permitted post-translational chemical modification of proteins with applications in diverse areas such as neurochemistry, radiochemistry, biomaterials fabrication and whole organism physiology.^{6,8–20} New amino acid analogues capable of protein incorporation followed by further chemical or biochemical elaboration should further expand these molecular approaches.^{10,12,21} Previous research has also shown that many fluorinated amino acid analogues are capable of undergoing facile residue-specific bioincorporation into proteins *in vivo*, due to the conservative size of the fluorine atom. These non-sterically demanding analogues have proven useful as biophysical probes (¹⁹F NMR) in protein structure/function^{22–25} and drug binding studies,^{26–28} and in redox protein modulation.²⁹ Based on these successes, the further application of fluorinated amino acid analogues to the area of biomolecular chemistry appears warranted.

It has been known for some time that the structural homologue of L-methionine, L-ethionine, can be readily incorporated into proteins, although its lack of interesting chemical functionality has relegated it to infrequent biochemical use (Fig. 1).^{2,30} It was hypothesized that the introduction of a fluorine atom into the ethyl moiety might result not only in a potentially interesting ¹⁹F NMR probe but may also provide a chemically reactive functional group capable of post-translational chemical modification if bioincorporation successfully occurs into cellular proteins. Previous studies have shown that the 2-fluoroethyl sulfide functionality is substantially more stable in aqueous solution than other halogenated analogues.^{31–34} As such, it was predicted that the 2-fluoroethyl methionine analogue might be sufficiently long-lived to allow for residue-specific bioincorporation at methionine positions with subsequent isolation of the fluorinated protein from a bacterial expression system. This known analogue, L-monofluoroethionine (L-MFE, *S*-(2-fluoroethyl)-L-homocysteine), the [¹⁸F] congener of which has been reported to be potentially useful in tumour imaging, was therefore synthesized and further investigated in this context (ESI Methods;



R = CH ₃	L-methionine
CHF ₂	L-difluoromethionine
CF ₃	L-trifluoromethionine
CH ₂ CH ₃	L-ethionine
CH ₂ CH ₂ F	L-monofluoroethionine
CH ₂ CH ₂ OH	L-hydroxyethylhomocysteine
CH=CH ₂	L-vinthionine

Fig. 1 Chemical structures of methionine analogues referred to in the text.

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Scheme S1†).^{32,35,36} L-MFE has been reported to be hydrolyzed in D₂O exclusively to S-(2-hydroxyethyl)-L-homocysteine (L-HEHC) relatively slowly with complete hydrolysis not being observed even after 7 days, although additional studies in H₂O indicated reduced stability.³² In our hands, L-MFE was indeed found to be acceptably stable with similar results being obtained in D₂O with only L-HEHC detected as a degradation product. Particularly pertinent was that in the presence of a thiol-based nucleophile, 2-mercaptoethanesulfonate, the formation of a sulfide adduct with L-MFE under basic aqueous conditions was detectable (pH 9) (ESI Scheme S2; Fig. S1 and S2†).

Based on the encouraging modulated reactivity of L-MFE, attempts to bioincorporate this analogue into a recombinant protein were investigated. Indeed L-MFE was successfully incorporated into bacteriophage lambda lysozyme (LaL), a model protein, utilizing protocols previously employed for the bioincorporation of several other unnatural methionine analogues such as L-difluoromethionine (L-DFM) and L-trifluoromethionine (L-TFM) into positions M1, M14 and

M107 of the protein (Fig. 2) (ESI Methods†).^{37–40} The resulting recombinant LaL proteins produced in the presence of L-MFE were fully characterized by ¹⁹F nuclear magnetic resonance and electrospray mass spectrometry. Protein isolation followed by ¹⁹F NMR (proton decoupled) spectroscopic analyses confirmed that L-MFE was indeed incorporated during protein biosynthesis as exhibited by the presence of the expected three ¹⁹F NMR resonances (Fig. 3a). Assignments of the resonances were accomplished by a combination of site directed mutagenesis (M107L) and paramagnetic line broadening of the ¹⁹F resonances at the solvent exposed M1 and M107 positions with gadolinium-ethylenediaminetetraacetate acid (Gd-EDTA) and knowledge of the molecular structure of the enzyme, a previously successful approach for resonance assignments for other fluorinated methionine analogues in this protein (Fig. 3a).^{37–40} Interestingly, the electrospray mass spectrum of the ensemble of isolated LaL proteins (wild type LaL *M_r* = 17 825 Da) was found to be complex (Fig. 3b), beyond what would be expected for incomplete substitution of each of the three methionine positions by L-MFE. Analysis of the detected ESI-MS protein masses (ESI Table S1†) indicated the possible presence of a new amino acid, L-vinthionine (S-vinyl-L-homocysteine), which would be formed by loss of HF from L-MFE at some point during protein biosynthesis. Based on the observed masses, only a minor presence of L-HEHC in the ensemble of proteins would be suggested (ESI Table S1†).

Control studies, however, demonstrated that L-MFE itself does not eliminate fluoride ion in buffer solution to produce L-vinthionine; only L-HEHC is detected over time in aqueous solutions as monitored by mass spectrometry and NMR, an observation that is consistent with a previous report for this amino acid.³² To confirm that L-MFE was partially processed into L-vinthionine during bioincorporation, a series of 1D and 2D proton NMR measurements were undertaken to provide further evidence for the existence of L-vinthionine in the MFE-labeled protein ensemble. In 1D proton NMR experiments on the amino acids themselves, a distinctly different NMR profile between L-MFE and L-vinthionine is present in the region between 5.0 ppm and 6.5 ppm (Fig. 4a), a region indicative of vinyl proton resonances. The 1D proton NMR spectrum for



Fig. 2 Ribbon diagram of bacteriophage lambda lysozyme with Met1, Met107 and Met14 shown as ball-and-sticks. Met 1 and Met107 are exposed to solvent (based on PDB 1D9U).

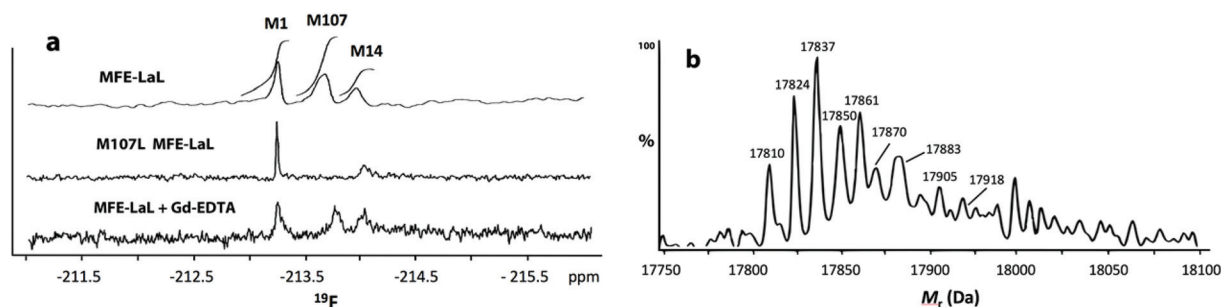


Fig. 3 ¹⁹F NMR and electrospray mass spectrum of isolated bacteriophage lambda lysozyme overproduced in the presence of L-MFE. (a) Resonances observed in the ¹⁹F NMR spectrum of MFE-labeled LaL were assigned by site-directed mutagenesis and paramagnetic line broadening experiments utilizing Gd-EDTA in concert with the known three-dimensional structure of the enzyme. (b) The ensemble of resulting proteins is a complex mixture as the deconvoluted ESI spectrum exhibits.



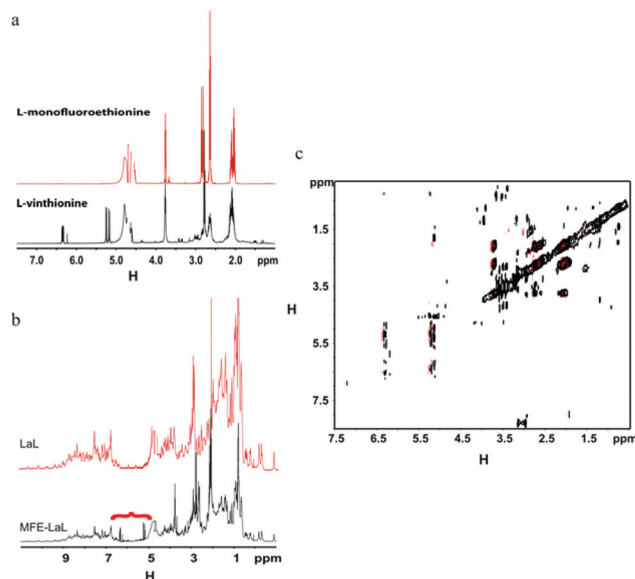


Fig. 4 ¹H NMR spectra of the amino acids L-MFE and L-vinthionine and the isolated bacteriophage lambda lysozyme overproduced in the presence of L-MFE. (a) ¹H NMR spectra of the amino acids L-MFE (in red) and L-vinthionine (in black) show differences in the low-field region. (b) ¹H NMR spectra of LaL (in red) and MFE-LaL (in black) show distinct differences in the low-field region between 5.0 ppm and 6.5 ppm as noted in red in the spectra for MFE-LaL. (c) Comparison of the NMR TOCSY spectra of L-vinthionine (in red) and denatured MFE-labeled LaL (in black) indicate similarities consistent with the presence of L-vinthionine residues also present in labeled LaL proteins.

LaL itself has no major proton resonances in this region, providing a simple way to confirm the presence of L-vinthionine in the labeled protein ensemble. Unique proton resonances were readily identified in the region between 5.0 ppm and 6.5 ppm in the 1D proton NMR spectrum of MFE-labeled LaL (Fig. 4b). Furthermore, analysis of 2-D TOCSY NMR experiments using short mixing times confirmed that the unique resonances in this region are indeed from vinthionine residues incorporated into the biosynthesized proteins (Fig. 4c).

These interesting sets of observations motivated an investigation into the substrate promiscuity of the *Escherichia coli* methionyl-tRNA synthetase. Recombinant *E. coli* methionyl-tRNA synthetase (ESI Methods†), isolated as previously reported,⁴¹ was studied with both L-MFE and L-vinthionine, in order to determine if L-vinthionine, which might be produced in *E. coli* in some fashion from supplied L-MFE present in the growth media, could be charged by the endogenous bacterial synthetase, resulting in a new misacylated tRNA^{Met}. ATP/pyrophosphate exchange assays,⁴¹ which have been utilized to determine charging activity of various amino acid analogues for methionyl-tRNA synthetase, were also undertaken with the analogues, including L-DFM, L-TFM and L-ethionine (ESI Methods†). Surprisingly although L-ethionine and L-MFE exhibited activity as substrates for the ATP exchange assay (L-ethionine: k_{cat} $8.4 \pm 0.6 \text{ s}^{-1}$, K_{m} $1.0 \pm 0.3 \text{ mM}$; L-MFE: k_{cat}

$5.0 \pm 0.2 \text{ s}^{-1}$, K_{m} $4.8 \pm 0.9 \text{ mM}$), L-vinthionine was completely inactive in the biochemical assay (ESI Table S2†). Additionally, attempts to express a labeled LaL in the absence of L-MFE but in the presence of L-vinthionine in *E. coli* under minimal media conditions as before met with no observable LaL production as determined by SDS PAGE analyses, confirming the lack of substrate capability of L-vinthionine for the bacterial methionyl-tRNA synthetase. This indicates that the direct conversion of L-MFE to L-vinthionine within the cell followed by charging of the resulting L-vinthionine by the bacterial synthetase is not a likely pathway for vinthionine incorporation into protein.

It was possible that L-vinthionine might have been generated during the activation process in *E. coli* or at a subsequent stage in protein biosynthesis possibly from charged MFE-tRNA^{Met}. It is interesting to note that in the case of methionyl-tRNA synthetase, an editing mechanism has been observed when L-homocysteine itself is supplied as substrate for the synthetase.⁴² It has been reported that the methionyl-tRNA synthetase charges L-homocysteine by reaction with ATP to form the aminoacyl-adenylate, but subsequent cyclization occurs which ejects L-homocysteine thiolactone, preventing misacylation of the tRNA^{Met}.⁴³ It was hypothesized that an unusual “editing” reaction occurred to a limited extent when L-MFE itself was being activated, resulting in elimination of fluoride ion, and producing an activated vinthionine-tRNA^{Met} which was utilized in protein biosynthesis. However, no evidence for conversion of L-MFE into observable L-vinthionine was evident in solution NMR experiments undertaken with L-MFE in the presence of both methionyl-tRNA synthetase and tRNA^{Met}. It was also observed that L-HEHC was insignificantly charged onto tRNA^{Met} (ESI Fig. S3 and S4†) in the presence of methionyl-tRNA synthetase and tRNA^{Met}.

From these experiments it appears that subsequent partial elimination of fluoride ion likely occurs at later stages in the protein biosynthetic process and/or occurs once MFE is incorporated into the protein structure. It is known that although β -hydroxy sulfides can undergo elimination to vinyl sulfides under harsh basic conditions,^{44,45} β -halo sulfides and even β -fluoro sulfides can more readily eliminate halide to form the vinyl sulfides.^{46,47} The presence of a number of basic side chains proximal to each of the methionine positions (<10 Å; Met1: Glu3, Lys84, Asp85, Lys89; Met14: His137, Lys145; Met107: Arg102, Arg110, Asp112, Asp118, Arg119) could potentially fill this chemical role.

Regardless of the actual cellular process in *E. coli* occurring for this particular analogue, it is clear that the introduction of an unnatural amino acid into a protein by bioincorporation also results, in this particular case (L-MFE), in a surprising secondary conversion, unambiguously determined to be L-vinthionine. There is currently intense interest in fabricating living cells and even whole animals that contain unnatural amino acids in their cellular proteins.^{6,15,16} Recently the ability of protein-attached vinyl sulfides to undergo click cycloadditions with *o*-quinolinone quinone methides has been reported, providing a unique approach to bioorthogonal ligation



strategies.⁴⁸ The vinyl sulfide functionality was introduced by chemical modification of lysine residues utilizing *N*-succinimidyl-2-(vinylthio)ethyl carbonate. Subsequent reactions with a variety of *o*-quinolinone quinone methides resulted in cycloaddition with the vinyl sulfide moiety. It has also been shown that vinyl sulfides, upon alkylation, can undergo nucleophilic modifications.⁴⁹ Since vinthionine cannot be directly incorporated as an unnatural amino acid into proteins under standard bioincorporation (this work), the indirect application of MFE to serve as a source of vinyl sulfide functionalities for bioorthogonal modification may find use in select problems in chemical biology. The development of dual bioorthogonal strategies^{50,51} to introduce two different reactive and/or biophysical probes into the same protein is also of current interest and the application of L-MFE as a single step strategy to accomplish dual labeling may also prove useful in select studies. For example, the incorporation of a highly sensitive ¹⁹F NMR nucleus located in the L-MFE side chain along with the presence of a potentially modifiable L-vinthionine in the same protein could permit the covalent modification or immobilization of medicinally relevant receptors and target enzymes to serve in drug screening by monitoring ligand binding by protein-observed ¹⁹F NMR.^{26,27} The added possibility that L-monofluoroethionine itself, and the subsequently generated L-vinthionine can partake in further chemical modification reactions such as nucleophilic substitution of the fluorine atom in L-MFE (ESI Scheme S2†) and/or click cycloadditions of the uncloaked vinyl sulfide in L-vinthionine with *o*-quinolinone quinone methides may be potentially useful in the area of single molecule fluorescence energy transfer (smFRET) which requires the presence of two different fluorophores on the same protein.^{50,52,53} Exploration of the chemistries that might occur on protein incorporated L-MFE and L-vinthionine are therefore of some interest.

Conclusions

Fluorinated amino acids can play enabling roles as biophysical probes in biological systems. The intriguing formation of L-vinthionine from L-MFE during *E. coli* protein biosynthesis further extends our knowledge of the chemistry that fluorine can undergo within a biological context. The capability of L-MFE to be converted into L-vinthionine during protein production may also prove useful as another strategy to introduce the vinyl sulfide moiety into proteins for applications in chemical biology. This indirect approach is the only known route to introduce this amino acid into proteins produced directly in *E. coli* without the requirement to develop more elaborate engineered amino acid tRNA synthetase/tRNA pairs,¹ or employ cell-free protein synthesis using either misacylated tRNA⁵⁴ or express protein ligation.²⁹ However L-MFE and L-Vin likely could be utilized in these approaches as well. The further exploration of the capabilities of incorporated L-MFE and L-vinthionine residues in proteins appears warranted.

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Notes and references

- 1 L. Wang, J. Xie and P. G. Schultz, *Annu. Rev. Biophys. Biomol. Struct.*, 2006, **35**, 225–249.
- 2 K. J. Rodgers and N. Shiozawa, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 1452–1466.
- 3 J. Guo, C. E. Melancon 3rd, H. S. Lee, D. Groff and P. G. Schultz, *Angew. Chem., Int. Ed.*, 2009, **48**, 9148–9151.
- 4 J. A. Johnson, Y. Y. Lu, J. A. Van Deventer and D. A. Tirrell, *Curr. Opin. Chem. Biol.*, 2010, **14**, 774–780.
- 5 J. T. Ngo and D. A. Tirrell, *Acc. Chem. Res.*, 2011, **44**, 677–685.
- 6 K. Lang and J. W. Chin, *Chem. Rev.*, 2014, **114**, 4764–4806.
- 7 P. Neumann-Staubitz and H. Neumann, *Curr. Opin. Struct. Biol.*, 2016, **38**, 119–128.
- 8 X. Chen and Y. W. Wu, *Org. Biomol. Chem.*, 2016, **14**, 5417–5439.
- 9 P. Adumeau, S. K. Sharma, C. Brent and B. M. Zeglis, *Mol. Imaging Biol.*, 2016, **18**, 153–165.
- 10 M. Zheng, L. Zheng, P. Zhang, J. Li and Y. Zhang, *Molecules*, 2015, **20**, 3190–3205.
- 11 G. Zhang, S. Zheng, H. Liu and P. R. Chen, *Chem. Soc. Rev.*, 2015, **44**, 3405–3417.
- 12 H. W. Shih, D. N. Kamber and J. A. Prescher, *Curr. Opin. Chem. Biol.*, 2014, **21**, 103–111.
- 13 C. S. McKay and M. G. Finn, *Chem. Biol.*, 2014, **21**, 1075–1101.
- 14 J. C. Knight and B. Cornelissen, *Am. J. Nucl. Med. Mol. Imaging*, 2014, **4**, 96–113.
- 15 T. S. Elliott, A. Bianco and J. W. Chin, *Curr. Opin. Chem. Biol.*, 2014, **21**, 154–160.
- 16 J. W. Chin, *Annu. Rev. Biochem.*, 2014, **83**, 379–408.
- 17 D. Zeng, B. M. Zeglis, J. S. Lewis and C. J. Anderson, *J. Nucl. Med.*, 2013, **54**, 829–832.
- 18 F. I. Hinz, D. C. Dieterich and E. M. Schuman, *Curr. Opin. Chem. Biol.*, 2013, **17**, 738–746.
- 19 S. Greiss and J. W. Chin, *J. Am. Chem. Soc.*, 2011, **133**, 14196–14199.
- 20 E. Strable and M. G. Finn, *Curr. Top. Microbiol. Immunol.*, 2009, **327**, 1–21.
- 21 P. Shieh and C. R. Bertozzi, *Org. Biomol. Chem.*, 2014, **12**, 9307–9320.
- 22 E. N. Marsh and Y. Suzuki, *ACS Chem. Biol.*, 2014, **9**, 1242–1250.



- 23 J. X. Yu, R. R. Hallac, S. Chiguru and R. P. Mason, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2013, **70**, 25–49.
- 24 H. Chen, S. Viel, F. Ziarelli and L. Peng, *Chem. Soc. Rev.*, 2013, **42**, 7971–7982.
- 25 J. L. Kitevski-LeBlanc and R. S. Prosser, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2012, **62**, 1–33.
- 26 K. E. Arntson and W. C. Pomerantz, *J. Med. Chem.*, 2016, **59**, 5158–5171.
- 27 N. K. Mishra, A. K. Urick, S. W. Ember, E. Schonbrunn and W. C. Pomerantz, *ACS Chem. Biol.*, 2014, **9**, 2755–2760.
- 28 X. Ge, C. A. MacRaid, S. M. Devine, C. O. Debono, G. Wang, P. J. Scammells, M. J. Scanlon, R. F. Anders, M. Foley and R. S. Norton, *J. Med. Chem.*, 2014, **57**, 6419–6427.
- 29 D. K. Garner, M. D. Vaughan, H. J. Hwang, M. G. Savelieff, S. M. Berry, J. F. Honek and Y. Lu, *J. Am. Chem. Soc.*, 2006, **128**, 15608–15617.
- 30 D. Gross and H. Tarver, *J. Biol. Chem.*, 1955, **217**, 169–182.
- 31 H. V. Vadi, C. S. Schasteen and D. J. Reed, *Toxicol. Appl. Pharmacol.*, 1985, **80**, 386–396.
- 32 T. Bourdier, C. J. R. Fookes, T. Q. Pham, I. Greguric and A. Katsifis, *J. Labelled Compd. Radiopharm.*, 2008, **51**, 369–373.
- 33 C. S. Schasteen and D. J. Reed, *Toxicol. Appl. Pharmacol.*, 1983, **70**, 423–432.
- 34 J. R. Sufrin, A. J. Spiess, D. L. Kramer, P. R. Libby, J. T. Miller, R. J. Bernacki, Y. Lee, R. T. Borchardt and C. W. Porter, *J. Med. Chem.*, 1991, **34**, 2600–2606.
- 35 G. H. Tang, M. F. Wang, X. L. Tang, L. Luo and M. Q. Gan, *Acta Pharm. Sin.*, 2003, **38**, 915–918.
- 36 G. Tang, M. Wang, X. Tang, L. Luo and M. Gan, *Nucl. Med. Biol.*, 2003, **30**, 509–512.
- 37 A. K. Leung, H. S. Duewel, J. F. Honek and A. M. Berghuis, *Biochemistry*, 2001, **40**, 5665–5673.
- 38 M. D. Vaughan, P. Cleve, V. Robinson, H. S. Duewel and J. F. Honek, *J. Am. Chem. Soc.*, 1999, **121**, 8475–8478.
- 39 H. Duewel, E. Daub, V. Robinson and J. F. Honek, *Biochemistry*, 1997, **36**, 3404–3416.
- 40 H. S. Duewel, E. Daub, V. Robinson and J. F. Honek, *Biochemistry*, 2001, **40**, 13167–13176.
- 41 M. D. Vaughan, P. B. Sampson, E. Daub and J. F. Honek, *Med. Chem.*, 2005, **1**, 227–237.
- 42 H. Jakubowski, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 4504–4508.
- 43 G. B. Fortowsky, D. J. Simard, M. M. Aboelnga and J. W. Gauld, *Biochemistry*, 2015, **54**, 5757–5765.
- 44 J. W. Cubbage, Y. Guo, R. D. McCulla and W. S. Jenks, *J. Org. Chem.*, 2001, **66**, 8722–8736.
- 45 R. Persson, M. J. Stchedroff, R. Gobetto, C. J. Carrano, M. G. Richmond, M. Monari and E. Nordlander, *Eur. J. Inorg. Chem.*, 2013, 2447–2459.
- 46 M. D. Winemiller and K. Sumpter, *Phosphorus, Sulfur Silicon Relat. Elem.*, 2008, **183**, 2309–2317.
- 47 G. R. Naumiec, L. Cai and V. W. Pike, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 225–228.
- 48 Q. Li, T. Dong, X. Liu and X. Lei, *J. Am. Chem. Soc.*, 2013, **135**, 4996–4999.
- 49 W. Qu, K. C. Catcott, K. Zhang, S. Liu, J. J. Guo, J. Ma, M. Pablo, J. Glick, Y. Xiu, N. Kenton, X. Ma, R. I. Duclos Jr. and Z. S. Zhou, *J. Am. Chem. Soc.*, 2016, **138**, 2877–2880.
- 50 A. Maruani, D. A. Richards and V. Chudasama, *Org. Biomol. Chem.*, 2016, **14**, 6165–6178.
- 51 D. M. Patterson and J. A. Prescher, *Curr. Opin. Chem. Biol.*, 2015, **28**, 141–149.
- 52 D. M. Patterson, L. A. Nazarova and J. A. Prescher, *ACS Chem. Biol.*, 2014, **9**, 592–605.
- 53 C. M. Haney, R. F. Wissner and E. J. Petersson, *Curr. Opin. Chem. Biol.*, 2015, **28**, 123–130.
- 54 C. J. Noren, S. J. Anthony-Cahill, M. C. Griffith and P. G. Schultz, *Science*, 1989, **244**, 182–188.

