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The crystal structure of isopenicillin N synthase with δ-(L-α-aminoadipoyl)-L-cysteinyl-D-methionine reveals thioether coordination to iron

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ABSTRACT

Isopenicillin N synthase (IPNS) catalyses cyclization of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N (IPN), the central step in penicillin biosynthesis. Previous studies have shown that IPNS turns over a wide range of substrate analogues in which the valine residue of its natural substrate is replaced with other amino acids. IPNS accepts and oxidizes numerous substrates that bear hydrocarbon sidechains in this position, however the enzyme is less tolerant of analogues presenting polar functionality in place of the valinyl isopropyl group. We report a new ACV analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-D-methionine (ACM), which incorporates a thioether in place of the valinyl sidechain. ACM has been synthesized using solution phase methods and crystallized with IPNS. A crystal structure has been elucidated for the IPNS:Fe(II):ACM complex at 1.40 Å resolution. This structure reveals that ACM binds in the IPNS active site such that the sulfur atom of the methionine thioether binds to iron in the oxygen binding site at a distance of 2.57 Å. The sulfur of the cysteinyl thiolate sits 2.36 Å from the metal.

Introduction

Isopenicillin N synthase (IPNS) catalyses the key step in penicillin biosynthesis: conversion of the linear tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV, **1**)[1] to bicyclic isopenicillin N (IPN, **2**) (Scheme 1) [1]. IPNS is a non-heme iron(II) oxidase (NHIO) [2,3], and is thought to generate a highly reactive iron(IV)-oxo (ferryl) species **3** during conversion of ACV to IPN [4,5].

IPNS has been the subject of considerable research interest due to the unique chemistry that it catalyses and to the clinical utility of β-lactam antibiotics [1]. A combination of solution-phase turnover experiments [6], spectroscopy [7] and more recently protein crystallography [5,8–10] has been applied to build a detailed picture of the IPNS active site and reaction mechanism.

Incubation experiments in solution have demonstrated that IPNS turns over a diverse range of analogues variant in the third position of the ACV tripeptide [6]. Analogues incorporating various hydrocarbon sidechains in place of the valinyl isopropyl group are substrates for IPNS, including AC-D-α-aminobutyrate (ACAb, **4**) [11], AC-D-isoleucine (ACI, **5**) [12], AC-D-methylcyclopropylglycine [13] and AC-D-vinylglycine [14]. In contrast, IPNS is much less accepting of polar sidechains in this position: none of AC-D-Ser **6**, AC-D-Cys **7** (both isosteres of ACAb) [6], AC-D-*O*-methylthreonine **8** (isosteric with ACI) [15], AC-D-Glu or AC-D-Asn [6] are turned over by the enzyme (Fig. 1).

δ-(L-α-Aminoadipoyl-L-cysteinyl-D-methionine (ACM, **9**) bears the thioether of methionine in place of the valinyl isopropyl group of the natural substrate ACV **1**; the interaction of ACM with IPNS has not previously been investigated. In this paper we report the synthesis of ACM **9**, crystallization of this new tripeptide with IPNS, and the crystal structure of the IPNS:Fe(II):ACM complex.

Results and discussion

Synthesis of ACM ***9***

ACM **9** was prepared from D-methionine **10** and protected dipeptide **11** [16] (Scheme 2). D-Methionine **10** was first converted to its benzhydryl ester **12** using *p*-toluenesulfonic acid to mask the

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[1] *Abbreviations used:* AC-, δ-(L-α-aminoadipoyl)-L-cysteinyl-; ACAb, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate; ACI, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-isoleucine; ACM, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-methionine; ACmC, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-*S*-methylcysteine; ACOmC, δ-(L-α-aminoadipoyl)-L-cysteinyl (1-(*S*)-carboxy-2-thiomethyl)ethyl ester; ACtaI, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-thia-*allo*-isoleucine; ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride; HOBt, 1-hydroxybenzotriazole hydrate; IPN, isopenicillin N; IPNS, isopenicillin N synthase; NHIO, non-heme iron(II) oxidase.

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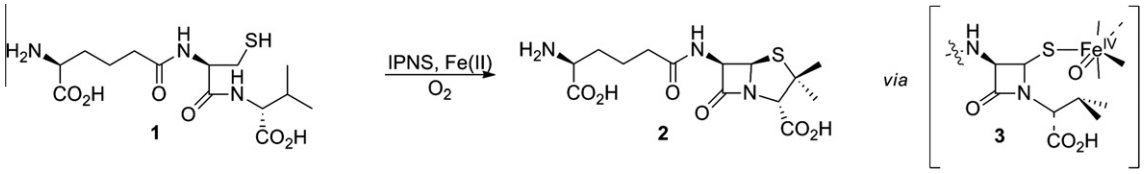


Scheme 1. The reaction of IPNS with its natural substrate ACV **1** to give IPN **2**, via a high-valent iron intermediate **3**.

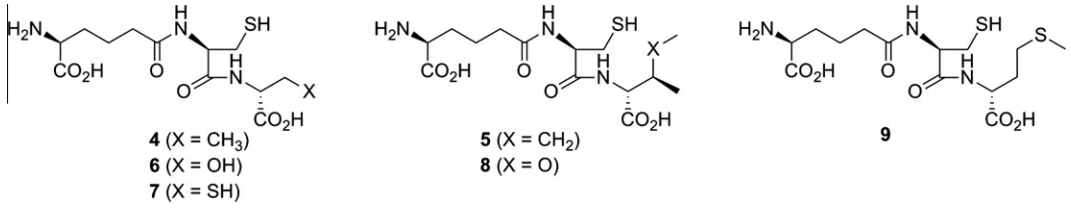
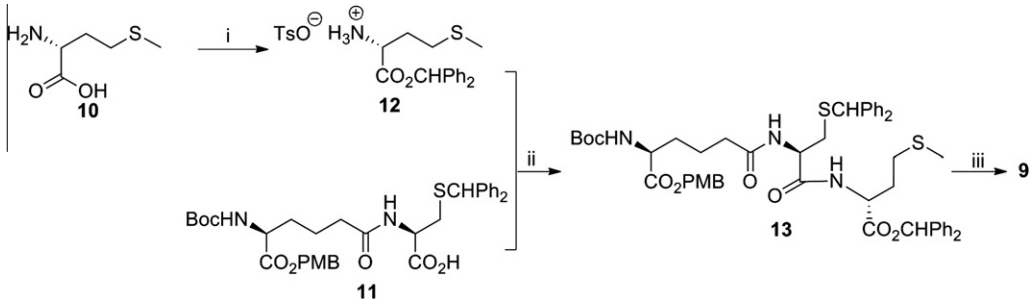


Fig. 1. Some of the many ACV analogues that have been incubated with IPNS in solution (AC-D-α-aminobutyrate (ACAb, **4**) [11], AC-D-isoleucine (ACI, **5**) [12], AC-D-Ser **6**, AC-D-Cys **7** [6] and AC-D-*O*-methylthreonine **8** [15]), and the tripeptide used in this study ACM **9**.



Scheme 2. Synthesis of tripeptide **9**. Reagents and conditions: (i) TsOH, H_2O, then Ph_2CN_2, Et_2O/MeCN, rt 3 h, 83%; (ii) EDCI, HOBt, Et_3N, CH_2Cl_2, rt 20 h, quant.; and (iii) TFA, anisole, reflux 1 h, quant.; reversed phase HPLC. Full experimental procedures and characterization data are available in the Supporting information, along with the NMR spectra of compounds **9**, **12** and **13**.

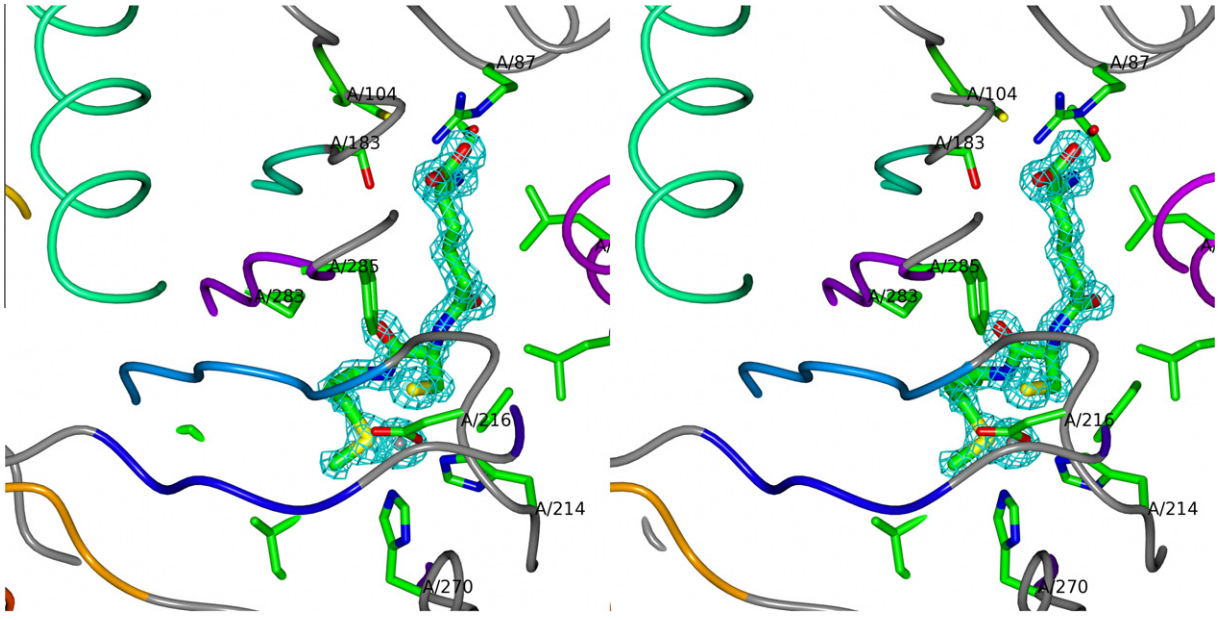


Fig. 2. Stereo image showing the active site of the anaerobic IPNS:Fe(II):ACM complex. A $2mF_o$–DF_c electron-density map is shown around the substrate analogue at 1σ.

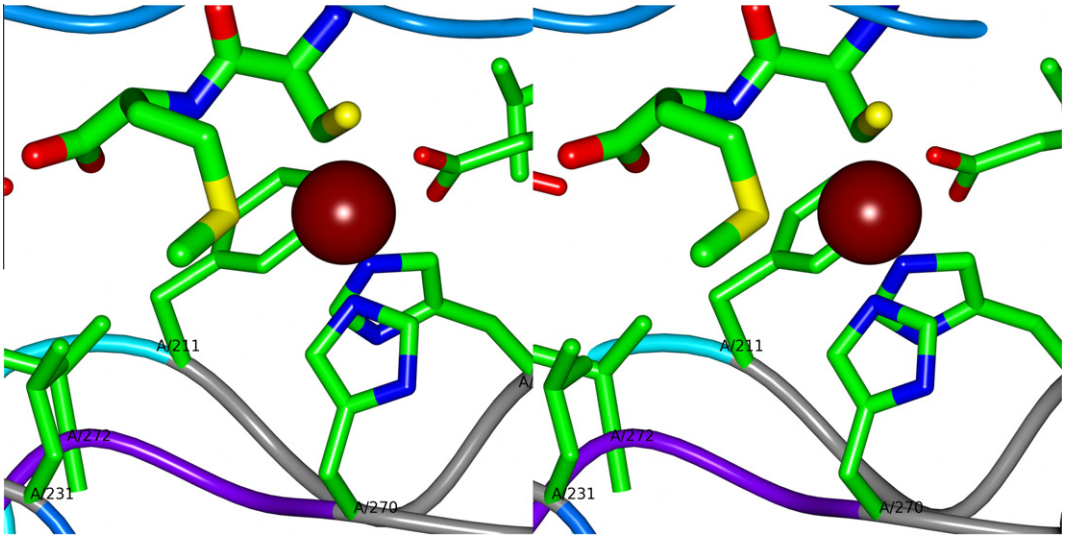


Fig. 3. Stereo image showing a close-up view of the iron binding environment in the IPNS:Fe(II):ACM complex.

Table 1
X-ray data collection and crystallographic statistics.

X-ray source	BW7B DESY, Hamburg	
Wavelength (Å)	0.8345	
PDB acquisition code	2y60	
Resolution (Å)	1.40	
Space group	$P2_12_12_1$	
Unit cell dimensions		
a (Å)	46.52	
b (Å)	70.88	
c (Å)	100.93	
Resolution shell (Å)	20.33–1.40	1.48–1.40
Total number of reflections	137,704	19,908
Number of unique reflections	55,964	8213
Completeness (%)	83.8	85.1
Average $I/(\sigma I)$	9.4	1.9
R_{merge} (%)[a]	7.9	43.0
R_{meas} (%)[b]	9.8	53.7
R_{cryst} (%)[c]	14.70	
R_{free} (%)[d]	19.93	
RMS deviation[e]	0.024, 2.1	
Average *B* factors (Å^2)[f]	11.6, 14.4, 12.0, 25.7	
Number of water molecules	386	

[a] $R_{merge} = \sum_j\sum_h|I_{h,j} - \langle I_h\rangle|/\sum_j\sum_h\langle I_h\rangle \times 100$.
[b] $R_{meas} = \sum_{hkl}\sqrt{N/(N-1)}\sum_i|I_i\,(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_I I_i\,(hkl) \times 100$ [40,41].
[c] $R_{cryst} = \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}| \times 100$.
[d] R_{free} = based on 5% of the total reflections.
[e] RMS deviation from ideality for bonds, followed by the value for angles.
[f] Average *B* factors in order: main chain; sidechain; substrate and iron; solvent (water).

amine and diphenyldiazomethane to trap the carboxylic acid [17]. Carbodiimide-promoted peptide coupling of **11** and **12** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and 1-hydroxybenzotriazole hydrate (HOBt) [18] gave the fully protected tripeptide **13**, and global deprotection with trifluoroacetic acid [19] afforded the target compound **9**. Purification by reversed-phase HPLC was required to generate material of sufficient purity for crystallization with the protein.

The structure of the IPNS:Fe(II):ACM complex

ACM **9** was crystallized with IPNS and iron(II) under anaerobic conditions following the previously reported procedure [20,21]. The crystal structure of the IPNS:Fe(II):ACM complex was solved to 1.40 Å resolution (Figs. 2 and 3, Table 1). Not unexpectedly, the overall structure of the protein is similar to the complexes of IPNS with its natural substrate [9] and substrate analogues [5,16,22–26]. ACM **9** binds to the protein in a similar manner to ACV **1** and analogues. The cysteinyl thiolate of the tripeptide ligates to iron opposite His270, and ACM is held by interactions with its amino and carboxylate groups: a salt bridge between the aminoadipoyl carboxylate and Arg87, and hydrogen bonding between the aminoadipoyl amino group and Thr331. The methionine carboxylate forms hydrogen bonds with the adjacent sidechains of Tyr189, Arg279 and Ser281.

The active site iron center is hexacoordinate. It is bound by the familiar three ligands from the protein (His214, Asp216 and His270, the defining '2-His-1-carboxylate' motif of the NHIO enzyme family [27]), a water ligand *trans* to His214, and two substrate-derived ligands. The cysteinyl thiolate of ACM ligates to iron opposite His270, and the methionine thioether binds *trans* to Asp216. The sulfur atoms are 2.36 Å (thiolate) and 2.57 Å (thioether) from iron, respectively.

In complexes of IPNS with ACV and related substrate analogues [9,22,24,26], iron is pentacoordinate: the valinyl isopropyl group sits within van der Waals contact of the metal and effectively reserves the site *trans* to Asp216 for oxygen (O_2, the co-substrate) [9]. Hexacoordinate iron has previously been observed in several IPNS:Fe(II):analogue complexes, in three general contexts. With less bulky substrate analogues such as AC-Gly, AC-D-Ala [28], AC-D-α-aminobutyrate [29] and AC-D-vinylglycine [16], a second water ligand can enter and bind to iron in the site opposite Asp216. LLL-Configured peptides and depsipeptides also bind to IPNS in a manner that permits an additional water ligand at iron, which is therefore hexacoordinate [30–32]. And sulfide ligation to iron has been observed in the complexes of IPNS with AC-D-*S*-methylcysteine (ACmC, **14**) [5], AC-(1-(*S*)-carboxy-2-thiomethyl)ethyl ester (ACOmC, **15**) [33] and AC-D-thia-*allo*-isoleucine (ACt*a*I, **16**) [34].

ACM **9** bears an additional methylene group between the α-carbon of its third residue and the methyl sulfide when compared to ACmC **14**, ACOmC **15** and ACt*a*I **16** (Fig. 4). Nonetheless IPNS easily accommodates the additional carbon atom of the methionine sidechain, which occupies a single conformation in which the two methylene groups are twisted back 'behind' the sulfur atom ('behind' in the orientation shown in Fig. 3). Although the thioether sulfur is not as tightly bound to iron as the free thiolate (sitting 2.57 Å from the metal, compared to 2.36 Å for the thiolate), the

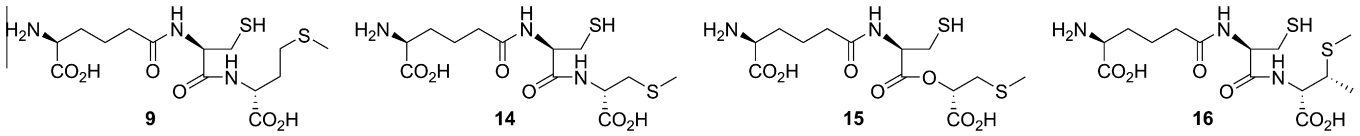


Fig. 4. ACV analogues that incorporate thioether functionality in their third position: ACM **9** (this study), AC-D-*S*-methylcysteine (ACmC, **14**) [5], AC-(1-(*S*)-carboxy-2-thiomethyl)ethyl ester (AC*O*mC, **15**) [33], AC-D-thia-*allo*-isoleucine (AC*t*a*l*, **16**) [34].

additional methylene unit between α-carbon and methyl sulfide allows closer approach of thioether to metal (2.57 Å) than is seen in the crystal structures of IPNS with ACmC **14**, AC*O*mC **15** (both 2.69 Å) [5,33] and AC*t*a*l* **16** (2.66 Å) [34].

Conclusions

The new tripeptide ACM **9** binds in the active site of IPNS, its cysteinyl thiolate tethered to iron in the site opposite His270, its methionyl thioether ligated opposite Asp216. This second substrate-derived ligand sits in the oxygen binding site, and renders the metal hexacoordinate. It is probable that the thioether would block oxygen from binding, preventing reaction under normal turnover conditions. This result offers further structural evidence for the failure of IPNS to turn over substrate analogues such as AC-D-Ser **6**, AC-D-Cys **7** and AC-D-*O*-methylthreonine **8**, which include polar amino acids in place of the valine residue of ACV **1**.

Experimental

Synthesis of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-methionine **9**

D-Methionine **10** was protected as the benzhydryl ester **12** using *p*-toluenesulfonic acid and diphenyldiazomethane [17], then coupled to previously reported dipeptide **11** [16] using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and 1-hydroxybenzotriazole hydrate (HOBt) under standard conditions [18]. Global deprotection with trifluoroacetic acid [19] and purification by reversed-phase HPLC [octadecylsilane 250 mm × 10 mm; 10 mM NH_4HCO_3 in water/methanol as eluant, running time: 0–6 min, 2.5%; 6–14 min, 25%; 14–20 min, 2.5% methanol, v/v; 4 mL min^{-1}; λ = 254 nm, five absorbance units full scale (AUFS); retention time 10.5–12 min] gave purified tripeptide **9**; δ_H (500 MHz, D_2O): 1.54–1.65 (2H, m, $^+H_3NCHCH_2C\underline{H}_2CH_2$), 1.72–1.81 (2H, m, $^+H_3NCHC\underline{H}_2CH_2CH_2$), 1.84–1.92 (1H, m, 1 of $C\underline{H}_2SCH_3$), 1.98 (3H, s, $SC\underline{H}_3$), 2.00–2.07 (1H, m, 1 of $C\underline{H}_2SCH_3$), 2.29 (2H, td, *J* 7.0, 2.5, $^+H_3NCHCH_2CH_2C\underline{H}_2$), 2.40 (1H, A of ABX, *J* 13.5, 7.5, 1 of $C\underline{H}_2SH$), 2.47 (1H, B of ABX, *J* 13.5, 5.0, 1 of $C\underline{H}_2SH$), 2.79 (2H, dd, *J* 7.5, 2.0, $CHC\underline{H}_2CH_2SCH_3$), 3.64 (1H, t, *J* 6.0, $^+H_3NC\underline{H}CH_2CH_2CH_2$), 4.31 (1H, *J* 9.0, 4.5, $NHC\underline{H}CH_2SH$), 4.41 (1H, t, *J* 6.5, $NHC\underline{H}CH_2CH_2SCH_3$).

Full experimental procedures and characterization data are available in the Supporting information, along with the NMR spectra of compounds **9**, **12** and **13**.

Crystallography and structure determination

Crystals of the IPNS:Fe(II):ACM complex were grown under anaerobic conditions as previously reported [20,21]. Crystals suitable for X-ray diffraction were selected using a light microscope, removed from the anaerobic environment and exchanged into cryoprotectant buffer (a 1:1 mixture of well buffer and saturated lithium sulfate in 40% v/v glycerol), then flash-frozen in liquid nitrogen [20,21].

Data were collected at Beamline BW7B of the Deutsches Elektronen-Synchrotron (DESY), Hamburg, Germany, and the temperature was maintained at 100 K using an Oxford Cryosystems Cryostream. Data were processed using MOSFLM [35] and the CCP4 suite of programs [36], then refined using REFMAC5 [37] and Coot for model building [38]. Initial phases were generated using co-ordinates for the protein from the previously published IPNS:Fe(II):ACV structure [9], and manual rebuilding of protein sidechains was performed as necessary. Crystallographic coordinates and structure factors have been deposited in the Worldwide Protein Data Bank under accession numbers 2y60. Figs. 2 and 3 were prepared using CCP4mg [39].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2011.09.014.

References

[1] J.E. Baldwin, C.J. Schofield, The biosynthesis of β-lactams, in: M.I. Page (Ed.), The Chemistry of β-Lactams, Blackie, Glasgow, 1992, pp. 1–78.
[2] M. Costas, M.P. Mehn, M.P. Jensen, L. Que, Chem. Rev. 104 (2004) 939–986.
[3] P.C.A. Bruijnincx, G. van Koten, R. Gebbink, Chem. Soc. Rev. 37 (2008) 2716–2744.
[4] J.E. Baldwin, Recent advances in the chemistry of the β-lactam antibiotics, in: A.G. Brown, S.M. Roberts (Eds.), Special Publication No. 52, The Royal Society of Chemistry, London, 1985, pp. 62–85.
[5] N.I. Burzlaff, P.J. Rutledge, I.J. Clifton, C.M.H. Hensgens, M. Pickford, R.M. Adlington, P.L. Roach, J.E. Baldwin, Nature 401 (1999) 721–724.
[6] J.E. Baldwin, M. Bradley, Chem. Rev. 90 (1990) 1079–1088.
[7] R.D.G. Cooper, Bioorg. Med. Chem. 1 (1993) 1–17.
[8] P.L. Roach, I.J. Clifton, V. Fulop, K. Harlos, G.J. Barton, J. Hajdu, I. Andersson, C.J. Schofield, J.E. Baldwin, Nature 375 (1995) 700–704.
[9] P.L. Roach, I.J. Clifton, C.M.H. Hensgens, N. Shibata, C.J. Schofield, J. Hadju, J.E. Baldwin, Nature 387 (1997) 827–830.
[10] A.C. Stewart, I.J. Clifton, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, ChemBioChem 8 (2007) 2003–2007.
[11] J.E. Baldwin, E.P. Abraham, R.M. Adlington, B. Chakravarti, A.E. Derome, J.A. Murphy, L.D. Field, N.B. Green, H.-H. Ting, J.J. Usher, J. Chem. Soc. Chem. Commun. (1983) 1317–1319.
[12] G.A. Bahadur, J.E. Baldwin, J.J. Usher, E.P. Abraham, G.S. Jayatilake, R.L. White, J. Am. Chem. Soc. 103 (1981) 7650–7651.
[13] J.E. Baldwin, R.M. Adlington, B.P. Domayne-Hayman, G. Knight, H.-H. Ting, J. Chem. Soc. Chem. Commun. (1987) 1661–1663.
[14] J.E. Baldwin, R.M. Adlington, A. Basak, S.L. Flitsch, A.K. Forrest, H.-H. Ting, J. Chem. Soc. Chem. Commun. (1986) 273–275.
[15] J.E. Baldwin, R.M. Adlington, A. Basak, S.L. Flitsch, S. Petursson, N.J. Turner, H.-H. Ting, J. Chem. Soc. Chem. Commun. (1986) 975–976.
[16] J.M. Elkins, P.J. Rutledge, N.I. Burzlaff, I.J. Clifton, R.M. Adlington, P.L. Roach, J.E. Baldwin, Org. Biomol. Chem. 1 (2003) 1455–1460.
[17] S. Wolfe, M.G. Jokinen, Can. J. Chem. 57 (1979) 1388–1396.
[18] J.C. Sheehan, J. Preston, P.A. Cruickshank, J. Am. Chem. Soc. 87 (1965) 2492–2493.
[19] M. Bodanzky, A. Bodanzky, The practice of peptide synthesis, in: K. Hafner, C.W. Rees, B.M. Trost, J.M. Lehn, P.v.R. Schleyer, R. Zahruchik (Eds.), Reactivity and Structure Concepts in Organic Chemistry, Springer-Verlag, Berlin, 1984, pp. 172–173.

[20] P.L. Roach, I.J. Clifton, C.M.H. Hensgens, N. Shibata, A.J. Long, R.W. Strange, S.S. Hasnain, C.J. Schofield, J.E. Baldwin, J. Hajdu, Eur. J. Biochem. 242 (1996) 736–740.
[21] P.J. Rutledge, N.I. Burzlaff, J.M. Elkins, M. Pickford, J.E. Baldwin, P.L. Roach, Anal. Biochem. 308 (2002) 265–268.
[22] J.M. Ogle, I.J. Clifton, P.J. Rutledge, J.M. Elkins, N.I. Burzlaff, R.M. Adlington, P.L. Roach, J.E. Baldwin, Chem. Biol. 8 (2001) 1231–1237.
[23] A.R. Grummitt, P.J. Rutledge, I.J. Clifton, J.E. Baldwin, Biochem. J. 382 (2004) 659–666.
[24] A. Daruzzaman, I.J. Clifton, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, ChemBioChem 7 (2006) 351–358.
[25] A.R. Howard-Jones, J.M. Elkins, I.J. Clifton, P.L. Roach, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, Biochemistry 46 (2007) 4755–4762.
[26] W. Ge, I.J. Clifton, A.R. Howard-Jones, J.E. Stok, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, ChemBioChem 10 (2009) 2025–2031.
[27] E.L. Hegg, L. Que Jr., Eur. J. Biochem. 250 (1997) 625–629.
[28] A.J. Long, I.J. Clifton, P.L. Roach, J.E. Baldwin, P.J. Rutledge, C.J. Schofield, Biochemistry 44 (2005) 6619–6628.
[29] A.J. Long, I.J. Clifton, P.L. Roach, J.E. Baldwin, C.J. Schofield, P.J. Rutledge, Biochem. J. 372 (2003) 687–693.
[30] A.R. Howard-Jones, P.J. Rutledge, I.J. Clifton, R.M. Adlington, J.E. Baldwin, Biochem. Biophys. Res. Commun. 336 (2005) 702–708.
[31] W. Ge, I.J. Clifton, J.E. Stok, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, Org. Biomol. Chem. 8 (2010) 122–127.
[32] W. Ge, I.J. Clifton, J.E. Stok, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, Biochem. Biophys. Res. Commun. 398 (2010) 659–664.
[33] W. Ge, I.J. Clifton, J.E. Stok, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, J. Am. Chem. Soc. 130 (2008) 10096–10102.
[34] I.J. Clifton, W. Ge, R.M. Adlington, J.E. Baldwin, P.J. Rutledge, ChemBioChem 12 (2011) 1881–1885.
[35] A.G.W. Leslie, Acta Cryst. D 55 (1999) 1696–1702.
[36] Collaborative Crystallography Project Number 4, Acta Cryst. D 50 (1994) 760–763.
[37] G.N. Murshudov, A.A. Vagin, E.J. Dodson, Acta Cryst. D 53 (1997) 240–255.
[38] P. Emsley, K. Cowtan, Acta Cryst. D 60 (2004) 2126–2132.
[39] L. Potterton, S. McNicholas, E. Krissinel, J. Gruber, K. Cowtan, P. Emsley, G.N. Murshudov, S. Cohen, A. Perrakis, M. Noble, Acta Cryst. D 60 (2004) 2288–2294.
[40] K. Diederichs, P.A. Karplus, Nat. Struct. Biol. 4 (1997) 269–275.
[41] M.S. Weiss, J. Appl. Cryst. 34 (2001) 130–135.