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Isopenicillin N Synthase Binds δ-(L-α-Aminoadipoyl)-L-Cysteinyl-D-Thia-*allo*-Isoleucine through both Sulfur Atoms

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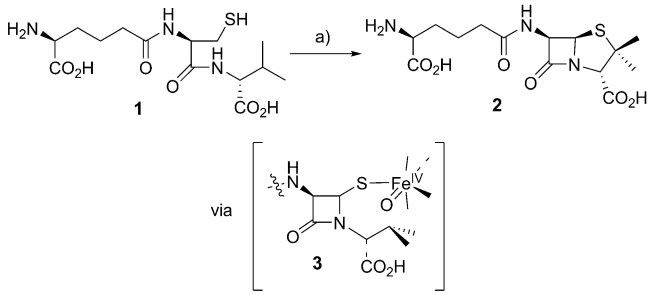
Isopenicillin N synthase (IPNS) catalyses the synthesis of isopenicillin N (IPN), the biosynthetic precursor to penicillin and cephalosporin antibiotics. IPNS is a non-heme iron(II) oxidase that mediates the oxidative cyclisation of the tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV) to IPN with a concomitant reduction of molecular oxygen to water. Solution-phase incubation experiments have shown that, although IPNS can turn over analogues with a diverse range of hydrocarbon side chains in the third (valinyl) position of its substrate, the enzyme is much less tolerant of polar residues in this position. Thus, although IPNS converts δ-L-α-aminoadipoyl-L-cysteinyl-D-isoleucine (ACI) and AC-D-*allo*-isoleucine (AC*a*I) to penam products, the isosteric sulfur-containing peptides AC-D-thiaisoleucine (ACtI) and AC-D-thia-*allo*-isoleucine (ACt*a*I) are not turned over. To determine why these peptides are not substrates, we crystallized ACt*a*I with IPNS. We report the synthesis of ACt*a*I and the crystal structure of the IPNS:Fe^{II}:ACt*a*I complex to 1.79 Å resolution. This structure reveals direct ligation of the thioether side chain to iron: the sulfide sulfur sits 2.66 Å from the metal, squarely in the oxygen binding site. This result articulates a structural basis for the failure of IPNS to turn over these substrates.

Introduction

Isopenicillin N synthase (IPNS) mediates the oxidative bicyclisation of δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV, **1**) to isopenicillin N (IPN, **2**), the central step in penicillin biosynthesis (Scheme 1).[1] IPNS is a non-heme iron(II) oxidase (NHIO) and



Scheme 1. The reaction of IPNS with its natural substrate ACV (**1**) to give IPN (**2**), via the high-valent iron intermediate **3**. a) IPNS, Fe^{II}, O_2.

reduces one molecule of oxygen to water while oxidizing ACV to IPN. NHIOs are thought to generate highly reactive iron(IV)-oxo (ferryl) species in their reaction cycles,[2,3] and there is good evidence that the conversion of ACV to IPN proceeds via an iron(IV)-oxo intermediate, **3**.[4,5] IPNS has attracted much attention over many years, due to the unique chemistry of the reaction that it catalyses and the clinical significance of the product that it forms.[1,5–9]

Solution-phase incubation studies show that IPNS tolerates considerable variation in the third residue of its tripeptide substrate ACV (**1**):[6] analogues incorporating a wide variety of hydrocarbon side chains in place of the isopropyl group of D-valine are turned over by IPNS. However IPNS is much less tolerant of polar side chains and heteroatoms in this position.[6] Thus, whereas δ-L-α-aminoadipoyl-L-cysteinyl-D-isoleucine (ACI, **4**) and AC-D-*allo*-isoleucine (AC*a*I, **5**) are converted by IPNS to the penam products **6** and **7** respectively (Scheme 2),[10–12] the isosteric sulfur-containing peptides AC-D-thiaisoleucine (ACtI, **8**) and AC-D-thia-*allo*-isoleucine (ACt*a*I, **9**) are not turned over.[6] To investigate the failure of IPNS to turn over such substrates, we have synthesised the tripeptide ACt*a*I **9**, crystallised it with IPNS, and solved the structure of the resulting complex.

Results and Discussion

Synthesis of ACt*a*I 9

ACt*a*I **9** was prepared from D-threonine (**10**) and protected dipeptide **15** (Scheme 3). D-Threonine was converted to (2*S*,3*R*)-methyl 3-(acetylthio)-2-(*tert*-butoxycarbonylamino)butanoate (**11**) as reported by Gross and co-workers.[13] The key step in this sequence is nucleophilic displacement of a tosylate leaving group by thioacetate. The thioester of **11** was cleaved by transesterification in alkaline methanol, and the resulting thiolate

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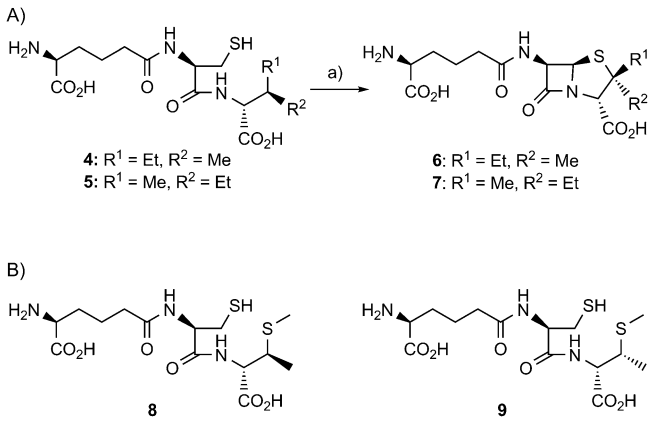
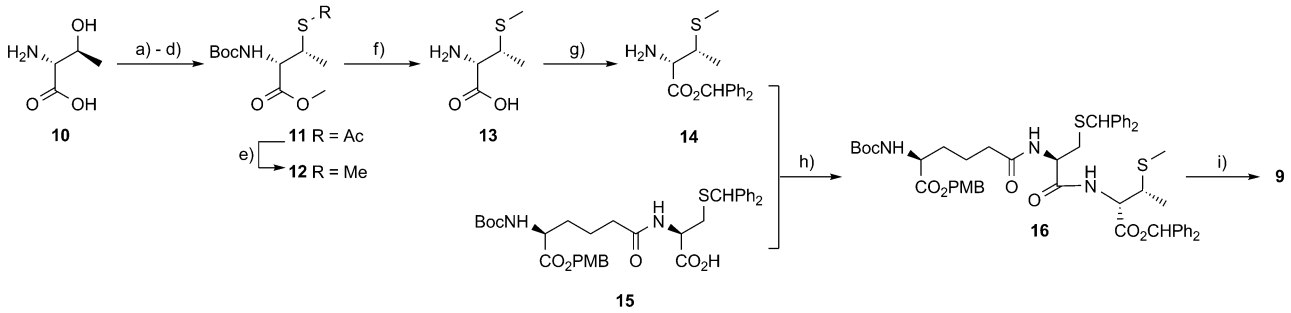


Scheme 2. A) The reaction of ACI (**4**) with IPNS gives penam **6**, while its epimer AC*a*I **5** gives the epimeric penam **7**.[10,11] (ACI also gives rise to small amounts of two epimeric 2-methyl-cepham products, in ca. 10% abundance relative to penam **6**.[12]); a) IPNS, Fe^{II}, O_2. B) In contrast, the isosteric sulfur-containing peptides ACtI (**8**) and ACt*a*I (**9**) are not turned over by IPNS.[6]

was trapped with methyl iodide to afford the methyl sulfide **12**. Two-step deprotection (neat TFA followed by 2.5 mol L^{-1} aqueous HCl) afforded the amino acid **13** in 13% yield over six steps from D-threonine. Protection of **13** as its benzhydryl ester **14**[14] and coupling to the previously reported dipeptide **15**[15] gave the fully protected tripeptide **16**. Global deprotection with trifluoroacetic acid[16] gave ACt*a*I **9** as its TFA salt, from which the pure tripeptide was obtained by reversed-phase HPLC.

Structure of the IPNS:Fe^{II}:ACt*a*I complex

Crystals of IPNS:Fe^{II}:ACt*a*I were grown according to the previously reported procedure.[17,18] The structure of the complex was solved to 1.79 Å resolution (Figures 1 and 2, Table 1). As is to be expected, the overall structure of the protein is not significantly different from that of the IPNS:Fe^{II}:ACV complex,[9] and the substrate analogue ACt*a*I binds to the active-site region in a similar orientation to the native substrate ACV and other substrate analogues.[5,9,15,19–24] Thus, the tripeptide is bound to iron through its cysteinyl thiolate, and tethered by interactions between its amino and carboxylate groups and the protein. The aminoadipoyl terminus forms a salt bridge from its carboxylate to protein residue Arg87, and hydrogen



Scheme 3. Synthesis of tripeptide **9**. Reagents and conditions: a) CH_3OH, $HCl_{(g)}$, 0 °C to RT, 23 h, 100%; b) $(Boc)_2O$, Et_3N, CH_2Cl_2, RT, 48 h, 77%; c) TsCl, DMAP, pyridine, 0–4 °C, 48 h, 68%; d) CH_3COSK, DMF, RT, 48 h, 78%; e) CH_3OH, 0.2 M $NaOH_{(aq)}$, CH_3I, RT, 2 h, 67%; f) TFA, RT, 30 min, then 2.5 M $HCl_{(aq)}$, reflux, 4 h, then DOWEX ion exchange, 48%; g) i: TsOH·H_2O, H_2O, RT, 5 min; ii: Ph_2CN_2, CH_3CN/Et_2O, RT, 4 h, 86%; h) Et_3N, EDCI, HOBt, CH_2Cl_2, RT, 48 h, 99%; i) TFA/anisole, reflux, 60 min, quantitative, then RP-HPLC. For details of steps a–d, see ref. [13], for details of steps e–i, see the Supporting Information.

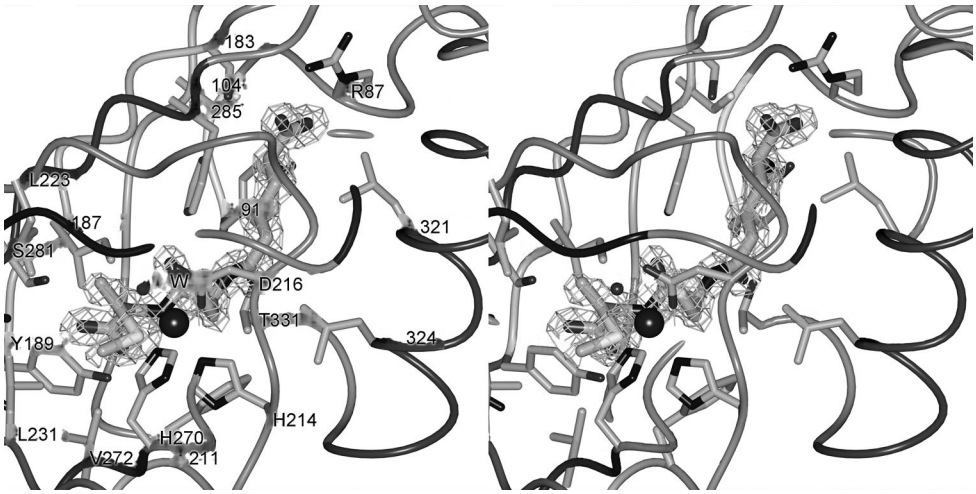


Figure 1. Stereo image showing the active site of the anaerobic IPNS:Fe^{II}:ACt*a*I complex. The large dark-grey sphere is iron(II); a $2mF_o-DF_c$ electron-density map is shown around the substrate analogue at 1σ. A colour version of this figure is available in the Supporting Information.

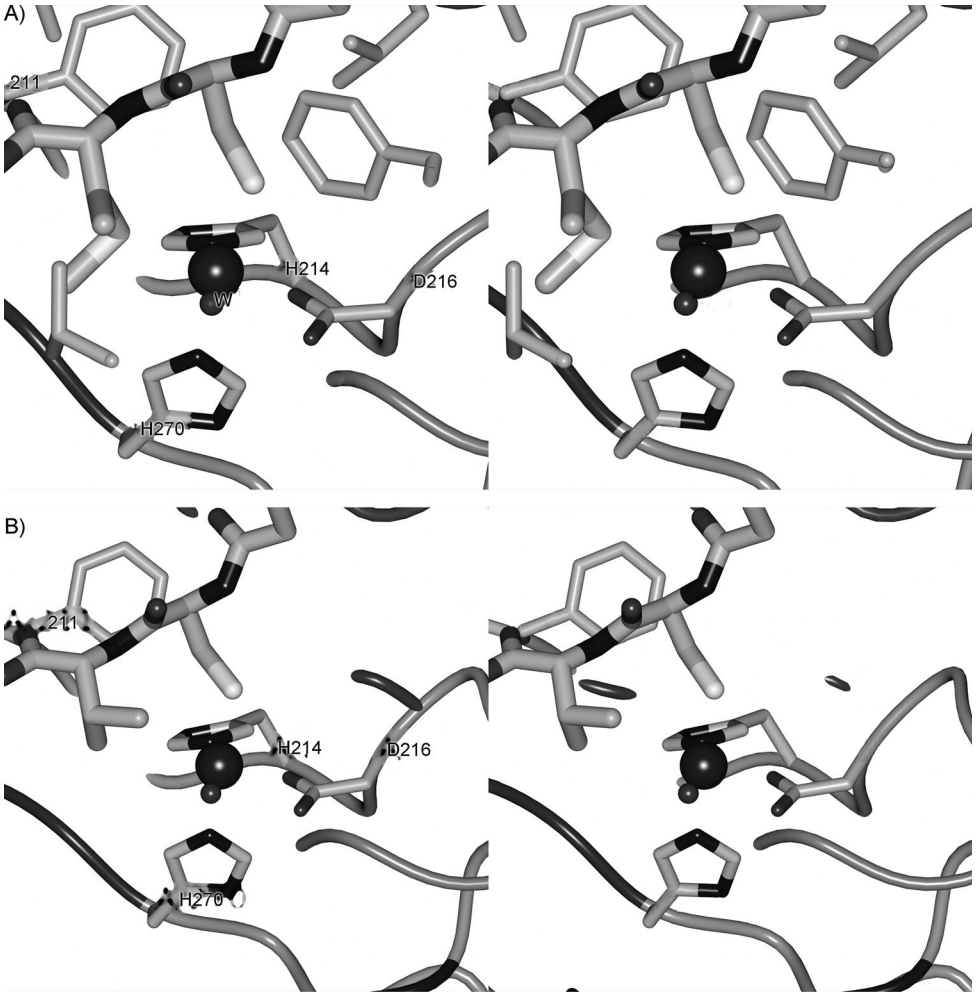


Figure 2. Stereo images showing close-ups of the iron binding environment in A) the IPNS:Fe^{II}:ACt*a*l complex, and B) the IPNS:Fe^{II}:ACV complex The large dark-grey sphere is iron(II). A colour version of this figure is available in the Supporting Information.

bonds from its amino group to Thr331. At the other end of the tripeptide, the thia-*allo*-isoleucine carboxylate is well positioned to hydrogen bond to several protein side chains, in particular Tyr189, Arg279 and Ser281.

However the active-site metal in the IPNS:Fe^{II}:ACt*a*l structure is hexacoordinate, a marked contrast to the IPNS:Fe^{II}:ACV complex in which iron is pentacoordinate. There are three ligands from the protein, His214, Asp216 and His270 (the defining "2-His-1-carboxylate" motif of enzymes in the NHIO family)[25] and a single water molecule opposite His214, plus two ligands from the substrate, the cysteinyl thiolate *trans* to His270, and the methylsulfide opposite Asp216, coordinated to iron at a distance of 2.66 Å (compared to 2.40 Å for the thiolate). In the IPNS:Fe^{II}:ACV complex, the valinyl isopropyl group sits in van der Waals contact with the metal, held by interactions with the side chains of Leu231, Val272, Pro283 and Leu223.[9] The valinyl isopropyl group thereby masks the site opposite Asp216, effectively reserving it for the dioxygen cosubstrate to bind and initiate the oxidative reaction.

Sulfide ligation to iron has previously been observed in the IPNS complexes with AC-D-*S*-methylcysteine (ACmC)[5] and δ-L-α-aminoadipoyl-L-cysteine (1-(*S*)-carboxy-2-thiomethyl)ethyl ester (AC*O*mC).[26] The iron centre is also hexacoordinate in IPNS complexes with less sterically demanding substrate analogues such as AC-Gly, AC-D-Ala and AC-D-α-aminobutyrate,[27,28] in which the smaller side chain allows a water molecule to bind iron in this site. Several LLL-configured substrate analogues have also recently been shown to bind in the IPNS active site in a way that allows an additional water ligand at iron, which is consequently hexacoordinate.[29–31]

Conclusions

It is evident that the affinity of sulfur for iron overrides any steric pressure caused by the branching methyl group on the thia-*allo*-isoleucine side chain, such that this side chain can twist as required to bring the second sulfur close to the metal. The branching methyl group on the β-carbon of this residue sits in a space that is occupied by the β-hydrogen atom of D-valine in the IPNS:Fe^{II}:ACV complex.[9] The methyl sulfide side chain of ACt*a*l **9** binds to iron in the putative oxygen binding site opposite Asp216. Presumably the additional ligand locks into this coordination site and blocks the cosubstrate from binding, preventing reaction under normal turnover condi-

Table 1. X-ray data collection and crystallographic statistics.

X-ray source	SRS, Daresbury, UK	
wavelength [Å]	1.488	
PDB ID	2y6f	
resolution [Å]	1.79	
space group	$P2_12_12_1$	
unit cell dimensions		
a [Å]	47.40	
b [Å]	73.35	
c [Å]	102.48	
resolution shell [Å]	19.9–1.79	1.89–1.79
total number of reflections	55650	7838
number of unique reflections	22362	3256
completeness [%]	91.3	93.9
average $I/\sigma[I]$	7.4	2.6
R_{merge} [%][a]	11.8	28.7
R_{meas} [%][b]	15.2	37.7
R_{cryst} [%][c]	17.80	
R_{free} [%][d]	22.01	
RMS deviation[e]	0.033, 1.9	
average *B* factors [Å^2][f]	19.1, 22.1, 13.3, 12.9	
number of water molecules	296	

[a] $R_{merge}=\Sigma_j\Sigma_h|I_{hj}-<I_h>|/\Sigma_j\Sigma_h<I_h>\times 100$; [b] $R_{meas}=\Sigma_{hkl}\sqrt{N/(N-1)}\ \Sigma_i\ |I_i(hkl)-I\ (hkl)|/\Sigma_{hkl}\Sigma_i I_i(hkl)\times 100$;[38,39] [c] $R_{cryst}=\Sigma||F_{obs}|-|F_{calcd}||/\Sigma|F_{obs}|\times 100$; [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; side chain; substrate and iron; solvent (water).

tions. This result provides a structural basis to explain the failure of IPNS to turn over substrate analogues such as ACtI **8** and ACt*a*I **9** that include polar valine analogues in the third position.

Experimental Section

Synthesis of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-thia-*allo*-isoleucine 9: D-Threonine (**10**) was converted to D-thia-*allo*-isoleucine ((2*S*,3*R*)-2-amino-3-(methylthio)butanoic acid, **13**) in six steps (Scheme 3); for details of steps a–d see ref. [13], for details of steps e and f see the Supporting Information. The amino acid **13** was protected as its benzhydryl ester **14** following Wolfe's protocol[14] and coupled to known dipeptide **15**[15] by using EDCI and HOBt.[32] TFA-mediated deprotection[16] and HPLC purification (octadecylsilane 250×10 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)) afforded **9** as a flocculent white powder; ^{1}H NMR (500 MHz, D_2O): δ = 1.14 (d, *J* = 7.0 Hz, 3H; $CHCH_3$), 1.59–1.74 (m, 2H; $CHCH_2CH_2CH_2$), 1.78–1.90 (m, 2H; $CHCH_2CH_2CH_2$), 2.08 (s, 3H; SCH_3), 2.38 (t, *J* = 7.0 Hz, 2H; $CHCH_2CH_2CH_2$), 2.88 (A of ABX, J_{AB} = 14.0 Hz, J_{AX} = 7.0 Hz, 1H; 1 of CH_2SH), 2.93 (B of ABX, J_{BA} = 14.0 Hz, J_{BX} = 5.5 Hz, 1H; 1 of CH_2SH), 3.28 (qd, *J* = 7.0, 4.0 Hz, 1H; $CHSCH_3$), 3.69 (m, 1H; $CHCH_2CH_2CH_2$), 4.51 (d, *J* = 4.0 Hz, 1H; $NHCHCHSCH_3$), 4.59 (X of ABX, J_{XA} = 7.0 Hz, J_{XB} = 5.5 Hz, 1H; $NHCHCH_2SH$). Further details of the protection, coupling and deprotection steps are available in the Supporting Information.

Crystallography and structure determination: Crystals of the IPNS:Fe^{II}:ACt*a*I complex were grown under anaerobic conditions, as previously reported.[17,18] Crystals suitable for X-ray diffraction were selected by 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% glycerol, *v*/*v*), then flash-frozen in liquid nitrogen.

Data were collected at the Synchrotron Radiation Source (SRS), Daresbury, UK, and the temperature was maintained at 100 K by using an Oxford Cryosystems Cryostream. Data were processed by using Denzo[33] and the CCP4 suite of programs,[34] then refined by using REFMAC5[35] and Coot for model building.[36] Initial phases were generated by using co-ordinates for the protein from the previously published IPNS:Fe^{II}:ACV structure,[9] and manual rebuilding of protein side chains was performed as necessary. Crystallographic coordinates and structure factors have been deposited in the Protein Data Bank under accession number 2y6f. Figures 1 and 2 were prepared with CCP4mg.[37]

Abbreviations: AC- = δ-L-α-aminoadipoyl-L-cysteinyl-, ACtI = δ-L-α-aminoadipoyl-L-cysteinyl-D-thiaisoleucine, ACt*a*I = δ-L-α-aminoadipoyl-L-cysteinyl-D-thia-*allo*-isoleucine, ACmC = δ-L-α-aminoadipoyl-L-cysteinyl-D-*S*-methylcysteine, AC*O*mC = δ-L-α-aminoadipoyl-L-cysteinyl (1-(*S*)-carboxy-2-thiomethyl)ethyl ester, ACV = δ-α-aminoadipoyl-cysteinyl-valine, DMAP = 4-dimethylaminopyridine, EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride, HOBt = 1-hydroxybenzotriazole hydrate, IPN = isopenicillin N, IPNS = isopenicillin N synthase, NHIO = non-heme iron(II) oxidase, TFA = trifluoroacetic acid, Ts = tosyl.

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[1] J. E. Baldwin, C. J. Schofield in *The Chemistry of β-Lactams* (Ed.: M. I. Page), Blackie, Glasgow, **1992**, p. 1.
[2] M. Costas, M. P. Mehn, M. P. Jensen, L. Que, *Chem. Rev.* **2004**, *104*, 939.
[3] P. C. A. Bruijnincx, G. van Koten, R. Gebbink, *Chem. Soc. Rev.* **2008**, *37*, 2716.
[4] J. E. Baldwin in *Special Publication No. 52* (Eds.: A. G. Brown, S. M. Roberts), The Royal Society of Chemistry, London, **1985**, p. 62.
[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* **1999**, *401*, 721.
[6] J. E. Baldwin, M. Bradley, *Chem. Rev.* **1990**, *90*, 1079.
[7] R. D. G. Cooper, *Bioorg. Med. Chem.* **1993**, *1*, 1.
[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* **1995**, *375*, 700.
[9] P. L. Roach, I. J. Clifton, C. M. H. Hensgens, N. Shibata, C. J. Schofield, J. Hadju, J. E. Baldwin, *Nature* **1997**, *387*, 827.
[10] G. Bahadur, J. E. Baldwin, L. D. Field, E.-M. M. Lehtonen, J. J. Usher, C. A. Vallejo, E. P. Abraham, R. L. White, *J. Chem. Soc. Chem. Commun.* **1981**, 917.
[11] G. A. Bahadur, J. E. Baldwin, J. J. Usher, E. P. Abraham, G. S. Jayatilake, R. L. White, *J. Am. Chem. Soc.* **1981**, *103*, 7650.
[12] J. E. Baldwin, R. M. Adlington, N. J. Turner, B. P. Domayne-Hayman, H.-H. Ting, A. E. Derome, J. A. Murphy, *J. Chem. Soc. Chem. Commun.* **1984**, 1167.
[13] J. L. Morell, P. Fleckenstein, E. Gross, *J. Org. Chem.* **1977**, *42*, 355.
[14] S. Wolfe, M. G. Jokinen, *Can. J. Chem.* **1979**, *57*, 1388.
[15] J. M. Elkins, P. J. Rutledge, N. I. Burzlaff, I. J. Clifton, R. M. Adlington, P. L. Roach, J. E. Baldwin, *Org. Biomol. Chem.* **2003**, *1*, 1455.

[16] M. Bodanzky, A. Bodanzky in *Reactivity and Structure Concepts in Organic Chemistry, Vol. 21* (Eds.: K. Hafner, C. W. Rees, B. M. Trost, J.-M. Lehn, P. v. R. Schleyer, R. Zahruchik), Springer, Berlin, **1984**, p. 172.
[17] 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.* **1996**, *242*, 736.
[18] P. J. Rutledge, N. I. Burzlaff, J. M. Elkins, M. Pickford, J. E. Baldwin, P. L. Roach, *Anal. Biochem.* **2002**, *308*, 265.
[19] 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.* **2001**, *8*, 1231.
[20] A. R. Grummitt, P. J. Rutledge, I. J. Clifton, J. E. Baldwin, *Biochem. J.* **2004**, *382*, 659.
[21] A. Daruzzaman, I. J. Clifton, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *ChemBioChem* **2006**, *7*, 351.
[22] A. C. Stewart, I. J. Clifton, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *ChemBioChem* **2007**, *8*, 2003.
[23] A. R. Howard-Jones, J. M. Elkins, I. J. Clifton, P. L. Roach, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *Biochemistry* **2007**, *46*, 4755.
[24] W. Ge, I. J. Clifton, A. R. Howard-Jones, J. E. Stok, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *ChemBioChem* **2009**, *10*, 2025.
[25] E. L. Hegg, L. Que Jr. , *Eur. J. Bioch.* **1997**, *250*, 625.
[26] W. Ge, I. J. Clifton, J. E. Stok, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *J. Am. Chem. Soc.* **2008**, *130*, 10096.
[27] A. J. Long, I. J. Clifton, P. L. Roach, J. E. Baldwin, C. J. Schofield, P. J. Rutledge, *Biochem. J.* **2003**, *372*, 687.
[28] A. J. Long, I. J. Clifton, P. L. Roach, J. E. Baldwin, P. J. Rutledge, C. J. Schofield, *Biochemistry* **2005**, *44*, 6619.
[29] A. R. Howard-Jones, P. J. Rutledge, I. J. Clifton, R. M. Adlington, J. E. Baldwin, *Biochem. Biophys. Res. Commun.* **2005**, *336*, 702.
[30] W. Ge, I. J. Clifton, J. E. Stok, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *Org. Biomol. Chem.* **2010**, *8*, 122.
[31] W. Ge, I. J. Clifton, J. E. Stok, R. M. Adlington, J. E. Baldwin, P. J. Rutledge, *Biochem. Biophys. Res. Commun.* **2010**, *398*, 659.
[32] J. C. Sheehan, J. Preston, P. A. Cruickshank, *J. Am. Chem. Soc.* **1965**, *87*, 2492.
[33] Z. Otwinowski, W. Minor, *Methods Enzymol.* **1997**, *276*, 307.
[34] Collaborative Crystallography Project Number 4, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1994**, *50*, 760.
[35] G. N. Murshudov, A. A. Vagin, E. J. Dodson, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **1997**, *53*, 240.
[36] P. Emsley, K. Cowtan, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 2126.
[37] L. Potterton, S. McNicholas, E. Krissinel, J. Gruber, K. Cowtan, P. Emsley, G. N. Murshudov, S. Cohen, A. Perrakis, M. Noble, *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2004**, *60*, 2288.
[38] K. Diederichs, P. A. Karplus, *Nat. Struct. Biol.* **1997**, *4*, 269.
[39] M. S. Weiss, *J. Appl. Crystallogr.* **2001**, *34*, 130.

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