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The crystal structure of an isopenicillin N synthase complex with an ethereal substrate analogue reveals water in the oxygen binding site

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ABSTRACT

Isopenicillin N synthase (IPNS) is a non-heme iron oxidase central to the biosynthesis of β-lactam antibiotics. IPNS converts the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N while reducing molecular oxygen to water. The substrate analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-*O*-methyl-D-threonine (ACmT) is not turned over by IPNS. Epimeric δ-(L-α-aminoadipoyl)-L-cysteinyl-*O*-methyl-D-*allo*-threonine (ACm*a*T) is converted to a bioactive penam product. ACmT and ACm*a*T differ from each other only in the stereochemistry at the β-carbon atom of their third residue. These substrates both contain a methyl ether in place of the isopropyl group of ACV. We report an X-ray crystal structure for the anaerobic IPNS:Fe(II):ACmT complex. This structure reveals an additional water molecule bound to the active site metal, held by hydrogen-bonding to the ether oxygen atom of the substrate analogue.

1. Introduction

Isopenicillin N synthase (IPNS) is a member of the non-heme iron oxidase family of enzymes [1–3]. IPNS uses an iron(II) cofactor and molecular oxygen to catalyse cyclisation of its tripeptide substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV, **1**) to bicyclic isopenicillin N (IPN, **2**), the central step in penicillin biosynthesis (Scheme 1) [4]. IPNS catalysis has been extensively studied using spectroscopy [5–8], crystallography [9–11], computational modeling [12,13], and turnover experiments with substrate analogues [4,14]. It is generally agreed that the IPNS reaction cycle proceeds via initial β-lactam formation and a high-valent iron-oxo intermediate [11,15].

A diverse array of tripeptide analogues have been incubated with IPNS in the parallel effort to access new antibiotic structures and characterize the enzyme mechanism [4,14]. The ACV analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-*O*-methyl-D-threonine (ACmT, **3**) contains a methyl ether in place of the valinyl isopropyl group of ACV. ACmT **3** is not turned over by IPNS but the epimeric tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-*O*-methyl-D-*allo*-threonine (ACm*a*T **4**) does react, and is converted by IPNS to 2-α-methoxy-2-β-methylpenam **5** (Scheme 2a) [16,17]. Penam **5** has antibiotic activity comparable to isopenicillin N **2** [16].

This result stands in contrast to incubation experiments with the isosteric tripeptides that contain D-isoleucine (ACI, **6**) and D-*allo*-isoleucine (AC*a*I, **7**) (Scheme 2b). ACI **6** and AC*a*I **7** are both cyclized to penicillin products by IPNS, with retention of configuration in the C–S bond-forming step [18,19]. However neither of the corresponding sulfur-containing compounds AC-D-thioisoleucine (ACtI, **8**) and AC-D-thio-*allo*-isoleucine (ACt*a*I, **9**) is turned over by the enzyme [14,20]. It was concluded in 1989 that the difference between the two series (**3** and **4** on the one hand, **6** and **7** on the other) "must derive from some property of the methoxy function," and proposed that "a hydrogen bond between the methoxy group and an active site group restricts rotation around C(2)–C(3) in the intermediate ... so the [intermediate] derived from the *allo*-threonine peptide can be cyclised, but that derived from the threonine [peptide] is restrained in a conformation in which the β-hydrogen atom cannot be attacked by the active species" [21].

Abbreviations: AC-, δ-L-α-aminoadipoyl-L-cysteinyl-; AC*a*I, AC-D-*allo*-isoleucine; ACI, AC-D-isoleucine; ACm*a*T, AC-*O*-methyl-D-*allo*-threonine; ACmT, AC-*O*-methyl-D-threonine; ACt*a*I, AC-D-thio-*allo*-isoleucine; ACtI, AC-D-thioisoleucine; ACV, AC-D-valine; IPN, isopenicillin N; IPNS, isopenicillin N synthase

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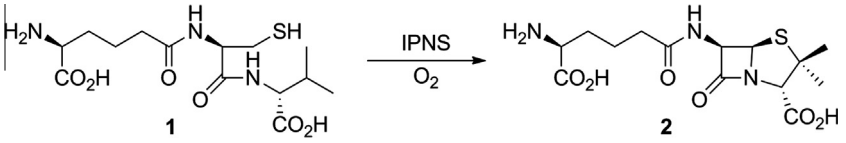
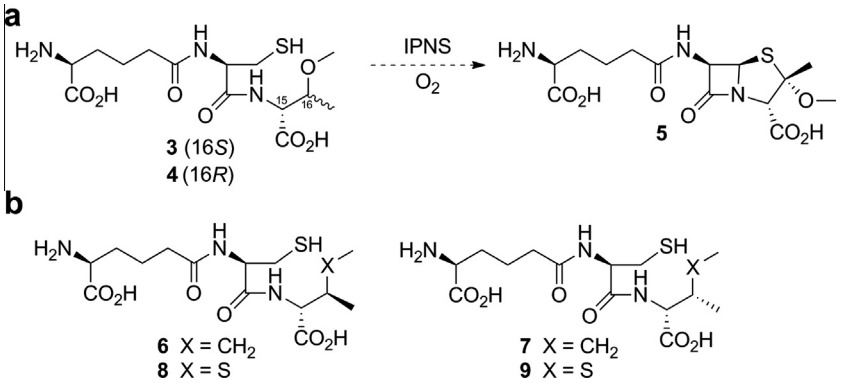


Scheme 1. The IPNS-catalysed reaction: conversion of the linear tripeptide ACV **1** to bicyclic IPN **2**, biosynthetic progenitor of all penicillin and cephalosporin antibiotics (L-AA = δ-(L-α-aminoadipoyl)).



Scheme 2. (a) The reaction of IPNS with the epimeric substrate analogues ACmT **3** and ACm*a*T **4**: ACmT **3** is not turned over by the enzyme, while ACm*a*T **4** gives a bioactive penam **5**. (b) Structures of the isosteric tripeptides ACI **6**, AC*a*I **7**, ACtI, **8** and AC*ta*I, **9** (L-AA = δ-(L-α-aminoadipoyl)).

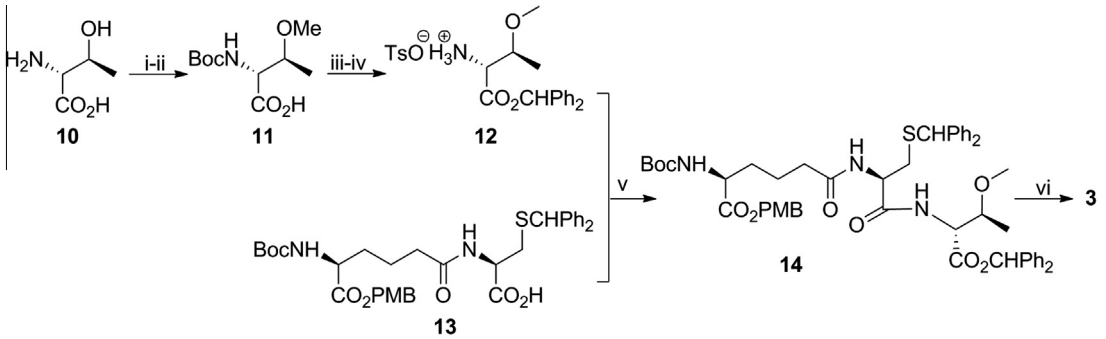
X-ray crystal structures of apo-IPNS [9] and the enzyme:substrate complex [10] paint a detailed structural picture of the enzyme active site. Time-resolved studies using high-pressure oxygenation to initiate reaction within crystalline IPNS have enabled step-by-step elucidation of the reaction mechanism on a structural level [11,22–25], while structures of the enzyme with ACV analogues reveal a range of binding modes and the interplay between steric and electronic effects at the IPNS active site [26–32]. Seeking. a structural explanation for the failure of ACmT **3** to react with IPNS, we have crystallised the protein with this substrate analogue and solved the crystal structure of the IPNS:-Fe(II):ACmT complex.

2. Materials and methods

2.1. Synthesis of δ-(L-α-aminoadipoyl)-L-cysteinyl-O-methyl-D-threonine *3*

D-Threonine **10** (Aldrich) was *N*-Boc-protected [33] then converted to *N*-Boc-*O*-methyl-D-threonine **11** using sodium *iso*-propoxide and methyl iodide in THF as described by Chen and Benoiton (Scheme 3) [34]. Although the percentage conversion in this etherification reaction is low (24–32% in our hands) unreacted starting material (45–49%) is readily recovered and recycled; the product **11** and residual starting material were isolated together after work-up and could be separated by partitioning the resultant oil between small volumes of DCM and water. *N*-Boc-*O*-methyl-D-threonine **11** was isolated from the DCM phase and further purified by crystallisation from chloroform.

The Boc group was removed using toluenesulfonic acid [35] and the resulting carboxylic acid converted to the benzhydryl ester **12** using diphenyldiazomethane [36] (Scheme 3). Protected amino acid **12** was coupled to previously reported dipeptide **13** [23] using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and 1-hydroxybenzotriazole hydrate (HOBt) [37] under standard conditions to give protected tripeptide **14**. Global deprotection with trifluoroacetic acid [38] gave the tripeptide **3** which was collected as the trifluoroacetate salt by precipitation from ether. Purification by reversed-phase HPLC (octadecylsilane 250 × 10 mm; λ = 254 nm, 5 AUFS; 4 mL/min, 10 mM NH_4HCO_3 in water/methanol as eluant: running time 0–5 min, 0% methanol; 5–12 min, gradient of 0–25% methanol; 13–18 min, 2.5% methanol; 18–20 min 0% metha-



Scheme 3. Synthesis of target tripeptide **3**; (i) NaOH, H_2O/^{t}BuOH, $(Boc)_2O$, rt, 23 h, 100%; (ii) NaO^{i}Pr, CH_3I, THF, 0 °C, 48 h, 32%; (iii) TsOH, toluene, 40 °C; (iv) Ph_2CN_2, CH_3CN/Et_2O, rt, 55% (over 2 steps); (v) EDCI, HOBt, Et_3N, DCM, rt, 20 h, 100%; (vi) TFA, anisole, reflux, 30 min, 100%; RP-HPLC.