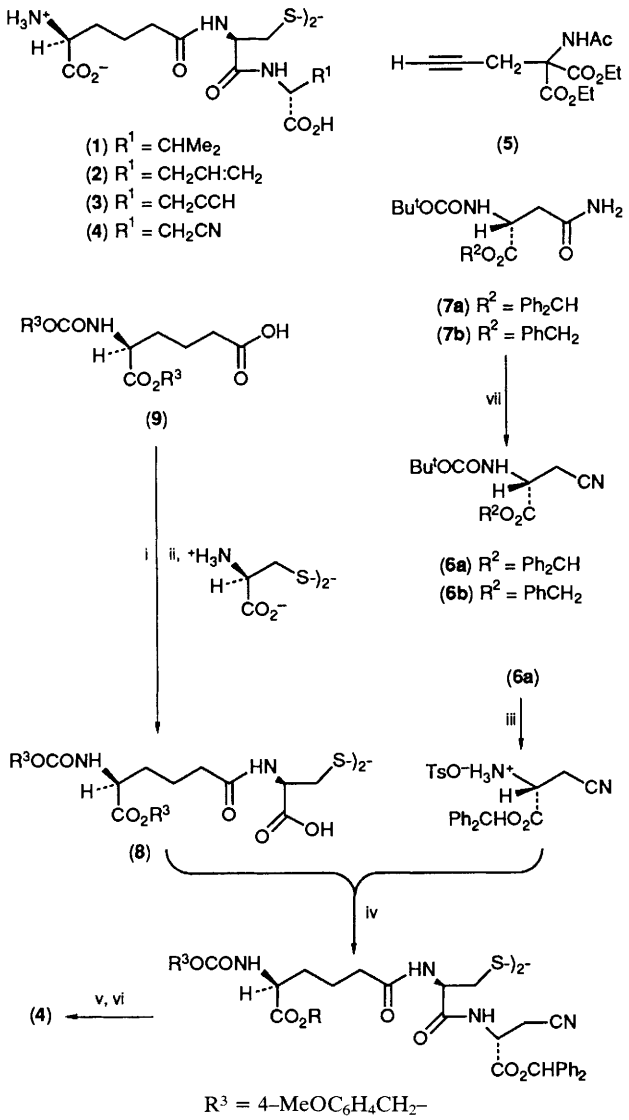
Formation of Novel Unsaturated Side Chain Penicillins with Isopenicillin N Synthase

Jack E. Baldwin,* Mark Bradley, Shaun D. Abbott, and R. M. Adlington

The Dyson Perrins Laboratory and the Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

Incubation of δ-L-α-aminoadipoyl-L-cysteinyl-D-propargylglycine (**3**) and δ-L-α-aminoadipoyl-L-cysteinyl-D-cyanoalanine (**4**) with isopenicillin N synthase resulted in the formation of three novel penicillin antibiotics, possessing unsaturated side chains (**10**), (**11**), and (**12**).

Many analogues of the natural substrate δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (**1**) have on incubation with isopenicillin N synthase (IPNS) given new β-lactam products.[1] Of particular interest is the analogue δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine (**2**), which gave six β-lactam products,[2] three of which were oxygenated species, oxygen being derived from the co-substrate O_2.[3] This surprising result illuminated the unexpected mono-oxygenase pathway for IPNS with unsaturated substrates and raised the fundamental question, *i.e.*, what active site species arising from the iron–dioxygen



Scheme 1. *Reagents and conditions*: i, $ClCO_2Bu^i/Et_3N$/tetrahydrofuran; iii, TsOH; iv, EEDQ/Et_3N; v, trifluoroacetic acid/anisole, 0 °C, 1 h; vi, reverse phase HPLC; vii, TsCl/pyridine. Ts = $OSO_2C_6H_4Me$.

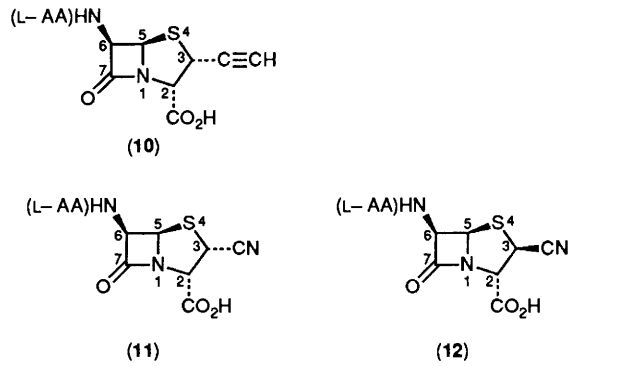


Table 1.

D-Valine substitute	Penicillins formed (β : α ratio)		Ref.
D-Norvaline	Ethyl	(>10 : 1)	8[a]
D-α-Aminobutyrate	Methyl	(7 : 1)	9
D-Allylglycine (**2**)	Vinyl	(4 : 1)	2
D-Allenylalanine	Allenyl	(1 : 2)	10
D-Propargylglycine	Ethynyl	(<1 : 15)	This work
D-Cyanoalanine	Cyano	(1 : 1)	This work

[a] The penicillin stereochemistry was only assigned after recent work involving a deuteriated version of the substrate, results of which will appear separately.

redox cycle could, on the one hand, abstract hydrogen from the valine β-position in (**1**), yet when presented with an alkenic functionality, as in (**2**), presumably in a similar spatial location, would proceed along this mono-oxygenase pathway? The proposal of an iron–oxene (Fe^{IV}=O) has been invoked to explain this duality of mechanism.[4] To explore the behaviour of triple bonds in this system the two tripeptides δ-L-α-aminoadipoyl-L-cysteinyl-D-propargylglycine (**3**) and δ-L-α-aminoadipoyl-L-cysteinyl-D-cyanoalanine (**4**) were synthesised and incubated with IPNS.

The D-propargylglycine functionality was synthesised *via* the diethylacetamidomalonate adduct (**5**), produced by alkylation of diethylacetamidomalonate with propargyl bromide using NaH, and resolved as the *N*-acyl compound using hog kidney acylase.[5] The amino acid was protected and converted to the tripeptide (**3**) by standard techniques.[6]

D-3-Cyanoalanine was produced in the *N*-t-butyloxycarbonyl, benzhydryl ester or *N*-t-butyloxycarbonyl, benzyl ester protected form (**6a**, **b**) (Scheme 1) by the method of Christie *et al.*[7] by dehydration of the similarly protected D-asparagines (**7a**,**b**) using toluene-*p*-sulphonyl chloride and pyridine. However, the nitrile functionality proved incompatible with standard deprotection techniques [refluxing with CF_3CO_2H/anisole for an acid labile protecting scheme; and selective removal with $Na/NH_3(l)$ for a benzyl protecting scheme], and so an alternative strategy was sought. Thus (**6a**) was deprotected using toluene-*p*-sulphonic acid in Et_2O/EtOH and coupled using ethyl 1,2-dihydro-2-ethoxy-1-quinoline carboxylate (EEDQ) with bis [*N*-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)]-L-cystine (**8**), produced by isobutylchloroformate mediated coupling of *N*-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzyl)-L-α-aminoadipic acid (**9**) and L-cystine. The protected tripeptide disulphide thus formed was smoothly deprotected at 0 °C with CF_3CO_2H/anisole, with no hydration of the nitrile to the amide observable by 500 MHz ^1H NMR and mass spectrometry.

Incubation of δ-L-α-aminoadipoyl-L-cysteinyl-D-propargylglycine (**3**) under standard conditions† produced, in

† The tripeptide (typically 1—2 mg) in NH_4HCO_3 buffer (3 ml, 25 mM) was pretreated with dithiothreitol (DTT) (100 μl, 100 mM solution) for 10 min at 27 °C, treated with the necessary cofactors (sequentially: 100 μl, 50 mM L-ascorbate solution; 50 μl catalase at one tenth dilution of standard Sigma C-100 supply; and 100 μl, 5 mM Fe^{2+} solution) and IPNS solution (typically 10—12 IU in 2 ml 25 mM NH_4HCO_3 buffer), divided into two portions, and incubated in air at 27 °C, 250 rpm shake rate for 40 min, with additional DTT (50 μl) and Fe^{2+} (50 μl) being added to each portion after 20 min. Acetone precipitation of the enzyme (70% acetone), centrifugation (12 000 rpm, 10 min), and evaporation of the supernatant *in vacuo*, gave the crude incubation mixture.

virtually quantitative yield, a mixture of three new β-lactam containing metabolites (ratio 15 : 1 : <0.5). The major product possessed antibiotic activity similar to isopenicillin N against the organisms *Staphylococcus aureus* and *Escherichia coli*, activity which was destroyed by the addition of β-lactamase I. This product was purified by reverse phase HPLC and characterised by 500 MHz ^{1}H NMR and mass spectrometry, as an acetylenic penicillin. By NOE experiments and by the magnitude of the coupling constant between H-2 and H-3 of 7 Hz, the relative stereochemistry of the penicillin acetylene group was assigned as α (**10**). Thus irradiation of the resonance associated with the H-2 proton gave an NOE to H-3 (10%) but no NOE to either H-6 or H-5. Irradiation of the resonance associated with H-3 gave an NOE to H-2 (8%) only, whilst irradiation of the resonances assigned to H-6 or H-5 gave no NOE to either H-2 or H-3.‡

Incubation of δ-L-α-aminoadipoyl-L-cysteinyl-D-cyanoalanine (**4**) under standard conditions gave only 10% conversion, leading to the formation of two β-lactam products (ratio 1 : 1), both of which displayed antibiotic activity against *S. aureus* and *E. coli*, which was destroyed by the addition of β-lactamase I. These products were purified by reverse phase HPLC and assigned by HPLC retention properties and 500 MHz ^{1}H NMR as the α and β penicillins (**11**) and (**12**)‡ (Table 1).

Provided that in each series both the α- and β-penam products are equistable to the conditions of incubation and work-up, then it is apparent that alkyl substituents (ethyl and methyl) preferentially form β-penams, whereas unsaturated entities (vinyl, allenyl, and ethynyl) assume increasingly α-oriented products. In contrast the highly polar cyano group shows no geometric preferences. Since our previous studies have provided evidence for a radical intermediate in the carbon–sulphur bond forming step, which in the formation of monosubstituted penams rotates faster than ring closure,§ then the results of Table 1 suggest a preferential binding of unsaturated substituents to the α-site and of saturated substituents to the β-site. This difference may arise from the juxtaposition of aromatic *vs.* aliphatic amino acid side chains in the α- and β-sites respectively. Not surprisingly the polar cyano group does not respond to such association, and its orientation probably results from electrostatic influences.

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‡ *Spectroscopic data* for (**10**): δ_H (500 MHz, D_2O) 1.66—1.96 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.38—2.41 (2H, m, CH_2CO), 2.91 (1H, d, *J* 2.5 Hz, HCC), 3.73—3.75 (1H, m, $CHCH_2$), 4.90 (1H, dd, *J* 7, 2.5 Hz, H-3), 5.53 and 5.66 (2H, ABq, *J* 4 Hz, H-5 and H-6); use of CD_3CN : D_2O (1 : 1) shifted the HOD peak, revealing δ_H 4.83 (1H, d, *J* 7 Hz, H-2); *m/z* (+ve argon FAB) 356 (MH^+). For (**11**) (more mobile isomer): δ_H (500 MHz, D_2O) 1.66—1.96 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.38—2.42 (2H, m, CH_2CO), 3.72—3.75 (1H, m, $CHCH_2$), 5.39 and 5.51 (2H, ABq, *J* 4 Hz, H-5 and H-6); H-2 and H-3 resonances obscured by residual solvent peak. For (**12**) (less mobile isomer): δ_H (500 MHz, D_2O) 1.66—1.96 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.38—2.42 (2H, m, CH_2CO), 3.72—3.75 (1H, m, $CHCH_2$), 5.34 and 5.38 (2H, ABq, *J* 4 Hz, H-5 and H-6); H-2 and H-3 resonances obscured by residual solvent peak.

§ A radical intermediate is thought most likely in the light of the result of incubating the two isomers δ-L-α-aminoadipoyl-L-cysteinyl-D-(3*R*) and (3*S*)-(2-amino-3-deuteriobutyrate) with IPNS which both give the same α-deuterio-β-methylpenam,[9] and the results with the D-cyclopropylalanine containing tripeptide.[11]