# Penicillin Biosynthesis: Active Site Mapping with L-α-Aminoadipoyl-(*C*-methyl-L-cysteinyl)-D-valine Variants

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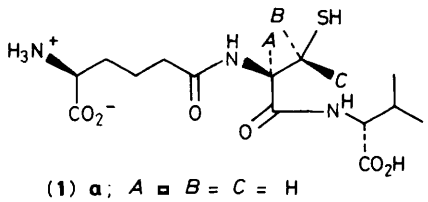
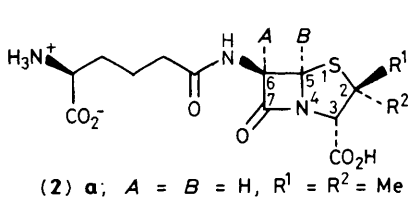
A series of structural variants of the cysteinyl moiety of the natural precursor of penicillins, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine, have been synthesised and their effectiveness as substrates for the enzyme isopenicillin N synthetase has been evaluated.

Although the enzyme isopenicillin N synthetase (IPNS) has been shown to convert tripeptides other than the natural substrate[1] δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (**1a**)† into β-lactam metabolites [*e.g.* (**1a**) → (**2a**)], studies of this reaction have so far been unrewarding when the L-cysteinyl moiety was replaced by an analogous amino acid. Thus although many valine substitutions provided substrates which were successfully cyclised to bicyclic β-lactams, replacement of L-cysteine by L-aminobutyric acid, L-serine, or *S*-methyl-L-cysteine [(**3a**—**c**), respectively] gave tripeptides which with IPNS were neither β-lactam-producing substrates nor inhibitors of the conversion (**1a**) → (**2a**) in mixed substrate experiments.[2] The importance of the cysteinyl moiety during penicillin biosynthesis followed from studies which demonstrated that the formation of an enzyme-bound monocyclic β-lactam such as (**4**) can be considered as the first irreversible step during the conversion (**1a**) → (**2a**) by IPNS.[3] Recently we have demonstrated that the conversion of (**1a**) into (**2a**) occurs with *complete retention* of the cysteinyl 3-pro-*R* hydrogen and with *complete loss* of the cysteinyl 3-pro-*S* hydrogen, even though this requires the energetically more demanding cleavage of a carbon–deuterium bond rather than a carbon–hydrogen bond; e.g. (**1b**) → (**2a**).[4] Taken together these

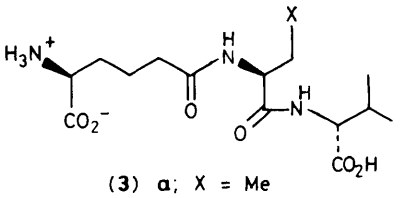
† δ-(L-α-Aminoadipoyl) = (5*S*)-5-amino-5-carboxypentanoyl.



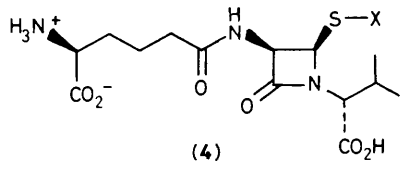
(1) **a**; *A* ▫ *B* = *C* = H
**b**; *A* = *B* = $^1$H, *C* = $^2$H
**c**; *A* = Me, *B* = *C* = H
**d**; *A* = *B* = H, *C* = Me
**e**; *A* = *C* = H, *B* = Me



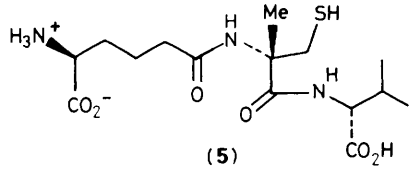
(2) **a**; *A* = *B* = H, $R^1$ = $R^2$ = Me
**b**; *A* = Me, *B* = H, $R^1$ = $R^2$ = Me
**c**; *A* = H, *B* = Me, $R^1$ = $R^2$ = Me
**d**; *A* ▫ *B* = H, $R^1$ = Et, $R^2$ = Me
**e**; *A* = *B* = H, $R^1$ = Me, $R^2$ = Et



(3) **a**; X = Me
**b**; X = OH
**c**; X = SMe



(4)



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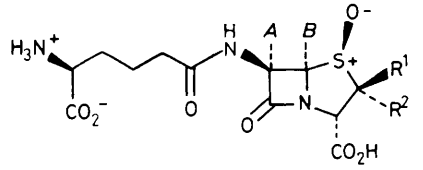


(6) **a**; *A* = *B* = H, $R^1$ = $R^2$ = Me
**b**; *A* = Me, *B* = H, $R^1$ = $R^2$ = Me
**c**; *A* = H, *B* = Me, $R^1$ = $R^2$ = Me
**d**; *A* = *B* = H, $R^1$ = Et, $R^2$ = Me
**e**; *A* = *B* = H, $R^1$ = Me, $R^2$ = Et

**Table 1.** Chemical shift differences on formation of sulphoxides (**6**).

| Oxidative conversion | Chemical shift difference (±0.02 p.p.m.) [$\delta_H$ (sulphoxide) − $\delta_H$ (penicillin)] 2α-Me | 2β-Me | 5-H | 6-H | 3-H | Ref. |
|---|---|---|---|---|---|---|
| (**2a**) → (**6a**) | −0.24[a] | +0.04[a] | −0.15[a] | +0.30[a] | +0.18 | 8a |
| (**2b**) → (**6b**) | −0.17 or −0.27[b] | +0.04 or +0.14[b] | −0.30 | +0.07[c] | +0.10[c] | |
| (**2c**) → (**6c**) | −0.12[b] | +0.04[b] | −0.12[d] | +0.27 | [e] | |
| (**2d**) → (**6d**) | −0.22 | | −0.24[a] | +0.46[a] | +0.07 | 14 |
| (**2e**) → (**6e**) | | +0.11 | −0.10[a] | +0.44[a] | +0.08 | 14 |

[a] Observed shift change proven by n.O.e.-based assignments of both C-2 methyl groups and β-lactam hydrogen before and after oxidation. [b] Relative shift changes unsupported by n.O.e. measurements. [c] 6-Me not 6-H. [d] 5-Me not 5-H. [e] Obscured.

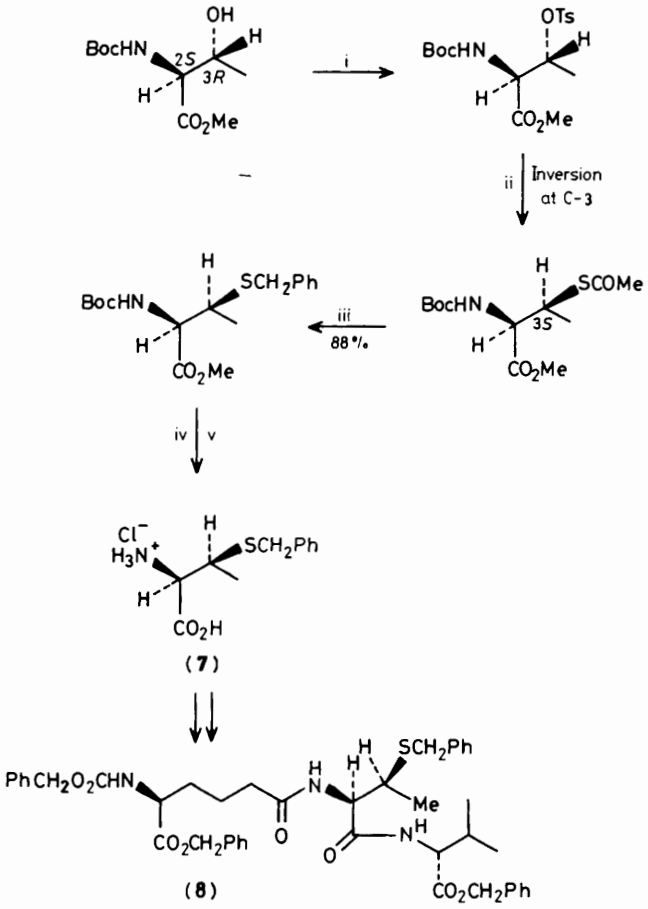
results imply that a C-3 thiol group and a C-3 pro-*S* hydrogen atom (or deuterium) are minimal requirements of the central amino acid of the tripeptide for activity with IPNS. In order to assess this requirement a series of *C*-methylated cysteinyl tripeptides have been synthesised and evaluated as substrates for IPNS.

First, replacement of the C-2 hydrogen by methyl gave both (2*R*)-2-methylcysteinyl and (2*S*)-2-methylcysteinyl tripeptides, (**1c**) and (**5**)‡ respectively. Incubation of (**1c**) (1 mg) with purified IPNS (5 International Units) under the usual conditions[7] gave, after protein precipitation and h.p.l.c. purification (reverse-phase octadecylsilane; 25mM $NH_4HCO_3$ as eluant), the 6α-methyl isopenicillin N (**2b**) (>60%, by n.m.r. calibration against internal standard), $\delta_H$ (500 MHz; $D_2O$)§ 1.49 (3H, s, 2-Me), 1.59 (3H, s, 2-Me), 1.68—1.78 and 1.89—1.97 (4H, 2 × m, $[CH_2]_2CH_2CO$), 1.71 (3H, s, 6-Me), 2.35—2.40 (2H, m, $CH_2CO$), 3.71—3.74 (1H, m, $CH[CH_2]_3$), 4.25 (1H, s, 3-H), and 5.47 (1H, s, 5-H); *m/z* (positive argon fast atom bombardment) 374 ($MH^+$). The assigned stereochemistries at C-3, C-5, and C-6 were consistent with both nuclear Overhauser enhancement (n.O.e.) to 5-H (9%) upon irradiation of 6-Me ($\delta_H$ 1.71), and the relative chemical shift changes observed upon formation of the β-sulphoxide (**6b**)¶ (Table 1). The purified penam (**2b**) showed no antibacterial

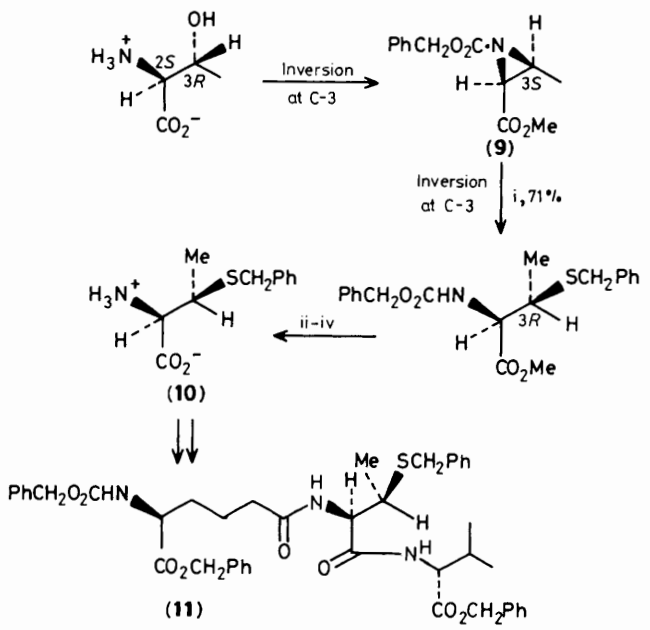
‡ Compounds (**1c**) and (**5**) were synthesised from racemic α-methylcysteine[5] following standard coupling procedures.[6] The corresponding fully benzyl-protected diastereoisomeric precursors were separated by chromatography followed by recrystallisation (from methanol). The absolute configurations of (**1c**) and (**5**) are assumed from their transformation with IPNS, but not proven.

§ $^1$H N.m.r. spectra are referenced to (2,2,3,3-$^2H_4$)-3-trimethylsilylpropanoate.

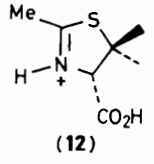
¶ The formation of β-sulphoxides from penam systems has been attributed to β-face selection resulting from complexation of the C-6 amido side chain with the oxidizing agent; see ref. 8a and references therein. For (**6b**) and (**6c**) such β-face selection should be enhanced by steric methyl group shielding of the α-face. 5-*epi*-Penicillins give a mixture of α- and β-sulphoxides.[8b]




**Scheme 1.** *Reagents:* i, toluene-4-sulphonyl chloride, pyridine, 0 °C; ii, $Me_3COSK$, DMF; iii, 0.2 M NaOH, $H_2O$, EtOH, $PhCH_2Cl$ (the mild thioacetyl hydrolysis and subsequent mild thiol alkylation conditions have literature precedent[10,11]); iv, $CF_3CO_2H$; v, HCl (aq.), reflux.




**Scheme 2.** *Reagents:* i, $PhCH_2SH$, $CH_2Cl_2$, $BF_3{\cdot}Et_2O$; ii, HBr, AcOH; iii, HCl (aq.), reflux; iv, Dowex 50W-X8(H) ion exchange.




activity against *Staphylococcus aureus* N.C.T.C. 6571 at a concentration of 35 μg in 100 μl of water (sample size 100 μl), but yielded a positive result in the β-lactamase induction test using *Bacillus licheniformis* and nitrocefin as the analytical assay.[9] Incubation of the diastereoisomer (**5**) with IPNS gave no detectable β-lactam product [$^1$H n.m.r. (500 MHz)].

For the synthesis of the (2*R*,3*S*)- and (2*R*,3*R*)-3-methylcysteinyl tripeptides (**1d** and **e**), L-threonine (2*S*,3*R*-isomer) served as the precursor of the L-cysteinyl moiety. For (**1d**), *retention* at C-2 and *inversion* at C-3 (Scheme 1) gave (2*R*,3*S*)-*S*-benzyl-3-methylcysteine hydrochloride salt (**7**), which was sequentially coupled following standard coupling procedures[6] to give a single diastereoisomerically pure protected tripeptide (**8**), then deprotected[6] ($Na/NH_3$) to give (**1d**). For (**1e**), *retention* at C-2 and *double inversion* at C-3 (Scheme 2) [*via* the *N*-Boc protected arizidine (**9**)][12] gave (2*R*,3*R*)-*S*-benzyl-3-methylcysteine (**10**), $[\alpha]_D^{20}$ +76.3 (*c* 1.1, 1 M HCl) [lit.,[13] −76.2 (*c* 1, 1 M HCl) for 2*S*,3*S* form], which was sequentially coupled[6] to give a single diastereoisomerically pure protected tripeptide (**11**), then deprotected to (**1e**). Incubation of (**1d**) (1 mg) with purified IPNS (5 International Units) under the standard conditions[7] gave, after protein precipitation, no detectable β-lactam-containing products {by $^1$H 500 MHz n.m.r. spectroscopy [threshold of detection 10 μg of (**2a**)] and β-lactamase induction assay[9]}. However incubation of (**1e**) under identical conditions gave, upon work-up and purification by h.p.l.c. (reverse-phase octadecylsilane column, 25 mM $NH_4HCO_3$) the 5α-methyl isopenicillin N (**2c**) (>50% by internal n.m.r. calibration), $\delta_H$ (500 MHz; $D_2O$)§ 1.62 (3H, s, 2-Me), 1.63 (3H, s, 2-Me), 1.65—1.95 (4H, m, $CHCH_2CH_2$), 2.05 (3H, s, 5-Me), 2.43 (2H, t, *J* 7 Hz, $CH_2CO$), 3.71 (1H, t, *J* 5.5 Hz, $CH[CH_2]_3$), 4.34 (1H, s, 3-H), and 5.12 (1H, s, 6-H); *m/z* (positive argon fast atom bombardment) 174 [$C_7H_{12}NO_2S^+$, ion (**12**)]. The assigned stereochemistries at C-5 and C-6 were consistent with both n.O.e. to 6-H (16%) upon irradiation of 5-Me ($\delta_H$ 2.05), and the relative chemical shift changes observed upon formation of a β-sulphoxide (**6c**).¶ The purified penam (**2c**) showed no antibacterial activity against *S. Aureus* (N.C.T.C. 6571) at a concentration of 300 μg in 100 μl of water, gave a positive result in the β-lactamase induction test using *B. licheniformis* and nitrocefin as the analytical assay,[9] and was stable to β-lactamase 1 from *Bacillus cereus* over 2 h at 25 °C.**

In summary, we have demonstrated that *C*-methylation of the central cysteinyl residue of the ACV thiol tripeptide (**1a**), can still give active substrates for IPNS, providing such modifications maintain a 2*R* absolute configuration and retain a hydrogen atom in the 3-pro-*S* position. It is surprising that IPNS can accommodate a significant increase in steric bulk so close to the crucial binding and catalytic site associated with the initial β-lactam formation. The so-produced penicillins (**2b** and **c**) were not antibacterially active towards the Gram-positive organism *S. aureus* (N.C.T.C. 6571) in a concentration at which isopenicillin N gave activity; these results are in agreement with the reported[15] lower activities of 6α-methyl

** In a control experiment under similar conditions, a 40-fold excess of penicillin G sodium salt was destroyed in less than 5 min (assay by 500 MHz $^1$H n.m.r.).

penicillins V and G when compared with penicillins V and G, respectively.

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