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Side Chain Specificity in the Enzymatic Synthesis of Penicillins

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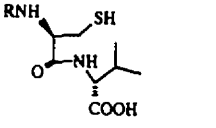
Abstract: *5-Carboxynaphth-1-oyl-, 4-carboxybenzoyl-, trans-5-carboxy-3-pentenoyl- and cis-5-carboxy-3-pentenoyl-L-cysteinyl-D-valine were shown to be converted by isopenicillin N synthetase to their corresponding penicillins in yields of 90%, 12%, 40% and 5%, respectively.*

Penicillins with arylamido side chains are potent antibiotics and continue to be of major clinical importance. Their direct *in vitro* enzymatic synthesis would avoid the present *in vivo* deacylation-acylation process used in their production. Previous studies in this laboratory have shown that structural variations of the δ-aminoadipoyl residue of the natural substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) (**1a**) of isopenicillin N synthetase (IPNS) require a 6-carbon chain or equivalent, terminating with a carboxyl function to be an efficient substrate.[1] Thus, δ-(D-α-aminoadipoyl)-(**1b**), adipoyl-(**1c**), and L-cysteine-S-acetyl-(**1d**), L-cysteinyl-D-valines were shown to be effective substrates. Peptides with arylamido side chains such as



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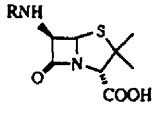

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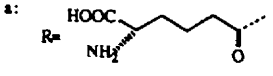
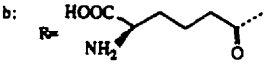
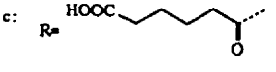
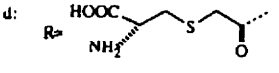
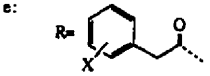
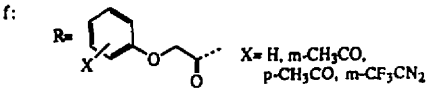


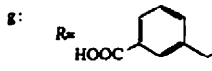


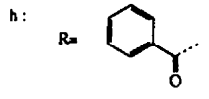












substituted phenylacetyls (**1e**) and phenoxyacetyls (**1f**) were also converted to penicillins but in very poor yields.[2-4] Their steady-state kinetic parameters, K_M and V_{max}, indicated that these substrates were binding to the enzyme active-site but that the catalytic events leading to formation of β-lactam products were retarded. Peptide (**1g**), with a *m*-carboxyphenylacetyl side chain, however, was an excellent substrate[5], suggesting that side chains incorporating aromatic groups with an appropriately placed acidic moiety may function as efficient substrates.

In this paper we describe the IPNS catalysed conversions of a series of peptides (**1i-l**) to penicillins (**2i-l**), that further probe the side chain specificity of this important enzyme. We reasoned that 5-carboxynaphthoyl-L-cysteinyl-D-valine (**1i**) would represent a totally rigid transoid side chain (Figure 1), and if an efficient substrate, would provide strong evidence that the natural side chain adopts a linear transoid conformation in the active-site. The tolerance for substrates with side chains unable to adopt a completely transoid conformation could be assessed by comparing the conversion of (**1i**) with 4-carboxybenzoyl-L-cysteinyl-D-valine (**1j**) and (*trans*-5-carboxy-3-pentenoyl)-L-cysteinyl-D-valine (**1k**) with its *cis* analogue (**1l**). A comparison of the aromatic side chains (**1i**), and (**1j**) with their olefinic equivalents (**1k**) and (**1l**) respectively, should provide a measure of the effect upon catalytic efficiency, of incorporating aromatic groups into the side chain.

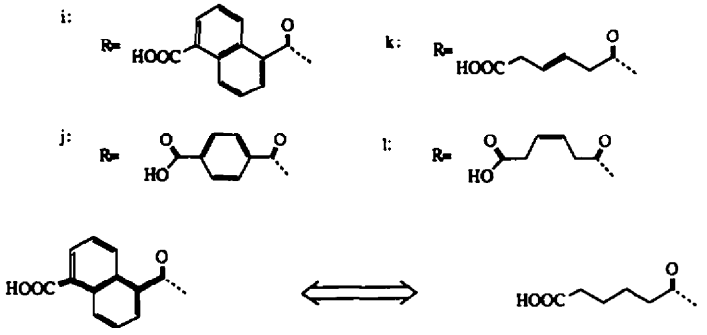
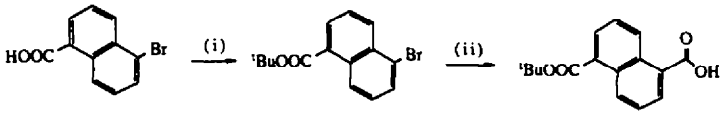


Figure 1.

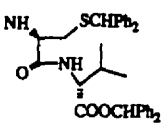
In the event, 5-*t*-butoxycarbonylnaphthoic acid (**5**) was obtained from 5-bromonaphthoic acid (**3**) (Scheme 1) and coupled with (**6**)[3] to give the protected peptide (**7**). Deprotection with trifluoroacetic acid/anisole proceeded smoothly to give (**1i**). Similarly, the mono *p*-methoxybenzyl ester (**8**) was coupled with (**6**) to produce peptide (**9**), which was readily deprotected upon trifluoroacetic acid/anisole treatment to give (**1j**). *trans*-3-Hexenedioic acid mono benzyl ester (**12**) was coupled with (**10**) to give the benzyl protected peptide (**13**) (Scheme 2), which was deprotected using sodium in liquid ammonia.[6] In the same manner, *cis*-3-hexenedioic acid[7] (**14**) was converted via (**15**) to the required peptide (**1l**).

Scheme 1.



3 4 5

(i) Cl_3CCNHO^tBu, 82%. (ii) BuLi/CO_2, 77%.



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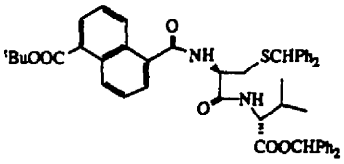

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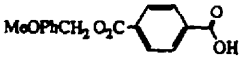

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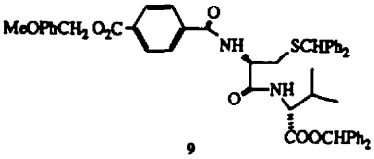

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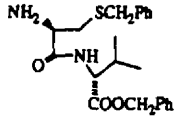

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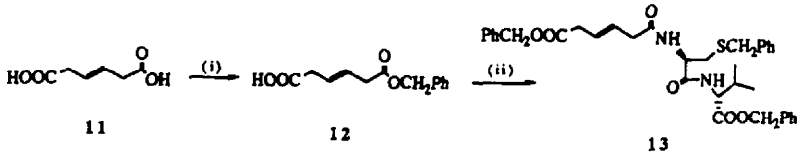
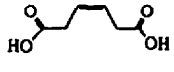
Scheme 2.



11 12 13

(i) $PhCH_2Br$, 25%. (ii) EEDQ, (10), 53%.



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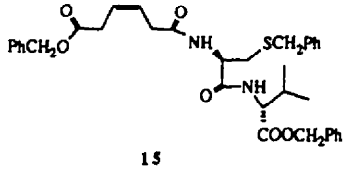

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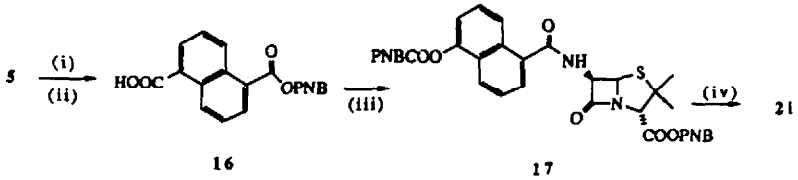
Scheme 3.



16 17

(i) NO_2PhCH_2Br/NEt_3. (ii) TFA/anisole, 80%.
(iii) $SOCl_2$/6-APA PNB ester, 60%. (iv) H_2/Pd/C, 63%.

Incubation of peptides (**1i-l**) with IPNS and the required cofactors gave the corresponding penicillins (**2i-l**) in the yields indicated in Table 1. The yields of conversion were determined by ^{1}H NMR and/or HPLC integration of the crude incubation mixtures and were calculated relative to remaining starting material. Competitive incubation experiments between the different substrates gave product ratios that agreed with these values. The penicillins were isolated by HPLC and characterised by ^{1}H NMR, mass spectroscopy and biological activity. In the case of (**2i**), the authentic material was synthesised (Scheme 3) and shown to be identical to the incubation product.

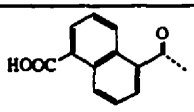
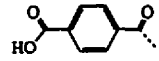
	R	% Conversion
i:		>90
j:		12
k:		40
l:		5

Table 1. Yields of Conversion for Peptides (**1i-l**) to Penicillins (**2i-l**) by IPNS.

The most striking result is the excellent conversion of peptide (**1i**). Since this is a totally rigid side chain, it presents strong evidence that the interaction of its carboxyl residue with the enzyme is at a fixed distance away from the events causing cyclisation and that this distance is best matched by a 6-carbon side chain in a linear transoid conformation. This distance requirement is strict; thus, peptides with 5 and 7 carbon side chains were apparently not substrates[1] and peptides (**1j**) and (**1l**), which both include a fixed *cis* bond junction in their side chains, were converted in significantly reduced yields. The conversion of (**1j**) was sufficiently high, however, to implicate its side chain carboxyl as still being an important binding interaction, since its unsubstituted analogue (**1h**) was not a substrate.[2] Examination of Table 1 also suggests that incorporation of aromatic groups into the side chain improves catalytic efficiency. This may be due to improved binding characteristics of the aromatic side chains; alternatively, the enhanced rigidity of these side chains may allow a more facile organisation of the cysteinyl-valine moiety for cyclisation to occur.

Acknowledgements: We thank the S.E.R.C. and Eli Lilly & Co. for financial support. We also acknowledge Mr J. Keeping and Mr J. Pitt for their excellent technical support.

EXPERIMENTAL

Melting points are uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were obtained as thin films or as cell solutions on a Perkin-Elmer 681 spectrophotometer; weak (w),

medium (m) or strong (s) bands were recorded. ^1H NMR spectra were recorded on Varian Gemini 200 MHz or Bruker AM 500 MHz spectrometers and referenced against the solvent or internal dioxan (δ 3.53 ppm). ^{13}C NMR spectra were recorded at 125.7 MHz on a Bruker AM 500 MHz spectrometer and were referenced to internal dioxan (δ 67.4 ppm). Mass spectra were recorded on VG Analytical Ltd. ZAB1F or VG20-250 mass spectrometers using Ammonia Desorption Chemical Ionisation (DCI, NH_3), Fast Atom Bombardment (FAB) or Electron Impact (EI). HPLC was carried out using either (i) dual Gilson 303 pumps, a Rheodyne 7125 injector and a Gilson holochrome detector or (ii) dual Waters 510 pumps, a Rheodyne 7125 injector and Waters 440 and 441 detectors set at either 218 or 254 nm. Microanalyses were performed by Mrs. V. Lamburn, Dyson Perrins Laboratory, University of Oxford.

5-Bromo-1-naphthoic acid (3): Following the procedure of Short and Wang,[8] a solution of 1-naphthoic acid (25 g, 0.145 mol) in hot glacial acetic acid was treated with bromine (23 g, 0.144 mol) to give (3) (16 g, 44%), mp 254-256° (lit.[8] 256°).

***t*-Butyl 5-bromo-1-naphthoate (4):** To the acid (3) (10.0 g, 39.8 mmol) in dry THF (80 ml) was added *t*-butyl 2,2,2-trichloroacetimidate[9] (17.0 g, 78 mmol) in dry cyclohexane (80 ml). The resulting mixture was treated with boron trifluoride etherate (2.0 ml) at room temperature, upon which there was a significant reduction in the amount of undissolved material. After stirring for 1 h, diethyl ether (100 ml) was added and the solution washed with saturated $NaHCO_3$ solution and water. After drying over $MgSO_4$, the solvent was evaporated to leave a clear oil which was purified by column chromatography (10% ether/hexane) to give the *t*-butyl ester (4) as a white solid (10.0 g, 82%), mp 112-115° (decomp.) TLC (20% ether/hexane) Rf. 0.80. ^1H NMR (200 MHz, $CDCl_3$) 1.69 (9H, s, ^tBu); 7.43 (1H, t, 8.5Hz); 7.61 (1H, t, 7.0Hz); 7.85 (1H, d, 6.5Hz); 8.12 (1H, d, 7.0Hz); 8.48 (1H, d, 8.5Hz); 8.84 (1H, d, 8.5Hz). ^{13}C NMR ($CDCl_3$) 166.7,s; 132.5,s; 132.3,s; 131.5,d; 130.3,d; 130.1,d; 127.5,d; 125.9,d; 125.8,d; 123.2,s; 81.9,s; 28.3,q. IR (film) 2980s; 2920s; 1710s; 1280br; 1135s. m/e (EI) 308, 306 $(M)^+$; 252, 250 $(M-^tBu)^+$; 172; 126; 57.

***t*-Butyl 5-carboxy-1-naphthoate (5):** A stirred solution of bromoester (4) (1.90 g, 6.2 mmol) in dry THF (70 ml) and hexane (30 ml) under an atmosphere of argon was cooled to -90°. A solution of butyllithium in hexane (1.43 M, 4.56 ml, 6.5 mmol) was added at a rate to keep the reaction below -90° (addition time 20 mins). The resulting yellow solution was stirred for another 30 mins at -90°, before being saturated with CO_2 gas. After stirring for another 10 mins at -90° the reaction was allowed to warm to room temperature, after which, it was quenched with saturated NH_4Cl solution. The organic layer was separated, dried and evaporated to leave a purple solid which was recrystallised from hexane/ether to give (5) as a white solid (1.30 g, 77%), mp 211-212°. TLC (ether) Rf. 0.12. ^1H NMR (200 MHz, $CDCl_3$) 1.70 (9H, s, ^tBu); 6.75 (1H, s, COOH); 7.67 (2H, t, 7.0Hz) 8.12 (1H, d, 7.0Hz), 8.42 (1H, d, 7.0Hz), 9.14 (1H, d, 9.0Hz); 9.27 (1H, d, 9.0Hz). ^{13}C NMR ($CDCl_3$) 177.7,s; 167.0,s; 131.9,d; 131.6,d; 130.2,s; 130.1,s; 130.0,d; 129.6,d; 126.5,d; 126.2,s; 125.9,d; 81.9,s; 28.3,q. IR (cell, CH_2Cl_2) 1730m; 1710s; 1130s. m/e (DCI, NH_3) 290 $(M+NH_4)^+$; 272 $(M)^+$; 234; 216; 172.

(5-*t*-Butoxycarbonylnaphth-1-oyl)-(S-diphenylmethyl-L-cysteinyl)-(D-valine benzhydryl ester) (7): A solution of the acid (5) (0.45 g, 1.65 mmol), S-diphenylmethyl-L-cysteinyl-D-

valine benzhydryl ester (**6**)[3] (0.95 g, 1.72 mmol) and 4-N,N-dimethylaminopyridine (0.21 g, 1.70 mmol) in dry CH_2Cl_2 (40 ml) was treated with dicyclohexylcarbodiimide (0.35 g, 1.70 mmol) and then stirred overnight. The resulting mixture was filtered, washed and the solvent removed. The crude material was purified by flash chromatography (50% hexane/ether) to give (**7**) as a white solid (0.97 g, 73%), mp 92-93°, $[\alpha]_D^{20}$= -27.3° (c 0.8, $CHCl_3$). TLC (50% hexane/ether) Rf. 0.40. ^1H NMR (200MHz, $CDCl_3$) 0.81 (3H, d, 6.5Hz, CMe); 0.93 (3H, d, 6.5Hz, CMe); 1.69 (9H, s, ^tBu); 2.20-2.36 (1H, m, $CHMe_2$); 2.90-3.02 (2H, m, CH_2S); 4.62-4.72 (1H, m, α-val); 4.80-4.92 (1H, m, α-cyst); 5.43 (1H, s, $SCHPh_2$); 6.68 (1H, d, 8.5Hz, NH); 6.77 (1H, d, 8.6Hz, NH); 6.91 (1H, s, $CHPh_2$); 7.28-7.36 (15H, m, ArH); 7.41-7.62 (8H, m, ArH); 8.11 (1H, d, 7.4Hz); 8.48 (1H, d, 8.5Hz); 8.99 (1H, d, 7.4Hz). IR (cell, $CHCl_3$) 3420m; 3000m; 1740m; 1705s; 1687s; 1660m; 1520s; 1500s. m/e (FAB) 829 $(M+Na)^+$; 167. $C_{50}H_{50}O_6N_2S$ requires: C 74.42; H 6.25; N 3.47. Found: C 74.35; H 6.50; N 3.41.

(5-Carboxynaphth-1-oyl)-L-cysteinyl-D-valine (1i): To the above peptide (**7**) (0.104 g, 0.13 mmol) was added anisole (0.4 ml) and trifluoroacetic acid (2 ml) and the solution stirred at 50° for 2 h. The solvent was removed *in vacuo* and the residue treated twice with toluene. The crude product was then taken up in EtOAc (60 ml) and washed once with 1 N HCl solution before being extracted with 10% $NaHCO_3$ solution (2 x 20 ml). The combined extracts were acidified with 2 N HCl solution and re-extracted with EtOAc, which was dried and evaporated to leave the deprotected peptide as a white solid. This material was triturated with EtOAc to give the pure product (0.046 g, 83%), mp 150-155° (decomp.) ^1H NMR (500 MHz, D_2O) 0.81 (3H, d, 6.5Hz, CMe); 0.83 (3H, d, 6.5Hz, CMe); 1.20 (1H, s, SH); 2.05-2.09 (1H, m, $CHMe_2$); 2.84-2.96 (2H, AB of ABX, CH_2S); 4.14 (1H, d, 5.5Hz, α-val); 4.72-4.74 (1H, m, α-cyst); 7.47-7.51 (2H, m); 7.60 (1H, d, 7.0Hz); 7.75 (1H, d, 7.0Hz); 8.08 (1H, d, 8.5Hz), 8.38 (1H, d, 8.5Hz). m/e (DCI, NH_3) 436 $(M+NH_4)^+$; 419 $(M+1)^+$; 401; 258; 199; 169.

4-Nitrobenzyl 5-carboxy-1-naphthoate (16): To the mono acid (**5**) (3.55 g, 13 mmol) in dry THF (60 ml) was added triethylamine (1.52 g, 15 mmol) and the solution stirred at room temperature for 30 mins. 4-Nitrobenzylbromide (3.67 g, 17 mmol) was added and the reaction refluxed until TLC showed it was consumed (2 days). The mixture was cooled to room temperature, filtered, washed with saturated $NaHCO_3$ solution, dried and the solvent removed to leave the solid diester (4.40 g). TLC (75% CH_2Cl_2/hexane) Rf 0.30. ^1H NMR (200 MHz, $CDCl_3$) 1.69 (9H, s, ^tBu); 5.55 (2H, s, OCH_2Ph); 7.59-7.77 (4H, m); 8.12 (1H, d, 8.0Hz); 8.29 (3H, d, 8.0Hz); 9.12 (2H, d, 8.0Hz). Without further purification, this material was added to a solution of anisole (4 ml) and TFA (15 ml) in dry CH_2Cl_2 (30 ml). After 3 h at room temperature, TLC showed consumption of the diester. The solvent was removed and the residue triturated with ethanol to leave the product (**16**) as a white solid (3.62 g, 80%). ^1H NMR (200 MHz, DMSO) 5.58 (2H, s, OCH_2Ph); 7.62-7.85 (4H, m); 8.12 (1H, d, 7.0Hz); 8.25 (3H, d, 7.0Hz); 8.91 (1H, d, 8.5Hz); 9.08 (1H, d, 8.5Hz). m/e (DCI, NH_3) 369 $(M+NH_4)^+$; 351 $(M)^+$; 216; 199; 122; 115; 106.

(2S,5R,6R)-1-Aza-3,3-dimethyl-6-(5'-(4''-nitrobenzyloxycarbonyl)naphth-1'-oylamido)-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid 4-nitrobenzyl ester (17): The acid (**16**) (3.0 g, 8.55 mmol) was treated with thionyl chloride (30 ml) and the mixture refluxed for 2 h. The excess thionyl chloride was then removed *in vacuo* and the residue dissolved in CH_2Cl_2 (50 ml). This solution

was added to a cooled, stirred solution of 6-aminopenicillanic acid 4-nitrobenzyl ester toluene sulfonic acid salt (5.4 g, 10 mmol) and triethylamine (3.03 g, 30 mmol) in CH_2Cl_2 (40 ml). After stirring for a few mins, the solvent was removed and the residue taken up in EtOAc, washed with saturated $NaHCO_3$ solution, 1N HCl solution, dried and the the solvent removed to leave a solid, which was recrystallised from acetone/hexane to give (**17**) as a pale yellow solid (3.5 g, 60%). TLC (ether) Rf 0.70. ^1H NMR (200 MHz, $CDCl_3$) 1.48 (3H, s, Me); 1.64 (3H, s, Me); 4.55 (1H, s, 2-H); 5.24-5.40 (2H, ABq, OCH_2Ph); 5.55 (2H, s, OCH_2Ph); 5.73 (1H, d, 4.0Hz, 5-H); 5.99 (1H, dd, 4.0, 8.5Hz, 6-H); 6.70 (1H, d, 8.5Hz, NH); 7.56-7.76 (7H, m); 8.26-8.33 (5H, m); 8.60 (1H, d, 8.5Hz); 9.10 (1H, d, 8.5Hz). IR (cell, $CDCl_3$) 1785m; 1750m; 1715m; 1670m; 1520s; 1350s. m/e (FAB) 685 $(M+1)^+$. $C_{34}H_{28}N_4O_{10}S$ requires C 59.64; H 4.12; N 8.18. Found: C 59.47; H 4.15; N 7.98.

(2S,5R,6R)-1-Aza-6-(5'-carboxynaphth-1'-oylamido)-3,3-dimethyl-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid (2i): To a solution of (**17**) (0.50 g, 0.73 mmol) in THF (40 ml) was added a solution of $NaHCO_3$ (0.123 g, 1.46 mmol) in water (40 ml) and 10% Pd/C (0.5 g). The reaction was hydrogenated for 6 h, after which the THF was evaporated and the remaining mixture filtered (celite) and freeze dried. The black residue was taken up in water, washed with EtOAc and the aqueous layer acidified to pH 3. The solution was then extracted with EtOAc, dried and the solvent removed to leave penicillin (**2i**) as the free acid (0.190 g, 63%). This material was converted to the disodium salt by dissolving it in a solution of $NaHCO_3$ (77 mg, 0.92 mmol.) in water and freeze drying, mp >200° ^1H NMR (500 MHZ, D_2O) 1.38 (3H, s, Me); 1.45 (3H, s, Me); 4.11 (1H, s, 2-H); 5.55-5.57 (2H, ABq, 4.0Hz, 5-H, 6-H); 7.42-7.48 (3H, m); 7.55 (1H, d, 7.5Hz); 7.96 (1H, d, 8.0Hz); 8.14 (1H, d, 8.0Hz). m/e (FAB) 415 $(M+1)^+$.

1,4-Dicarboxybenzene mono 4-Methoxybenzyl ester (8): 1,4-Dicarboxybenzene (5.32 g, 32 mmol) and dicyclohexylamine (12 ml, 64 mmol) were dissolved in dry DMF (40 ml) and heated to 70° for 20 mins. 4-Methoxybenzyl chloride (5.0 g, 32 mmol) and a few crystals of sodium iodide were added and the heating continued overnight. The mixture was cooled, diluted with EtOAc, filtered, the organic layer washed repeatedly with water and then extracted with 5% $NaHCO_3$ solution. Acidification to pH 9 precipitated a white solid (0.20 g), shown to be the mono-ester (**8**) contaminated with some starting diacid, which was directly used for the next reaction. ^1H NMR (200 MHz, acetone-d_6) 3.76 (3H, s, OCH_3); 5.27 (2H, s, OCH_2); 6.94 (2H, d, 7.5Hz, MeOArH); 7.39 (2H, d, 7.5Hz, MeOArH); 8.02 (4H, s).

(4-(4'-Methoxybenzyloxycarbonyl)-benzoyl)-(S-diphenylmethyl-L-cysteinyl)-(D-valine benzhydryl ester) (9): A suspension of the impure mono-ester (**8**) (0.20 g), S-diphenylmethyl-L-cysteinyl-D-valine benzhydryl ester (**6**)[3] (0.30 g, 0.54 mmol) and EEDQ (0.172 g, 0.70 mmol) in CH_2Cl_2 (20 ml) were stirred overnight. The CH_2Cl_2 was evaporated and the residue taken up in EtOAc, washed with 1N HCl and 10% $NaHCO_3$ solutions, dried and the solvent removed. The crude product was purified by column chromatography (60% ether/hexane) to give (**9**) as a white solid (0.28 g), mp 141-144° (decomp.) $[\alpha]_D^{20} = -11.4°$ (c 0.8, $CHCl_3$). TLC (60% ether/hexane) Rf 0.30. ^1H NMR (200 MHz, $CDCl_3$) 0.77 (3H, d , 6.5Hz, CMe); 0.91 (3H, d, 6.5Hz, CMe); 2.20-2.32 (1H, m, $CHMe_2$); 2.84-2.97 (2H, AB of ABX, CH_2S); 3.84 (3H, s, OMe); 4.64-4.74 (2H, m, α-cyst, α-val); 5.34 (2H, s, CH_2OPh); 5.36 (1H, s, $SCHPh_2$); 6.68 (1H, d, 8.5Hz, NH); 6.90 (1H, s, $CHPh_2$); 6.94 (2H, d, 8.5Hz, MeOArH); 7.01 (1H, d, 8.0Hz, NH); 7.20-7.50

(22H, m); 7.78 (2H, d, 8.5Hz, ArHCON); 8.10 (2H, d, 8.5Hz, ArHCOO). m/e (FAB) 821 $(M+1)^+$. $C_{50}H_{48}O_7N_2S$ requires: C 73.15; H 5.89; N 3.41. Found: C 73.20; H 6.26; N 3.14.

4-Carboxybenzoyl-L-cysteinyl-D-valine (1j): The peptide (**9**) (0.100 g) was deprotected with trifluoroacetic acid using the method described for compound (**1i**) to give (**1j**) as a white solid, mp >200°. ^1H NMR (500 MHz, acetone-d_6) 0.94 (3H, d, 6.5Hz, CMe); 0.98 (3H, d, 6.5Hz, CMe); 2.16-2.25 (1H, m, $CHMe_2$); 2.95-3.00 (1H, m, A of ABX, CHS); 3.06-3.12 (1H, m, B of ABX, CHS); 4.41-4.45 (1H, m, α-val); 4.82-4.86 (1H, m, α-cyst); 7.56 (1H, d, 8.5Hz, NH); 8.01 (3H, d, 8.0Hz, NH, ArH); 8.09 (2H, d, 8.0Hz, ArH). m/e (FAB) 735 (disulfide, $M+1)^+$.

***trans*-3-Hexenedioic acid monobenzyl ester (12):** *trans*-3-Hexenedioic acid (5.76 g, 0.04 mol) and dicyclohexylamine (5 ml) in DMF (40 ml) were stirred at 70° for 20 mins. Benzyl bromide (4.76 ml, 0.04 mol) was added and the suspension stirred for another 20 mins. The reaction was then cooled, diluted with EtOAc, filtered, washed with water and extracted with 5% $NaHCO_3$ solution. The aqueous phase was acidified to pH 2 with H_2SO_4 (5 M), extracted with ether, dried and the solvent evaporated to leave a solid which was recrystallised from ether/hexane to give (**12**) as a white solid (2.0 g, 21%), mp 40-41°. ^1H NMR (500Mz, $CDCl_3$) 3.16 (4H, t, 5.5Hz, CH_2CO); 5.14 (2H, s, OCH_2Ph); 5.62-5.86 (2H, m, CCH); 7.32-7.42 (5H, m, ArH). IR (cell, $CDCl_3$) 1735s; 1710s. m/e (DCI, NH_3) 252 $(M+NH_4)^+$; 235 $(M+1)^+$; 108; 91.

(*trans*-5-Benzyloxycarbonyl-3-pentenoyl)-(S-benzyl-L-cysteinyl)-(D-valine) benzyl ester (13): A solution of mono-ester (**12**) (0.165 g, 0.70 mmol), S-benzyl-L-cysteinyl-D-valine benzyl ester toluenesulfonic acid salt (0.38 g, 0.64 mmol), triethylamine (0.09 ml, 0.64 mmol) and EEDQ (0.160 g, 0.64 mmol) were stirred in CH_2Cl_2 overnight. The solvent was removed, EtOAc added and the solution washed with 10% $NaHCO_3$ solution, 10% HCl, H_2O and dried. Evaporation of the solvent left a solid residue which was recrystallised from ether/hexane to give a white solid (0.21 g, 53%), mp 67-68°, $[\alpha]_D^{20}$= -5.1° (c 0.8, $CHCl_3$). TLC (ether) Rf 0.65. ^1H NMR (500 Mz, $CDCl_3$) 0.86 (3H, d, 6.5Hz, CMe); 0.92 (3H, d, 6.5Hz, CMe); 2.18-2.22 (1H, m, $CHMe_2$); 2.68-2.72 (1H, A of ABX, CHS); 2.87-2.90 (1H, B of ABX, CHS); 2.99 (2H, d, 7.0Hz, CH_2CO); 3.16 (2H, d, 6.5Hz, CH_2CO); 3.80 (2H, s, SCH_2); 4.48-4.52 (1H, m, α-cys); 4.54-4.56 (1H, m, α-val); 5.13 (2H, s, OCH_2); 5.12-5.20 (2H, ABq, OCH_2); 5.68-5.71 (1H, m, CCH); 5.73-5.77 (1H, m, HCC); 6.45 (1H, d, 7.0Hz, NH); 6.69 (1H, d, 8.5Hz, NH); 7.06-7.39(15H, m, ArH). IR (cell, $CDCl_3$) 1730s; 1670m. m/e (DCI, NH_3) 617 $(M+1)^+$; 495; 234; 208; 108; 91. $C_{35}H_{41}O_6N_2S$ requires: C 68.04; H 6.69; N 4.54. Found: C 68.15; H 6.63; N 4.54.

(*trans*-5-Carboxy-3-pentenoyl)-(L-cysteinyl)-(D-valine) (1k): Liquid ammonia (20 ml) was distilled, from sodium, into a solution of (**13**) (78 mg), in dry THF (2 ml). Small pieces of sodium were added until a blue colour persisted. After 2 mins, a few crystals of ammonium acetate were added to discharge the blue colour, the ammonia was removed with a stream of argon and the THF evaporated to leave an oil which was dissolved in water (15 ml) and washed with EtOAc. The aqueous phase was then acidified to pH 8.6 and oxygen bubbled through for 3 h. Freeze drying left the crude material which was purified by HPLC (Reverse phase ODS; 6% methanol/20 mM ammonium bicarbonate; flow rate 4 ml/min; retention times, thiol 9 min, disulfide 18 min) to give (**1k**) as the thiol (5 mg) and disulfide (12 mg). ^1H NMR (500 MHz, D_2O) 0.67 (3H,

d, 6.5Hz, CMe); 0.72 (3H, d, 6.5Hz, CMe); 1.91-1.95 (1H, m, $CHMe_2$); 2.78 (2H, d, 7.0Hz, CH_2CO); 2.81-2.83 (1H, A of ABX, CHS); 2.93 (2H, d, 6.0Hz, CH_2CO); 3.02-3.06 (1H, B of ABX, CHS); 3.91 (1H, d, 5.4Hz, α-val); 4.52-4.54 (1H, m, α-cyst); 5.39-5.44 (1H, m, CCH); 5.56-5.63 (1H, m, HCC). ^{13}C NMR 18.0; 19.8; 31.6; 39.6; 40.0; 42.0; 53.9; 61.5; 125.2; 131.2; 172.0; 175.8; 178.9; 180.5. m/e (FAB) 691.

***cis*-3-Hexenedioic acid monobenzyl ester (14):** *cis*-3-Hexenedioic acid was synthesised using the method of Benington and Morin[7] and mono-benzylated using the procedure for (**12**) to give (**14**) as a clear oil, in 37% yield. ^1H NMR (200 MHz, $CDCl_3$) 3.14-3.22 (4H, m, CH_2CO); 5.12 (2H, s, OCH_2Ph); 5.76-5.92 (2H, m, CCH); 7.32-7.42 (5H, m, ArH). m/e (DCI, NH_3) 252 $(M+NH_4)^+$; 235 $(M+1)^+$; 108; 91.

(*cis*-5-Benzyloxycarbonyl-3-pentenoyl)-(S-benzyl-L-cysteinyl)-(D-valine) benzyl ester (15): Using the procedure described for compound (**13**), the *cis*-monobenzyl ester (**14**) was coupled to S-benzyl-L-cysteinyl-D-valine benzyl ester toluenesulfonic acid salt. The product was purified by flash chromatography (80% ether/hexane) to give (**15**) as a white solid in 60% yield, mp 90-92°, $[\alpha]_D^{20}$=-8.5° (c 0.8, $CHCl_3$). TLC (ether) Rf 0.80. ^1H NMR (200 MHz, $CDCl_3$) 0.85 (3H, d, 6.5Hz, CMe); 0.91 (3H, d, 6.5Hz, CMe); 2.12-2.30 (1H, m, $CHMe_2$); 2.67-2.92 (2H, AB of ABX, SCH_2); 3.03 (2H, d, 6.5Hz, CH_2CO); 3.18 (2H, d, 6.0Hz, CH_2CO); 3.77 (2H, s, SCH_2); 4.47-4.59 (2H, m, α-cyst, α-val); 5.14 (2H, s, OCH_2); 5.08-5.12 (2H, ABq, OCH_2); 5.77-5.88 (2H, m, CCH); 6.67 (1H, d, 7.0Hz, NH); 6.76 (1H, d, 9.0Hz, NH); 7.32-7.37 (15H, m, ArH). IR (cell, $CDCl_3$) 1740s; 1670s; 1500m. m/e (FAB) 617$(M+1)^+$. $C_{35}H_{41}O_6N_2S$ requires: C 68.15; H 6.54; N 4.54. Found: C 67.79; H 6.72; N 4.68.

(*cis*-5-Carboxy-3-pentenoyl)-L-cysteine-D-valine (1l): The benzyl protected *cis*-tripeptide (**15**) was deprotected using the conditions described for the *trans* analogue (**1k**). Purification using the same HPLC conditions gave (**1l**) in the disulfide form. ^1H NMR (500 MHz, D_2O) 0.66 (3H, d, 6.5, CMe); 0.71(3H, 6.5Hz, CMe); 1.89-1.94 (1H, m, $CHMe_2$); 2.77-2.80 (1H, A of ABX, CHS); 2.83 (2H, d, 8.0Hz, CH_2CO); 2.98 (2H, d, 7.5Hz, CH_2CO); 3.02-3.05 (1H, B of ABX, CHS); 3.90 (1H, d, 5.5Hz, α-val); 4.51 (1H, t, 5.0Hz, α-cyst); 5.44-5.47 (1H, m, CCH); 5.60-5.64 (1H, m, HCC). ^{13}C NMR 18.0; 19.8; 31.6; 35.0; 36.4; 39.4; 53.8; 61.5; 124.2; 129.9; 172.1; 175.5; 178.9; 180.6. m/e (FAB) 691 $(M+1)^+$.

Incubation of tripeptides with IPNS.

General procedure: To the tripeptide (1.0 mg) was added IPNS (10 I.U. in 50 mM NH_4HCO_3 solution, 3.5 ml), ascorbate (50 mM, 100 μL), iron sulfate (5 mM, 100 μL) and dithiothreitol (100 mM, 100 μL) and the solution split in half and shaken (27°, 250 rpm). After 15 mins and 30 mins, extra aliquots of dithiothreitol (100 mM, 100 μl) were added. After one hour the incubation was stopped, acetone (10 ml) added and the supernatant collected after centrifugation. The acetone was evaporated and the residue freeze dried. The sample was purified by HPLC (the fractions collected at dry ice temperature to inhibit ammonolysis) and the penicillin product identified by ^1H NMR, mass spectroscopy and biological activity.

(2S,5R,6R)-1-Aza-6-(5'-carboxynaphth-1'-oylamido)-3,3-dimethyl-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid (2i): HPLC; reverse phase ODS; eluant, 3%

acetonitrile/10 mM ammonium bicarbonate; flow rate, 3ml/min; retention time, 13 mins. ^{1}H NMR and m/e identical to authentic material described above. Active against *Staphylococcus aureus* and *Escherichia coli*, with activity about 1% of Pen G.

(2S,5R,6R)-1-Aza-6-(4'-carboxybenzoylamido)-3,3-dimethyl-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid (2j): HPLC; reverse phase ODS; eluant 20 mM ammonium bicarbonate; flow rate, 1ml/min; retention time, 16 mins. ^{1}H NMR (500 MHz, D_2O) 1.38 (3H, s, Me); 1.50 (3H, s, Me); 4.13 (1H, s, 2-H); 5.48 (1H, d, 4.0Hz, 5-H); 5.51 (1H, d, 4.0Hz, 6-H); 7.65 (2H, d, 8.0Hz, ArH); 7.77 (2H, d, 8.0Hz, ArH). m/e (FAB) 387 $(M+Na)^+$. Active against *S. aureus*.

(2S,5R,6R)-1-Aza-6-(*trans*-5'-carboxy-3'-pentenoylamido)-3,3-dimethyl-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid (2k): HPLC; reverse phase ODS; eluant, 20 mM ammonium bicarbonate; flow rate, 1ml/min; retention time, 14 mins. ^{1}H NMR (500 MHz, D_2O) 1.29 (3H, s, Me); 1.40 (3H, s, Me); 2.74 (2H, d, 6.0Hz, CH_2CO); 2.86 (2H, d, 6.5Hz, CH_2CO); 4.01 (1H, s, 2-H); 5.22 (1H, d, 4.0Hz, 5-H); 5.33 (1H, d, 4.0Hz, 6-H); 5.36-5.38 (1H, m, CCH); 5.51-5.56 (1H, m, HCC). m/e (FAB) 343 $(M+1)^+$. Active against *S. aureus*.

(2S,5R,6R)-1-Aza-6-(*cis*-5'-carboxy-3'-pentenoylamido)-3,3-dimethyl-7-oxo-4-thiabicyclo(3.2.0)heptane-2-carboxylic acid (2l): HPLC; reverse phase ODS; eluant, 20 mM ammonium bicarbonate; flow rate, 1 ml/min; retention time, 13 mins. ^{1}H NMR (500 MHz, D_2O) 1.28 (3H, s, Me); 1.40 (3H, s, Me); 2.78 (2H, d, 7.5Hz, CH_2CO); 2.94 (2H, d, 7.5Hz, CH_2CO); 4.01 (1H, s, 2-H); 5.22 (1H, d, 4.0Hz, 5-H); 5.32 (1H, d, 4.0Hz, 6-H); 5.40-5.42 (1H, m, CCH); 5.60-5.62 (1H, m, HCC). m/e (FAB) 365 $(M+Na)^+$. Active against *S. aureus*.

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