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FURTHER EVIDENCE FOR THE INVOLVEMENT OF A MONOCYCLIC β-LACTAM IN THE ENZYMATIC CONVERSION OF δ-L-α-AMINOADIPOYL-L-CYSTEINYL-D-VALINE INTO ISOPENICILLIN N.

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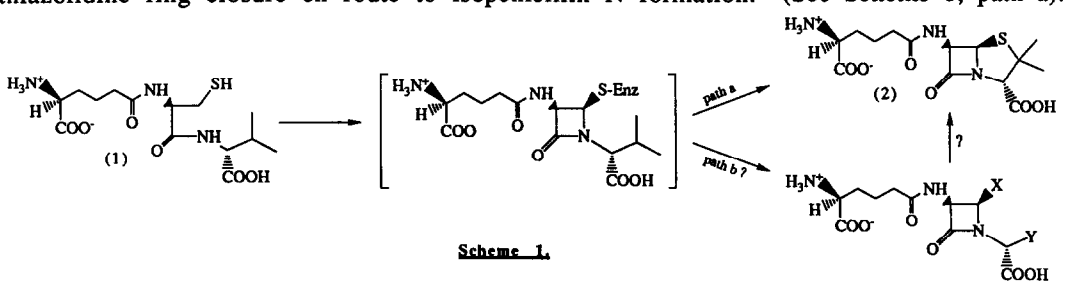
Abstract: Incubation of δ-L-α-aminoadipoyl-L-[3-^{13}C]cysteinyl-D-[3-^{2}H]valine with Isopenicillin N Synthase (IPNS) resulted in the observation of a 'shunt metabolite', which we believe is formed from the collapse of an enzyme bound monocyclic β-lactam intermediate. Chemical studies into the origin of the shunt metabolite suggest its formation occurred after initial β-lactam ring closure. Further chemical studies on the decomposition pathway of a free thiol monocyclic β-lactam have indicated it is not the source of the shunt metabolite, as upon decomposition the major product formed retains sulphur in the form of an ene-thiol dehydrocysteine.

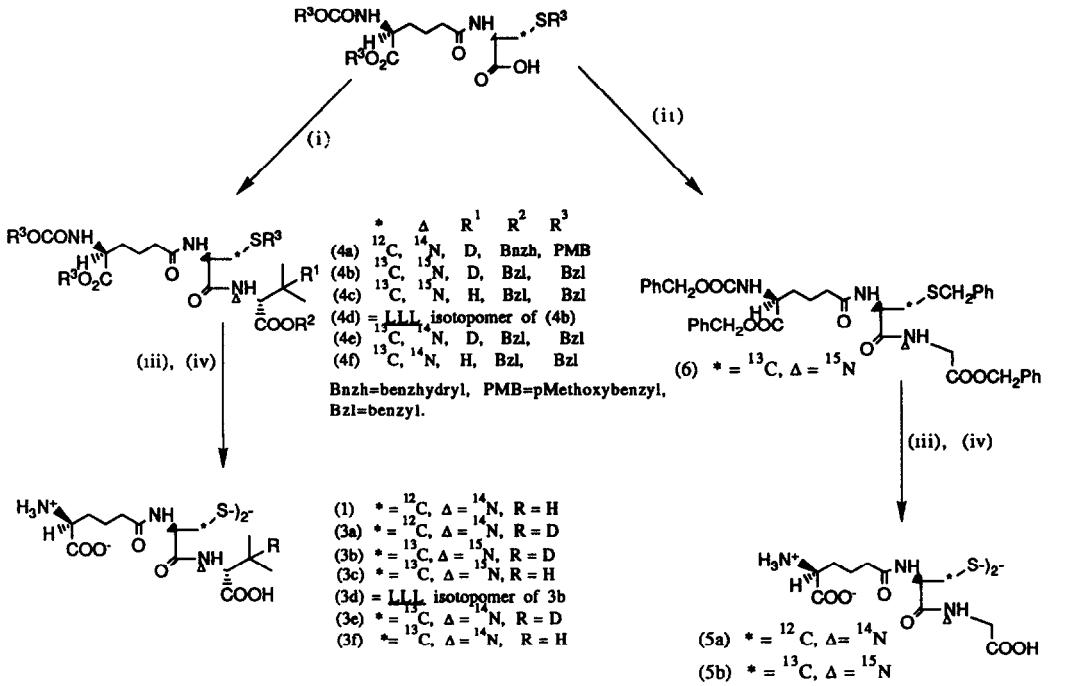
Isopenicillin N Synthase (IPNS) is the enzyme which catalyses the cyclization of the Arnstein tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (LLD-ACV) (1) into isopenicillin N (2).[1,2] This process has been shown to occur in a step wise fashion[3,4] each closure exhibiting a marked primary kinetic isotope effect.[5] Evidence to date strongly suggests that initial closure of the β-lactam ring occurs, followed by thiazolidine ring closure en route to isopenicillin N formation.[6] (See Scheme 1, path a).



Scheme 1.

The stepwise nature of this reaction suggested to us that it may be possible to observe a monocyclic intermediate or other products formed as a result of leakage of the partially processed substrate from the enzyme before thiazolidine ring closure occurred (See Scheme 1, path b). To further increase the possibility of such

observations it was deemed adventitious to retard, by means of kinetic isotope effects, or even prevent thiazolidine ring closure by presenting IPNS with modified substrates. Thus two analogues were synthesised firstly by the incorporation of deuterium instead of hydrogen at the C-3 position of valine, e.g. (3a), and secondly the δ-L-α-aminoadipoyl-L-cysteinyl-glycine (5a) tripeptide which by its nature would be unable to participate in thiazolidine ring closure. An informative method for observation of a shunt pathway between LLD-ACV (1) and Isopenicillin N (2) would be to follow the fate of the cysteinyl residue of ACV through the enzymatic process. Thus initially a series of specifically labelled tripeptides LLD-A-[3-^{13}C]C-[^{15}N, 3-^{2}H]V (3b), LLD-A-[3-^{13}C]C-[^{15}N]V (3c), LLL-A-[3-^{13}C]C-[^{15}N, 3-^{2}H]V (3d) were prepared from their benzyl protected precursors (4b-d)[7,8] (see scheme 2).



Scheme 2.

(i) EEDQ/NEt_3/and appropiate valine ester, tosylate salt.
(ii) EEDQ/NEt_3/glycine benzyl ester, tosylate salt.
(iii) Tripeptide (4a): TFA/Anisole, Tripeptides (4b->4f, 6): Na/NH_3(l).
(iv) Hg(II), H_2S, O_2/pH 8-9, reverse phase HPLC.

Incubation of the tripeptides (3b and 3c) with IPNS under identical conditions[9], followed by examination of their C-13 n.m.r. spectra in D_2O after protein precipitation and freeze drying gave the n.m.r. spectra shown (Figure 1). Control experiments with the tripepide (3d) and with denatured enzyme were also obtained for comparison. The major difference observed between the spectra was the presence of the resonances at δ_C 89 in the case of (3b), which were approximately 1-5% of the intensity of the corresponding isopenicillin N product. This result was consistently achieved when using both native fungal and recombinant enzymes[9], with several separately prepared samples of the tripeptide (3b). The nature of the signals at δ_C 89 (approximately 1:1 intensity, $\Delta\delta_C$ 7Hz) suggested that a ^{13}C-^{15}N coupling may be responsible for the δ_C 89 apparent doublet. This idea was dismissed after synthesis and incubation of the tripeptide δ-L-α-aminoadipoyl-L-[3-^{13}C]cysteinyl-D-[3-^{2}H]valine, (LLD-A-[3-^{13}C]C-[3-^{2}H]V, (3e)), [and (3f) as a control], as the splitting of the δ_C 89 signals was still present in the absence of the ^{15}N label. It became clear that the signals were not the components of a doublet, but instead probably represented a 1:1 mixture of diastereotopic compounds.

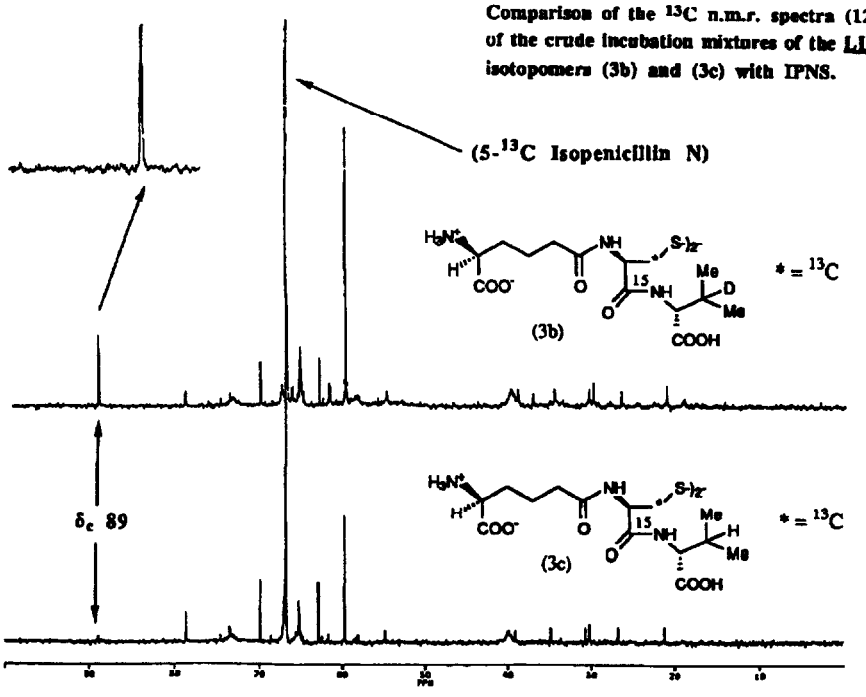
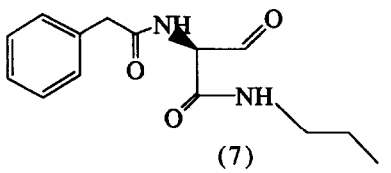
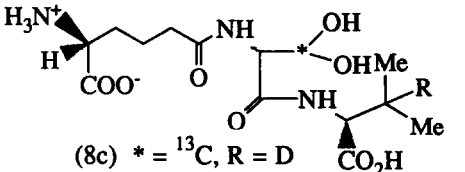


Figure 1
Comparison of the ^{13}C n.m.r. spectra (126MHz) of the crude incubation mixtures of the LLD-ACV isotopomers (3b) and (3c) with IPNS.

In order to further investigate the nature of the δ_C 89 signals the tripeptide δ-L-α-aminoadipoyl-L-[3-^{13}C]cysteinyl-[^{15}N]glycine (5b) was synthesised from (6) (see scheme 2), and incubated with IPNS under identical conditions again this incubation showed a signal at δ_C 89, although this time only as one singlet. As expected the major component in the n.m.r. spectrum (BB, C-13) was that of starting material. Literature searches[10] revealed that the most likely moiety responsible for δ_C 89 would be a hydrated aldehyde (i.e. -C<u>H</u>(OH)$_2$). In support of this idea the C-13 n.m.r. of incubation mixtures (CW mode) indicated that one hydrogen atom was indeed attached to each of the carbons giving rise to the δ_C 89 signals. Model studies were therefore undertaken in order to identify the molecular structure of the shunt metabolite. One such model was the aldehyde (7) derived from the ring opening of Penicillin G sulphone by propylamine, followed by treatment with HCl and extraction.[11] The "aldehyde (7)" actually exists in several forms (i.e aldehyde/hydrate/enol) and could be observed to change (by C-13 n.m.r) from the aldehyde to the hydrate when water was added to a DMSO solution of the aldehyde (7). If D_2O was added the resonance assigned to C<u>H</u>α in the C-13 n.m.r spectrum broadened and disappeared, suggesting exchange of the C<u>H</u>α proton with solvent. Furthermore, the H-1 n.m.r showed the hydrate resonance -C<u>H</u>(OH)$_2$, which appeared as a singlet when D_2O was added to the DMSO solution of the aldehyde. When a DEPT experiment (D_2O) was carried out on the incubation mixtures (observing the signals at δ_C 89), no protons were observed on the carbon atom α to the carbons responsible for the δ_C 89 signals, in agreement with the findings of the work on the model compounds. The exact structure of the 'shunt metabolite' responsible for the δ_C 89 signals observed from (3e) was therefore tentatively assigned as being that shown in structure (8c).



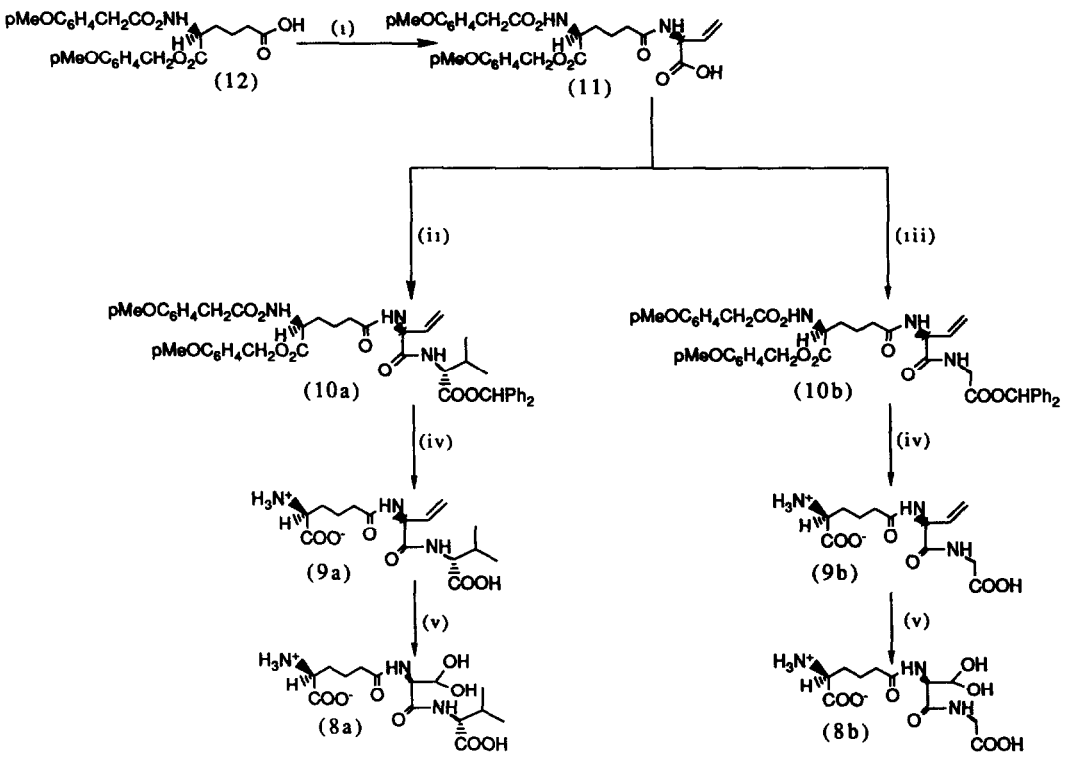
(7)



(8c) * = ^{13}C, R = D

(8d) * = ^{12}C, R = H

A totally synthetic sample of this material was prepared by carrying out an ozonolysis ($MeOH/H_2O$, -50°C), followed by a DMS work up of the tripeptide (9a), which was prepared by standard methodology from the acid labile protected tripeptide (10a) (scheme 3). Similarly (9b) was converted to (8b).



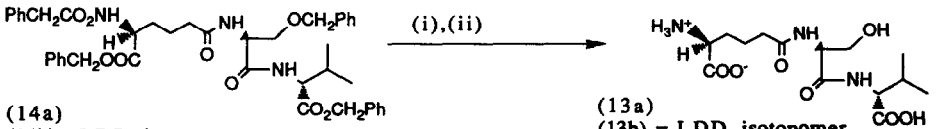
Scheme 3.

(i) isobutylchloroformate/NEt_3/L-vinylglycine, (ii) EEDQ/NEt_3/D-valine benzhydryl ester, tosylate salt. (iii) EEDQ/NEt_3/glycine benzhydryl ester, tosylate salt, (iv) TFA/Anisole, (v) O_3, MeOH/H_2O,-50°C/DMS.

Access to an authentic sample (8a) of the shunt metabolite enabled a thorough spectroscopic comparison to be carried out, all the data obtained for the authentic material was in complete agreement with that obtained from the enzymatically derived shunt compound. Noticably the hydrated aldehydes (8a,8b) obtained via ozonolysis of the tripeptides (9a, and 9b), gave the signals at δ_C 89 (BB) as two and one resonance respectively, in agreement with the enzyme derived shunt metabolitesfrom (3e) and (5b).

HPLC purification of a crude incubation mixture of LLD-A[3-^{13}C]C-[3-^{2}H]V (3e) with IPNS (ODS, mobile phase 20mM NH_4HCO_3), gave a fraction with the same retention time as a synthetic sample of the hydrate which contained the resonances at δ_C 89. This sample also gave a H-1 n.m.r. which showed signals that were identified as the

hydrate $^{13}C\underline{H}(OH)_2$. Treatment of this hydrate (obtained both synthetically and enzymatically) with sodium borohydride gave two new signals with equal intesities at δ_C 62 which were identical to the signals obtained for the -$\underline{C}H_2OH$ carbons from the two tripeptides δ-L-α-aminoadipoyl-L-serinyl-D-valine (13a) and δ-L-α-aminoadipoyl-D-serinyl-D-valine (13b) (These were prepared from the fully benzyl protected tripeptides (14a,14b) by hydrogenation, see scheme 4).



(14a)
(14b)= LDD isotopomer.

(13a)
(13b) = LDD isotopomer of (13a)

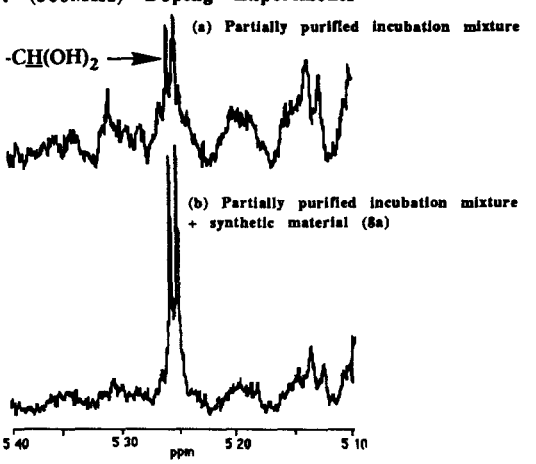
Scheme 4.

(i) H_2/10% Pd/C/THF/H_2O, (ii) Reverse phase HPLC.

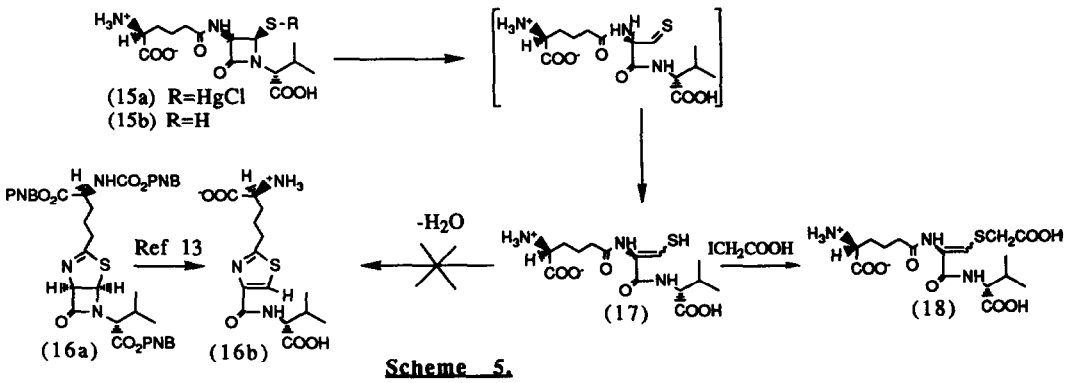
In order to further establish the identical nature of the synthetically and enzymatically derived hydrate, a second HPLC system was employed (μ-bondpack-NH_2, mobile phase 25% MeOH/ 75% (0.05% aq HCOOH)), here again both samples were shown to have identical retention times. Isolation of the "hydrate window" from the HPLC of a crude incubation mixture of δ-L-α-aminoadipoyl-L-cysteinyl-[3-^{2}H]-D-valine (3a) allowed a ^{1}H n.m.r. of the partially purified shunt metabolite (8d) to be obtained. When the sample was doped with the synthetic material (8a) the resonances associated with the hydrate proton -$C\underline{H}(OH)_2$ were enhanced in intensity as anticipated (see figure 2).

Figure 2.

^{1}H n.m.r. (500MHz) Doping Experiments



In order to determine whether the shunt metabolite was derived from an enzyme free mercaptoazetidinone (15b), a synthetic sample of the unstable thiol (15b) was prepared[12] by liberating it from its stable mercury(II) salt (15a), following treatment with H_2S. The mercaptoazetidinone was then exposed to conditions identical to those encountered in IPNS incubations. Upon investigation of the total decomposition products, the C-13 n.m.r. spectrum showed no signs of a δ_C 89 signal, while the proton n.m.r. showed that the major decomposition product gave rise to a singlet at δ_H 8.11. Unsuccessful attempts to directly isolate the decomposition product giving rise to the δ_H 8.11 signal, was followed by the preparation of an authentic sample of the suspected decomposition product, the thiazole (16b) (see scheme 5).



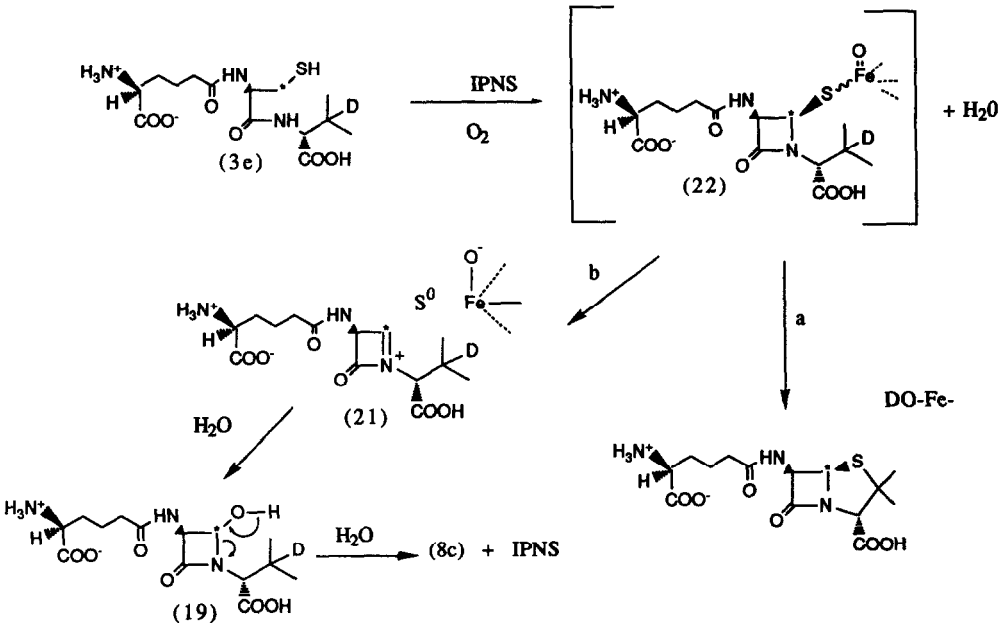
Scheme 5.

Having the relevant spectroscopic and HPLC data it became clear that the decomposition product was not the thiazole, and on this basis the ene-thiol (17) was considered. Thus iodoacetic acid was added to the crude decomposition mixture in order to trap out the putative ene-thiol species. This approach proved successful and allowed HPLC isolation and characterization of the alkylated dehydrocysteine containing tripeptide (18), as a major decomposition product of mercaptoazetidinone (15b).

A possible route whereby the monocyclic enzyme-bound β-lactam intermediate gives rise to the shunt metabolite and yet completes the stoichiometric reduction of dioxygen to water in each catalytic cycle is shown in scheme 6.

As already discussed, the deuterium isotope effect competitively slows carbon-sulphur bond formation to Isopenicillin N (path a), thus promoting fragmentation

(path b) to the imminium ion (21), atomic sulphur and iron(II). Quenching this imminium ion gives rise to the hydroxy-β-lactam.* It has already been shown that such 4-hydroxyazetidinones are unstable with respect to ring opening,[15] forming aldehydes and their derived enols analogous to those we have observed as the shunt metabolite (8).



Scheme 6.

More recently[16] we have shown in our laboratory that incubation of the tripeptide δ-L-α-aminoadipoyl-L-homocysteinyl-D-valine (20) with IPNS gave rise to analogous hydroxylated γ-lactams, this result is readily interpreted on the basis of scheme 6.

In conclusion we have observed the formation of a shunt type metabolite from IPNS as it transforms its natural substrate into isopenicillin N. The nature of the shunt metabolite has been assigned to that of the hydrated aldehyde (8), which we believe is derived from the release of a hydroxy-β-lactam (19) formed as a result of a diversion of the IPNS enzyme from its normal catalytic pathway.

* Such a species has also recently been proposed in the biosynthesis of Clavulanic acid.[14]

Experimental.

Machine details.

^1H nuclear magnetic resonance spectra were recorded on Bruker AM 500 (500MHz), Bruker WH 300 (300MHz), Bruker AM 250 (250MHz) or Varian Gemini 200 (200MHz) spectrometers. ^{13}C nuclear magnetic resonance spectra were recorded on Bruker AM 500 (126MHz) or Varian Gemini 200 (50MHz), multiplicities were assigned by off resonance proton decoupling or DEPT spectrum editing. [All chemical shifts (δ) are expressed in parts per million (ppm)]. ^1H spectra were referenced internally to residual $C\underline{H}Cl_3$ (δ_H 7.27) and $C\underline{H}D_2SOCD_3$ (δ_H 2.50) in $CDCl_3$ or CD_3SOCD_3 respectively, while aqueous solutions (D_2O) were referenced internally to TSP (δ_H 0.00). ^{13}C spectra were referenced internally to $\underline{C}DCl_3$ (δ_C 77.00), dioxan for aqueous solutions (δ_C 67.00) and $CD_3S\underline{O}CD_3$ (δ_C 39.70). Unless otherwise stated the pD of aqueous solutions was 6-7, and were HOD suppressed. High performance (pressure) liquid chromatography (HPLC) was performed on one of two systems depending on sample quantity:

(1). Preparative scale. (>5mg).

Two Gilson model 303 pumps, Rheodyne 7125 injector (1000µl loop), Gilson model HM holochrome detector (set at 214nm), and a 250x10.0mm i.d. column packed with Zorbax® hypersil ODS (particle size 5µm), with a pre-column (30x10.0mm i.d. packed with hypersil ODS). Flow rates were typically 3-4ml/min, with computer controlled gradient operation.

(2). Semi-analytical scale. (<1mg).

Two Waters model 510 pumps, Rheodyne 7125 injector (200µl loop), Waters model 441 and model 440 absorbance detectors (in series, λ= 214 and 254nm respectively), with a 250x4.6mm i.d. column packed with Zorbax® hypersil ODS (particle size 5µm), and a pre-column (15x4.6mm i.d. packed with hypersil ODS). Flow rates were typically 1ml/min, and were controlled with a Waters automated gradient controller. All solvents and samples were filtered (0.5µm) and rigorously degassed before use. Mass spectrometry was performed on a V.G. Mass Lab. 20-250 Quadrupole (ACE (alternative electron impact/chemical ionization)), FAB (+ve argon fast atom bombardment), a V.G. Micromass ZAB-IF, DCI (desorption chemical ionization), FAB (+ve argon) spectrometers. Optical rotations were run on a Perkin-Elmer 241 polarimeter. Micro analysis were performed on a Carlo-Erba Strumentazone Elemental analyser, model 1106. Flash chromatography was performed using Merck silica gel 60. Analytical TLC was performed on commercial Merck silica gel 60 F_{254} aluminium backed plates (0.2mm thickness). Where appropriate reagents and solvents were purified by standard methods.

DL-[3-^{13}C]-S-benzyl cysteine was prepared from ^{13}C paraformaldehyde (90 atom %, Amersham) obtained from an aqueous solution of ^{13}C formaldehyde (9.1% w/v) using, with only slight variation, the method of Upson et al.[17] The S-benzyl-DL-[3-^{13}C]-cysteine was resolved via N-acetylated S-benzyl-DL-[3-^{13}C]-cysteine using hog kidney acylase to give, after ion exchange chromatography, S-benzyl-L-[3-^{13}C]-cysteine.[18] DL-[3-^2H]valine and DL-[^{15}N,3-^2H]valine were prepared by the method of

Baldwin *et al.*[19] and where necessary were resolved using the N-acetyl derivative and hog kidney acylase[18]. After treatment with this enzyme the various N-acetyl D-valines were extracted into EtOAc and crystallised (x2). The amino acids D-valine, DL-[^{15}N,3-^{2}H]valine, D-[^{14}N,3-^{2}H]valine and [^{15}N]glycine were all converted to their benzyl ester ammonium tosylate salts by treatment with benzyl alcohol and TsOH according to usual literature procedures[20]. Additionally D-valine, D-[3-^{2}H]valine and glycine were prepared as their benzhydryl ester ammonium tosylate salts using diphenyldiazomethane[21]. S-4-methoxybenzyl-L-cysteine was prepared by the literature method[22]. L-vinylglycine was prepared from L-methionine in five steps by the method of Rapoport *et al.*[23] in an overall yield of 23% with no observable racemisation. LLD-ACV is a literature compound[7] and was used as a reference for comparison.

General procedures.

(A). EEDQ Couplings of Dipeptides to Protected Amino acids.[7]

Equimolar quantities of EEDQ, NEt_3, benzyl or benzhydryl protected amino acid tosylate salts, and the free carboxylic acid dipeptides (typically 0.3mmol) were dissolved in dry DCM (5ml), with a small quantity of Na_2SO_4 and strirred overnight under Ar(g). EtOAc (30ml) was added, and the solution washed sequentially with $NaHCO_3$ (saturated solution, 30ml), HCl (1M, 30ml), and NaCl (saturated solution, 30ml), dried (Na_2SO_4), filtered, and the solvent removed *in vacuo*, to give the crude fully protected tripeptide. These were purified by column chromatography on silica gel as described for the individual tripeptides.

(B). Acid deprotections of Tripeptides.

The fully protected tripeptide (typically 100mg, 0.1mmol), was dissolved in freshly distilled TFA (2ml) and dry anisole (400µl), and the mixture refluxed under Ar(g) for 15min. After cooling the TFA was removed by azeotroping with toluene (3x5ml), and the residue was partitioned between water (10ml) and EtOAc (10ml). The aqueous layer was washed with further EtOAc (10ml), before freeze drying, to give the tripeptide as its TFA salt. Treatment was then as described for each of the individual tripeptides.

(C) Na/NH_3(l) deprotections of Benzyl protected Tripeptides.[7]

The benzyl protected tripeptides (typically 50mg, 0.05mmol) were dissolved in freshly distilled THF (2ml), to which sodium dried liquid ammonia (30ml) was added by distillation, at -35°C. Small pieces of cleanly cut sodium metal were added to the mixture at -35°C until the blue colour persisted for 10min. At this time the reaction was quenched by the addition of a small quantity of NH_4OAc, with rapid stirring. The ammonia was removed under a stream of Ar(g), at room temperature, and the remaining solvent was removed under high vacuum. This residue was dissolved in 10% AcOH (20ml), washed with EtOAc (2x20ml), before the dropwise addition of 5% $Hg(OAc)_2$ in 10% AcOH. The resulting white precipitate was collected by centrifugation, washed with water (2x20ml), MeOH (20ml), and water (20ml), suspended in water

(2ml), and the free thiol liberated by passing H_2S(g) through the solution for 5 min. The solution was centrifuged, before filtration through washed celite, and freeze drying. The residue was taken up in water (10ml), the pH adjusted to 9-10, and O_2 bubbled through the solution for 4h. This solution was freeze-dried, before the residue was purified by reverse phase HPLC (stationary phase ODS, mobile phase 25mM NH_4HCO_3/MeOH).

(D). Dipeptide Formation with Isobutylchloroformate.[7]

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipic acid, or N-4-methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-L-α-aminoadipic acid (typically 0.5mmol) was dissolved in freshly distilled THF (6ml), together with NEt_3(1eq), and cooled to -15°C for 15min. Isobutyl chloroformate (1eq) was added and the mixture stirred at -15°C for 30min. To this mixture the amino acid (1eq, L-vinylglycine, or S-protected-L-cysteine) in water (6ml) and NEt_3 (2eq), cooled to 0°C, was added rapidly in one portion. The mixture was vigorously stirred at room temperature for 1h. Water (10ml) was added, and the solution washed with Et_2O (2x20ml), followed by acidification to pH 1-2 (1M HCl), and the white precipitate extracted with EtOAc (3x50ml). The organic layer was washed with NaCl (saturated solution, 30ml), dried (Na_2SO_4), filtered, and the solvent removed in vacuo, to give the crude dipeptide as a foam or oil. These were purified by crystallization where appropiate.

(E). Incubation Conditions of IPNS with Substrates.

IPNS was typically available in Tris buffer, which was exchanged with 50mM NH_4HCO_3 before use by the passage through an equilibrated Sephadex G-25 gel filtration column. IPNS, which typically had an activity of 5 I.U./ml, was used with a substrate ratio of approximately 1mg/ml of IPNS solution. The substrate (1mg) was dissolved in 50mM NH_4HCO_3 (3ml), to which was added sequentially DTT (100μl, 100mM), ascorbic acid (100μl, 50mM), and Iron (II) sulphate (100μl, 5mM), followed by the enzyme solution. The mixture was divided into two, to aid aeration, and shaken at 250 r.p.m. at 27°C for 10min. Additional DTT (100μl, 100mM) and Iron(II)sulphate (100μl, 5mM) were added, and the mixture shaken for a further 10min, after which time the enzyme was precipitated by the addition of acetone (20ml), the mixture centrifuged (10,000g, 4°C), and the clear supernatant decanted. The solvent was removed in vacuo, and the residue freeze-dried, to give the "crude incubation mixture".

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-S-benzyl-L-[3-^{13}C]cysteinyl-[^{15}N]glycine.benzylester (6).

^{15}N-Glycine benzylester (0.035g, 0.22mmol), was coupled to benzyl protected LL-'A[3-^{13}C]' (1eq), using IIDQ (1eq, 0.082g), in DMF (5ml) with slight modification of general procedure A.[7] Purification by column chromatography on silica gel [eluant EtOAc/Petrol (2:1) Rf 0.4], gave the title compound, which was recrystallized from

EtOAc/Petrol to give a white solid (0.09g, 56%); δ_H (500MHz, $CDCl_3$), 7.38-7.22 (20H, complex m, ArH), 6.82 (1H, 2xt, J 93, 5Hz, $CH_2{}^{15}N\underline{H}$), 6.24 (1H, d, J 7Hz, NH), 5.58 (1H, d, J 8Hz, NH), 5.20-5.08 (6H, m, 3xABq, 3x$C\underline{H}_2$Ar), 4.54-4.49 and 4.44-4.38 (2H, 2xm, 2x$C\underline{H}\alpha$), 4.10-4.06 and 4.00-3.95 (2H, AB part of ABX system, J 18, 6, 5Hz, $C\underline{H}_2{}^{15}NH$), 3.74 (2H, d, J 6Hz, $^{13}CSC\underline{H}_2Ar$), 3.04-3.00, 2.87-2.83, 2.76-2.71 and 2.59-2.55 (2H, AB part of ABMX system, J 143, 141.5, 14, 7, 6Hz, $^{13}C\underline{H}_2S$), 2.26-2.12 (2H, 2xm, $C\underline{H}_2CO$), 1.93-1.80 and 1.77-1.66 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$); δ_C (126MHz, $CDCl_3$), 172.54, 172.05, 170.60 and 169.28 (4xs, 2x amides, 2x esters), 156.09 (s, urethane), 138.04, 136.23, 135.08 and 135.04 (4xs, Ar$\underline{C}$-1), 128.99-127.28 (d, Ar$\underline{C}$H's), 67.21, 67.16 and 67.04 (3xt, 3x$\underline{C}H_2Ar$), 53.6 (d, $\underline{C}H\alpha$ of aminoadipoyl), 51.97 (d of d, J 48Hz, $\underline{C}H\alpha$-^{13}C), 41.40 (d of t, J 17Hz, ^{15}NH-$\underline{C}H_2$), 36.58 (t, S$\underline{C}H_2$), 35.26 (t, $\underline{C}H_2CO$), 33.11 (t, $^{13}\underline{C}H_2$), 31.91 (t, $\underline{C}H_2CH_2CH_2CO$), 21.25 (t, $CH_2\underline{C}H_2CH_2$); m/z (+ve argon FAB), MH^+ 728.

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-S-benzyl-L-[3-^{13}C]cysteinyl-D-[3-^{2}H]valine, benzylester (4e).

D-[3-^{2}H]Valine benzyl ester, tosylate salt (0.045g, 0.11mmol), was coupled with 'LL-A[3-^{13}C]C'[7] according to general method A. Purification by column chromatography on silica gel [eluant DCM/EtOAc (3:1) Rf 0.5] gave the title compound as a white powder (0.052g, 58%); δ_H (500MHz, $CDCl_3$), 7.36-7.21 (20H, complex m, ArH), 6.75 (1H, d, J 8.5Hz, NH), 6.24 (1H, d, J 7Hz, NH), 5.58 (1H, d, J 8.5Hz, NH), 5.21-5.06 (6H, m, 3xABq, 3x$C\underline{H}_2$Ar), 4.53-4.49 (2H, 1xd and 1xm, J 9Hz, $C\underline{H}\alpha$ of valinyl and cysteinyl), 4.41-4.38 (1H, m, $C\underline{H}\alpha$ of aminoadipoyl), 3.76 (2H, d, J 4Hz, S$C\underline{H}_2$Ar), 3.01-2.97, 2.83-2.79, 2.72-2.68 and 2.55-2.51 (2H, AB part of ABMX system, J 142, 141, 14, 7, 6Hz, $^{13}C\underline{H}_2S$), 2.20-2.09 (2H, 2xm, $C\underline{H}_2CO$), 1.92-1.86 and 1.73-1.64 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.89 and 0.83 (6H, 2xs, $\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ 770; m.p. 109-112°C; $[\alpha]_D^{20}$ –18° (*c* = 1, $CHCl_3$).

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-S-benzyl-L-[3-^{13}C]cysteinyl-D-[^{15}N,3-^{2}H]valine, benzylester (4b) and N-benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-S-benzyl-L-[3-^{13}C]cysteinyl-L-[^{15}N,3-^{2}H]valine, benzylester (4d).

The diastereoisomeric mixture was prepared according to general method A, using benzyl protected LL-A[3-^{13}C][7] and DL-[^{15}N,3-^{2}H]valine benzyl ester, tosylate salt (0.071g, 0.17mmol). The resulting mixture of diastereoisomers was separated by column chromatography on silica gel [eluant EtOAc/Petrol (1:1) Rf 0.5 and 0.45], to give as the less polar component benzyl protected LLD-A[3-^{13}C]C[^{15}N,3-^{2}H]V (4b), (0.052g, 40%); δ_H (500MHz, $CDCl_3$), 7.41-7.18 (20H, complex m, ArH), 6.76 (1H, 2xd, $^2J_{NH}$ 92Hz, J 8.5Hz, ^{15}NH), 6.26 (1H, d, J 7Hz, NH), 5.59 (1H, d, J 8Hz, NH), 5.19-5.04 (6H, m, 3xABq, 3x$C\underline{H}_2$Ar), 4.51 (1H, d, J 9Hz, $C\underline{H}\alpha$ of valinyl), 4.45 (1H, m, $C\underline{H}\alpha$ of aminoadipoyl), 3.78 (2H, d, J 4Hz, S$C\underline{H}_2$Ar), 3.02-2.98, 2.82-2.78, 2.73-2.69 and 2.54-2.49 (2H, AB part of ABMX system, J 142, 141, 14, 7, 6Hz, $^{13}C\underline{H}_2S$), 2.26-2.09 (2H,

2xm, $C\underline{H}_2CO$), 1.92-1.86 and 1.73-1.64 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.89 and 0.84 (6H, 2xs, $\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ 771; m.p. 107-110°C; $[\alpha]_D^{20}$ –18° (*C* = 1, $CHCl_3$). Benzyl protected LLL-A[3-^{13}C]C[^{15}N,3-^{2}H]V (4d) (more polar component), (0.050g, 38%); δ_H (500MHz, $CDCl_3$), 7.41-7.18 (20H, complex m, ArH), 6.86 (1H, 2xd, $^2J_{NH}$ 90Hz, J 9Hz, ^{15}NH), 6.32 (1H, d, J 7Hz, NH), 5.57 (1H, d, J 8Hz, NH), 5.19-5.04 (6H, m, 3xABq, 3x$C\underline{H}_2Ar$), 4.53 (1H, d, J 9Hz, C$\underline{H}\alpha$ of valinyl),4.41 (1H, m, C$\underline{H}\alpha$ of aminoadipoyl), 3.78 (2H, d, J 4Hz, $SC\underline{H}_2Ar$), 3.03-2.98, 2.85-2.81, 2.74-2.70 and 2.58-2.52 (2H, AB part of ABMX system, J 142, 141, 14, 7, 6Hz, $^{13}C\underline{H}_2S$), 2.23-2.09 (2H, 2xm, $C\underline{H}_2CO$), 1.92-1.86 and 1.73-1.64 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.89 and 0.83 (6H, 2xs, $\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ 771.

N-4-Methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-L-α-aminoadipoyl-S-4-methoxybenzyl-L-cysteinyl-D-(3-^{2}H)valine, benzhydrylester (4a).

N-4-Methoxybenzyloxycarbonyl-α-4-methoxybenzyl-δ-L-α-aminoadipoyl-S-4-methoxybenzyl-L-cysteine (0.21g, 0.3mmol), was coupled to D-(3-^{2}H) valine benzhydryl ester (1.1eq, 0.1g) with EEDQ (0.076g, 1eq) according to general method A, to give after purification by column chromatography on silica gel [eluant EtOAc/Petrol (1:1)], the title compound, (0.21g, 77%); δ_H (500MHz, $CDCl_3$), 7.32-7.24 and 6.89-6.77 (24H, 2xm, NH, $C\underline{H}Ph_2$ and ArH), 6.29 (1H, d, J 7Hz, NH), 5.50 (1H, d, J 12Hz, NH), 5.16-5.03 (4H, 2xABq, 2x$C\underline{H}_2Ar$), 4.64 (1H, d, J 12Hz, C$\underline{H}\alpha$ of valine), 4.57-4.51 and 4.38-4.36 (2H, 2xm, 2x$C\underline{H}\alpha$), 3.79 and 3.76 (9H, 2xs, 3xMeO), 3.72 (2H, s, $SC\underline{H}_2Ar$), 2.89-2.79 and 2.70-2.60 (2H, AB part of ABX system), 2.15-2.09 (2H, 2xm, $C\underline{H}_2CO$), 1.92-1.82 and 1.79-1.65 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.88 and 0.76 (6H, 2xs, Me_2CD); δ_C (50MHz, $CDCl_3$), 172.38, 170.38, 170.86 and 170.47 (4xs, 2x ester, 2x amides), 160.04, 159.78 and 159.03 (3xs, 3xAr$\underline{C}$-4), 156.44 (s, 1x urethane), 140.06 and 139.93 (2xs, $\underline{Ph}C$-1), 130.34-127.07 (d's, Ar$\underline{C}$H's), 113.98-114.17 (3xd, 3xAr$\underline{C}$-3), 77.98 (d, $\underline{C}HPh_2$), 67.01 and 66.83 (2xt, 2xO$\underline{C}H_2Ar$), 55.18 (q, 3xMeO), 57.18, 53.65 and 52.06 (3xd, 3x$\underline{C}H\alpha$), 35.71 (t, $CH\underline{C}H_2S$), 35.20 (t, $\underline{C}H_2CO$), 33.09 (t, $S\underline{C}H_2Ar$), 31.67 (t, $\underline{C}HCH_2CH_2$), 21.07 (t, $CH_2\underline{C}H_2CH_2$), 18.85 and 17.10 (2xq, 2xMe_2CD); m.p. 45-50°C; m/z (+ve argon FAB), MNa^+ 957; $C_{52}H_{58}N_3O_{11}SD$ requires C 66.79, H 6.36, N, 4.49, S 3.43% : found C 66.81, H 6.61, N 4.39, S 3.29%; m.p. 45-50°C; $[\alpha]_D^{20}$ –0.2° (*C* = 1.07, CH_2Cl_2).

δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-(3-^{2}H)valine disulphide (3a).

(N-4-Methoxybenzyloxycarbonyl)-α-4-methoxybenzylester–δ-L-α-aminoadipoyl-S-4-methoxybenzyl-L-cysteinyl-D-(3-^{2}H)valine, benzhydryl ester (4a) (0.089g, 0.1mmol), was deprotected, with TFA (2ml) and anisole (200μl) according to general method B. The residue was taken up in water (10ml), the pH adjusted to 9-10, and O_2 bubbled through the solution for 2h. This crude disulphide was purified by reverse phase HPLC (Stationary Phase ODS, mobile phase 25m$\underline{M}$ NH_4HCO_3/20%MeOH) to give the title compound as a white powder (29mg, 89%); δ_H (500MHz, D_2O, pH 6), 4.06 (1H, s, C$\underline{H}\alpha$ of valine), 3.71 (1H, dd J 2x6Hz, C$\underline{H}\alpha$ of aminoadipoyl), 3.21-3.17 and 2.99-2.96 (2H, AB part of ABX system, J 14, 9, 5Hz, $C\underline{H}_2S$), 2.39 (2H, dd, J 2x7Hz, $C\underline{H}_2CO$), 1.90-1.83 and

1.81-1.64 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.89 and 0.84 (6H, 2xs, Me_2CD), $C\underline{H}\alpha$ of cysteine under HOD suppression; δ_C (126MHz, D_2O), 178.42, 176.26, 174.79 and 171.63 (4xs, 2x acids, 2x amides), 61.16, 54.87 and 53.13 (3xd, 3x$\underline{C}H\alpha$), 39.17 (t, $\underline{C}H_2S$), 35.20 (t, $\underline{C}H_2CO$), 30.23 (t, $\underline{C}H_2CH_2CH_2CO$), 21.29 (t, $CH_2\underline{C}H_2CH_2$), 19.36 and 17.58 (2xq, 2x$\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ (disulphide) 727, MH^+ (thiol) 365.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-D-(^{15}N,3-^{2}H)valine disulphide (3b).[7]

(N-Benzyloxycarbonyl)-α-benzylester-δ-(L-α-Aminoadipoyl)-S-benzyl-L-(3-^{13}C)cysteinyl-D-(^{15}N,3-^{2}H)valine benzylester (4b) (0.052g, 0.07mmol) was deprotected according to general method C,[7] to give, after oxidation to the disulphide and purification by reverse phase HPLC (mobile phase 20mM NH_4HCO_3/10% MeOH) the title compound as a white powder (16mg, 63%); δ_H (500MHz, D_2O), 4.11 (1H, s, $C\underline{H}\alpha$ of valine), 3.75 (1H, dd J 2x6Hz, $C\underline{H}\alpha$ of aminoadipoyl), 3.38-2.85 (2H, AB part of ABMX system, J 142, 142, 14, 7, 6Hz, $CH^{13}C\underline{H}_2S$), 2.39 (2H, dd, J 2x7Hz, $C\underline{H}_2CO$), 1.94-1.80 and 1.78-1.62 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.90 and 0.87 (6H, 2xs, $\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ (disulphide) 731, MH^+ (thiol) 367.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-D-(^{15}N)valine disulphide (3c).

(N-Benzyloxycarbonyl)-α-benzylester-δ-(L-α-Aminoadipoyl)-S-benzyl-L-(3-^{13}C)cysteinyl-D-(^{15}N)valine benzylester (4c)[7] (0.072g, 0.09mmol), was deprotected according to general method C, with subsequent oxidation to the disulphide, purification being accomplished by reverse phase HPLC (mobile phase 20mM NH_4HCO_3/20%MeOH), to give the title compound as a white powder (26mg, 76%); δ_H (500MHz, D_2O), 4.07 (1H, s, $C\underline{H}\alpha$ of valine), 3.72 (1H, dd, J 2x6Hz, $C\underline{H}\alpha$ of aminoadipoyl), 3.37-3.33, 3.20-3.04 and 2.86-2.82 (2H, AB part of ABMX system, J 143, 142, 14, 9, 5Hz, $CH^{13}C\underline{H}_2S$), 2.40 (2H, dd, J 2x7Hz, $C\underline{H}_2CO$), 2.13-2.06 (1H, m, $C\underline{H}Me_2$),1.93-1.82 and 1.76-1.66 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.90 and 0.87 (6H, 2xd, J 7Hz, $\underline{Me}_2CH$); m/z (+ve argon FAB), MH^+ (disulphide) 729, MH^+ (thiol) 366.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-L-(^{15}N,3-^{2}H)valine disulphide (3d).

(N-Benzyloxycarbonyl)-α-benzylester-δ-(L-α-Aminoadipoyl)-S-benzyl-L-(3-^{13}C)cysteinyl-L-(^{15}N, 3-^{2}H)valine benzylester (4d) (0.050g, 0.07mmol) was deprotected according to method C to give, after oxidation to the disulphide and purification by reverse phase HPLC (mobile phase 50mM NH_4HCO_3/25% MeOH), the title compound as a white powder (17mg, 72%); δ_H (500MHz, D_2O), 4.09 (1H, s, $C\underline{H}\alpha$ of valine), 3.57-3.54 (1H, m, $C\underline{H}\alpha$ of aminoadipoyl), 3.46-2.81 (2H, AB part of ABMX system, J 142, 141, 14, 7, 6Hz, $CH^{13}C\underline{H}_2S$), 2.39 (2H, dd, J 2x7Hz, $C\underline{H}_2CO$), 1.88-1.63 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.88 and 0.86 (6H, 2xs, $\underline{Me}_2CD$); m/z (+ve argon FAB), MH^+ (disulphide) 731.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-D-(3-^{2}H)valine disulphide (3e).
(N-Benzyloxycarbonyl)-α–benzylester-δ-(L-α-Aminoadipoyl)-S-benzyl-L-(3-^{13}C)Cysteinyl-D-(3-^{2}H)Valine benzylester (4e) (0.052g, 0.07mmol), was deprotected according to general method C, followed by oxidation to the disulphide, and purification being accomplished by reverse phase HPLC (mobile phase 20mM NH_4HCO_3/20%MeOH), to give the title compound as a white powder (19mg, 76%); δ_H (500MHz, D_2O), 4.07 (1H, s, CHα of valine), 3.72 (1H, dd, J 2x6Hz, CHα of aminoadipoyl), 3.37-3.33, 3.20-3.04 and 2.86-2.82 (2H, AB part of ABMX system, J 143, 142, 14, 9, 5Hz, $CH^{13}CH_2S$), 2.40 (2H, dd, J 2x7Hz, CH_2CO), 1.93-1.82 and 1.76-1.66 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.90 and 0.87 (6H, 2xs, Me_2CD); δ_C (126MHz, D_2O) 177.94, 176.29, 174.71 and 171.74 (4xs, 2x amides, 2x acids), 61.09 and 54.94 (2xd, 2xCHα), 53.11 (dd, J 37Hz, $^{13}CH_2CH$), 39.16 (t, $^{13}CH_2$), 35.23 (t, CH_2CO), 30.26 (t $CHCH_2CH_2$), 21.35 (t, $CH_2CH_2CH_2CH$), 19.22 and 17.61 (2xq, MeCD); m/z (+ve argon FAB), MH^+ (disulphide) 729, MH^+ (thiol) 366.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-D-valine disulphide (3f).
The tripeptide (N-benzyloxycarbonyl)-α–benzylester-δ-(L-α–Aminoadipoyl)-S-benzyl-L-(3-^{13}C)cysteinyl-D-valine benzylester (4f)[7] (0.10g, 0.1mmol), was deprotected according to general method C, to give the title tripeptide thiol, which was oxidized to the disulphide form by adjusting the pH of the solution to 9-10, and bubbling O_2 through for 4h. This was purified by reverse phase HPLC (mobile phase 20mM NH_4HCO_3/20%MeOH), to give the title compound as a white powder (24mg, 67%); δ_H (500MHz, D_2O), 4.07 (1H, d, J 5Hz, CHα of valine), 3.73 (1H, dd, J 2x6Hz, CHα of aminoadipoyl), 3.37-3.23, 3.15-3.04 and 2.87-2.82 (2H, AB part of ABMX system, J 143, 142, 14, 9, 5Hz $^{13}CH_2S$), 2.40 (2H, dd, J 2x7Hz, CH_2CO), 2.13-2.06 (1H, m, $CHMe_2$), 1.94-1.81 and 1.78-1.64 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.93 and 0.89 (6H, 2xd, J 7Hz, $CHMe_2$); δ_C (126MHz, D_2O), 178.11, 176.29, 174.74 and 171.70 (4xs, 2x amides, 2x acids), 61.04 and 54.87 (2xd, 2xCHα), 53.14 (dd, J 37Hz, $^{13}CH_2CH$), 39.18 (t, $^{13}CH_2$), 35.23 (t, CH_2CO), 31.10 and 30.26 (t and d, $CHCH_2CH_2$ and $CHMe_2$), 21.34 (t, $CH_2CH_2CH_2$), 19.34 and 17.73 (2xq, MeCH); m/z (+ve argon FAB), MH^+ (disulphide) 727, MH^+ (thiol), 365.

δ-(L-α-Aminoadipoyl)-L-(3-^{13}C)cysteinyl-(^{15}N)glycine thiol (5b).
The protected tripeptide (6) (0.089g, 0.12mmol) was deprotected according to general method C, without subsequent oxidation to give after work up the title compound as a gummy solid (0.023g, 59%); δ_H (500MHz, D_2O, pH 6), 4.56-4.54 (1H, m, X part of ABMX system, CHα of cysteine), 3.79 (2H, d, A_2 part of A_2X system, J 1.5Hz, CH_2 of glycine), 3.75 (1H, dd. J 2x6Hz, CHα of aminoadipoyl), 3.11-3.05 and 2.81-2.77 (2H, AB part of ABMX system, J 144, 144, 14, 7, 5.5Hz, CH_2S), 2.43 (2H, dd, J 2x7Hz, CH_2CO), 1.93-1.87 and 1.77-1.67 (4H, 2xm, $CH_2CH_2CH_2CO$); δ_C (126MHz, D_2O), 176.24, 173.23 and 172.82 (3xs, 2x acids, 2x amides), 55.83 (d, CHα of aminoadipoyl), 53.36 (d of d, J 35Hz, CHα of cysteine), 41.46 (d of t, J 13Hz, $CH_2{}^{15}NH$), 34.77 (t, CH_2CO), 29.50 (t,

$\underline{C}H_2CH_2CH_2CO$), 26.50 (t, $^{13}\underline{C}H_2S$), 20.93 (t, $CH_2\underline{C}H_2CH_2$); m/z (+ve argon FAB), MH^+ 324.

Preparation of N-benzyloxycarbonyl-serine aldehyde propylamide (7).
Penicillin G sulphone[24] (0.10g, 0.3mmol) was stirred in n-propylamine (5ml) at room temperature for 2h, before removal of the solvent in vacuo. The residue was taken up in HCl (1M,10ml), saturated with brine (10ml), and extracted with EtOAc (3x15ml). The combined organic layers were washed with brine (saturated solution, 20ml), dried (Na_2SO_4), filtered, and the solvent removed in vacuo, to give a clear gum (0.061g, 84%); whose ^1H and ^{13}C n.m.r. spectra were observed in different solvents.

(a).
δ_H (200MHz, $CDCl_3$), 9.49 (1H, s, HCO), 7.47-7.20 (5H, m, ArH), 7.03 (1H, d, J 8Hz, NH), 6.80-6.73 (1H, unresolved t, $N\underline{H}CH_2$), 4.98 (1H, d, J 8Hz, CHα), 3.67 (2H, s, $C\underline{H}_2Ar$), 3.16 (2H, overlapping 2xt, $NHC\underline{H}_2$), 1.50-1.46 (2H, m, $C\underline{H}_2Me$), 0.86 (3H, t, $\underline{Me}CH_2$); δ_C (50MHz, $CDCl_3$), 196.15 (d, $H\underline{C}O$), 172.45 and 163.62 (2xs, 2x amides), 134.28 (s, $Ar\underline{C}$-1), 129.52-126.99 (3xd, 3x$Ar\underline{C}H$), 63.17 (d, $\underline{C}H\alpha$), 42.85 and 41.53 (2xt, $\underline{C}H_2Ar$ and $NH\underline{C}H_2$), 22.28 (t, $\underline{C}H_2Me$), 11.03 (q, $\underline{Me}CH_2$); m/z (DCI, NH_3) MH^+ (263, 100%), MNH_4^+ (280, 15%).

(b).
δ_H (500MHz, CD_3SOCD_3), 9.50 (0.5H, s, HCO), 8.21 and 7.73 (0.5H, 2xs, $C\underline{H}OH$, (E) and (Z)), 7.30-7.12 (7H, m, ArH and 2xNH), 5.08 (1H, d, CHα), 3.60 and 3.52 (2H, s, $C\underline{H}_2Ar$), 3.08-3.05 (2H, 3xm, $NHC\underline{H}_2$), 1.49-1.47 (2H, m, $C\underline{H}_2Me$), 0.86-0.72 (3H, t, $\underline{Me}CH_2$); δ_C (126MHz, $CD_3SO_2CD_3$), 196.15 (d, $H\underline{C}O$), 172.15, 171.32, 170.06, 169.93, 165.40, 164.71, 158.83 and 146.66 (6xs, 2xd, 6x amides and 2xC=$\underline{C}$HOH), 136.42 and 136.35 (2xs, 2x$Ar\underline{C}$-1), 129.59-126.33 (d's, $Ar\underline{C}H$'s), 110.02 and 106.54 (2xs, $\underline{C}$=CHOH), 63.38 (d, $\underline{C}H\alpha$), 42.52, 42.06, 41.99, 41.49, 40.63 and 40.39 (6xt, 3x$\underline{C}H_2Ar$ and 3x$NH\underline{C}H_2$), 22.44, 22.22 and 21.94 (3xt, 3x$\underline{C}H_2Me$), 11.26 and 11.20 (q's, $\underline{Me}CH_2$). (data represents mixture of aldehyde (50%) and enols (Z) and (E) (50%)).

(c).
δ_C (126MHz, CD_3SOCD_3 and D_2O (5:1)), 195.69 (d, $H\underline{C}O$), 172.02, 171.29, 171.04, 170.67, 169.40, 165.47, 147.33 (6xs, 1xd, 6xamides and 1xC=$\underline{C}$HOH), 135.82 and 135.45 (2xs, 2x$Ar\underline{C}$-1), 129.48-126.44 (d and s, $Ar\underline{C}H$ and $Ar\underline{C}$), 109.02 (s, $\underline{C}$=CHOH), 88.85 (d, $\underline{C}H(OH)_2$), 41.94, 41.43, 40.80, 40.25, (4xt, $\underline{C}H_2Ar$ and $NH\underline{C}H_2$), 22.20 and 21.94 (2xt, 2x$\underline{C}H_2Me$), 11.22 and 10.04 (q's, $\underline{Me}CH_2$). (data represents mixture of aldehyde/enol/hydrate).

(d).
δ_C (126MHz, CD_3SOCD_3 and H_2O (5:1)), as for (c) except 59.71 (d, $\underline{C}H\alpha$) is observed.

(e).
δ_C (126MHz, CD_3SOCD_3 and excess D_2O (1:1)), 172.08, 169.50 (2xs, 2x amides), 135.14 (s, $Ar\underline{C}$-1), 129.31-126.68 (d's, $Ar\underline{C}H$'s), 88.85 (d, $\underline{C}H(OH)_2$), 41.83 and 40.38, (2xt, $\underline{C}H_2Ar$ and $NH\underline{C}H_2$), 21.66 (t, 2x$\underline{C}H_2Me$), 10.76 (q, $\underline{Me}CH_2$), (data represents hydrate).

$C_{14}H_{18}N_2O_3$ 1/3 H_2O requires C 62.67, H 7.01, N 10.44%, found C 62.67, II 7.41, N 10.77%. The 2,4 dinitrophenylhydrazone derivative was prepared in the usual manner, and recrystallised from water/EtOH to give; δ_H (300MHz, $CDCl_3/CD_3OD$), 9.03 (1H, d, J 2.6Hz, ArCH-3), 8.20 (1H, dd, J 9.5, 2.6Hz, ArCH-5), 7.55 (1H, d, J 4Hz, CH=N), 7.50 (1H, d, J 9.5Hz, ArCH-6), 7.35-7.32 (5H, m, ArH), 5.15 (1H, d, J 4Hz, CHα), 3.64-3.56 (2H, m, CH_2Ar), 3.14 (2H, t, J 7Hz, $NHCH_2$), 1.52-1.45 (2H, tq J 7Hz, CH_2CH_2), 0.87 (3H, t, J 7Hz, $MeCH_2$); $C_{20}H_{22}N_6O_6$ requires 54.29, H 5.01, N 19.00%, found C 54.12, H 5.11, N 19.13%.

Preparation of N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzylester)-δ-L-α-aminoadipoyl-L-vinylglycine (11).

Diprotected aminoadipic acid [N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzylester)-δ-L-α-aminoadipic acid], (0.20g) and 1 equivalent of triethylamine (67μl), were dissolved in freshly distilled THF (6ml), and treated with isobutyl chloroformate (1eq, 60μl) and L-vinylglycine according to general method D. to give upon work up the title compound as a white foam, which was crystallized from Et_2O/Petrol to give the title compound (0.18g, 75%); $[\alpha_D]^{20}$ = -6.7° (c=1, acetone); δ_H (500MHz, $CDCl_3$), 7.29-7.25 and 6.90-6.84 (8H, $2xA_2B_2$ systems, overlapping 2xAr), 6.98 (1H, d, J 7Hz, NH), 5.97-5.89 (1H, 8 line multiplet, $CH=CH_2$), 5.58 (1H, d, J 7Hz, NH), 5.34 (1H, d, J 17Hz, trans-HCH=CH), 5.30 (1H, d, J,10Hz, cis-HCH=CH), 5.10-4.98 (5H, $2xCH_2Ar$ and CHα of Vinylglycine), 4.40 (1H, m, $CHCH_2$), 3.78 (6H, s, 2xMeO), 2.42-2.25 (2H, m, CH_2CO), 1.96-1.80 and 1.75-1.64 (4H, $CH_2CH_2CH_2CO$); δ_C (50MHz, $CDCl_3$), 173.49, 173.07 and 172.64 (3xs, 2x amides, 1x acid, 1x ester), 159.94 and 159.79 (2xs, 2xArC-4,), 156.79 (s, -urethane), 132.05 (d, $CH=CH_2$), 130.31-127.46 (d and s, ArCH, and ArC-1), 118.05 (t, $CH_2=CH$), 113.99 (2xd, 2xArC-3), 67.10 and 66.99 (2xt, OCH_2Ar), 55.18 (2xq, 2xMe), 54.47 and 53.46 (2xd, 2xCHα), 35.01 (t, CH_2CO), 31.34 (t, CH_2CH), 21.16 (t, CH_2CH_2CO); m/z (+ve argon FAB), MNa^+ (551), MH^+ (529).

Preparation of N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzylester)-δ-L-α-aminoadipoyl-L-vinylglycine-D-valine, benzhydrylester (10a).

The dipeptide N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzylester)-δ-L-α-aminoadipoyl-L-vinylglycine (LL-AVinylglycine) (11), (0.245g, 0.45mmol) was coupled to the free amine of the benzhydryl ester of D-Valine (1.1eq) with EEDQ, according to general method A, to give after purification by column chromatography on silica gel [eluant DCM/EtOAc (2:1) Rf 0.6] the title compound as a white foam (0.28g,77%); δ_H (500MHz, $CDCl_3$), 7.36-7.24 and 6.90-6.84 (19H, 2xm, ArH and $CHPh_2$), 6.74 (1H, d, J 9Hz, NH), 6.49 (1H, d, J 8Hz, NH), 5.89-5.83 (1H, complex m, 8 lines, $CH=CH_2$), 5.50 (1H, d, J 8Hz, NH), 5.35 (1H, d, J 17Hz, trans-HCH=CH), 5.25 (1H, d, J 10Hz, cis-HCH=CH), 5.08-4.97 (5H, m, $2xOCH_2Ar$ and CHα), 4.65 (1H, 4 lines, X part of ABX system, J 2x9Hz, CHα), 4.35-4.34 (1H, m, CHα), 3.78 and 3.76 (9H, s, 3xMeO), 2.25-2.12 (3H, 2xm, CH_2CO and CHβ of valine), 1.81-1.64 (4H, 2xm, $CH_2CH_2CH_2CO$),

0.87 and 0.76 (6H, 2xd, J 7Hz, $\underline{Me_2}CH$); δ_C (126 MHz, $CDCl_3$), 172.13, 170.71, 169.75, (4xs, 2x esters, 2x amides), 159.76, 159.66 (2xs, 2x Ar$\underline{C}$-4), 156.12 (s, O$\underline{C}$ONH), 139.53-139.36 (2xs, Ph$\underline{C}$-1), 133.74 (d, $\underline{C}H=CH_2$), 130.11-126.96 (d and s, Ar$\underline{C}$H and Ar$\underline{C}$-1), 118.52 (t, $\underline{C}H_2=CH$), 114.02-113.91 (2xd, Ar$\underline{C}$-3), 78.14 (d, $\underline{C}HPh_2$), 66.97 and 66.80 (2xt, $\underline{C}H_2Ar$), 57.20 and 55.63 (2xd, 2x$\underline{C}H\alpha$), 55.21 (3xq, 3xMeO), 53.52 (d, $\underline{C}H\alpha$), 35.32 (t, $\underline{C}H_2CO$), 31.80 (t, $CH\underline{C}H_2CH_2$), 31.35 (d, $\underline{C}HMe_2$), 21.33 (t, $\underline{C}H_2CH_2CO$), 19.02 and 17.25 (2xq, 2xMe); m/z (+ve argon FAB), MH^+ (794); $C_{45}H_{51}N_3O_{10}$ requires C 68.08, H 6.47, N 5.29% : found C 68.34, H 6.77, N 5.01%; m.p. 97-103°C.

Preparation of N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzylester)-δ-L-α-aminoadipoyl-L-vinylglycine-glycine, benzhydrylester (10b).

The title compound was prepared in an analogous manner to the valinyl analogue, with LL-AVinylglycine (0.27g, 0.55mmol), EEDQ, and glycine benzhydryl ester, to give after purification by column chromatography on silica gel [eluant DCM/EtOAc (1:1) Rf 0.55] the title compound as a white solid, (0.21g, 55%); m.p 115-119°C; δ_H (500MHz, $CDCl_3$), 7.35-7.26 and 6.91-6.86 (19H, 2xm, ArH and $\underline{C}HPh_2$), 6.58 (1H, d, J 5Hz, NH), 6.41 (1H, d, J 7Hz, NH), 5.88-5.81 (1H, complex m, 8 lines $\underline{C}H=CH_2$), 5.47 (1H, d, J 8Hz, NH), 5.38 and 5.28 (2H, 4 lines, J 17Hz, 10Hz, $\underline{C}H_2=CH$), 5.47-5.09 (5H, m, 2x$\underline{C}H_2O$ and $\underline{C}H\alpha$ of vinylglycine), 4.35-4.34 (1H, m, $\underline{C}H\alpha$), 4.19-4.09 (2H, AB part of ABX system, $\underline{C}H_2$ of glycine), 3.79 (6H, s, 2xMeO), 2.31-2.13 (2H, m, $\underline{C}H_2CO$), 1.90-1.75 and 1.72-1.60 (4H, 2xm, $\underline{C}H_2\underline{C}H_2CH_2CO$); δ_C (50MHz, $CDCl_3$), 172.53, 170.49, 169.03 (4xs, 2x amides, 2x esters), 159.96 and 159.75 (2xs, 2xAr$\underline{C}$-4), 156.44 (s, O$\underline{C}$ONH), 139.61 (s, Ph$\underline{C}$-1), 133.40 (d, $\underline{C}H=CH_2$), 130.31-127.05 (d and s, Ar$\underline{C}$H and Ar$\underline{C}$-1), 118.52 (t, $\underline{C}H_2=CH$), 114.06-113.97 (2xd, Ar$\underline{C}$-3), 78.14 (d, $\underline{C}HPh_2$), 66.99 and 66.78 (2xt, $\underline{C}H_2Ar$), 55.60 (d, $\underline{C}H\alpha$), 55.19 (3xs, 3xMeO), 53.52 (d, $\underline{C}H\alpha$), 41.51 (t, $\underline{C}H_2$ of glycine), 35.10 (t, $\underline{C}H_2CO$), 31.52 (t, $\underline{C}H_2CH_2CH_2CO$), 21.12 (t, $CH_2\underline{C}H_2CH_2$); $C_{42}H_{45}N_3O_{10}$ requires C 67.10, H, 6.03, N 5.59%, found C 66.84, H 6.10, N 5.27%; m/z (+ve argon FAB), MH^+ 752.

Preparation of δ-L-α-aminoadipoyl-L-vinylglycine-D-valine (9a).

The protected tripeptide (10a) (0.086g, 0.11mmol) was deblocked with TFA/Anisole (5:1) according to general procedure B, to give after purification by reverse phase HPLC (mobile phase 20m$\underline{M}$ NH_4HCO_3/20% MeOH), the title compound (0.028g, 77%); δ_H (500MHz, D_2O, pH 6), 5.96-5.89 (1H, 8 lines, $\underline{C}H=CH_2$), 5.42 (1H, d, J 17Hz, trans-$\underline{H}$CH=CH), 5.38 (1H, d, J 10Hz, cis-$\underline{H}$CH=CH), 4.92 (1H, d, J 7Hz, $\underline{C}H\alpha$ of vinylglycine), 4.72 (1H, d, J 6Hz, $\underline{C}H\alpha$ of valine), 3.72 (1H, t, J 5Hz, $\underline{C}H\alpha$ of $\underline{L}$-α-AA), 2.38 (2H, dd, J 2x7Hz, $\underline{C}H_2CO$), 2.20-2.09 (1H, m, $\underline{C}HMe_2$), 1.94-1.63 (4H, 2xm, $\underline{C}H_2\underline{C}H_2CH_2CO$), 0.89 and 0.87 (6H, 2xd, J 5Hz, $\underline{Me_2}CH$); δ_C (126MHz, D_2O), 177.22, 175.84, 174.60, and 172.16 (4xs, 2x acids, 2x amides), 132.31 (d, $\underline{C}H=CH_2$), 119.96 (t, $CH=\underline{C}H_2$), 60.17, 56.88 and 54.81 (3xd, 3x$\underline{C}H\alpha$), 35.11 (t, $\underline{C}H_2CO$), 31.01 (t, $\underline{C}H_2CH_2CH_2CO$), 30.23 (d, $\underline{C}HMe_2$), 21.28 (t, $CH_2\underline{C}H_2CH_2CO$), 19.13 and 17.57 (2xq, $\underline{Me_2}CH$); m/z (+ve argon FAB), MH^+ 344.

Preparation of δ-L-α-aminoadipoyl-L-vinylglycine-glycine (9b).

This was prepared in an analogous manner to (9a) from the tripeptide (10b) (0.088g, 0.12mmol), to give after purification by reverse phase HPLC (mobile phase

20mM NH_4HCO_3), the title compound (0.031g, 91%); δ_H (500MHz, D_2O, pH 6), 5.97-5.91 (1H, 8 lines, CH=CH_2), 5.42 (1H, d, J 17Hz, trans-HCH=CH), 5.39 (1H, d, J 10Hz, cis-HCH=CH), 4.92 (1H, d, J 6Hz, CHα of vinylglycine), 3.79-3.70 (3H, m, AB system of glycine CH_2 and CHα of L-α-AA), 2.39 (2H, dd, J 2x7Hz, CH_2CO), 1.90-1.83 and 1.74-1.65 (4H, 2xm, $CH_2CH_2CH_2CO$); δ_C (126MHz, D_2O), 176.53, 176.03, 174.79, and 172.16 (4xs, 2x acids, 2x amides), 132.20 (d, CH=CH_2), 120.00 (t, CH=CH_2), 56.65 and 54.90 (2xd, 2xCHα), 43.86 (t, CH_2 of glycine), 35.21 (t, CH_2CO), 30.25 (t, $CH_2CH_2CH_2CO$), 21.30 (t, $CH_2CH_2CH_2CO$); m/z (+ve argon FAB), MH^+ 302, MNa^+ 324.

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-O-benzyl-D-serinyl-D-valine, benzylester (14b).

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-Aminoadipoyl-O-benzyl-D-Serine, was prepared according to general method D from dibenzyl protected δ-L-α-aminoadipic acid (0.29g)[7] , and O-benzyl-D-serine (0.15g), to give a colourless gum (0.34g 78%); δ_H (200MHz, $CDCl_3$), 9.30 (1H, vbr s, HOOC), 7.43-7.26 (15H, complex m, ArH), 6.79 (1H, d, J 6Hz, NH), 5.82 (1H, d, J 7Hz, NH), 5.21-5.05 (4H, 2xABq, 2xArCH_2O_2C-), 4.78-4.47 (1H, m, CHα of aminoadipoyl), 4.44 (2H, s, OCH_2Ar), 4.42-4.38 (1H, m, CHα of serine), 3.81-3.75 and 3.71-3.65 (2H, AB part of ABX system, CH_2O), 2.25-2.14 (2H, m, CH_2CO), 1.96-1.61 (4H, 2xm, $CH_2CH_2CH_2CO$). This material (0.34g, 0.6mmol) was coupled to D-valine benzyl ester, tosylate salt (0.23g, 0.6mmol), according to general method A, and the crude material was purified by column chromatography on silica gel [eluant DCM/EtOAc (9:1) Rf 0.4], to give the title compound as an oil (0.23g, 75%), $[\alpha_D]^{20}$= -7.5° (c=1.06, DCM); δ_H (500MHz, $CDCl_3$), 7.41-7.28 (20H, m, ArH), 7.13 (1H, d, J 8Hz, NH), 6.39 (1H, d, J 6Hz, NH), 5.52 (1H, d, J 8Hz, NH), 5.21-5.05 (6H, m, 3xCH_2Ar), 4.62-4.37 (5H, m, 3xCHα and CH_2Ar), 3.91-3.86 and 3.51-3.45 (2H, AB part of ABX system J 9, 8, 4Hz, CH_2OCH_2Ar), 2.29-2.10 (2H, m, CH_2CO), 1.91-1.80 (3H, m, $CHMe_2$ and $CH_2CH_2CH_2CO$), 1.69-1.65 (2H, m, CH_2CH_2CO), 0.87 and 0.77 (6H, 2xd, J 7Hz, Me_2CH); δ_C (50MHz, $CDCl_3$), 172.63, 172.42, 171.69 and 170.53 (4xs, 2x esters, 2x amides), 156.29 (s, urethane), 137.40, 136.39 and 135.44 (3xs, 3xArC-1), 128.76-128.12 (d's, ArC-H), 73.51, 69.45, 67.15, 67.06 and 66.96 (5xt, 4xArCH_2 and $CHCH_2OCH_2$), 57.36, 53.65 and 52.17 (3xd, 3xCHα), 35.29 (t, CH_2CO), 31.63 (t, $CH_2CH_2CH_2$), 30.90 (d, $CHMe_2$), 21.07 (t, $CH_2CH_2CH_2$), 18.86 and 17.27 (2xq, 2xMe_2CH); m/z (+ve argon FAB), MH^+ 752;.oil.

N-Benzyloxycarbonyl-α-benzyl-δ-L-α-aminoadipoyl-O-benzyl-L-serinyl-D-valine, benzylester (14a).

This tripeptide was prepared in an analogous manner to its diastereoisomer, to give after purification by column chromatography on silica gel [eluant DCM/EtOAc (9:1)], the title compound as a white solid, which was recystallized from DCM/Petrol, (0.31g, 89%); δ_H (500MHz, $CDCl_3$), 7.54-7.27 (20H, m, ArH), 6.88 (1H, d, J 7Hz, NH), 6.39 (1H, d, J 7Hz, NH), 5.58 (1H, d, J 8Hz, NH), 5.16-5.04 (6H, m, 3xCH_2Ar), 4.65-4.36 (5H, m, 3xCHα and CH_2Ar), 3.91-3.84 and 3.52-3.45 (2H, AB part of ABX system, J 7, 4, 4Hz, CH_2OCH_2Ar), 2.23-2.11 (2H, m, CH_2CO), 1.90-1.80 (1H, br m, $CHMe_2$), 1.69-1.49 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.87 and 0.76 (6H, 2xd, J 7Hz, Me_2CH);

m/z (+ve argon FAB) MH^+ 752; $[\alpha_D]^{20} = 1.4^o$ (c=1.05, DCM); $C_{43}H_{49}N_3O_9$ requires, C 68.69, H 6.57, N 5.59%, found C 68.64, H 6.50, N 5.09%; m.p. 113-118°C.

δ-(L-α-Aminoadipoyl)-L-serinyl-D-valine (13a).

N-Benzyloxycarbonyl-α-benzylester-δ-L-α-aminoadipoyl-O-benzyl-L-serinyl-D-valine, benzylester (14a) (0.1g, 0.13mmol), was dissolved in THF (8ml) and water (8ml), and Pd/C (10%, 20mg) was added. The system was evacuated and flushed with hydrogen gas (x3) before stirring overnight at 40°C. The solution was then filtered through washed celite, and the THF removed in vacuo. The aqueous layer was washed with DCM (30ml), and EtOAc (30ml) before freeze drying. The material recovered was then purified to homogeneity by reverse phase HPLC (mobile phase 25mM NH_4HCO_3), to give the title compound as a white powder in quantitative yield. δ_H (500MHz, D_2O), 4.30 (1H, dd, J 2x5Hz, CHα of serine), 3.92 (1H, d, J 6Hz, CHα of valine), 3.67 (2H, d, J 5Hz, CH_2O), 3.56 (1H, t, J 5Hz, CHα of aminoadipoyl), 2.25 (2H, dd, J 2x7Hz, CH_2CO), 1.94-1.91 (1H, m, $CHMe_2$), 1.73-1.63 and 1.56-1.45 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.72 and 0.68 (6H, 2xd, J 7Hz, Me_2CH); δ_C (126MHz, D_2O), 178.41, 176.47, 174.76 and 171.53 (4xs, 2x acids, 2x amides), 61.65 (t, CH_2OH), 60.87, 56.11 and 54.77 (3xd, 3xCHα), 35.09 (t, CH_2CO), 31.00 (d, $CHMe_2$), 30.16 (t, $CH_2CH_2CH_2CO$), 21.19 (t, $CH_2CH_2CH_2$), 19.72 and 17.49 (2xq, 2xMe_2CH); m/z (+ve argon FAB), MH^+ 348.

δ-(L-α-Aminoadipoyl)-D-serinyl-D-valine (13b).

This title compound was prepared in an analogous manner to its diastereoisomer (13a) from (14b), to give the title compound as a gummy solid (35mg, 84%); δ_H (500MHz, D_2O), 4.31 (1H, dd, J 2x6Hz, CHα of serine), 3.93 (1H, d, J 6Hz, CHα of valine), 3.70-3.63 (2H, 8 lines, AB part of ABX system, J 11, 7, 5Hz, CH_2O), 3.56 (1H, dd, J 2x5Hz, CHα of aminoadipoyl), 2.23 (2H, dd, J 2x7Hz, CH_2CO), 1.93 (1H, m, $CHMe_2$), 1.73-1.67 and 1.56-1.48 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.73 and 0.71 (6H, 2xd, J 7Hz, Me_2CH); δ_C (126MHz, D_2O), 178.06, 176.49, 174.77 and 171.70 (4xs, 2x acids, 2x amides), 61.49 (t, CH_2OH), 60.76, 56.04 and 54.88 (3xd, 3xCHα), 35.15 (t, CH_2CO), 30.91 (d, $CHMe_2$), 30.22 (t, $CH_2CH_2CH_2CO$), 21.34 (t, $CH_2CH_2CH_2$), 19.01 and 17.59 (2xq, 2xMe_2CH); m/z (+ve argon FAB), MH^+ 348.

Data for ozonolysis products.

δ-(L-α-Aminoadipoyl)-DL-[2-amino-1-carboxyl-propan-3-al]-D-valine (8a).

The tripeptide δ-(L-α-aminoadipoyl)-L-vinylglycine-D-valine (0.02g, 0.05mmol) (9a), was dissolved in MeOH/H_2O (3:1), (15ml), and cooled to -50°C. Ozone was bubbled through the solution until the development of a pink/blue colouration, and continued through the solution for 30min. Oxygen was bubbled through the solution for 15min, followed by Ar(g) for 1h at -50°C. Dimethylsulphide (1ml) was added, and Ar(g) bubbled through the solution for a further 4h, during which time the solution was warmed to room temperature. Water (20ml) was added, before

removal of the solvent in vacuo, then further water (5ml) was added before freeze drying the resulting solution. This crude material was purified by reverse phase HPLC (stationary phase μ-bondpack, mobile phase 75% (0.05% HCOOH(aq))/25% MeOH) or (stationary phase ODS, mobile phase 10mM NH_4HCO_3), to give the title compound as a white solid and as an epimeric mixture of hydrates; δ_H (500MHz, D_2O, pH 4), 5.26 and 5.25 (1H, 2xs, $C\underline{H}(OH)_2$), 4.13 and 4.11 (1H, 2xd, $C\underline{H}\alpha$ of valinyl), 3.72 and 3.70 (1H, 2xt, $C\underline{H}\alpha$ of aminoadipoyl), 2.41-2.37 (2H, m, $C\underline{H}_2CO$), 2.12-2.07 (1H, m, $C\underline{H}Me_2$), 1.88-1.64 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.94-0.89 (6H, m, $\underline{Me}_2CH$); δ_C (126MHz, D_2O), 177.00-169.50 (4xs, 2x acids, 2x amides), 89.14 and 89.06 (2xd, $\underline{C}H(OH)_2$), 59.63 and 54.81 (2xbr d, $\underline{C}H\alpha$ of aminoadipoyl, and $\underline{C}H\alpha$ of valinyl), 35.13 (t, $\underline{C}H_2CO$), 30.83 and 30.74 (2xt, $\underline{C}H_2CH_2CH_2CO$), 30.14 and 30.09 (2xd, $\underline{C}HMe_2$), 21.33 and 21.24 (2xt, $CH_2\underline{C}H_2CH_2$), 18.96 and 18.91 (2xq, $\underline{Me}_2CH$), 17.56 (2xq, $\underline{Me}_2CH$), δ_C (126MHz, H_2O/10% D_2O) data as for above except new signal at 58.59); m/z (+ve argon FAB), (ex $(COOH)_2$/ $HOCH_2CHOHCH_2OH$) MH^+ (hydrate) 364, MH^+ (enol/aldehyde) 346. δ_H (500MHz, D_2O, pH > 8.5), 8.11 and 7.72 (1H, 2xs, enolic H (E) and (Z)), 4.03 and 3.97 (1H, 2xd, $C\underline{H}\alpha$ of valinyl), 3.10-3.00 (1H, br m, $C\underline{H}\alpha$ of aminoadipoyl), 2.27-2.21 and 2.17-2.04 (2H, 2xm, $C\underline{H}_2CO$), 2.02-1.84 (1H, 2xm, $C\underline{H}Me_2$), 1.55-1.30 (4H, br m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.78-0.62 (6H, m (2xd)x2, $\underline{Me}_2CH$); δ_C (126MHz, D_2O, pH>8.5), 177.00-169.50 (4xs, 2x acids, 2x amides), 169.4 and 168.9 (2xd, enolic (E) and (Z) C=$\underline{C}H(OH)$), 60.00 and 56.2 (2xbr d, $\underline{C}H\alpha$ of aminoadipoyl, and $\underline{C}H\alpha$ of valinyl both doubled up), 35.9 and 35.8 (t, $\underline{C}H_2CO$), 34.2 and 34.1 (2xt, $\underline{C}H_2CH_2CH_2CO$), 30.1 (2xd, $\underline{C}HMe_2$), 22.2 (2xt, $CH_2\underline{C}H_2CH_2$), 19.4 and 19.5 (2xq, $\underline{Me}_2CH$), 17.8 (2xq, $\underline{Me}_2CH$).

δ-(L-α-Aminoadipoyl)-DL-[2-amino1-carboxyl-propan-3-al]-glycine (8b).

The tripeptide δ-(L-α-aminoadipoyl)-L-vinylglycine-glycine (0.02g, 0.06mmol) (9b) was ozonolysed in an analogous manner to (9a), to give after purification by reverse phase HPLC the title compound as a powdery solid; δ_H (500MHz, D_2O, pH 4), 5.22 (1H, very tight 2xs, Δδ 2Hz (pH dependant), $C\underline{H}(OH)_2$), 3.81-3.76 (2H, d, $C\underline{H}_2$ of glycine), 3.67-3.64 (1H, m, $C\underline{H}\alpha$ of aminoadipoyl), 2.36-2.33 (2H, m, $C\underline{H}_2CO$), 1.82-1.77 and 1.66-1.59 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$); δ_C (126MHz, D_2O), 176.30-171.73 (4xbr s, 2x acids, 2x amides), 89.15 (1xd, $\underline{C}H(OH)_2$), 54.99 (br d, $\underline{C}H\alpha$ of aminoadipoyl), 43.27 (t, $\underline{C}H_2$ of glycine) 35.15 and 34.83 (2xt, $\underline{C}H_2CO$), 30.22 (2xt, $\underline{C}H_2CH_2CH_2CO$), 21.31 and 21.21 (2xt, $CH_2\underline{C}H_2CH_2$).

Observation of hydrate resonances.

The tripeptides (3a-f, 5b) were incubated with IPNS under the standard incubation conditions (method (E)), to give the crude incubation mixtures. These were dissolved in D_2O (400μl) and examined by both ^1H and ^{13}C n.m.r (at 500 and 126MHz respectively), typically collecting 45,000 transients for each overnight ^{13}C experiment. (Spectral differences were observed for tripeptides (3b),(3e), and gave; δ_C (126MHz, D_2O, pH 7-8), 89.10 and 89.14 (2xd, $^{13}\underline{C}H(OH)_2$, Δδ 5-11Hz (pH dependent), J_{CH} 167Hz), DEPT analysis indicated the absence of protons 2 or 3 bonds

distant to this centre).[Tripeptide (5b) treated in an identical manner gave δ_C (126MHz, D_2O) 89.14 (d, $^{13}\underline{C}H(OH)_2$).]

Enzyme used for denatured controls was prepared by heat treating (100°C for 1min), and the mixtures were treated identically to above, no signals in the region of the C-13 spectra of interest were observed. Purification of the crude incubation mixtures from the tripeptides (3a,b,e,5b) was accomplished by reverse phase HPLC (a) stationary phase ODS, mobile phase 20m<u>M</u> NH_4HCO_3, or (b) stationary phase μ-bondpack-NH_2, mobile phase 75% (0.05% HCOOH)/25% MeOH, and treated as indicated below.

<u>Reduction of Hydrate (8c)</u>

The tripeptide (3e) (10mg) was incubated with IPNS under standard incubation conditions. The residue after protein precipitation and freeze-drying was purified by reverse phase HPLC (system (a)) and the fraction that contained the resonances at δ_C 89 coincided with that of the synthetic material. This was dissolved in water (1ml) and treated with $NaBH_4$ (ca 200μg). This mixture was stirred for 3h, before quenching, freeze-drying, and examination by C-13 n.m.r. (126MHz, D_2O, 45,000 transients). Examination of the C-13 spectra showed 2 resonances at δ_C 62 (2xt, $\underline{C}H_2O$, Δδ 20Hz). [The proton spectra before reduction had corresponding signals at δ_H 5.25 (d, J_{AX} 167Hz, J Hz $^{13}C\underline{H}(OH)_2$, and $\Delta\delta_H$ 3Hz). Reduction of a synthetic sample of the hydrate (8a) was carried out in an analogous manner, purified by HPLC (as above), and gave n.m.r. and m/z data identical to that obtained from a 1:1 mixture of the two synthetic tripeptides (13a,b).

<u>Doping experiments.</u>

Tripeptide (3a) (15mg) was incubated with IPNS according to general method E. The crude incubation mixture was examined by ^1H n.m.r. (500MHz); signals assigned to the hydrate resonance (-$C\underline{H}(OH)_2$); δ_H (500MHz, D_2O) 5.22 (2xs, Δδ 3Hz, $C\underline{H}(OH)_2$) increased in intensity when a small portion of the sample was doped with synthetic material (8a). The remaining material was purified by reverse phase HPLC (system (a) or (b)), isolating a fraction that had identical retention times to that of the synthetic sample. Examination of this fraction by ^1H n.m.r. spectroscopy (3000 transients) clearly showed resonances associated with the hydrate resonances. These were enhanced when doped with synthetic material (8a).

<u>Investigation of Hydrate stability.</u>

The hydrated aldehyde (8a or 8b) was incubated under normal conditions (general method E), followed by the usual work up. H-1 n.m.r. showed the presence of the intact hydrate, indicating its stability to incubation conditions.

<u>Preparation and alkylation of ene-thiol (17).</u>

The azetidinone (15a)[12](8mg, 0.013mmol) was dissolved in dilute HCl (1ml, pH 2) at room temperature before bubbling H_2S through the solution for 45s. The resulting black precipitate was immediately removed by filtration and the colourless solution of mercaptoazetidinone was degassed under high vacuum for 90s in order to remove excess H_2S. The thiol was then rapidly transfered to a solution containing the co-factors required by IPNS (19ml 25m<u>M</u> NH_4HCO_3, DTT (7.7mg), ascorbic acid (4.4mg), and $FeSO_4.7H_2O$ (1.4mg). After allowing the solution to stand at room

temperature in the presence of O_2 for 1h the decomposition mixture was freeze-dried. The crude solid (containing (17)) was re-dissolved in D_2O, prior to H-1 and overnight C-13 n.m.r. spectra being obtained. δ_H (500MHz, D_2O), 8.11 (1H, s, C=CHSH), 4.14 (1H, d, J 6Hz, CHα of valinyl), 3.70 (1H, dd, J 6.5, 6Hz, CHα of aminoadipoyl), 2.43-2.41 (2H, m, CH_2CO), 2.14-2.10 (1H, m, $CHMe_2$), 1.96-1.71 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.98-0.88 (6H, m, $CHMe_2$); δ_C (126MHz, D_2O), 179.19, 175.91, 175.98 and 166.47 (4xs, 2x amides, 2x acids), 151.50 (d, C=CHSH), 125.66 (s, C=CSH), 60.80 (d, CHα of valinyl), 55.05 (d, CHα of aminoadipoyl), 35.39 (t, CH_2CO), 31.61 (t, CH_2CH_2CH), 30.61 (d, $CHMe_2$), 21.61 (t, $CH_2CH_2CH_2CO$), 19.31 and 17.70 (2xq, 2xMe). The solution of ene-thiol was treated with a large excess of iodoacetic acid (15eq) and the pH adjusted to 7.8 before allowing to stand overnight. The crude mixture was freeze-dried prior to purifying the alkylated ene-thiol using reverse phase HPLC (ODS, mobile phase 87% (0.05% HCOOH(aq))/13% MeOH, to give for (18); δ_H (500MHz, D_2O), 7.45 (1H, s, $C=CHSCH_2$), 4.17 (1H, d, J 6Hz, CHα of valinyl), 3.75 (1H, dd, J 2x6Hz, CHα of aminoadipoyl), 3.61 (2H, s, SCH_2COO), 2.50-2.46 (2H, m, CH_2CO), 2.12-1.97 (1H, m, $CHMe_2$), 1.94-1.72 (4H, 2xm, $CH_2CH_2CH_2CO$), 0.89 and 0.84 (6H, m, $CHMe_2$); δ_C (126MHz, D_2O), 178.60, 176.51, 175.93, 174.95 and 164.47 (5xs, 2x amides, 3x acids), 139.26 (d, $C=CHSCH_2$), 123.65 (s, C=CHSH), 61.24 (d, CHα of valinyl), 55.00 (d, CHα of aminoadipoyl), 38.61 (t, SCH_2COO), 35.28 (t, CH_2CO), 31.44 (d, $CHMe_2$), 30.52 (t, $CH_2CH_2CH_2CO$) 21.61 (t, $CH_2CH_2CH_2CO$), 19.31 and 17.71 (2xq, 2xMe); m/z (+ve argon FAB) MNa^+ 442, MNa_2^+ 464, MNa_3^+ 486.

Preparation of Thiazole (16b)[13]

Tri(PNB)-protected thiazole (16a)[25] (7mg, 0.009mmol), was dissolved in MeOH (1ml), before adding HCl (1M, 0.25ml), and heating at reflux for 2h. After cooling the reaction mixture was concentrated in vacuo and the residual acidic solution was neutralized with $NaHCO_3$ (saturated solution), and extracted with DCM (10ml). After drying (Na_2SO_4), the organic phase was concentrated and a portion of this crude product was hydrogenated overnight at room temperature (1atm), in THF/Water (1:1), with (Pd/C (10%) 2.5mg). The reaction was then filtered through a celite pad before concentrating, and purification of the thiazole (16) by reverse phase HPLC (mobile phase 40% (0.05% HCOOH(aq))/60% MeOH); δ_H (500MHz, D_2O), 7.95 (1H, s, C=CHS), 4.19 (1H, d, J 6Hz, CHα of valinyl), 3.61 (1H, dd, J 6, 5Hz, CHα of aminoadipoyl), 2.96 (2H, dd, J 2x7Hz, CH_2CNS), 2.12-2.04 (1H, m, $CHMe_2$), 1.82-1.69 (4H, 2xm, $CH_2CH_2CH_2CN$), 0.84 and 0.82 (6H, dd, J 2x7Hz, $CHMe_2$); δ_C (126MHz, D_2O), 177.5, 175.0, 173.5 (3xs 2x amides, 2x acids), 163.5 (s, thiazole C-2),148.0 (s, thiazole C-4), 126.0 (d, thiazole C-5), 61.0 (d, CHα of valinyl), 55.3 (d, CHα of aminoadipoyl), 33.5 (t, CH_2CO), 31.6 (t, $CH_2CH_2CH_2CO$), 30.5 (d, $CHMe_2$), 25.8 (t, $CH_2CH_2CH_2CO$), 19.5 and 18.1 (2xq, 2xMe); m/z (+ve argon FAB) MH^+ 344.

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