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NEW PENICILLINS FROM ISOPENICILLIN N SYNTHASE.

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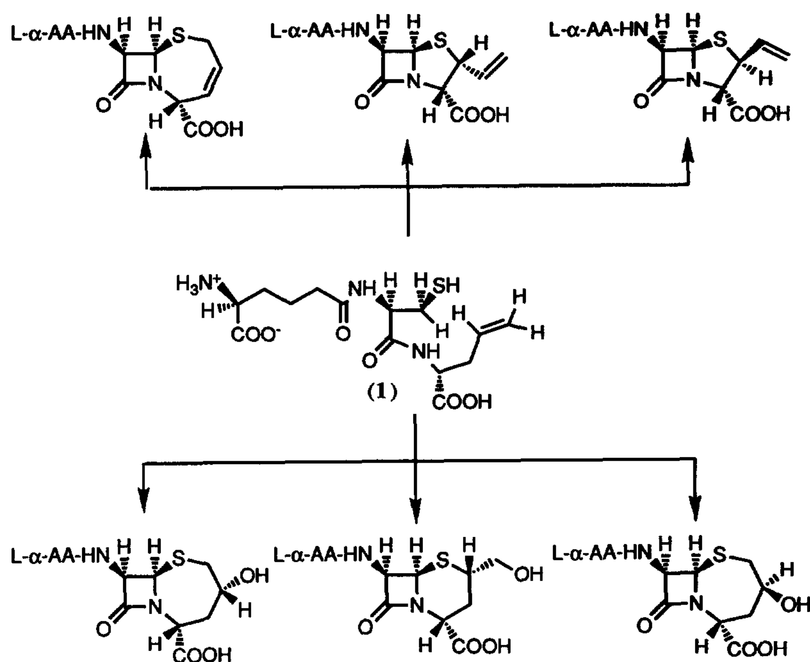
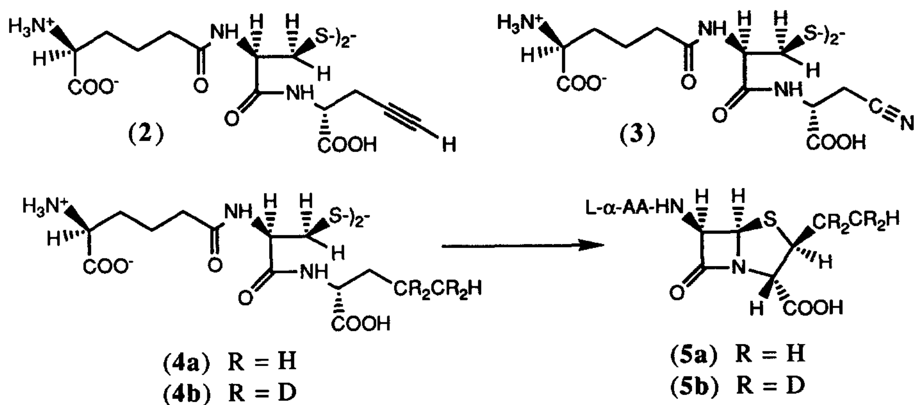
Abstract: The three tripeptides δ-L-α-aminoadipoyl-L-cysteinyl-D-propargylglycine, δ-L-α-aminoadipoyl-L-cysteinyl-D-cyanoalanine and δ-L-α-aminoadipoyl-L-cysteinyl-D-[4,4,5,5-$^{2}H_4$]-norvaline were synthesised and incubated with the enzyme Isopenicillin N Synthase (IPNS). All incubation mixtures contained biologically active products, and led to the isolation of four new penicillins (β-ethyl, α-acetylenic, α- and β-nitrile) following purification by reverse phase HPLC. The stereochemistries of formation of monosubstituted penicillins with IPNS are rationalised.

It has been shown that the enzyme Isopenicillin N Synthase (IPNS) exhibits a broad degree of substrate flexibility, catalysing the bicyclisation of numerous modified natural substrate analogues and enabling many new and interesting β-lactam containing products to be isolated, characterised and tested for biological activity.[1] These results have led to a unified theory of second ring closure to be proposed which covers all the various valinyl substrate analogues,[2] while opening up the possibilities of active site modelling/mapping of the IPNS enzyme from product ratios, stereochemistries and acceptabilities with respect to both steric and electronic constraints of these analogues.

The allylglycine containing tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-allylglycine (1) has been the substrate *par excellence* for IPNS. It has been shown to be converted to six novel β-lactam containing metabolites by this enzyme,[3] including three products which were derived by oxygenation of the olefin by a monooxygenase type action of this most remarkable enzyme.[4]
By analogy the propargylic containing tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-propargylglycine (LLD-ACPg) (2) was considered to be an interesting analogue, while the nitrile containing tripeptide (3) would offer a comparison between the electronic and polar constraints of the nitrile/propargylic systems.



Finally the tripeptide (**4b**) was prepared with the aim of allowing the isolation and characterisation of the penam (**5b**) by utilising a primary kinetic isotope effect to bias its formation. Formation of the penam (**5a**) from the protio analogue (**4a**) had been assumed[5] due to the biological activity of incubation mixtures of the tripeptide with IPNS, but it had never been possible to isolate and characterise this metabolite due to insufficient quantities of material, a cepham product predominating.

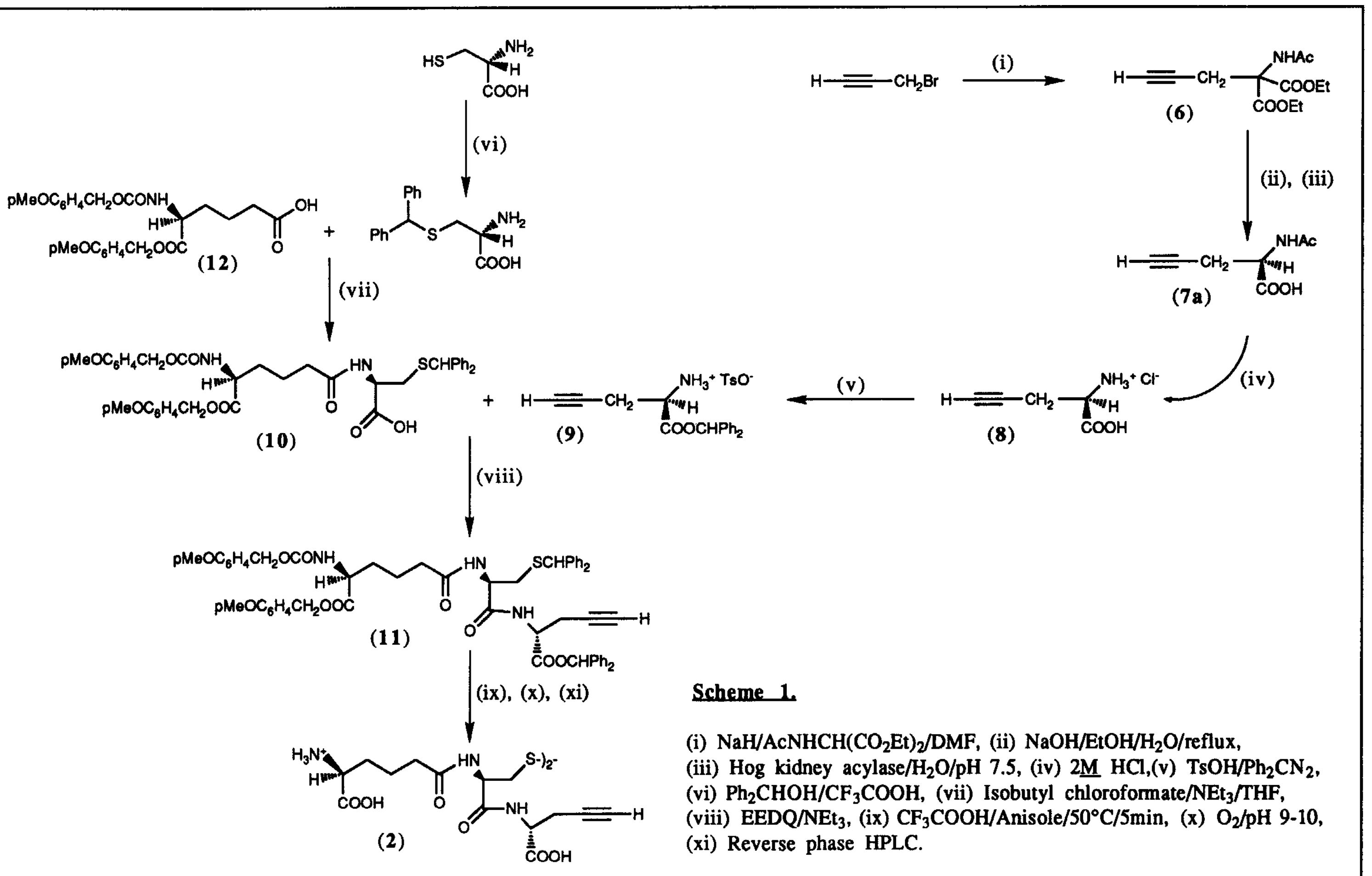


Synthesis of the Tripeptides.

The acetylene containing tripeptide (**2**) was prepared as shown in Scheme 1. Thus diethylacetamidomalonate was treated with sodium hydride in DMF and alkylated with propargyl bromide.[6] The resulting crystalline derivative (**6**) was saponified and decarboxylated (NaOH/EtOH/H_2O), and the resulting DL-N-acetyl-propargylglycine (**7a,b**) was resolved with hog kidney acylase to give, after recrystallization, D-N-acetyl propargylglycine (**7a**). This was deprotected in 2M HCl to give the amino acid (**8**); more vigorous conditions resulted in substantial loss of material. The free amino acid (**8**) was converted to the benzhydrylester (**9**) with diphenyldiazomethane.[7,8] Coupling with EEDQ[9] to the protected dipeptide LL-AC ((N-4-methoxybenzyloxy-carbonyl)-(α-4-methoxybenzylester)-δ-(L-α-aminoadipoyl)-S-benzhydryl-L-cys-teine) (**10**) gave the diastereomerically pure protected tripeptide (**11**) shown. All the protecting groups were removed under relatively mild conditions (TFA/anisole, 4:1, 50°C, 5min),[10] the thiol oxidised to the disulphide (O_2, pH 9-10) to give, after purification by reverse phase HPLC, the tripeptide (**2**).

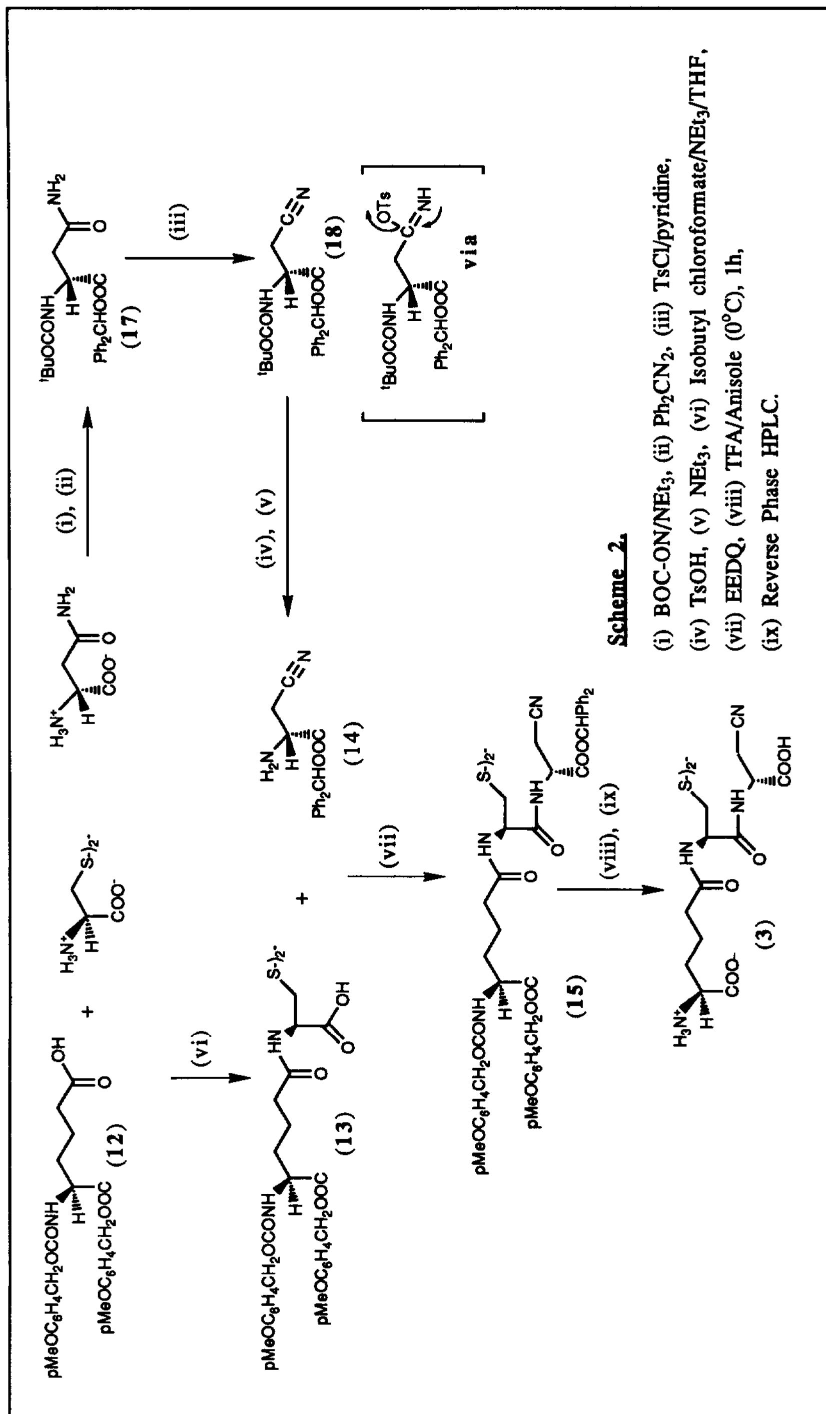
Attempts to prepare the tripeptide (**3**) in a similar manner failed due to the hydrolysis of the nitrile functionality upon treatment with TFA/anisole under conditions required to remove the benzhydryl sulphur protecting group.[11] The use of the fully benzyl protected tripeptide N-benzyloxycarbonyl-α-benzylester-δ-(L-α-amino-adipoyl)-S-benzyl-L-cysteinyl-D-cyanoalanine benzylester in the hope of selective titration of the benzyl groups, by Na/NH_3, also failed. Thus the method shown in Scheme 2 was utilised, providing the required tripeptide (**3**) in a homogeneous form and in moderate yield. Thus N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxy-benzyl)-δ-L-α-aminoadipic acid (2eq) (**12**) was coupled to L-cystine using isobutyl chloroformate and the resultant dipeptide disulphide (**13**) treated with EEDQ[9] and 4eq of D-cyanoalanine benzhydrylester (**14**), to give the fully protected tripeptide disulphide (**15**). This was smoothly deprotected at 0°C with TFA/anisole (4:1) without any observable hydrolysis of the nitrile functionality, to give the desired tripeptide (**3**). D-Cyanoalanine benzhydrylester (**14**) was prepared by slight modification of the method of Rapoport[12] *via* the N-^{t}butyloxycarbonyl benzhydrylester of D-asparagine (**17**) with toluene-4-sulphonylchloride and pyridine to give N-^{t}butyl-oxycarbonyl-D-cyanoalanine benzhydrylester (**18**) followed by treatment with toluene-4-sulphonic acid to remove the ^{t}butyloxycarbonyl protecting group.[13]

The tripeptide (**4b**) was prepared in a similar manner to that of tripeptide (**2**) as shown in Scheme 3. The deuterated amino acid (4,4,5,5-$^2H_4$)-DL-norvaline (**19**) was prepared *via* deuteration of the alkylated adduct (**6**) using Wilkinsons catalyst and D_2(g). The deuterated adduct (**20**) was deacylated, de-esterified and decarboxylated by reflux in HCl (3M) to give the racemic amino acid (**19**) which was protected in the usual manner to give the benzhydryl ester tosylate salt (**21**).[8] Subsequent treat-



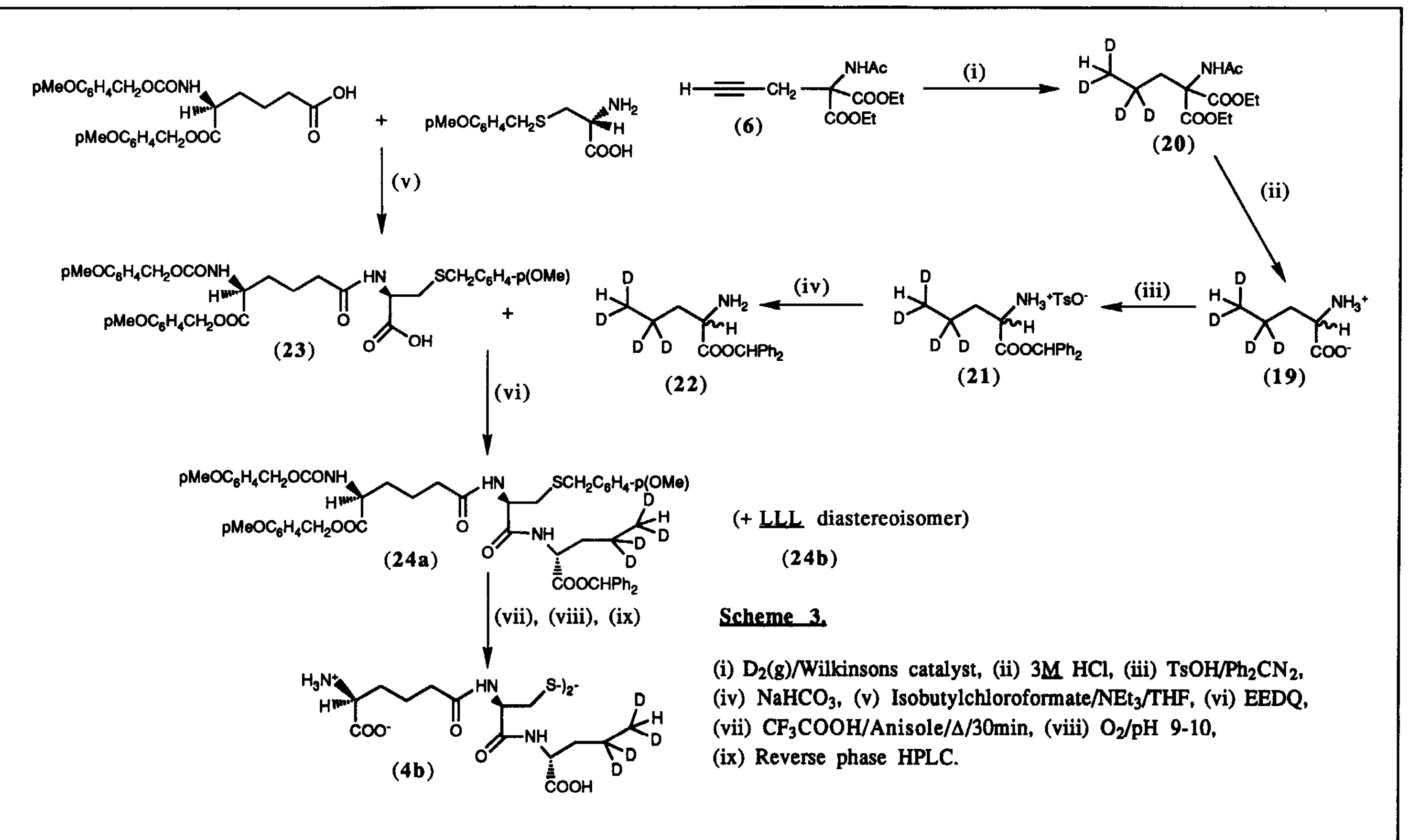
Scheme 1.

(i) NaH/AcNHCH($CO_2Et)_2$/DMF, (ii) NaOH/EtOH/H_2O/reflux, (iii) Hog kidney acylase/H_2O/pH 7.5, (iv) 2M HCl,(v) TsOH/Ph_2CN_2, (vi) Ph_2CHOH/CF_3COOH, (vii) Isobutyl chloroformate/NEt_3/THF, (viii) EEDQ/NEt_3, (ix) CF_3COOH/Anisole/50°C/5min, (x) O_2/pH 9-10, (xi) Reverse phase HPLC.



Scheme 2.

(i) BOC-ON/NEt_3, (ii) Ph_2CN_2, (iii) TsCl/pyridine, (iv) TsOH, (v) NEt_3, (vi) Isobutyl chloroformate/NEt_3/THF, (vii) EEDQ, (viii) TFA/Anisole (0°C), 1h, (ix) Reverse Phase HPLC.



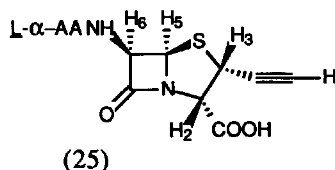
Scheme 3.

(i) D_2(g)/Wilkinsons catalyst, (ii) 3M HCl, (iii) TsOH/Ph_2CN_2, (iv) $NaHCO_3$, (v) Isobutylchloroformate/NEt_3/THF, (vi) EEDQ, (vii) CF_3COOH/Anisole/Δ/30min, (viii) O_2/pH 9-10, (ix) Reverse phase HPLC.

ment with $NaHCO_3$ gave the free amine (**22**). This was coupled to the protected dipeptide N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxybenzyl)-δ-L-amino-adipoyl-S-(4-methoxybenzyl)-L-cysteine (**23**) with EEDQ to give a separable pair of diastereoisomers LLL and LLD-N-(4-methoxybenzyloxycarbonyl)-α-(4-methoxy-benzyl)-δ-(α-aminoadipoyl)-S-(4-methoxybenzyl)-cysteine-(4,4,5,5-$^2H_4$)-norvaline (**24a,b**). The less polar isomer was was identified as the LLD isotopomer by comparison with authentic[5] (non deuterated) material and deprotected (TFA/anisole 5:1, Δ, 30min) to give, after oxidation to the disulphide and purification by reverse phase HPLC, the tripeptide (**4b**).

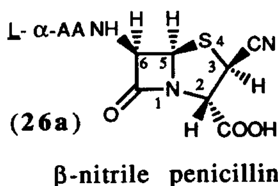
Incubation of the Tripeptides with IPNS.

Incubation of the tripeptide (**2**) with the enzyme IPNS under standard incubation conditions[14,15] led to the generation, (in virtually quantitative yield), of a mixture of 3 new β-lactam containing metabolites (ratio 15:1:trace), the major one of which displayed biological activity comparable to isopenicillin N against both *S.aureus* and *E.coli*, but was destroyed by the addition of β-lactamase I.[16] The two minor metabolites could not be purified to homogeneity. They were both resistant to β-lactamase, and showed no biological activity against the organisms *S.aureus* and *E.coli*. Isolation of the major new β-lactam containing metabolite by reverse phase HPLC led to its characterisation as the α-acetylenic penicillin (**25**). The stereochemistry was assigned both by n.O.e experiments and the magnitude (7.0Hz) of the coupling constants between H_2 and H_3.[17]

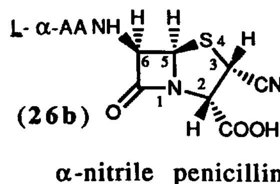


(25)

Incubation of the nitrile containing tripeptide (**3**) with the IPNS enzyme gave (*ca.* 10% conversion under conditions giving quantitative conversion of LLD-ACV to isopenicillin N) a 1:1 ratio of β-lactam containing metabolites. Each product displayed biological activity against both *S.aureus* and *E.coli*, which was destroyed by the addition of β-lactamase I. These were purified by reverse phase HPLC and assigned as the β and α nitrile penicillins (**26a,b**).



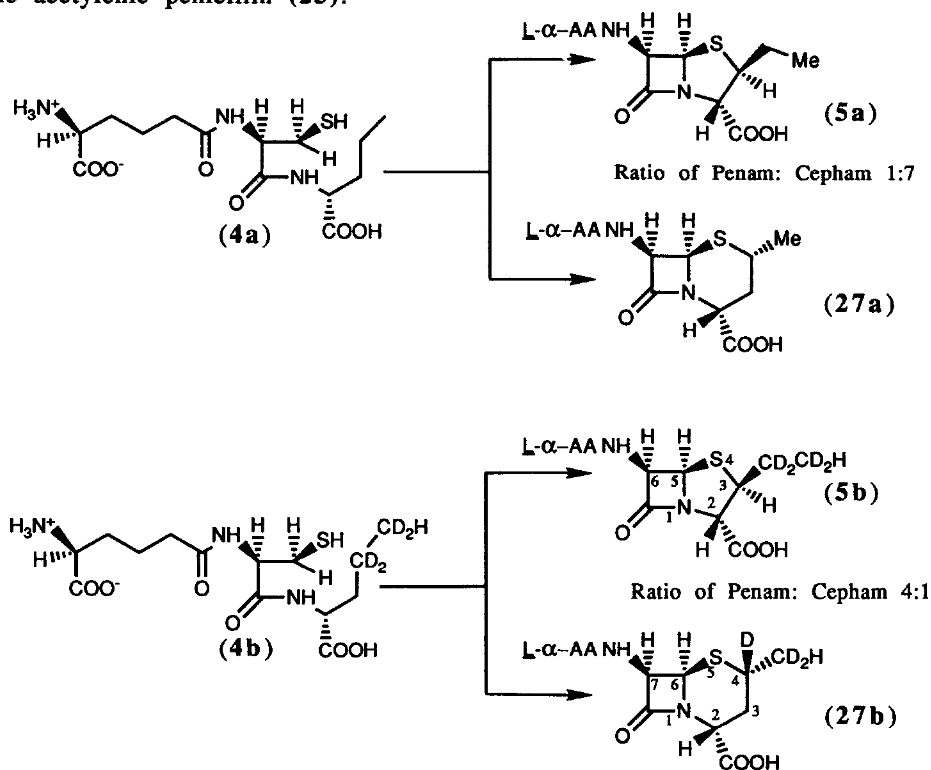
(**26a**) β-nitrile penicillin



(**26b**) α-nitrile penicillin

The formation of these penicillins show that the nitrile functionality is accepted by the IPNS enzyme as a substrate, albeit with low conversion. This low conversion could well be the result of the polarity of the nitrile (compare for example with the O-methylserine valinyl analogue which also gave only low conversion)[18] and thus poor resultant binding.

Incubation of the tripeptides (4) with IPNS provided the penams (5) and cephams (27) with the expected bias towards penam formation with the deuterated tripeptide (4b). This led to the isolation of the β-ethyl penam (5b), the stereochemistry being determined both by n.O.e. experiments and coupling constant arguments between H_2 and H_3,[17] in clear contrast to the α stereochemical preference determined for the acetylenic penicillin (25).



In this work we have described the isolation and determination of the stereochemistry of the α-acetylenic penicillin (25) and of the 1:1 ratio of the α:β nitrile penicillins (26a) and (26b). These results are combined in Table 1 with a series of mono-substituted penicillins which have been isolated following incubations of tripeptide analogues of LLD-ACV. Bearing in mind that we must by needs assume equal stability of all penam products to the incubation and NMR procedures, it is

Table 1

Monosubstituted penicillins isolated from substrate analogues of LLD-ACV upon incubation with IPNS. The ratios quoted represent those determined by ^{1}H NMR integration after incubation, but before purification (any rationale from this data has to assume equal stability of both product isomers to the incubation conditions).

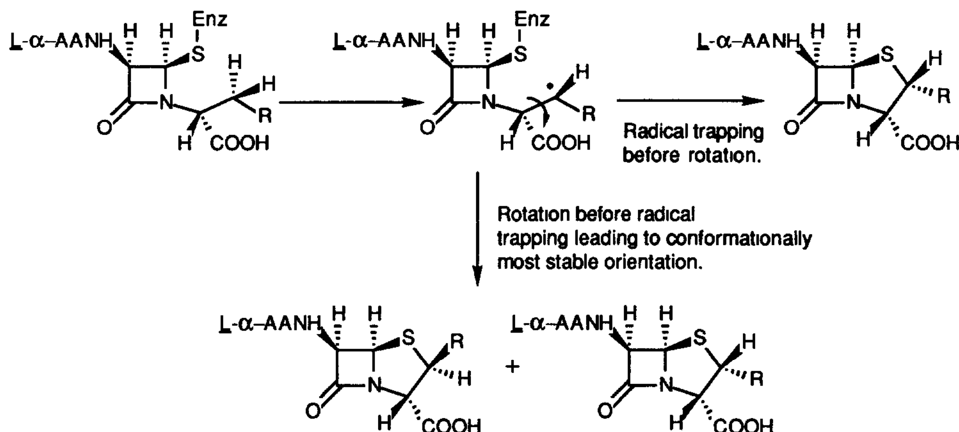
Analogue LLD-ACR: R =	α-Penicillin Isolated	β-Penicillin Isolated	α:β ratio	Ref
—HN, H, COOH	None Observed	L-α-AANH, H, H, S, Et, N, H, O, H, COOH	100% β	
—HN, H, COOH	L-α-AANH, H, H, S, H, N, Me, O, H, COOH	L-α-AANH, H, H, S, Me, N, H, O, H, COOH	1:9	20
—HN, H, COOH	L-α-AANH, H, H, S, H, N, O, H, COOH	L-α-AANH, H, H, S, N, H, O, H, COOH	1:4	3
—HN, H, CH:C:CH_2, COOH	L-α-AANH, H, H, S, H, N, CH:C:CH_2, O, H, COOH	L-α-AANH, H, H, S, CH:C:CH_2, N, H, O, H, COOH	2:1	19
—HN, H, COOH	L-α-AANH, H, H, S, H, N, O, H, COOH	None Observed	100% α†	
—HN, H, CN, COOH	L-α-AANH, H, H, S, H, N, CN, O, H, COOH	L-α-AANH, H, H, S, CN, N, H, O, H, COOH	1:1	

† Three products were observed in the crude mixture in the ratio 15(α-penicillin):1:trace

interesting to see if we can rationalise the observed stereochemistries in terms of both enzymic and chemical considerations.

In Table 1 the various stereochemistries of the isolated monosubstituted penicillins are shown. It becomes apparent that alkyl penicillins tend to have predominantly β-stereochemistry, while with the unsaturated systems α-orientation begins to become favoured. It is possible to view this effect as arising from active site constraints which play a role in maintaining and stabilising the stereochemical orientation of the substituent group, after radical type hydrogen atom abstraction from the C-3 position of the valinyl moiety of the tripeptide analogue. [Radical formation is considered to be the most likely possibility considering the results of the two specifically deuterated aminobutyrate containing tripeptides δ-L-α-aminoadipoyl-L-cysteinyl-D-(3R)-(2-amino-3-deuterobutyrate) and δ-L-α-aminoadipoyl-L-cysteinyl-D-(3S)-(2-amino-3-deuterobutyrate) which both gave the same β-methyl-α-deutero penam].[20]

The considerations must then be a balance between bond rotation and radical trapping, which ultimately leads to penicillin formation, in which the lifetimes and transition states for various conformations are dictated by active site binding energies and non-binding repulsion effects, with the sulphur atom ultimately attacking from the same face as that from which the hydrogen atom was abstracted.



Thus ethyl and methyl monosubstituted penicillins both have the β-orientation for the alkyl group, indicative of favourable active site interactions with these groups. This view is also supported by the results obtained from incubation of the two deuterated aminobutyrate containing tripeptides, in which the β-methyl-α-deutero penam was the major isolated product from both of these tripeptides.[20] The β-stereochemistry was also found for the major penicillin product isolated from incubation of the allylglycine containing tripeptide, although 20% of the α-vinyl penicillin was

observed. The argument for this is that it is possible that the production of the α-penicillin reflects the small difference in binding potentials between the α and β-sites, and the lifetime of the radical is sufficiently long for complete population of both according to their relative binding energies. (Transition state energies can likewise be expected to reflect these differences).

From the allene containing tripeptide[19] the major isomer isolated was the α-penicillin (65%), while the proportion is increased to *ca*. 100% for the α-acetylenic penicillin. This clearly suggests enhanced binding for the unsaturated moieties in the α-position relative to that for the β-site.
The differences between these observed stereochemistries of unsaturated and saturated side chains may arise from interactions with aromatic and aliphatic amino acid side chains in the α- and β-sites respectively. The nitrile penicillin was produced in a 1:1 ratio of α:β penicillins, and suggests that the lifetime of any radical intermediate is sufficiently long to allow equilibrium between the two conformations, suggesting that neither is particularly favourable in binding terms. This is interesting with respect to the α-acetylenic penicillin as the nitrile and acetylene groups are structurally very similar. Thus the bias must essentially be on electronic grounds (polarity) alone, the polar nitrile group being insensitive to the different binding in the two sites, a factor which may have also influenced the relatively poor conversion when compared with the acetylene containing tripeptide.

Acknowledgements.
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Experimental.
The general experimental methods are as previously described.[21]

Preparation of Diethyl-α-acetamido-α-propargylmalonate (6).[6]
Diethylacetamidomalonate (4.5g, 20mmol), dissolved in warm DMF (15ml), was added dropwise under an argon atmosphere to a cooled suspension of NaH (0.6g, 25mmol) in DMF (0°C) over 30min. Freshly distilled propargyl bromide (2.2ml, 25mmol), was then added dropwise over 1h to the stirred solution at room temperature. The mixture was heated at 60°C for 3h, after which time a small quantity of ethanol was added (2ml), and the solution filtered through celite to remove NaBr. The solvent was removed in vacuo, to give a thick orange oil. This was taken up in Et_2O (50ml), washed with water (3x50ml), dried (Na_2SO_4), filtered and the solvent removed in vacuo to give a colourless oil. Crystallisation from Et_2O/Petrol gave the title compound as white needles. (5.0g, 95%), m.p. 88°C (Lit.,[6] 91-92°C), [eluant EtOAc/Petrol (8:2), Rf 0.8]; δ_H (500MHz, $CDCl_3$), 7.03 (1H, s, NH), 4.29 (4H, 2xq, J 7Hz,

CH2O), 3.28 (2H, d, J 2.5Hz, CH2CCH), 2.07 (3H, s, MeCO), 1.95 (1H, t, J 2.5Hz, HCC), 1.30 (6H, t, J 7Hz, CH_3CH_2); δ_C (50MHz, $CDCl_3$), 169.50 (s, esters), 166.84 (s, amide), 78.22 (br s, 2 bond HCC coupling, CCH), 71.34 (d, CCH), 65.16 (s, CH_2CNH), 62.88 (t, MeCH_2), 23.59 (t, CH_2CCH), 22.71 (q, MeCO), 13.73 (q, MeCH_2); m/z (ammonia DCI), MH^+ (256, 100%); ν_{max} ($CHCl_3$), 3410 m, 3300 m, 3000-2880 m, 1740 s, 1670 s; $C_{12}H_{17}NO_5$ requires C 56.46, H 6.71, N 5.51%: found C 56.47, H 6.74, N 5.35%.

Preparation of N-Acetyl-DL-2-aminopent-4-ynoic acid (7a,b).[6]
Diethyl-α-acetamido-α-propargylmalonate (**6**) (1.0g, 3.9mmol) was dissolved in water (10ml) and EtOH (10ml), to which was added NaOH (0.4g, 10mmol), and the mixture refluxed for 2h. The solution was cooled, washed with EtOAc (20ml), acidified to pH 1-2 with HCl (1M), and extracted with EtOAc (3x30ml). The combined organic layers were dried (Na_2SO_4), filtered, and the solvent removed in vacuo to give the title compound as a white solid, which was crystallised from EtOAc, (0.54g, 90%), m.p. 136°C. (Lit.[6] 137°C). [eluant BuOH/Water/AcOH, 4:1:1, Rf 0.6]; δ_H (500MHz, D_2O), 4.39 (1H, t, J 6Hz, CHα), 2.61-2.59 (2H, m, CH_2CH), 2.27 (1H, dd, J 3Hz, HCC), 1.88 (3H, s, MeCO); δ_C (126MHz, $CDCl_3$), 175.06 and 174.77 (2xs, HOOC- and MeCO-), 80.43 (s, -CCH), 72.94 (d, HCC-), 52.71 (d, CHα), 22.98 (t, CH_2), 22.62 (q, Me); m/z (ammonia DCI), MH^+ (156, 100%); ν_{max} (nujol), 3320 s, 3290 s, 1720 s, 1590 s, 1540 s, 1370 s.

Preparation of N-Acetyl-D-2-aminopent-4-ynoic acid (7a).
N-Acetyl-DL-propargylglycine (0.3g, 1.9mmol), was dissolved in water (50ml), and the pH adjusted to 7.5 with NH_4OH (1M). Resolution with Hog kidney acylase (*ca.* 40000 units) followed the general literature procedure,[22] after which the solution was boiled for 1-2 min, filtered through celite, and the water removed in vacuo; δ_H (500MHz, D_2O), 4.18 (0.5H, dd, J 2x6Hz, AcNHCH-), 3.76 (0.5H, dd, J 2x5.5Hz, H_2NCH-), 2.72-2.70 (1H, 8 line m, AcNHCHCH_2), 2.54-2.52 (1H, 4 lines, J 2.5, 6Hz, H_2NCHCH_2), 2.38 (0.5H, dd, J 2.5Hz, HCC-), 2.23 (0.5H, t J 2.5Hz, HCC), 1.90 (1.5H, s, MeCO). This mixture was taken up in water (5ml), acidified to pH 1 with HCl (1M), and extracted with EtOAc (3x100ml). The organic layers were dried (Na_2SO_4), filtered, and the solvent removed in vacuo, to give a pale brown solid, (0.149g, 50%, or 100% recovery of the D-isomer). This was crystallised from EtOAc/petrol to give the title compound as white plates. $C_7H_9NO_3$ requires C 54.19, H, 5.85, N 9.03%, found, C 54.04, H 5.94, N 8.72%.

Preparation of D-2-aminopent-4-ynoic acid (8).
N-Acetyl-D-propargylglycine (**7a**) (0.14g, 1mmol), was dissolved in water (20ml) to which was added HCl (11M, 5ml). The solution was refluxed for 3h, cooled to room temperature, washed with EtOAc (2x20ml), and the solvent removed in vacuo. Water (2x20ml) was added and re-evaporated (x2), to give the title compound as its HCl salt (quantitative). The free salt was purified by precipitation from water by the addition of acetone, or from iPrOH/Et_2O, to give white crystals (needles), m.p. 180-185°C (lit.[6] 195°C); $[\alpha_D]^{20}$ +29° (c=0.95, H_2O) (Lit.[6] value for L-isomer $[\alpha_D]^{20}$ -35.0° (c=1.0, H_2O)); δ_H (500MHz, D_2O), 4.15 (1H, t, J 6Hz, CHα), 2.83 (2H, dd, J 6, 2.5Hz, CH_2), 2.41 (1H, t, J

2.5Hz, HCC-); δ_C (126MHz, D_2O), 171.74 (s, HOOC), 77.53 (s, HCC), 75.11 (d, HCC), 52.53 (d, CHα), 21.12 (t, CH_2); m/z (ammonia DCI), MH^+ (113, 100%).

Preparation of D-2-aminopent-4-ynoic acid benzhydrylester, (toluene-4-sulphonate salt) (9).

The amino acid HCl salt (8) (0.08g, 0.5mmol) was treated with diphenyldiazomethane according to the general literature procedure[8] with the exception of the use of acetonitrile as solvent instead of DMF. The white solid was purified by crystallisation from DCM/Petrol to give the title compound as a white powder (0.22g, 95%); $[\alpha_D]^{20}$ +24° (c=1.2, DCM); δ_H (500MHz, $CDCl_3$), 7.64 and 6.99 (4H, A_2B_2 system, J 8Hz, TsO), 7.30-7.23 (10H, m, ArH), 6.84 (1H, s, $CHPh_2$), 4.34 (1H, dd, J 5, 4Hz, CHα), 2.94-2.79 (2H, 16 lines AB part of ABMX system, J 18, 5, 4, 2.6, 2.5Hz, CH_2), 2.29 (3H, s, Me), 1.80 (1H, dd, J 2.6, 2.5Hz, HCC); δ_C (50MHz, $CDCl_3$), 168.05 (s, ester), 141.47 and 140.22 (2xs, 2xPhC-1), 129.54-126.63 (d and s, ArCH, and ArC-4 of tosylate), 80.56 (d, $CHPh_2$), 76.32 (s, CCH), 75.13 (d, CCH), 52.17 (d, CHα), 20.75 (q, Me), 20.64 (t, CH_2CH); m/z: no MH^+ observed, Ph_2CH^+ (167, 100%), $TsOH_2^+$ (172, 24%).

Preparation of N-4-methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-(L-α-aminoadipoyl)-L-cysteinyl-S-benzhydryl-D-2-aminopent-4-ynoic acid benzhydrylester (11).

N-(4-Methoxybenzyloxycarbonyl)-α-4-methoxybenzylester-δ-(L-α-aminoadipoyl)-S-benzhydryl-L-cysteine[21] (10) (0.13g, 0.2mmol), D-propargylglycine benzhydryl ester, (toluene-4-sulphonate salt) (9) (0.09g, 1eq), triethylamine (36μl, 1eq), and EEDQ (0.05g, 1eq), were stirred in dry DCM (5ml), for 24h, under an atmosphere of Ar(g), and in the presence of Na_2SO_4 (50mg). EtOAc (30ml) was added and the solution washed sequentially with HCl (1M, 30ml), $NaHCO_3$ (30ml, saturated solution), brine (30ml, saturated solution). The organic layer was dried (Na_2SO_4), filtered and the solvent removed in vacuo. The residue was purified by column chromatography on silica gel [eluant 1:1 EtOAc/Petrol, Rf 0.7], to give the title compound as a white foam, (90mg, 46%); δ_H (500MHz, $CDCl_3$), 7.44-7.19 and 6.88-6.85 (28H, complex m, ArH), 6.95 (1H, d, J 8Hz, NH), 6.91 (1H, s, $CHPh_2$), 6.18 (1H, d, J 7Hz, NH), 5.43 (1H, d, J 8Hz, NH), 5.26 (1H, s, $SCHPh_2$), 5.08-4.98 (4H, complex m, $2xCH_2Ar$), 4.76 (1H, 6 lines, dt, J 8, 5, 5Hz, CHα), 4.53 (1H, dt, J 8, 6.5, 6.5Hz, CHα), 4.35-4.34 (1H, m,CHα), 3.79 (6H, s, 2xMeO), 2.81-2.70 (4H, 8 lines, AB part of ABX system SCH_2, and 16 lines AB part of ABMX system CH_2CCH), 2.18-2.06 (2H, m, CH_2CO), 1.92 (1H, t, J 2.5Hz HCC), 1.81-1.63 (4H, 2xm, $CH_2CH_2CH_2CO$); δ_C (126MHz, $CDCl_3$), 172.44, 172.10,170.11 and 169.01 (4xs, 2x amides, 2x esters), 159.87 and 159.67 (2xs, 2xArC-4), 156.12 (1xs, CO_2NH), 141.09, 139.37 and 139.24 (3xs, PhC-1), 129.96-127.26 (d and s, ArCH's and ArC's), 114.04 and 113.95 (2xd, 2xArC-3), 78.71 (d, $CHPh_2$), 78.05 (s, CCH), 72.27 (d, HCC), 67.01 and 66.87 (2xt, $2xCH_2Ar$), 55.28 (q, Me), 54.62, 53.65, 52.02 and 51.11 (4xd, 3xCHα, and $SCHPh_2$), 35.29 (t, CH_2CO), 34.29 (t, CH_2S), 31.84 (t, $CH_2CH_2CH_2CO$), 22.14 ($CH_2CH_2CH_2CO$), 21.29 (t, CH_2CCH); m/z (+ve argon FAB) MNa^+ (998).

Preparation of δ-(L-α–aminoadipoyl)-L-cysteinyl-D-2-aminopent-4-ynoic acid disulphide (2).

The protected tripeptide (11) (70mg, 0.07mmol) was dissolved in freshly distilled TFA (2ml) and dry anisole (400μl), and the mixture heated at 50°C under Ar(g) for 5min. After cooling the TFA was removed by azeotroping with toluene (3 x 5ml), and the residue partitioned between water (10ml) and EtOAc (10ml). The aqueous layer was washed with further EtOAc (10ml), before freeze drying to give the tripeptide TFA salt as a white powder (28mg, 84%); m/z (+ve argon FAB) MH^+ (360). This material was then oxidised to the disulphide by bubbling O_2 through a slightly alkaline aqueous solution of the thiol and purified by reverse phase HPLC; δ_H (500MHz, D_2O), 4.65 (1H, dd, X part of ABX system, J 7, 5Hz, $C\underline{H}\alpha$), 4.59 (1H, dd, J 7, 6Hz, $C\underline{H}\alpha$), 4.03 (1H, dd, J 2x6Hz, $C\underline{H}\alpha$), 3.00-2.90 (2H, 8 lines, AB part of ABX system, J 14, 7, 5Hz, $SC\underline{H}_2$), 2.86-2.80 (2H, complex AB part of ABMX system, J 10, 7, 6, 2.5Hz, $CHC\underline{H}_2CCH$), 2.47 (1H, dd, J 2.5, 2.5Hz, $\underline{H}CC$), 2.43 (2H, dd, J 2x6Hz, $C\underline{H}_2CO$), 2.03-1.93 and 1.83-1.73 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$); δ_C (126MHz, D_2O) 176.40, 174.34 and 171.53 (4xs, 2x acids, 2x amides), 80.92 (s, $HC\underline{C}$), 71.94 (d, $H\underline{C}C$), 54.80, 53.62 and 52.99 (3xd, 3x$\underline{C}H\alpha$), 39.17 (d, CH_2S), 35.10 (t, $\underline{C}H_2CO$), 30.17 (t, $\underline{C}H_2CH_2CH_2CO$), 25.93 (t, $\underline{C}H_2CCH$), 21.25 (d, $CH_2\underline{C}H_2CH_2$).

Incubation of δ-(L-α–aminoadipoyl)-L-cysteinyl-D-2-aminopent-4-ynoic acid disulphide (2) with IPNS.

The tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-2-aminopent-4-ynoic acid disulphide (2) was incubated with IPNS (2ml, 5 I.U./ml) under standard conditions.[14,15] The crude mixture was examined by ^1H n.m.r. spectroscopy (500MHz) which showed the presence of at least 3 β-lactam containing products in a ratio of approximately 15:1:trace. The crude mixture displayed biological activity comparable to that of similarily biosynthesised isopenicillin N against *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* in standard 'hole plate' bioassays. This activity was lost in a portion of the crude mixture treated with β-lactamase enzyme. The crude mixture was purified by reverse phase HPLC (Stationary phase ODS, mobile phase 25mM NH_4HCO_3), to give in a homogeneous state the α-acetylenic penicillin (25); δ_H (500MHz, D_2O), 5.66 and 5.53 (2H, ABq, J 4Hz, H-5 and H-6), 4.90 (1H, dd, J 7, 2.5Hz, H-3), 3.75-3.73 (1H, m, $C\underline{H}CH_2$), 2.91 (1H, d, J 2.5Hz, $\underline{H}CC$), 2.41-2.38 (2H, m, $C\underline{H}_2CO$), 1.96-1.66 (4H, 2xm, $C\underline{H}_2C\underline{H}_2CH_2CO$); addition of CD_3CN (D_2O/CD_3CN (1:1) allowed the observation of the resonance associated with H-2 (due to the shift of the HOD resonance to higher field), δ_H 4.83 (1H, d, J 7Hz, H-2). The relative stereochemistry was determined by n.O.e experiments on this CD_3CN/D_2O sample. Thus irradiation of the resonance associated with H-2 gave a n.O.e (10%) to H-3, and no n.O.e to either H-6 or H-7. Irradiation of the resonance associated with H-3 gave an n.O.e (8%) to H-2 only. Irradiation of H-6 or H-7 did not give an n.O.e to either H-2 or H-3. This suggests that the relative configuration of the acetylenic group is α. This is also supported by the value of the coupling constant of 7Hz for protons H-2 and H-3; m/z (+ve argon FAB), MH^+ (356).

Preparation of N-^{t}Butyloxycarbonyl-D-asparagine, benzhydrylester (17). D-asparagine monohydrate (2.00g, 13.3mmol) was treated with BOC-ON (3.93g, 16.0mmol) in H_2O (10ml) and 1,4-dioxane (10ml) with slight modification of the literature procedure.[23] Thus the reaction was stirred at room temperature for 20h, and then partitioned between H_2O (50ml) and EtOAc (50ml). The aqueous phase was acidified to pH 1-2 with HCl (1M) and extracted with EtOAc (5 x 50ml). The extract was treated with MeOH to dissolve suspended acid, dried (Na_2SO_4), filtered and evaporated *in vacuo* to give N-^{t} butyloxycarbonyl -D-asparagine as a white crystalline solid (2.48g, 80%); δ_H (500MHz, d_6-DMSO) 7.37 and 6.83 (2H, 2 x bs, NH_2), 6.72 (1H, d, J 7Hz, NH), 2.48-2.37 (2H, AB part of ABX system, CH_2), 1.30 (9H, s, ^{t}Bu), (H_α signal obscured by HOD peak); δ_C (125.7MHz, d_6-DMSO), 178.6 and 176.8 (2xs, 1x acid, 1x amide), 160.3 (s, urethane), 83.7 (s, Me_3C), 55.9 (d, CHα), 42.2 (t, CH_2), 33.7 (q, Me_3); m/z (NH_3, DCI), 250 (MNH_4^+, 20%), 233 (MH^+, 64%), 232 (M^+, 72%), 194 (54%), 176 ((OCOAsn)H^+, 100%), 150 (35%), 133 (73%), 104 (32%), 87 (75%). Diphenyldiazomethane (1.18g, 6.07mmol) was added in small portions to a vigorously stirred suspension of N-^{t}butyloxycarbonyl- D-asparagine (1.41g, 6.1mmol) in CH_3CN (100ml), until the purple colouration persisted. Excess diphenyldiazomethane was quenched with glacial acetic acid (1ml), and the solvent removed *in vacuo*. Purification by column chromotography on silica gel [eluant DCM/EtOAc (9:1-3:2)] gave the title compound as a white crystalline solid (1.09g, 45%), m.p. 159.5-161.0°C (from PhMe); $[\alpha]_D^{20}$ +27.3° (c=0.89 in DMF) [Lit. value[24] $[\alpha]_D^{20}$ +30.2° (c=1.00 in DMF)]; $C_{22}H_{26}N_2O_5$ requires C 66.30, H 6.60, N 7.05%: found C 66.65, H 6.45, N 6.75%; ν_{max} (Nujol mull) 3460m (NH), 3360-3200m (NH_2), 1740s (CO), 1700s (CO), 1675s (CO), 1610m (CO), 1580s and 1165s cm^{-1}; δ_H (300MHz, $CDCl_3$), 7.35-7.27 (10H, m, Ph_2), 6.89 (1H, s, C**H**Ph_2), 5.80 (1H, d, J 8Hz, NH), 5.54 and 5.31 (2H, 2 x bs, NH_2), 4.66-4.61 (1H, m, CHα), 2.99-2.92 and 2.79-2.72 (2H, AB part of ABX system, CH_2), 1.44 (9H, s, ^{t}Bu); m/z (NH_3, DCI), 399 (MH^+, 11%), 184 (10%),176 (23%), 167 (Ph_2CH^+, 100%), 87 (19%).

N-^{t}Butyloxycarbonyl-3-cyano-D-alanine, benzhydrylester (18).[12]
Dry pyridine (0.3ml) and TsCl (289mg, 1.6mmol) were added to a solution of di-protected D-asparagine (17) (281mg, 0.7mmol) in DCM (3ml) and the reaction was stirred in a sealed flask for 40 h. Saturated $NaHCO_3$ solution (3ml) was added, and the mixture stirred for a further 1h. The reaction was diluted with DCM (7ml), and the phases separated. The aqueous phase was extracted with DCM (7ml), and the combined organic phases were dried (Na_2SO_4), filtered and evaporated *in vacuo* to give a yellow oil. Purification by column chromotography [silica gel; gradient elution DCM:EtOAc (99:1-98:2)] gave the title compound as a white solid (240mg, 90%), m.p. 90-91°C (from Et_2O/petrol); $[\alpha]_D^{20}$ +2.9 (c=0.9 in $CHCl_3$); $C_{22}H_{24}N_2O_4$ requires C 69.45, H 6.35, N 7.35% found: C 69.30, H, 6.35, N 7.15%; ν_{max} (Nujol mull) 3370s (NH), 2250 w (CN), 1745s (CO), 1680s (CO), 1515s (CO), 1295s, and 1230m cm^{-1}; δ_H (500MHz, $CDCl_3$), 7.42-7.31 (10H, m, Ph_2CH), 6.95 (s, C**H**Ph_2), 5.50 (1H, d, J 8Hz, NH), 4.57-4.58 (1H, m, C**H**α), 2.97 (2H, very tight AB part of ABX system), 1.46 (9H, s, Me_3C); δ_C (62.9MHz, $CDCl_3$), 168.2 (s, COO), 154.8 (s, OCON), 138.8 (quaternary Ph carbons), 127.1-128.7 (CH of Ph groups), 116.1 (s, CN), 80.9 (s, Me_3C), 79.4 (d, CHPh_2), 50.5 (d, CHα), 28.2 (q, 3 x Me), 21.5 (t, CH_2(2)); m/z (NH_3, DCI), 398 (MNH_4^+, 10%), 167 (Ph_2CH^+, 100%).

3-Cyano-D-alanine, benzhydrylester (14)
A solution of protected amino acid (18) (240mg, 0.63mmol) in Et_2O (7ml) and EtOH (7ml) was treated with TsOH (0.11g, 1eq). The reaction was stirred at room temperature for 5min followed by removal of the solvents *in vacuo* at 40°C. The residue was repeatedly dissolved in Et_2O (7ml) and EtOH (7ml) and solvent evaporated *in vacuo*, using a warm water bath, until reaction was complete.[13] The residue was partitioned between HCl (0.3M) (20ml) and EtOAc (10ml), and the aqueous layer was then basified with solid $NaHCO_3$ and extracted with EtOAc (5x15ml). The combined extracts were dried (Na_2SO_4), filtered and evaporated *in vacuo* to give the title compound as a yellow oil (0.15g, 82%); δ_H (500MHz, $CDCl_3$), 7.70-7.06 (10H, m, ArH), 6.95 (s, CHPh2), 3.91-3.85 (1H, AB part of ABX system, J 5, 7, 17Hz CH_2CN), 2.07-1.73 (2H, br s, NH_2).

Preparation of bis(N-4-methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-(L-α-aminoadipoyl))-L-cystine (13).
N-4-Methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-L-α-aminoadipic acid (0.2g, 0.45mmol, 2eq) (12), was dissolved in THF (10ml) at -15°C with NEt_3 (2eq, 64μl) for 15min. Isobutylchloroformate (60μl, 2eq) was added, and the mixture stirred at -15°C for 30min. L-cystine (0.05g, 0.22mmol) in water (6ml) and NEt_3 (to effect solution of the amino acid disulphide,100μl), cooled to 0°C, was added in one portion to the vigorously stirred solution, and the mixture stirred at room temperature for 1h. Work up according to the general literature method[9] gave the title compound as a gum (0.2g, 88%); δ_H (x0.5) (200MHz, $CDCl_3$), 8.31-7.92 (1H, br s, CO_2H), 7.39-7.12 and 6.95-6.78 (8H, 2xm, ArH), 5.80 (1H, d, J 8Hz, NH), 5.45 (1H, d, J 7Hz, NH), 5.14-4.97 (4H, m, $2xCH_2Ar$), 4.84-4.82 (1H, m, CHα), 4.37-4.35 (1H, m, CHα), 3.76 (6H, 2xs, 2xMeO), 3.26-3.11 (2H, br m, CH_2S-)2-), 2.40-2.20 (2H, br m, CH_2CO), 1.92-1.55 (4H, 2xbr m, $CH_2CH_2CH_2CO$); m/z (+ve argon FAB) (MH^+, 1095). This material was used directly without further purification.

Preparation of N-4-methoxybenzyloxycarbonyl-α-4-methoxybenzylester-δ-(L-α-aminoadipoyl))-L-cystinyl-D-cyanoalanine benzhydrylester (15).
The crude dipeptide disulphide (13) (0.14g, 0.13mmol) was mixed with EEDQ (2eq, 0.07g), and D-cyanoalanine benzhydrylester (14) (4eq, 0.154g), in DCM (10ml) under Ar(g) with Na_2SO_4 (50mg) and stirred overnight. Work up according to the method previously described gave a very insoluble residue, which was purified by column chromatography on silica gel [eluant DCM/EtOAc (1:3), Rf 0.5], to give the title compound as a white solid (0.082g, 24% from L-cystine); δ_H (x0.5) (500MHz, $CDCl_3$), 9.06 (1H, d, J 7Hz, $NHCHCH_2S$-)2-), 7.46-7.18 and 6.94-6.83 (19H, 2xm, $CHPh_2$ and ArH), 6.42 (1H, d, J 8Hz, NH), 5.54 (1H, d, J 8Hz, NH), 5.49-5.47 (1H, m, CHα of cystine), 5.08-5.01 (4H, 2xm, $2xCH_2Ar$), 4.89-4.87 (1H, m, CHα), 4.37-4.34 (1H, br m, CHα), 3.79 and 3.77 (6H, 2xs, 2xMeO), 3.00-2.73 (4H, 2x overlapping AB parts of ABX systems, CH_2CHS- and CH_2CN), 2.15-2.05 (2H, m, CH_2CO), 1.76-1.51 (4H, 2xm, $CH_2CH_2CH_2CO$); δ_C (50MHz, $CDCl_3$), 173.24, 172.38, 170.98 and 167.79 (4xs, 2x amides, 2x esters), 159.94 and 159.75 (2xs, 2xArC-4), 156.41 (s, urethane), 139.15 and 139.01 (2xs, 2xPhC-1), 130.27-127.07 (d and s, ArCH and ArC), 116.32 (s, CN),

114.07 and 113.97 (2xd, ArCH-3), 79.20 (d, CHPh$_2$), 66.94 and 66.78 (2xt, CH$_2$Ar), 55.19 (2xq, 2xMe), 53.44, 52.98 and 48.98 (3xd, 3xCHα), 34.96 (t, CH$_2$CO), 31.45 (t, CH$_2$S-), 29.56 (t, CH$_2$CH$_2$CH), 20.67 (t, CH$_2$CN), 20.60 (t, CH$_2$CH$_2$CO); m/z (+ve argon FAB), MNa$^+$ (833, free thiol -SH), M$_2$Na$^+$ (1641, disulphide).

Preparation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-cyanoalanine-disulphide (3).

The tripeptide disulphide (**15**) (0.035g, 22μmol) was deprotected in TFA/anisole (4:1, 5ml) at 0°C for 1h under Ar(g). The majority of the solvent was removed *in vacuo* at 0°C, and then under high vacuum at 0°C. The resulting material was then partitioned between water/EtOAc (2x10ml), and the aqueous layer washed with further EtOAc (10ml), before freeze-drying to give a white crystalline solid, which was purified by reverse phase HPLC to give the title compound in virtually quantitative yield; ν_{max} (CaF$_2$ cells, D$_2$O), 2260 (CN); δ_H (500MHz, D$_2$O), 3.87 (1H, t, J 6Hz, CHα), 3.10-2.82 (4H, 2 overlapping AB parts of ABX systems, δ_{A1}=3.08, δ_{A2}=2.84, δ_{B1}=2.98, δ_{B2}=2.89, J_A 7, 9Hz, J_B 5, 8Hz, CH$_2$-S-, and CH$_2$CN), 2.26-2.24 (2H, m, CH$_2$CO), 1.89-1.49 (4H, 2xm, CH$_2$CH$_2$CH$_2$CO). δ_H (500MHz, D$_2$O/CD$_3$CN quoting 5-3ppm region), 4.68 (1H, X part of ABX system, dd, J 5, 8Hz, CHα), 4.60 (1H, X part of ABX system, dd, J 7, 9Hz, CHα), 3.80 (1H, X part of ABX system, 4 lines, J 6, 6Hz, CHα); δ_C (126MHz, D$_2$O), 176.14, 172.86, 172.43 (4xs, 2x amides, 2x acids), 118.74 (s, CN), 53.49, 52.93 and 49.67 (3xd, 3xCHα), 39.10 (t, CH$_2$S), 34.96 (t, CH$_2$CO), 29.69 (t, CH$_2$CH), 21.06 (t, CH$_2$CN), 20.44 (t, CH$_2$CH$_2$CH$_2$CO); m/z (+ve argon FAB), MH$^+$ (719). No amide derived from hydrolysis of the nitrile was observed by m/z or ^{1}H n.m.r. analysis.

Incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-cyanoalanine disulphide (3) with IPNS.

The tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-cyanoalanine disulphide (2-3mg) was incubated with IPNS (2ml, 5 I.U./ml) under usual incubation conditions.[14,15] Analysis by ^{1}H n.m.r. (500MHz) spectroscopy showed the presence of 2 sets of β-lactam signals (2xABq) in approximately 10% conversion (under conditions giving 100% conversion of LLD-ACV). These two metabolites were both labile to β-lactamase I[16] and were isolated by purification by reverse phase HPLC (stationary phase ODS, mobile phase 25mM NH$_4$HCO$_3$). Fraction A (β-nitrile penicillin (**26a**)); δ_H (500MHz, D$_2$O), 5.38 and 5.34 (2H, ABq, J 4Hz, H-5 and H-6), 5.23 (1H, d, J 2Hz, H-3), 4.33 (1H, d, J 2Hz, H-2), 3.75-3.72 (1H, m, CHα of aminoadipoyl), 2.42-2.38 (2H, m, CH$_2$O), 1.96-1.66 (4H, 2xm, CH$_2$CH$_2$CH$_2$CO).

Fraction B (α-nitrile penicillin (**26b**)); δ_H (500MHz, D$_2$O), 5.51 and 5.39 (2H, ABq, J 4Hz, H-5 and H-6), 3.75-3.72 (1H, m, CHα of aminoadipoyl), 2.42-2.38 (2H, m, CH$_2$O), 1.96-1.66 (4H, 2xm, CH$_2$CH$_2$CH$_2$CO); H-2 and H-3 obscured by residual solvent peak, but observable by the addition of acetonitrile (*ca.* 50% volume), 4.95 (1H, d, J 5Hz, H-3), 4.43 (1H, d, J 5Hz, H-2).

Preparation of DL-2-amino-[4,4,5,5-^{2}H$_4$]-pentanoic acid (19).

Diethyl-α-acetamido-α-propargylmalonate (**6**) (0.6g, 2.4mmol) was dissolved in dry toluene (20ml), which was thoroughly degassed with N$_2$(g). Wilkinsons catalyst was

added (30mg) and the system was evacuated and filled with an atmosphere of D_2(g) (x3). This mixture was stirred overnight, after which time the solution was filtered through silica, washing through with ether. The solvents were removed in vacuo to leave an orange solid. This was purified by column chromatography on silica gel [eluant petrol/EtOAc (4:1-4:2)] Rf (petrol/EtOAc 4:1) 0.3, to give the reduced adduct as a yellow powder (0.56g, 94%); δ_H (300MHz, $CDCl_3$), 6.77 (1H, s, NH), 4.27 (4H, q, 2xCH_2CH_3), 2.25 (2H, s, CD_2CH_2), 2.05 (3H, s, MeCO), 1.29 (6H, t, 2xCH_3CH_2), 0.87 (1H, br s, CHD_2); δ_C (50MHz, $CDCl_3$), 169.10 and 168.38 (2xs, 1x ester, 1x amide), 66.40 (s, C-$(CO_2Et)_2$), 62.21 (t, $MeCH_2$), 33.81 (t, CD_2CH_2), 22.72 (q, MeCO), 15.84 (m, CD_2), 13.69 (q, $MeCH_2$), 12.75 (m, CD_2H). This material (**20**) was taken up in water (40ml) and HCl (10ml, 11M) and refluxed for 3h. After cooling the solution was washed sequentially with DCM (30ml) and EtOAc (30ml) followed by removal of the solvent in vacuo. Water was again added (2x15ml) and again removed in vacuo (x2). The residue was freeze-dried before crystallisation from acetone/MeOH to give the title compound as a white solid (0.26g, 89%); δ_H (500MHz, D_2O, pH 5), 3.86 (1H, t, J 6Hz, $CH\alpha$), 1.72-1.61 (2H, AB part of ABX system, CH_2CH), 0.66 (1H, br s, CD_2H); δ_C (50MHz, D_2O), 173.28 (s, acid), 53.38 (d, $CH\alpha$), 32.11 (t, CH_2), 17.40 (m, CD_2), 12.62 (m, CD_2H); m/z (NH_3, DCI), 122(MH^+, 100%), 123 (12%), 120 (1%).

Preparation of DL-2-amino-[4,4,5,5-$^2H_4$]-pentanoic acid benzhydrylester, tosylate salt (21).

DL-2-amino-[4,4,5,5-$^2H_4$]-pentanoic acid (**19**) was treated with diphenyldiazomethane according to the general literature procedure[8] to give the title compound as a white cystaline solid (82%); δ_H (300MHz, $CDCl_3$), 8.26 (3H, vb s, $^+NH_3$), 7.66 and 6.96 (4H, A_2B_2 system, J 8Hz Ar of TsO), 7.30-7.22 (10H, m. Ph_2), 6.83 (1H, s, Ph_2CH), 4.05 (1H, m, $CH\alpha$), 2.29 (3H, s, Me), 1.73 (2H, m, CH_2), 0.63 (1H, br s, CD_2H); m/z (NH_3, DCI) MH^+ (288, 100%).

N-(4-Methoxybenxyloxycarbonyl)-α-(4-methoxybenzyl)-δ-(L-α-amino-adipoyl)-S-(4-methoxybenzyl)-L-cysteinyl-D-2-amino-4,4,5,5-tetra-deuteropentanoic acid, benzhydryl ester (24a).

The tosylate salt (**21**) (241mg, 0.52mmol) was partitioned between EtOAc (20ml) and saturated $NaHCO_3$ (20ml), and the aqueous phase was extracted with EtOAc (20ml). The combined organic phases were washed with saturated $NaHCO_3$ solution (20ml), dried (Na_2SO_4), filtered and evaporated in vacuo to give the free amine (**22**) (151mg, 100%). A solution of the amine (**22**) (151mg, 0.52mmol), protected dipeptide (**23**) (361mg, 0.54mmol), and EEDQ (130mg, 0.52mmol) in DCM (7ml), with some drying agent (Na_2SO_4, ~10mg), was stirred at room temperature, under argon, overnight. Solvent was evaporated in vacuo, and the residue partitioned between EtOAc (60ml) and $NaHCO_3$ solution (25% sat., 40ml). The organic phase was washed with 1M HCl (30ml) and saturated NaCl solution (30ml), dried (Na_2SO_4), filtered and evaporated in vacuo, to give the crude material as a yellow oil, which was purified by column chromotography [silica gel; gradient elution EtOAc:petrol (30-40°C fraction from petroleum) (2:3 - 1:1)] to give the title compound (**24a**) (0.11g, 46%); δ_H (200MHz, $CDCl_3$) 7.30-7.23 (16H, m, ArH), 6.89-6.82 (7H, m, 6 x ArH & NH), 6.81 (1H, s, $CHPh_2$), 6.33 (1H, d, J 7.5Hz, NH), 5.55 (1H, d, J 8Hz, NH), 5.03 and 5.08 (4H, 2 x s, 2

x $O\underline{CH}_2Ar$), 4.63-4.73 (1H, m, $C\underline{H}\alpha$), 4.59-4.49 (1H, m, $C\underline{H}\alpha$), 4.39-4.32 (1H, m, $C\underline{H}\alpha$), 3.78 (6H, s, 2 x $OCH_2C_6H_4OC\underline{H}_3$), 3.75 (3H, s, $SCH_2C_6H_4OC\underline{H}_3$), 3.70 (2H, s, $SC\underline{H}_2Ar$), 2.87-2.78 and 2.71-2.64 (2H, AB part of ABX system, CH_2S), 2.17-2.08 (2H, m, CH_2CO), 1.88-1.60 (6H, m, $C\underline{H}_2CH_2CH_2CO$ & CH_2CD_2), 0.80 (1H, br s, CHD_2); δ_C (50MHz, $CDCl_3$), 172.7, 170.2, 160.0 and 156.4 (4xs, 2 x CONH & 2 x COO), 139.6-139.8 (quaternary aromatic C's), 127.1-130.3 and 114.0 (aromatic CH's), 78.2 ($\underline{C}HPh_2$), 66.8 & 67.0 (2 x $O\underline{C}H_2Ar$), 52.0, 52.3 & 53.5 (3xd, 3 x $\underline{C}H_\alpha$), 55.2 (q's, 3 x OMe), 35.7-28.6 (4xt, $\underline{C}H_2CH_2\underline{C}H_2CO$, $CH\underline{C}H_2S$ & $S\underline{C}H_2Ar$), 21.1 (t, $\underline{C}H_2CH_2CO$), 14.0 (m, CD_2H); m/z [FAB(MCA)] MH^+ (938, 100%), 937 (3%), 936 (4%).

δ-(L-α-aminoadipoyl)-L-cysteinyl-D-2-amino-4,4,5,5-tetradeuteropentanoic acid, disulphide (4b)

N-(4-Methoxybenxyloxycarbonyl)-α-(4-methoxybenzyl)-δ-(L-α-aminoadipoyl)-*S*-(4-methoxybenzyl)-L-cysteinyl-D-2-amino-4,4,5,5-tetradeuteropentanoic acid, benzhydryl ester (**23**) (113mg, 0.12mmol) was deprotected as previously described for the acetylenic containing tripeptide only using more vigorous conditions (30min reflux TFA/anisole). Work up and oxidation gave, on freeze drying, the crude title compound (**4b**) as a white solid which was purified by reverse phase HPLC (46mg, 79%); δ_H (500MHz, D_2O), 4.23-4.20 (1H, m, H_α), 3.78-3.76 (1H, m, H_α), 3.26-3.21 and 3.04-3.00 (2H, AB part of ABX system, CH_2S), 2.43 (2H, t, J 7Hz, CH_2CO), 1.96-1.66 (6H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$ & CH_2CD_2), 0.87 (1H, s, CHD_2), one H_α signal obscured by HOD peak; m/z [FAB(MCA)] 733 (MH^+).

Incubation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-2-amino-4,4,5,5-tetradeuteropentanoic acid (4) with IPNS

The tripeptide (4) (1.9mg) was incubated with IPNS (2ml, 5 I.U./ml) under standard conditions.[14,15] The 500MHz ^1H NMR spectrum of the crude incubation mixture showed two sets of resonances in the β-lactam region, in the ratio of 4:1 [δ_H 5.43:δ_H 5.29 & 5.24 (2 x d)]. After addition of 5μl of a solution of β-lactamase enzyme the resonance at δ_H 5.43 was absent from the spectra. The crude mixture displayed biological activity comparable to that of similarily biosynthesised isopenicillin N against *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* in standard 'hole plate' bioassays. However, no activity was seen in 'hole plate' bioassay with *E. coli* grown on a medium containing β-lactamase enzyme.

Separation of β-lactam products by reverse phase HPLC (ODS; mobile phase 25mM NH_4HCO_3 4.0ml/min) gave products with retention times of 11.5 and 13min, corresponding to the penam (**5b**) and cepham (**27b**) respectively.

(**5b**) ν_{max} (KBr disc) 1772m cm^{-1} (CO of β-lactam ring in penam); δ_H (500MHz, D_2O), 5.44 (2H, very tight AB system, H-5 and H-6), 4.59 (1H, d, J 3Hz, H-2), 4.02 (1H, d, J 3Hz, H-3), 3.71 (1H, t, J 6Hz, H_α of L-α-AA), 2.46 (2H, t, J 7Hz, CH_2CO), 1.94-1.73 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 0.98 (1H, bs, CHD_2); m/z [FAB(MCA)] 364 (MH^+).

(**27b**) δ_H (500MHz, D_2O), 5.34 and 5.28 (2H, 2 x d, H-6 and H-7), 4.40 (1H, t, J 7.5Hz, H-2), 3.62-3.60 (1H, m, H_α of L-α-AA), 2.44 (2H, t, J 7Hz, CH_2CO), 2.41-2.37 and 2.10-2.05 (2H, AB part of ABX system, 2xH-3), 1.85-1.72 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 1.34 (1H, bs, CHD_2); m/z [FAB(MCA)] 363 (MH^+).

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