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The Enzymatic Synthesis of Isotopically Labelled Penicillin Ns With Isopenicillin N Synthase

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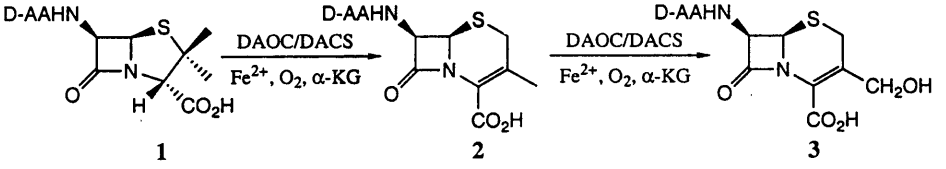
Summary

The preparation of isotopically labelled penicillin Ns using a chemico-enzymatic approach is described. This route involves the chemical synthesis of variously labelled D,L,D,-aminoadipoyl-cysteinyl-valine tripeptides *via* well established facile protocols and concludes with the conversion of these tripeptides directly into penicillin Ns by the action of recombinant isopenicillin N synthase. Milligram quantities of isotopically labelled penicillin Ns, which would otherwise represent very challenging and expensive synthetic targets, are readily accessible from this route.

Keywords : Preparation, Isotopically, Labelled, Penicillin Ns

Our studies concerning the biosynthesis of cephalosporin C in the fungus *Cephalosporium acremonium* have focused on the enzymatic ring expansion of penicillin N **1** to the first of the natural cephalosporins, deacetoxycephalosporin C (DAOC, **2**)[1]. This interesting reaction is catalysed by the bifunctional enzyme deacetoxycephalosporin C / deacetylcephalosporin C synthase (DAOC/DACS) which is also responsible for the hydroxylation of DAOC to deacetylcephalosporin C (DAC, **3**) (Scheme 1).



1 **2** **3**

D-AA = δ-(D-α-Aminoadipoyl)
α-KG = α-Ketoglutarate

Scheme 1

CCC 0362–4803/98/121145–19$17.50

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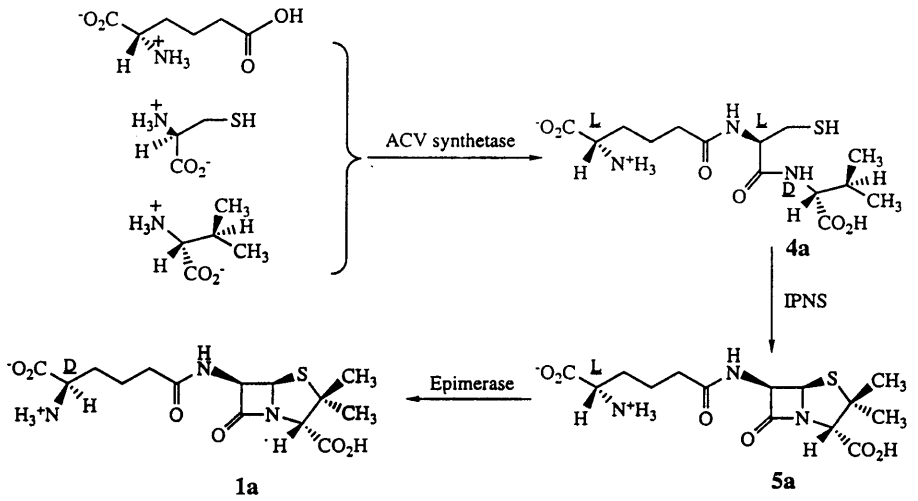
DAOC/DACS has an approximate molecular weight of 40KDa and requires molecular oxygen and α-ketoglutarate as co-substrates, ferrous iron, dithiothreitol and ascorbate for optimal activity[2,3].

To pursue our ideas regarding the mechanism of action of this enzyme we needed a variety of isotopically labelled penicillin N s containing either deuterium and/or ^{13}C isotopic labels[4]. Preparation of these penicillins by total synthesis would be a major undertaking, not least when the inherent cost of introducing a ^{13}C-label into a complex synthetic target such as penicillin N is considered. As a result of these and other considerations, an alternative approach to the preparation of the labelled penicillins involving partial enzymic synthesis was sought. We thus turned our attention back to the β-lactam biosynthetic pathway and to the biosynthesis of penicillin N **1a** itself. The biosynthetic route to penicillin N **1a** (Scheme 2) proceeds with the incorporation of the amino acids L-α-aminoadipic, L-cysteine and L-valine into a tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (L,L,D-ACV, **4a**) by the action of the enzyme ACV synthetase. This tripeptide is then cyclised to isopenicillin N **5a** by the much studied enzyme, isopenicillin N synthase (IPNS), which has a molecular weight of approximately 38KDa and requires molecular oxygen as a co-substrate and ferrous iron, dithiothreitol and ascorbate as co-factors[5]. Finally an epimerase enzyme is responsible for the inversion of the side-chain configuration [i.e isopenicillin N **5a** to penicillin N **1a**][6].

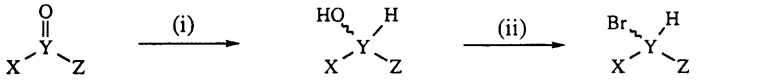


Scheme 2

It appeared that, in principle, it would be possible to prepare the desired labelled penicillins by synthesis and incubation of the corresponding labelled tripeptides. However, the side-chain of

the natural substrate for IPNS, δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (L,L,D-ACV, 4a) is of opposite configuration to that required for ring expansion by DAOC/DACS[7]. The enzyme responsible for the *in vivo* inversion of the side-chain configuration [i.e isopenicillin N **5a** to penicillin N **1a**] (Scheme 2) was not available to us in sufficient quantities to prepare the desired milligram amounts of penicillin N **1a**. Tripeptides with D-configured side-chains are, however, substrates for IPNS[8] thus we proposed to incubate labelled D,L,D-ACV **6** tripeptides with IPNS in order to enzymatically synthesise the required penicillin Ns.

The majority of the desired penicillins contained labels which were in positions derived from the valinyl residue of the tripeptide. We could, therefore, introduce labels into penicillin N simply by synthesising the specifically labelled valines followed by incorporation of these into the final precursor tripeptide. This approach gave rise to the largest degree of flexibility and ease of synthesis. Thus standard protocols[9] were employed for the preparation of the protected dipeptide, (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentan-amido-S-*p*-methoxybenzyl-L-cysteine **7**. Labelled racemic valine benzhydryl esters were typically prepared *via* alkylation of O'Donnell's imine **11**[10] with variously labelled isopropylbromides **10a-d** prepared from the corresponding labelled propanones **8a-d** by $LiAlH_4$ reduction and PBr_3 bromination (Scheme 3). The resulting imines **12a-e** were then hydrolysed with dilute and concentrated hydrochloric acid and finally esterified with diphenyldiazomethane.



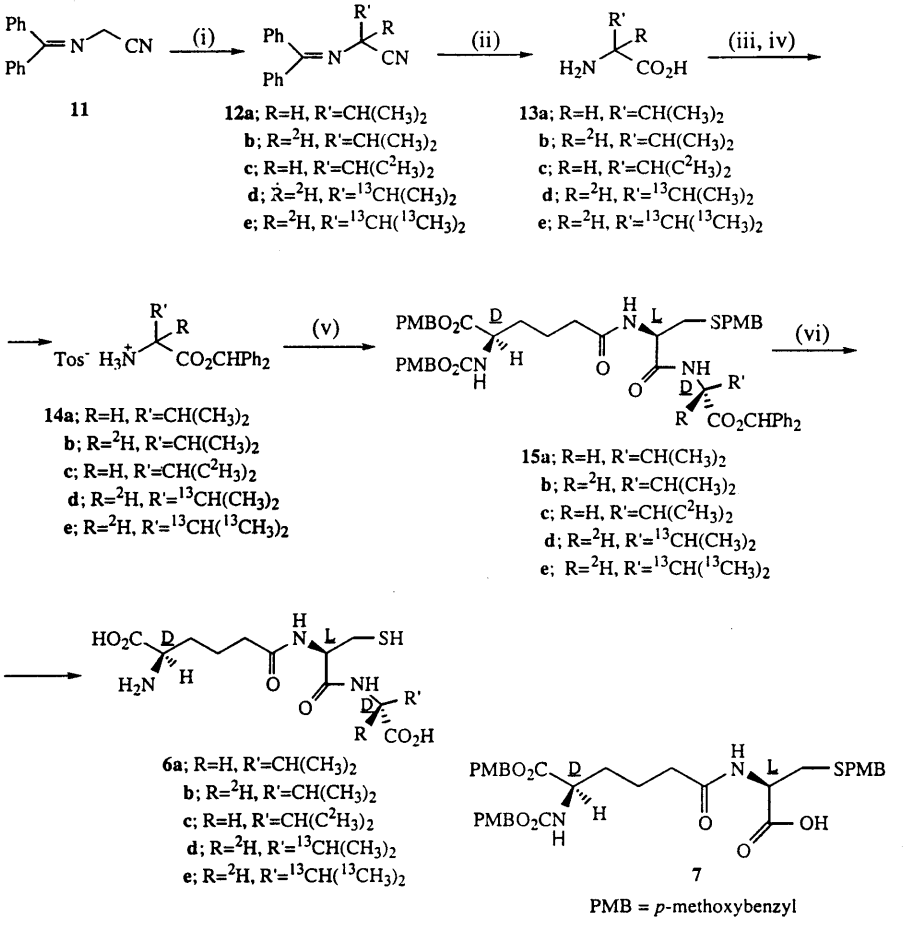
8a; X=Z=CH_3, Y=C
b; X=Z=C^2H_3, Y=C
c; X=Z=CH_3, Y=^{13}C
d; X=Z=$^{13}CH_3$, Y=^{13}C

9a; X=Z=CH_3, Y=C
b; X=Z=C^2H_3, Y=C
c; X=Z=CH_3, Y=^{13}C
d; X=Z=$^{13}CH_3$, Y=^{13}C

10a; X=Z=CH_3, Y=C
b; X=Z=C^2H_3, Y=C
c; X=Z=CH_3, Y=^{13}C
d; X=Z=$^{13}CH_3$, Y=^{13}C

Reagents i) $LiAlH_4$, diglyme 0°C, then ethane-1,2-diol quench and distillation; ii) PBr_3, -10°C, then distillation.

Scheme 3



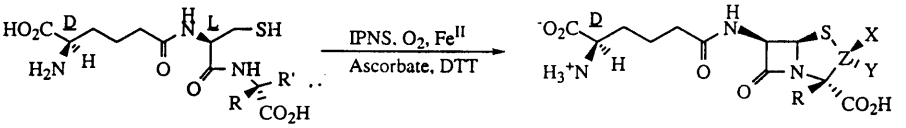
Reagents: (i) 50% NaOH or NaOD, benzyltriethylammonium chloride, toluene, $(CH_3)_2CHBr$ or labelled isopropylbromide, 0°C, 24 hours; (ii) 1N HCl 12 hours r.t, then 6N HCl, 12 hours, reflux; (iii) 1 eq. *p*-toluene -sulphonic acid, THF/H_2O, then lyophilisation; (iv) Ph_2CN_2, MeCN, (v) $NaHCO_3$;EEDQ, DCM, (**7**); (vi) TFA anisole, reflux 30 minutes.

Scheme 4

The dipeptide **7** and labelled valine benzhydryl esters **14a-e** were then coupled with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) to give the fully protected tripeptide **15a-e** as a mixture of DLD- and DLL-ACV diastereomers (Scheme 4).

Separation by chromatography and deprotection by refluxing in anhydrous trifluoroacetic acid containing anisole gave the desired DLD-tripeptides **6a-e** as their ammonium trifluoroacetate salts. These were generally incubated with IPNS, without further purification, under previously described conditions[9] (Scheme 5). Purification of the incubation mixture by HPLC (reverse phase

C_{18} column eluting with 0.75% MeCN in 25 mM aqueous NH_4HCO_3) gave the labelled penicillin N s **1a-e** in typically 30% isolated yield.



6a; R=H, R'=$CH(CH_3)_2$
b; R=^{2}H, R'=$CH(CH_3)_2$
c; R=H, R'=$CH(C^2H_3)_2$
d; R=^{2}H, R'=$^{13}CH(CH_3)_2$
e; R=^{2}H, R'=$^{13}CH(^{13}CH_3)_2$

1a; R=H, X=Y=CH_3, Z=C
b; R=^{2}H, X=Y=CH_3, Z=C
c; R=H, X=Y=C^2H_3, Z=C
d; R=^{2}H, X=Y=CH_3, Z=^{13}C
e; R=^{2}H, X=Y=$^{13}CH_3$, Z=^{13}C

Scheme 5

In conclusion, the availability of the enzyme isopenicillin N synthase and the synthesis of the correspondingly labelled D,L,D-ACV tripeptides **6a-e** has enabled the preparation of a variety of isotopically labelled penicillin N s, unavailable to us by alternative synthetic methodology, to be achieved.

Acknowledgements

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Experimental

Standard chemical procedures as previously reported[9] were used. Flash chromatography was performed with Merck Kieselgel 60, 230-400 mesh. Preparative plate chromatography was performed with silica gel (HF254) coated onto glass plates. Thin layer chromatography was performed with Merck silica gel 60 F254 pre-coated onto aluminium plates. Infra-red spectra were recorded on a Perkin Elmer 681 infra red spectrometer or Perkin Elmer 1750 fourier-transform spectrometer (absorbances recorded as: s strong, m medium, w weak and b broad).

Melting points were recorded on a Buchi 510 apparatus and are uncorrected.

^{1}H-NMR spectra were either recorded at 300 MHz on a Bruker WH 300 spectrometer or at 500 MHz on a Bruker AM 500 spectrometer and are referenced internally to either TMS (samples in $CDCl_3$ $\delta_{ref.}$ = 0.0 ppm) or TSP (samples in D_2O, $\delta_{ref.}$ = 0.0 ppm) unless otherwise indicated.

^{13}C-NMR spectra were either recorded at 62.38 MHz on a Bruker AM 250 spectrometer or at 125.77 MHz on a Bruker AM 500 MHz spectrometer and are referenced internally to either $CDCl_3$ ($\delta_{ref.}$ = 77.0 ppm) or 1,4-dioxan (for samples in D_2O, $\delta_{ref.}$ = 67.3 ppm).

Mass spectra in the electron-impact (E.I.) mode or chemical ionisation (C.I.) mode were recorded on a VG Micromass 30F spectrometer. Samples requiring field desorption chemical ionization (F.D.) were run on either a ZAB 1F spectrometer or VG 20-250 spectrometer. Fast atom bombardment (F.A.B.), thermaspray (T.S.P.) and HPLC mass spectra were run on a VG 20-250 spectrometer.

High performance liquid chromatography (HPLC) of crude incubation mixtures was performed with two Waters M-510 A pumps, a Rheodyne 7125 injector, a Waters 441 detector set at 218 nm (unless otherwise stated) and columns packed with Hypersil 5 ODS (250 x 4.6mm internal diameter). Preparative scale HPLC was performed using two Gilson 303 pumps, a Rheodyne 7125 injector, a Gilson HM holochrome variable wavelength detector set at 220 nm and columns packed with Zorbax ODS (250 x 9.4 mm internal diameter).

Propan-2-ol (9a)

To a stirred suspension of $LiAlH_4$ (164 mg, 4.3 mmol) in diglyme (10 ml, dried over CaH_2) under argon at 0°C, was slowly added acetone **8a** (500 mg, 8.6 mmol). The resulting solution was stirred at 0°C for 1hour and then diethylene glycol (15 ml) slowly added. The product was distilled from the reaction mixture and the fraction distilling between 79-89°C collected to give **9a** as a colourless liquid (585 mg, contained solvent), identical to an authentic standard by NMR.

(1,3-$^2H_6$)-Propan-2-ol (9b)

(1,3-$^2H_6$)-Propan-2-ol was prepared as for **9a** except that $^2H_6$-acetone **8b** (99.96% atom D, 10.0 g, 156 mmol) and $LiAlH_4$ (2.5 g, 66 mmol) in diglyme (50 ml, dried over CaH_2) were used. The product was distilled from the reaction mixture and the fraction boiling between 79-105°C collected to give **9b** (10.0 g, 151 mmol, 97% yield). δ_H (500MHz, $CDCl_3$) 3.95 (br.s, $C\underline{H}(C^2H_3)_2$).

(1,2,3-$^{13}C_3$)-Propan-2-ol (9d)

As for **9a** except that (1,2,3-$^{13}C_3$)-propanone **8d** (500 mg, 8.2 mmol, 99.2 atom %^{13}C, supplied by Merck Sharpe and Dhome Isotopes Ltd) was used. The product was distilled from the reaction mixture and the fraction distilling between 60-120°C collected and used without further purification. δ_H (500MHz, $CDCl_3$) 1.06-1.09 and 1.31-1.34 (2 x 3H, 2 x m, J ^{13}C-^1H 130 Hz, $^{13}CH(^{13}C\underline{H}_3)_2$, 1.84 (1H, br.s, $^{13}CHO\underline{H}$), 3.85-3.90 and 4.14-4.18 (1H, 2 x m, J ^{13}C-^1H 142 Hz, $^{13}C\underline{H}(^{13}CH_3)_2$); δ_C (125.77MHz, $CDCl_3$) 25.25 (dq, J ^{13}C-^{13}C 38Hz and J ^{13}C-^1H 78Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 64.29 (dt, J ^{13}C-^1H 108Hz , J ^{13}C-^{13}C 38Hz , $^{13}\underline{C}H(^{13}CH_3)_2$).

2-Bromopropane (10a)

To a stirred solution of **9a** (585 mg) prepared as described above, at -10°C was added dropwise phosphorus tribromide (2.33 g, 8.6 mmol). The resulting solution was stirred at room temperature overnight and the product **10a** isolated by distillation, the fraction boiling between 59-62°C collected (1.169 g, contained solvent). δ_H (500MHz,$CDCl_3$) 1.70 (6H, d, J 7Hz, 2 x $C\underline{H}_3$), 4.29 (1H, septet, J 7 Hz , $C\underline{H}Me_2$).

(1,3-$^2H_6$)-2-Bromopropane (10b)

(1,3-$^2H_6$)Propan-2-ol **9b** (9.5 g, 144 mmol) and phosphorus tribromide (24.9 g, 92 mmol) were used. The fraction boiling between 50-62 °C was collected to give **10b** (14.0 g, 109 mmol, 76% yield). δ_H (500MHz,$CDCl_3$) 4.25 (1H, s, $C\underline{H}(C^2H_3)_2$)

2-Bromo-(2-^{13}C)-propane (10c)[17]

2-Bromo-(1,2,3-$^{13}C_3$)-propane (10d)

As for compound **10a** except that crude (1,2,3-$^{13}C_3$)-propan-2-ol **9d** (780 mg, *ca* 60% pure by ^1H-NMR) and phosphorus tribromide (2.23 g, 8.2 mmol) were used. The product was distilled from the reaction mixture, the fraction distilling between 40-85°C being collected, to give **10d** (835 mg, 6.63 mmol, 81% from (1,2,3-$^{13}C_3$)-propanone **8d**) which was shown to be pure by ^1H-NMR δ_H (500MHz, $CDCl_3$); 1.49-1.60 and 1.82-1.85 (2 x 3H, 2 x m, J ^{13}C-^1H 130 Hz, $^{13}CH(^{13}C\underline{H}_3)_2$, 4.11-4.17 and 4.41-4.47 (1H, 2 x m, J ^{13}C-^1H 153 Hz, $^{13}CHBr$); δ_C (125.77MHz,

$CDCl_3$) 28.47 (dq, J ^{13}C-^{13}C 37Hz and J ^{13}C-^{1}H 130Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 45.55 (dt, J ^{13}C-^{1}H 153Hz, J ^{13}C-^{13}C 37Hz , $^{13}\underline{C}H(^{13}CH_3)_2$).

2-[N-(Diphenylmethylene)amino]-3-methylbutyronitrile (12a)[10,16]

N-(Diphenylmethylene)aminoacetonitrile **11** (1.00 g, 4.55 mmol), benzyltriethylammonium chloride (100 mg, 0.44 mmol), NaOH (1.10 g as a 50% aqueous solution) and toluene (1ml) were stirred together at 0°C in a round bottom flask sealed with a rubber septum. 2-Bromopropane (540 mg, 4.39 mmol) in toluene (1ml) was added *via* a syringe over a period of 1-2 hours at 0°C. The solution was stirred at room temperature overnight and was then poured into a separating funnel containing dichloromethane (60ml) and water (80ml). The layers were separated and the aqueous layer extracted with DCM (3 x 30ml). The organic layers were combined, washed with water (20ml), brine (20ml), dried (anhydrous Na_2SO_4), filtered and the solvent evaporated. Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave **12a** (800 mg, 3.0 mmol, 67%) as an oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25; δ_H (500MHz, $CDCl_3$) 1.00 and 1.12 (2 x 3H, 2 x d, J 7.5 Hz, 2 x CH_3), 2.11-2.16 (1H, m, $C\underline{H}Me_2$), 3.98 (1H, d, J 6Hz, $C\underline{H}CHMe_2$), 7.20-7.82 (10H, m, Ar$\underline{H}$); δ_C(125.77MHz, $CDCl_3$); 18.22 and 18.65 (2 x q, 2 x CH_3), 33.26 (d, $\underline{C}HMe_2$), 59.18 (d, $\underline{C}HCHMe_2$), 118.51(s, CN), 127.16-138.37 (m, Ph), 172.57 (s, $Ph_2\underline{C}$=N); υ_{max} ($CHCl_3$) 2948 (s), 2177 (w, CN), 1620 (s), 1455 (m), 1243 (s); *m/z* (E.I.) 261 (5%), 262 (M^+, 8), 263 (MH^+, 12), 264 (3).

2-[N-(Diphenylmethylene)amino]-(2-^{2}H)-3-methylbutyronitrile (12b)[9]

2-[N-(Diphenylmethylene)amino]-3-($^2H_3$-methyl),4-($^2H_3$)-butyronitrile (12c)

As for compound **12a** except that N-(diphenylmethylene)aminoacetonitrile **11** (9.17 g, 41.7 mmol), benzyltriethylammonium chloride (840 mg, 3.7 mmol), NaOH (5.18 g as a 50% aqueous solution), toluene (10ml) and (1, 3-$^2H_6$)-2-bromopropane **10b** (6.45 g, 50.0 mmol) in toluene (10ml) were used. Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave **12c** (5.3 g, 20 mmol, 47% yield) as a pale yellow oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.50.; δ_H(300MHz, $CDCl_3$) 2.11 (1H, d, J 6, $C\underline{H}(C^2H_3)_2$), 3.99 (1H, d, J 6, $C\underline{H}CH(C^2H_3)_2$), 7.11-7.58 (10H, m, 10 x ArH); δ_C (125.77MHz, $CDCl_3$) 17.80 (2 x sept, 2 x $\underline{C}^2H_3$), 32.93 (d, $\underline{C}H(C^2H_3)_2$), 59.23 (d, $\underline{C}HCH(C^2H_3)_2$), 118.66 (s, $\underline{C}$N), 127.29-138.49 (m, $\underline{Ph}$), 172.67 (s, $Ph_2\underline{C}$=N); υ_{max}

($CHCl_3$) 2228(w), 1619(s), 1458(m); *m/z* (DCI, NH_3), 269 (MH^+, 100%), 270 (34), 271 (6), 272 (1).

2-[N-(Diphenylmethylene)amino]-(2-^{2}H, 3-^{13}C)-3-methylbutyronitrile (12d)

As for compound **12a** except that N-(diphenylmethylene)aminoacetonitrile **11** (887 mg, 4.03 mmol), benzyltriethylammonium chloride (100 mg, 0.4 mmol), NaOD (1.8 g as a 50% aqueous solution), toluene (1ml) and 2-(2-^{13}C)-bromopropane **10c** (500 mg, 4.03 mmol, 92.2 atom % ^{13}C supplied by Merck Sharpe and Dhome Isotopes Ltd) in toluene (1ml) were used. Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave **12d** (840 mg, 3.18 mmol, 79% yield) as a pale green oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.50.; δ_H (500MHz, $CDCl_3$) 0.86-0.94 and 1.03-1.05 (6H, 2 x m, $^{13}CH(C\underline{H}_3)_2$), 1.85-1.94, 2.04-2.08 and 2.15-2.22 (1H, 3 x m, J ^{13}C-^1H 131Hz, $^{13}C\underline{H}(CH_3)_2$), 7.11-7.85 (10H, m, ArH); δ_C(125.77MHz, $CDCl_3$) 18.50 and 18.79 (2 x d, $^{13}CH(\underline{C}H_3)_2$), 33.41 (d, J ^{13}C-^1H 131Hz, $^{13}\underline{C}HMe_2$), 118.81 (s, Ph_2C=N), 127.43-138.62 (m, Ph), 172.85 (s, CN).

2-[N-(Diphenylmethylene)amino]-2-(^{2}H), 3-^{13}C, 3'-(^{13}C methyl),4-^{13}C-butyronitrile (12e)

Procedure as for **12a** except that N-(diphenylmethylene)aminoacetonitrile **11** (1.46 g, 6.6 mmol), benzyltriethylammonium chloride (150 mg, 0.7 mmol), NaOD (2mls of *ca* 50% solution), toluene (10ml) and 2-bromo(1,2,3-$^{13}C_3$)-propane **10d** (835 mg, 6.6 mmol) in toluene (10ml). Chromatography [flash silica, with petrol/diethyl ether (9:1, v/v)] gave **12e** (665 mg, 2.5 mmol, 38% yield) as a pale yellow oil. T.l.c. [petrol/diethyl ether (9:1,v/v)] Rf 0.25. δ_H (500MHz, $CDCl_3$); 0.86-1.26 (6H, 4 x m, J ^{13}C-^1H 126Hz, $^{13}CH(^{13}C\underline{H}_3)_2$), 1.96-2.05 and 2.23-2.31 (1H, 2 x m, J ^{13}C-^1H 130Hz, $^{13}C\underline{H}(^{13}CH_3)_2$), 7.20-7.66 (10H, m,10 x Ar$\underline{H}$); δ_C (125.77MHz, $CDCl_3$) 18.47 and 18.89 (2 x dq, J ^{13}C-^{13}C 36Hz, J ^{13}C-^1H 126Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 33.43 (dt, J ^{13}C-^1H 130Hz and J ^{13}C-^{13}C 36Hz, $^{13}\underline{C}H(^{13}CH_3)_2$); υ_{max} ($CHCl_3$); 2228(w), 1619(s), 1458(m); *m/z* (DCI, NH_3) 265 (4%), 266 (MH^+ for $^{12}C_{15}{}^{13}C_3H_{18}N_2$, 16), 267 ($MH^+$ for $^{12}C_{15}{}^{13}C_3H_{17}{}^2HN_2$, 100), 268 (17), 269 (2).

D/L-Valine (13a)[10]

To the imine **12a** (796 mg, 3.0 mmol) in diethyl ether (10 ml) was added 1N hydrochloric acid (25ml) and the mixture vigorously stirred for 12 hours. The organic and aqueous layers were separated and the aqueous phase washed with diethyl ether (3 x 10ml). Concentrated hydrochloric acid (25 ml) was added and the resulting solution refluxed for 12 hours. The solution was

evaporated and the residue dissolved in a small quantity of water and purified by chromatography [ion-exchange, Dowex 1x8-400 acetate form (eluting with water)]. Column fractions were assayed with ninhydrin and the relevant fractions evaporated to dryness to give racemic **13a** (244 mg, 2.1 mmol, 70%) as white solid. δ_H (500MHz, D_2O, HOD suppressed) 0.99 (3H, d, *J* 8 Hz, $CHC\underline{H}_3$), 1.04 (3H, d, *J* 8 Hz, $CHC\underline{H}_3$), 2.25-2.31 (1H, m, $CHC\underline{H}CMe_2$), 3.60 (1H, d, *J* 4Hz, $C\underline{H}CHMe_2$); *m/z* (C.I., NH_3) 118 (MH^+, 100%), 119 (6), 120 (1).

D/L-(2-^{2}H)-Valine (13b)[9]

D/L-Di-($^2H_3$-methyl)-Valine (13c)

As for **13a** except that 2-[N-(diphenylmethylene)amino]-3-($^2H_3$)-methyl,4-($^2H_3$)-butyronitrile **12c** (5.03 g, 18.8 mmol) gave di-($^2H_3$-methyl)-valine **13c** (1.375 g, 11.2 mmol, 60% yield). δ_H (500MHz, D_2O, HOD suppressed), 2.31 (1H, br.s, $C\underline{H}CD_3$) 3.92 (1H, d, *J* 4Hz,$C\underline{H}CHCD_3$); δ_C (62.5 MHz, D_2O) 16.55 and 17.78 (2 x sept., 2 x $\underline{C}D_3$), 29.36 (d, $\underline{C}HCD_3$), 61.07 (d, $\underline{C}HNH_3$), 174.87 (s, $\underline{C}O_2$); *m/z* (CI, NH_3) 122 (1%), 123 (6), 124 (MH^+,100), 125 (6), 126 (1).

D/L-(2-^{2}H, 3-^{13}C)-Valine (13d)

Procedure as for compound **13a** except that 2-[N-(diphenylmethylene)amino]-(2-^{2}H,3-^{13}C)-3-methylbutyronitrile **12d** (840 mg, 3.18 mmol) were used to give (2-^{2}H, 3-^{13}C)-valine **13d** (374 mg, 3.14 mmol, 99% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.99 and 1.04 (2 x 3H, 2 x m, $^{13}CH(C\underline{H}_3)_2$), 2.14, 2.27 and 2.40 (1H, 3 x m, $J\ ^{13}C\text{-}^1H$ 131Hz, $^{13}C\underline{H}(CH_3)_2$ and $^{12}C\underline{H}(CH_3)_2$); δ_C(125.77MHz, D_2O) 17.34 and 18.62 (2 x d, $^{13}CH(\underline{C}H_3)_2$), 29.67 (d, $J\ ^{13}C\text{-}^1H$ 131Hz, $^{13}\underline{C}H(CH_3)_2$); *m/z* (FAB) 118 (2%), 119 (17), 120 (MH^+, 100), 121 (5), 122 (1).

D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-Valine (13e)

Procedure as for compound **13a** except that 2-[N-(diphenylmethylene)amino]-(2-^{2}H, 3-^{13}C), 3'-(^{13}C methyl),4-^{13}C-butyronitrile **12e** (665 mg, 2.5 mmol) was used. Chromatography [ion exchange with Dowex 1 x 8-400 acetate form, eluting with water] gave **13e** (266 mg, 2.2 mmol, 88% yield) as a white solid. δ_H (500MHz, D_2O, HOD suppressed) 0.87-0.94 and 1.12-1.19 (2 x 3H, 2 x m, $J\ ^{13}C\text{-}^1H$ 127 Hz, $^{13}CH(^{13}C\underline{H}_3)_2$), 2.15-2.22 and 2.42-2.48 (1H, 2 x m, $J\ ^{13}C\text{-}^1H$

132 Hz, $^{13}C\underline{H}(^{13}CH_3)_2$); δ_C (125.77MHz, $CDCl_3$) 17.45 and 18.28 (2 x dq, J ^{13}C-^{13}C 35Hz, J ^{13}C-^{1}H 127Hz, $^{13}CH(^{13}\underline{C}H_3)_2$), 29.66 (dt, J ^{13}C-^{1}H 132Hz and J ^{13}C-^{13}C 35Hz, $^{13}\underline{C}H(^{13}CH_3)_2$); *m/z* (FAB) 121 ($MH^+$ for $^{12}C_2{}^{13}C_3H_{11}NO_2$, 2%), 122 ($MH^+$ for $^{12}C_2{}^{13}C_3H_{10}{}^{2}HNO_2$. 100).

D/L-Valine Benzhydryl Ester Ammonium Tosylate Salt (14a)[9]

To the free amino acid **13a** (100 mg, 0.85 mmol) in acetone/water (2:1 v/v, 10 ml) was added a solution of *p*-toluenesulphonic acid (163 mg, 0.85 mmol) in THF (10 ml). The resulting solution was evaporated to dryness and the solid residue suspended in MeCN (20 ml). To the stirred suspension was added diphenyldiazomethane (165 mg, 0.85 mmol) in MeCN (5 ml), followed by the dropwise addition of a diphenyldiazomethane solution in MeCN until a pale pink colouration persisted. After 30 minutes the white precipitate was filtered off and washed with petrol (10 ml) to give the product (**14a**) (360 mg, 0.79 mmol, 93%). δ_H (500MHz, $CDCl_3$) 0.80-0.84 (6H, m, $CH(C\underline{H}_3)_2$), 2.18-2.28 (1H, m, $CHC\underline{H}Me_2$ and 3H, s, $ArCH_3$) 3.99 (1H, *ca* t, *J* 4Hz, $C\underline{H}CHMe_2$), 6.85 (1H, s, $C\underline{H}Ph_2$), 6.98-7.65 (14H, m, $Ar\underline{H}$).

D/L-(2-^{2}H)Valine Benzhydryl Ester Ammonium Tosylate Salt (14b)[9]

D/L-di-($^2H_3$-methyl)-Valine Benzhydryl Ester, Ammonium tosylate salt (14c).

As for D/L-valine benzhydryl ester, ammonium tosylate salt except that (**13c**) (0.5 g, 4.1 mmol), in acetone/water (2:1 v/v, 10 ml), *p*-toluenesulphonic acid (779 mg, 4.1 mmol) and diphenyldiazomethane (1.0-1.5 eq) were used to give (**14c**) (1.68 g, 3.64 mmol, 90% yield); m.p.174-5 °C.; Found; C 64.88, H 6.30 and N 2.87. $C_{25}H_{23}{}^{2}H_6NO_5S$ - requires C 65.05, H 6.33 and N 3.03% ; δ_H (500MHz,$CDCl_3$) 2.20 (1H, br.d, *J* 4Hz, $CHC\underline{H}(C^2H_3)_2$), 2.28 (3H, s, $ArC\underline{H}_3$), 3.95 (1H, *ca*.t, *J* 4Hz, $C\underline{H}CH$), 6.85 (1H, s, $C\underline{H}Ph_2$), 6.97-7.64 (14H, m, ArH).

D/L-(2-^{2}H, 3-^{13}C)-Valine Benzhydryl Ester, Ammonium Tosylate Salt (14d).

Procedure as for (**14a**) except that (2-^{2}H, 3-^{13}C)-valine (**13d**) (200 mg, 1.68 mmol) , p-toluenesulphonic acid (335 mg, 1.75 mmol) and diphenyldiazomethane (330 mg, 1.75 mmol) was used to give (**14d**) (709 mg, 1.55 mmol, 92% yield); m.p 173-4°C; Found C 65.46, H 6.55, N 2.95%. $C_{24}{}^{13}CH_{28}{}^{2}HNO_5S$ - requires C 65.64, H 6.39 and N 3.06%. δ_H (500MHz, $CDCl_3$) 0.82-0.85 (6H, m, $CH\underline{Me}_2$), 2.06-2.12, 2.21-2.28 and 2.30-2.48 (4H, 3 x m, $C^2H^{13}C\underline{H}Me_2$ and 3H, s, $ArC\underline{H}_3$), 6.86 (1H, s, $C\underline{H}Ph_2$), 6.96-7.64 (14H, m, $Ar\underline{H}$).

D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-Valine Benzhydryl Ester, Ammonium Tosylate Salt (14e).

Procedure as for (**14a**) except that (2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (**13e**) (66.5 mg, 0.55 mmol), p-toluenesulphonic acid (105 mg, 0.55 mmol) and diphenyldiazomethane (104 mg, 0.55 mmol) was used to give (**14e**) (245 mg, 0.53 mmol, 97% yield); δ_H (500MHz, $CDCl_3$) 0.65-0.71 (3H, m, J ^{13}C-^{1}H 136 Hz, $^{13}CH(^{13}CH_3)_2$), 0.91-0.97 (3H; m, J ^{13}C-^{1}H 136 Hz, $^{13}CH(^{13}CH_3)_2$), 2.04-2.10, and 2.33-2.36 (1H, 2 x m, J ^{13}C-^{1}H 130 Hz, $^{13}CH(^{13}CH_3)_2$), 2.27 (3H, s, $ArCH_3$), 6.865(1H, s, $CHPh_2$), 6.95-7.64 (14H, m, ArH).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonyl-pentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-valine Benzhydryl Ester (15a)[9]

The ammonium tosylate salt of the D-valine benzhydryl ester [13] (77 mg, 0.17 mmol) was suspended in saturated aqueous $NaHCO_3$ (10 ml) and the free amine extracted into EtOAc (3 x 20 ml). The combined organic layers were dried (anhydrous Na_2SO_4), filtered and evaporated to dryness to give the free amine which was used without further purification. To the amine (46 mg, 0.16 mmol) in dichloromethane (2 ml) was added (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (**7**)[14,15] (116 mg, 0.17 mmol), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (47 mg, 0.19 mmol) and anhydrous Na_2SO_4 (*ca* 5 mg). The resulting mixture was stirred at room temperature under argon for 24 hours. The solution was then evaporated to dryness and the residue partitioned between EtOAc (50 ml) and water (50 ml) in a separating funnel. The layers were separated and the organic phase washed with 2N HCl (20 ml), saturated aqueous $NaHCO_3$ (20 ml) and brine (20 ml). The solution was dried (anhydrous Na_2SO_4), filtered and evaporated to dryness. Chromatography [flash silica, with dichloromethane/ ethyl acetate (gradient 4:1 to 1:3, v/v)] gave **15a** (125 mg, 0.13 mmol, 84%) as a white amorphous solid. T.l.c. [ethyl acetate/ petrol (1:1, v/v)] Rf 0.45; δ_H (500MHz, $CDCl_3$) 0.76 (3H, d, J 7Hz, $CHCH_3$), 0.87 (3H, d, J 7Hz, $CHCH_3$), 1.62-1.86 (4H, m, $CH_2CH_2CH_2CO$), 2.05-2.25 (3H, m, CH_2CO and $CH(CH_3)_2$), 2.67, 2.82 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.70 (2H, s, SCH_2Ar), 3.74 (3H, s, SCH_2ArOCH_3), 3.78 (6H,s, 2 x CH_2ArOCH_3), 4.28-4.35 (1H, m, $CHCH_2CH_2$), 4.51 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $CHCH_2S$), 4.62-4.64 (1H, m, $CHCHMe_2$), 5.00, 5.02 (2H, ABq, J 12Hz, OCH_2Ar), 5.07 (2H, s, OCH_2Ar), 5.45 and 6.29 (2H, 2 x d, J 8Hz, 2 x NH), 6.79-6.89 and 7.16-7.31 (23H, 2 x m,

CHPh$_2$ and ArH); δ_C (125.77MHz, CDCl$_3$); 17.24 (q, CHCH$_3$) 19.02 (q, CHCH$_3$), 21.24 (t, CH$_2$CH$_2$CH$_2$), 31.09 (d, CHMe$_2$), 31.77 (t, CH$_2$CH$_2$CH$_2$), 33.18, 35.27, 35.82 (3 x t, SCH$_2$Ar, CH$_2$S, and CH$_2$CO), 52.14 and 53.59 (2 x d, NHCHCH$_2$ and NHCHCHS), 55.17 (q, ArOCH$_3$), 57.31 (d, CHCHMe$_2$), 66.74 and 66.92 (2 x t, CH$_2$Ar), 77.93 (d, CHPh$_2$), 113.86, 113.96 and 114.04 (3 x d, Ar), 126.89-139.57 (m, Ar), 156.09, 158.76, 159.52 and 159.73 (4 x s, COMe of ArOMe), 170.24, 170.56, 172.06 and 172.48 (4 x C=O); υ_{max} (CHCl$_3$) 3020, 1736, 1682 and 1515 (all s); *m/z* (F.D.) 933 (M$^+$).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonyl-pentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H)-valine Benzhydryl Ester (15b)[9]

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonyl-pentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-[di-(^{2}H$_3$ -methyl)]-valine Benzhydryl Ester (15c).

As for compound (**15a**) except that the ammonium tosylate salt of D, L-di-(^{2}H$_3$-methyl)-valine benzhydryl ester (**14c**) (507 mg, 1.10 mmol) was used to give the free amine which was then coupled with (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonyl-S-*p*-methoxybenzyl-L-cysteine (**7**) (735 mg, 1.10 mmol) with EEDQ (306 mg, 1.22 mmol) and anhydrous Na$_2$SO$_4$ (*ca* 5 mg) to give (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteinyl-D-[di-(^{2}H$_3$ -methyl)]-valine benzhydryl ester (**15c**) (393 mg, 0.42 mmol, 76% yield) as a viscous oil. δ_H (500MHz, CDCl$_3$) 1.26-1.81 (4H, m, CH$_2$CH$_2$CH$_2$), 2.05-2.20 (3H, m, CH$_2$CO and CH(CD$_3$)$_2$), 2.65, 2.82 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH$_2$S), 3.70 (2H, s, SCH$_2$Ar), 3.77 (3H, s, SCH$_2$ArOMe), 3.78 (6H, s, 2 x CH$_2$ArOMe), 4.28-4.34 (1H, m, CHCH$_2$CH$_2$), 4.50 (1H, X of ABX , J_{AX} 7 and J_{BX} 6Hz, CHCH$_2$S), 4.62 (1H, dd, CHCHMe$_2$), 5.00, 5.03 (2H, ABq, *J* 12Hz, OCH$_2$Ar), 5.07 (2H, s, OCH$_2$Ar), 5.44 (1H,d, *J* 8Hz, NH), 6.24 (1H, d, *J* 8Hz, NH), 6.77-6.90 and 7.16-7.32 (23H, 2 x m, CHPh$_2$ and ArH); δ_C (125.77MHz, CDCl$_3$) 16.20 and 18.10 (2 x br m, CH(CD$_3$)$_2$), 21.21 (t, CH$_2$CH$_2$CO), 30.51 (d, CH(CD$_3$)$_2$), 31.63 (t, CHCH$_2$CH$_2$), 33.27, 35.20 and 35.74 (3 x t, CH$_2$CO, CH$_2$SAr and SCH$_2$Ar), 52.10 and 53.50 (2 x d, NHCHCH$_2$S and CHCH$_2$CH$_2$), 55.05 (q, ArOMe), 57.36 (d, CHCH(CD$_3$)$_2$), 66.61 and 66.79 (2 x t, CH$_2$Ar), 77.81 (d, CHPh$_2$), 113.76, 113.86, and 113.91 (3 x d, Ar), 126.79-139.51 (10 x s, Ar), 156.10, 158.65,

159.45 and 159.64 (4 x s, $ArO_2\underline{C}NH$ and $ArO_2\underline{C}HN$ of ArOMe), 169.62, 170.43, 172.01 and 172.31 (4 x s, $\underline{C}$=O); υ_{max} ($CHCl_3$) 2959(w), 1734(m), 1718(m), 1515(s) and 1223(s); *m/z* (FAB) 940 (MH^+).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonyl-pentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H,3-^{13}C)-valine Benzhydryl Ester (15d).

As for compound (**15a**) except that the ammonium tosylate salt of D/L-(2-^{2}H,3-^{13}C)-valine benzhydryl ester (**14d**) (242 mg, 0.53 mmol) was used to give the free amine which was then coupled to [(5R)-5-N-*p*-methoxybenzyloxy-carbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteine (**7**) (352 mg, 0.53 mmol), with EEDQ (146 mg, 0.58 mmol) and anhydrous Na_2SO_4 (*ca* 10 mg). Chromatography [preparative plate with EtOAc/Hexane (1:1, v/v)] gave (**15d**) (175 mg, 0.19 mmol, 70% yield). δ_H (500MHz, $CDCl_3$) 0.74-0.77 and 0.86-0.89 (6H, 2 x m, $^{13}CH(C\underline{H}_3)_2$), 1.55-1.85 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2$), 2.04-2.26 (3H, m, CH_2CO and $^{13}C\underline{H}(CH_3)_2$), 2.65, 2.84 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.72 (2H, s, $SC\underline{H}_2Ar$), 3.76 (3H, s, $SCH_2ArO\underline{Me}$), 3.79 (6H, s, 2 x $CH_2ArO\underline{Me}$), 4.34 (1H, br.s, $C\underline{H}CH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $C\underline{H}CH_2S$), 5.00, 5.02 (2H, ABq, J 12Hz, $CC\underline{H}_2Ar$), 5.44 (1H, br.s, NH), 6.23 (1H, br.s, NH), 6.78-6.90 and 7.23-7.32 (23H, 2 x m, $C\underline{H}Ph_2$ and $Ar\underline{H}$); υ_{max} ($CHCl_3$) 3020(s), 1725(s), 1670(m) and 1520(s).

[(5R)-5-N-*p*-Methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine Benzhydryl Ester (15e).

Procedure as for (**15a**) except that the ammonium tosylate salt of D/L-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (**14e**) (92 mg, 0.20 mmol) was used to give the free amine which was then coupled to (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoxybenzyl-L-cysteine (**7**) (136 mg, 0.20 mmol), with EEDQ (56 mg, 0.22 mmol) and anhydrous Na_2SO_4 (*ca* 5 mg). Chromatography [flash silica with EtOAc/hexane (2:3, v/v)] gave (5R)-5-N-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido-S-*p*-methoybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (**15e**) (45 mg, 0.05 mmol, 48% yield). T.l.c. [ethyl acetate/ petrol (3:2, v/v)] Rf 0.30 (DLL-diastereomer, Rf 0.25). $[\alpha_D^{20}]$ = - 5.5 [c = 0.33, $CHCl_3$]; δ_H (500MHz, $CDCl_3$) 0.62-0.65, 0.73-0.76, 0.86-0.90 and 0.98-1.01 (6H, 4 x m, $^{13}CH(^{13}C\underline{H}_3)_2$), 1.57-1.88 (4H, m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.04-2.38 (3H, m, CH_2CO and $^{13}C\underline{H}(^{13}CH_3)_2$), 2.63-2.67, 2.82-2.85 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J

J_{BX} 6Hz, CH_2S), 3.71 (2H, s, SCH_2Ar), 3.75 (3H, s, SCH_2ArOMe), 3.78 (6H,s, 2 x CH_2ArOMe), 4.29-4.35 (1H, m, $CHCH_2CH_2$), 4.49 (1H, X of ABX, J_{AX} 7 and J_{BX} 6Hz, $CHCH_2S$), 5.00 and 5.03 (2H, ABq, J 12Hz, OCH_2Ar), 5.08 (2H, s, OCH_2Ar), 5.43 (1H, d, J 8Hz, NH), 6.23 (1H, d, J 7Hz, NH), 6.77 (1H, s, $CHPh_2$), 6.81-6.90 and 7.22-7.32 (22H, 2 x m, ArH); υ_{max} ($CHCl_3$) 3020 (s), 2960 (m), 1720 (s), 1515 (m), 1448 (s), 1250 (s).

[(5R)-5-amino-5-carboxypentanoyl]-L-cysteinyl-D-valine (6a)[9]

To the dry fully protected tripeptide (**15a**) (70 mg, 0.075 mmol) under argon was added anisole (0.4 ml) and freshly distilled trifluoroacetic acid (2.0 ml). Immediately upon addition of the acid, the stirred solution turned pink and the solution was heated under reflux for 30 minutes. Evaporation of this mixture gave a residue which was re-dissolved in dry toluene and once more evaporated to dryness. The solid material was partitioned between EtOAc (5 ml) and water (5 ml). After separation, the aqueous phase was washed with EtOAc (2 x 5 ml) and lyophilised to give the deprotected tripeptide (**6a**) as it's ammonium trifluoroacetate salt (26 mg, 0.055 mmol, 73%). δ_H (500MHz, D_2O, HOD suppressed) 0.92-0.99 (6H, m, $CH(CH_3)_2$), 1.65-2.02 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.16-2.24 (1H, m, $CH(CH_3)_2$), 2.41 (2H, *ca* t, J 7Hz, CH_2CO), 2.88, 2.94 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.98 (1H, *ca* t, J 6Hz, $CHCH_2S$), 4.28 (1H, d, J 6Hz, $CHCHMe_2$), 4.57 (1H, *ca* t, J 7Hz, $CH_2CHNH_3^+$); δ_C(125.77MHz, D_2O) 18.02 (q, $CHCH_3$), 19.23 (q, $CHCH_3$), 21.45 (t, $CH_2CH_2CH_2CO$), 26.15 (t, $CH_2CH_2CH_2CO$), 30.00 (t, CH_2CH_2CO), 30.68 (d, $CH(CH_3)_2$), 35.29 (t, CH_2SH), 53.74 (d, $CHCH_2S$), 56.28 (d, $CHNH_3^+$), 59.18 (d, $CHCO_2H$), 172.64, 172.95, 175.62 and 176.37 (4 x s, 4 x C=O); *m/z* (FAB) 362 (6%), 363 (10), 364 (MH^+,100), 365 (23), 366 (10).

[(5R)-5-amino-5-carboxypentanoyl]-L-cysteinyl-D-(2-^{2}H)valine (6b)[9]

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-[di-($^2H_3$-methyl)]-valine (6c).

As for compound (**6a**) except that (**15c**) (34.4 mg, 0.037 mmol) was used to give (**6c**) as it's ammonium trifluoroacetate salt (10.1mg, 0.021 mmol, 57% yield); δ_H (500MHz, D_2O, HOD suppressed) 1.65-2.01 (4H, 2 x m, $CH_2CH_2CH_2$), 2.20 (1H, m, $CH(CH_3)_2$), 2.42 (2H, *ca* t, J 7Hz, CH_2CO), 2.98, 2.95 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.97 (1H, *ca*. t, J 6Hz, $CHCH_2S$), 4.57 (1H, *ca*. t, J 7Hz, $CH_2CH^+NH_3$); δ_C(125.77MHz, D_2O) 17.20 and 18.60 (2

x br. m, 2 x $\underline{C}D_3$), 21.42 (t, $CH_2\underline{C}H_2CH_2$), 26.19 (t, $\underline{C}H_2CH_2CH_2$), 29.92 (t, $CH_2CH_2\underline{C}H_2$), 30.24 (d, $CH\underline{C}H(CD_3)_2$), 35.23 (t, $\underline{C}H_2SH$), 53.58 (d, $\underline{C}HCH_2S$), 56.28 (d, $\underline{C}HCH(CD_3)_2$), 59.07 (d, $\underline{C}HCH_2CH_2$), 172.69, 172.74, 175.60 and 176.34 (4 x s, $\underline{C}$=O); *m/z* (FAB) 368 (5%), 369 (7), 370 (MH^+, 100), 371 (20), 372 (8), 373 (2).

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H,3-^{13}C)-valine (6d).

As for (**6a**) except that [(5R)-5-*N*-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzyloxycarbonylpentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3-^{13}C)-valine benzhydryl ester (**15d**) (115 mg, 0.12 mmol) was used to give [(5R)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3-^{13}C)-valine (**6d**) as it's ammonium trifluoroacetate salt (47 mg, 0.098mmol, 82% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.90-0.97 (6H, m, $^{13}CH(C\underline{H}_3)_2$), 1.64-2.02 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.05, 2.19, 2.34 (1H, 3 x m, $J\,^{13}C\text{-}^1H$ 130Hz, $^{13}CHMe_2$), 2.42 (2H, *ca* t, 7 Hz, CH_2CO), 2.87, 2.95 (2H, AB part of ABX, J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.96 (1H, *ca.* t, *J* 6Hz, $C\underline{H}CH_2S$), 4.56 (1H, *ca.* t, *J* 7Hz, $CH_2C\underline{H}^+NH_3$); *m/z* (FAB) 365 (MH^+ for $^{12}C_{14}H_{24}{}^2HN_3O_6S$, 22%), 366 ($MH^+$ for $^{13}C^{12}C_{13}H_{24}{}^2HN_3O_6S$, 100), 367 (21), 368 (1).

[(5R)-5-Amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (6e).

As for compound (**6a**) except that [(5R)-5-*N*-*p*-methoxybenzyloxycarbonyl-5-amino-5-*p*-methoxybenzylcarboxypentanamido]-S-*p*-methoxybenzyl-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine benzhydryl ester (**15e**) (30 mg, 0.032 mmol) was used to give [(5R)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H, 3,4,4'-$^{13}C_3$)-valine (**6e**) as it's ammonium trifluoroacetate salt (15 mg, 0.031 mmol, 97% yield). δ_H (500MHz, D_2O, HOD suppressed) 0.78-0.85 and 1.04-1.11 (6H, 2 x m, $J\,^{13}C\text{-}^1H$ 126Hz, $^{13}CH(^{13}C\underline{H}_3)_2$), 1.54-1.98 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2CH_2CO$), 2.04-2.34 (1H, 2 x m, $^{13}C\underline{H}(^{13}CH_3)_2$), 2.42 (2H, *ca* t, 7 Hz, CH_2CO), 2.88, 2.93 (2H, AB part of ABX , J_{AB} 14, J_{AX} 7 and J_{BX} 6Hz, CH_2S), 3.85-3.87 (1H, *m*, $C\underline{H}CH_2S$), 4.56 (1H, *ca.* t, *J* 6Hz, $CH_2C\underline{H}NH_3$); *m/z* (FAB) 366 (8%), 367 (22), 368 (MH^+ for $^{12}C_{11}{}^{13}C_3H_{24}{}^2HN_3O_6S$, 100), 369 (54), 370 (13), 371 (4).

Penicillin N (1a)

Partially purified isopenicillin N synthetase enzyme[9] (2-3 ml, *ca* 1.0 International Units) in TRIS-HCl buffer (50 mM ; pH 7.4) was exchanged into ammonium bicarbonate buffer (3.5 ml, 50 mM,

pH 7.8) on a pre-equilibrated sephadex column (PD-10) in a cold-room at 4°C. To an aqueous solution of the tripeptide (**6a-e**) (720 µl, ca 10 mM) was added dithiothreitol (80 µl, 2 mM), L-ascorbate (80 µl, 1 mM, ferrous sulphate (80 µl, 0.1 mM) and catalase (40 µl). The pH was adjusted to 7.8 by the addition of 1N NaOH solution and the enzyme solution (3.5 ml) added giving a total volume of 4.5 ml. This solution was divided into two aliquots, each of ca 2.3 ml and incubated at 27°C and 250 rpm for 10 minutes after which time, more dithiothreitol (10 µl, 2 mM) was added to each aliquot. After a further 30 minutes, the incubation was quenched by the addition of acetone to 70% (v/v). The precipitated protein was spun down by centrifugation (15 Krpm, 2 minutes, 0°C) and purified by chromatography (h.p.l.c., Gilson system, solvent; 0.75% MeCN in 25mM aqueous NH_4HCO_3; flow rate 4.0 ml min^{-1}, retention time 8.0 min) to give pure penicillin N (**1a**) (*ca* 1mg); δ_H (500MHz, D_2O, HOD suppressed) 1.51 (3H, s, CH_3), 1.63 (3H, s, CH_3) 1.64-1.95 (4H, 2 br. m, $CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.77 (1H, ca t, *J* 6Hz, $CHCH_2CH_2$), 4.34 (1H, s, $CHCO_2H$), 5.47, 5.56 (2H, ABq, *J* 4.5Hz, CHCHS). δ_C (125.77MHz, D_2O) 21.67 (t, $CH_2CH_2CH_2CO$), 27.22 (q, α-CH_3), 30.80 (t, $CHCH_2CH_2$), 31.11 (q, β-CH_3), 32.27 (t, CH_2CO), 55.28 , 57.43 (2 x d, CHCHS), 58.74 (d, $^+H_3NCHCO_2^-$), 73.91 (d, $CHCO_2H$), 161.05, 175.21, 175.58 and 176.63 (4 x s, C=O); *m/z* (NH_3 C.I.) of N-ethoxycarbonyl, dimethyl ester derivative[11,12] 460 (100, MH^+), 461 (24), 462(10).

[3-^{2}H]Penicillin N (1b)[9]

Di-($^2H_3$ -methyl)-penicillin N (1c)

Procedure as for (**1a**) except that (5R)-5-amino-5-carboxypentanamido-L-cysteinyl-D-[di-($^2H_3$-methyl)]-valine (**6c**) (*ca* 10 mg) was used to give, after chromatography [Hplc, Gilson system, eluting with 0.75% MeCN in 5mMolar aqueous NH_4HCO_3, retention time 8 minutes], di-($^2H_3$-methyl)-penicillin N (**1c**) (3.5 mg) as determined by ^{1}H-NMR calibration[9]. δ_H (500MHz, D_2O, HOD suppressed) 1.67-2.02 (4H, 2 x m, $CH_2CH_2CH_2$), 2.43 (2H, *ca* t, *J* 7Hz, CH_2CO), 3.81 (1H, *ca* t, *J* 6Hz, $CHCH_2CH_2$), 4.34 (1H, s, $CHCO_2H$), 5.50, 5.59 (2H, ABq, *J* 4Hz, CHCHS); *m/z* (NH_3 C.I.) of N-ethoxycarbonyl, dimethyl ester derivative[11,12] 466 (100, MH^+), 467 (24), 468 (10).

(2R,5R,6R)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylate (1d).

[(5R)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H,3-^{13}C)valine (**6d**) (14 mg) was incubated with IPNS (*ca* 30 I.U.) as described above. Chromatography [hplc Gilson system, 0.75% MeCN in 5mMolar aqueous NH_4HCO_3, retention time 8 minutes], gave (2R,5R,6R)-1-aza-(2-^{2}H,3-^{13}C)-3,3-dimethyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylate (**1d**) (2.8 mg). δ_H (500MHz, D_2O, HOD suppressed) 1.51 (3H, d, J ^{13}C-^{1}H 4 Hz,$^{13}CHCH_3$), 1.63 (3H, d, J ^{13}C-^{1}H 4 Hz,$^{13}CHCH_3$), 1.65-1.95 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, J 7Hz, CH_2CO), 3.77 (1H, *ca* t, J 6Hz, $CHCH_2CH_2$), 5.47, 5.56 (2H, ABq, J 4.5Hz, CHCHS).

(2R,5R,6R)-1-Aza-(2-^{2}H,3-^{13}C)-3,3-di-^{13}C-methyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3,2,0]heptane-2-carboxylate (1e).

[(5R)-5-amino-5-carboxypentanamido]-L-cysteinyl-D-(2-^{2}H,3,4,4'-$^{13}C_3$)valine (**6e**) (10 mg) was incubated with IPNS (*ca* 30 I.U.) as described above to give (2R,5R,6R)-1-aza-(2-^{2}H,3-^{13}C)-3,3-di-^{13}C-methyl-6-[(5R)-5-amino-5-carboxypentanamido]-7-oxo-4-thiabicyclo[3,2,0] heptane-2-carboxylate (**1e**) (*ca*. 2mg) which was used without further purification. δ_H (500MHz, D_2O, HOD suppressed) 1.39 and 1.65 (3H, dm, J ^{13}C-^{1}H 135 Hz, $^{13}CH(^{13}CH_3)_2$, 1.51 and 1.77 (3H, dm, J ^{13}C-^{1}H 135 Hz, $^{13}CH(^{13}CH_3)_2$, 1.65-1.95 (4H, 2 x m, $CH_2CH_2CH_2CO$), 2.40 (2H, *ca* t, J 7Hz, CH_2CO), 3.77 (1H, *ca* t, J 6Hz, $CHCH_2CH_2$), 5.47, 5.56 (2H, ABq, J 4.5Hz, CHCHS).

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