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Synthesis of (2R,3S)[4-$^2H_3$] Valine: Application to the Study of the Ring Expansion of Penicillin N by Deacetoxycephalosporin C Synthase from *Streptomyces clavuligerus*

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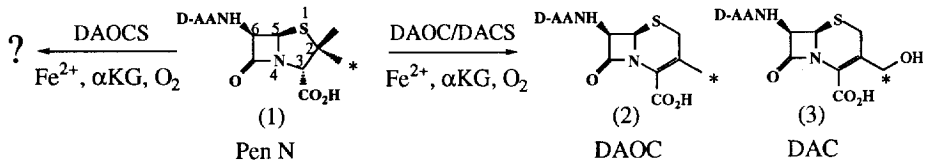
Abstract: A new synthesis of (2R,3S)[4-$^2H_3$] valine was developed, and used to synthesise (2R) [2'-$^2H_3$]-penicillin N *via* a chemico-enzymatic synthesis. This labelled penicillin was then used to demonstrate the stereospecific ring expansion of penicillin N to deacetoxycephalosporin C by deacetoxycephalosporin C synthase from *Streptomyces clavuligerus.*

The ring expansion of penicillin N (pen N) to deacetoxycephalosporin C (DAOC) is a major rate-limiting step in the biosynthesis of cephalosporin C[1,2,3]. In fungal systems, such as *Cephalosporium acremonium,* the ring expansion to DAOC and the subsequent hydroxylation to form deacetylcephalosporin C (DAC) are catalysed by a single, bifunctional enzyme, termed deacetoxycephalosporin C/deacetylcephalosporin C synthase (DAOC/DACS)[4,5,6] (scheme 1). In contrast, in bacteria, such as *Streptomyces clavuligerus* and *Streptomyces lactamdurans*, these two processes are catalysed by two distinct and separable enzymes[7], termed deacetoxycephalosporin C synthase (DAOCS), and deacetylcephalosporin C synthase (DACS), respectively.



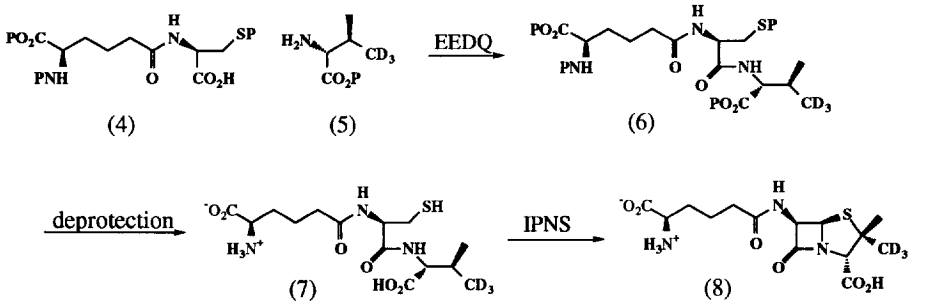
αKG = 2-oxoglutarate

Scheme 1

Mechanistic studies on DAOC/DACS have led to a proposed mechanism for the ring expansion in *Cephalosporium acremonium*[8,9]. The recent purification of DAOCS from *Streptomyces clavuligerus*[7], and the subsequent cloning and over-expression of the encoding gene in *E. coli*[10,11,12] have enabled studies to be carried out on the substrate specificity of the ring expansion[13], these results indicate that there are some differences in the substrate specificity of the ring expansion in the fungal and bacterial systems.

In order to investigate further the mechanism of the ring expansion in *Streptomyces clavuligerus*, we wished to synthesise 2'-isotopically labelled pen N, to incubate this with DAOCS from *Streptomyces*, and to analyse the specificity of the incorporation of the isotopic label into the resulting DAOC, a step known to occur stereospecifically in species of *Cephalosporium*[14].

The enzyme isopenicillin N synthase (IPNS) has previously been used to synthesise labelled penicillin Ns from labelled D-aminoadipoyl-L-cysteinyl-D-valine (D,L,D-ACV)[14,15], thus a synthesis of stereospecifically 2'-labelled pen N (8) was designed, requiring 4-deuterated D-valine with known C3 stereochemistry (scheme 2):

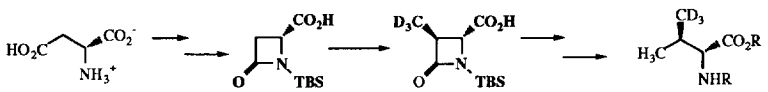


(4) (5) (6) (7) (8)

Scheme 2

Synthesis of (2R,3S)[4-$^2H_3$] valine

Previously (2S,3S)-[4-^{13}C]-valine has been synthesised by enzymatic methods[16], which required racemisation at C2 and subsequent resolution when used in a cell-free study of cephalosporin biosynthesis by DAOC/DACS[14]. Other syntheses[17,18] produced C2 racemic mixtures of labelled valines directly. Thus there was a necessity for a new synthesis of isotopically labelled R-valine with defined C3 stereochemistry. We designed a synthesis starting from R-aspartic acid, proposing the required C2-C3 stereochemistry by *trans*- alkylation of a β-lactam, a transformation known to occur stereospecifically with other related electrophiles[19,20](scheme 3).

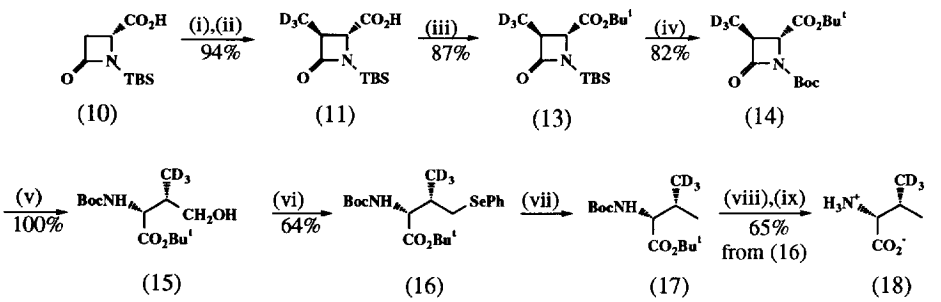


(9) (10) (11) (12)

Scheme 3

Thus β-lactam (10) was prepared from R-aspartic acid following the procedure of Birkofer[21]. This was successfully *trans*-alkylated, by treatment with two equivalents of lithium diisopropylamide, then excess CD_3I (3 eq.). No *cis*-alkylation could be detected by ^1H nmr. Esterification was carried out using *tert*-butyltrichloroacetimidate with catalytic boron trifluoride, according to the protocol of Jackson[22], to provide *tert*-butyl ester (13). The *tert*-butyldimethylsilyl group was then substituted for a *tert*-

butoxycarbonyl group to give (14). Sodium borohydride reduction proceeded smoothly at 0°C (adding the sodium borohydride in small portions), to give alcohol (15), which was deoxygenated *via* radical reduction of selenide (16). Deprotection using *p*-toluenesulphonic acid gave (2R,3S) [4-$^2H_3$] valine, which was purified by ion exchange chromatography (scheme 4).



(10) (11) (13) (14)

(15) (16) (17) (18)

(i) $((CH_3)_2CH)_2NLi$ (2 eq.), THF, 0°C; (ii) CD_3I (3 eq.), 0°C; (iii) $Cl_3C(C{=}NH)OBu^t$, $BF_3.OEt_2$, CH_2Cl_2, C_6H_{12}; (iv) Boc_2O, DMAP, CH_3CN; (v) $NaBH_4$, MeOH, 0°C; (vi) N-(Phenylseleno)-phthalimide, Bu_3P, CH_2Cl_2; (vii) Ph_3SnH, toluene, reflux; (viii) TsOH, EtOH, Et_2O; (ix) ion exchange.

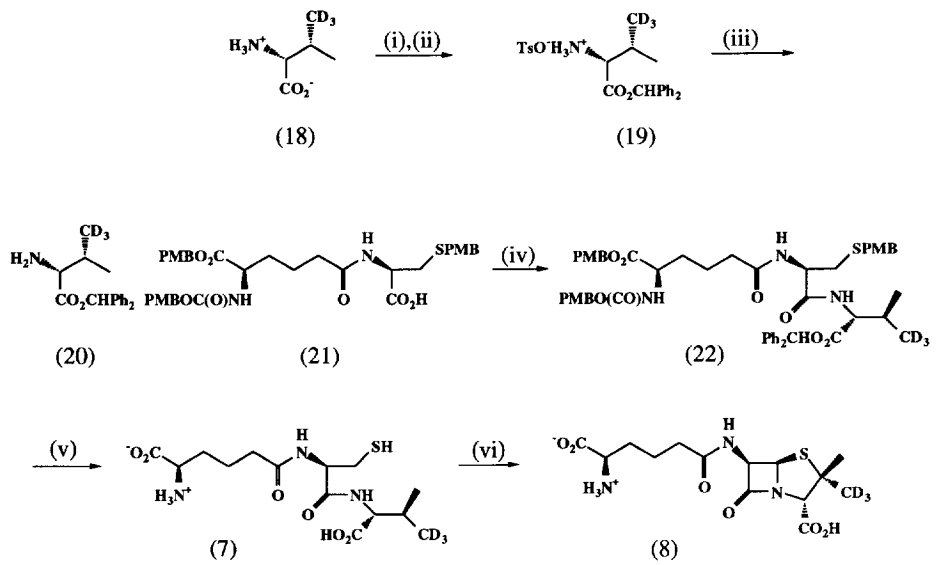
Scheme 4

The ^1H nmr spectrum of the resulting $^2H_3$-valine (18) had just one doublet centred at δ 0.74 showing that the material was stereochemically pure at C-3 (compare with the ^1H nmr spectrum of unlabelled valine which has two resolved doublets).

Conversion of (2R,3S)[4-$^2H_3$] valine to (2R) [2'-$^2H_3$] penicillin N

The procedure chosen for the synthesis of the isotopically labelled D,L,D-ACV was to couple a suitable (2R, 3S)-[4-$^2H_3$] valine derivative with a suitable D,L-AC derivative, all the protecting groups being compatible with an acidic deprotection. Thus (2R, 3S)-[4-$^2H_3$] valine benzhydryl ester was synthesised by protection of the amino group of (2R, 3S)-[4-$^2H_3$] valine as its *p*-toluene sulphonate salt, followed by treatment with diphenyl diazomethane and extraction into organic solution from aqueous base (scheme 5). The resulting (2R, 3S)-[4-$^2H_3$] valine benzhydryl ester (20) was coupled with tri-*p*-methoxybenzyl-protected D,L-AC, using EEDQ as the coupling agent. The fully protected D,L,D-ACV (22) was purified by chromatography (silica gel), then deprotected (trifluoroacetic acid), to give D-α-aminoadipoyl-L-cysteinyl-(2R, 3S)-[4-$^2H_3$] valine (7). This was then converted through to (2R)

[2'-$^2H_3$] pen N (8) by incubation with *Cephalosporium acremonium* IPNS, purified from a recombinant source[23] (scheme 5):



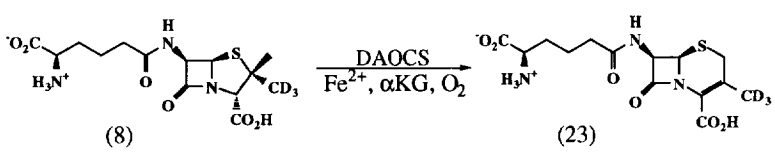
PMB = 4-methoxybenzyl

(i) TsOH, H_2O, CH_3CN; (ii) Ph_2CN_2; (iii) $NaHCO_3$, H_2O, EtOAc; (iv) EEDQ, CH_2Cl_2; (v) CF_3CO_2H, C_6H_5OMe, reflux; (vi) IPNS, Fe^{2+}, O_2.

Scheme 5

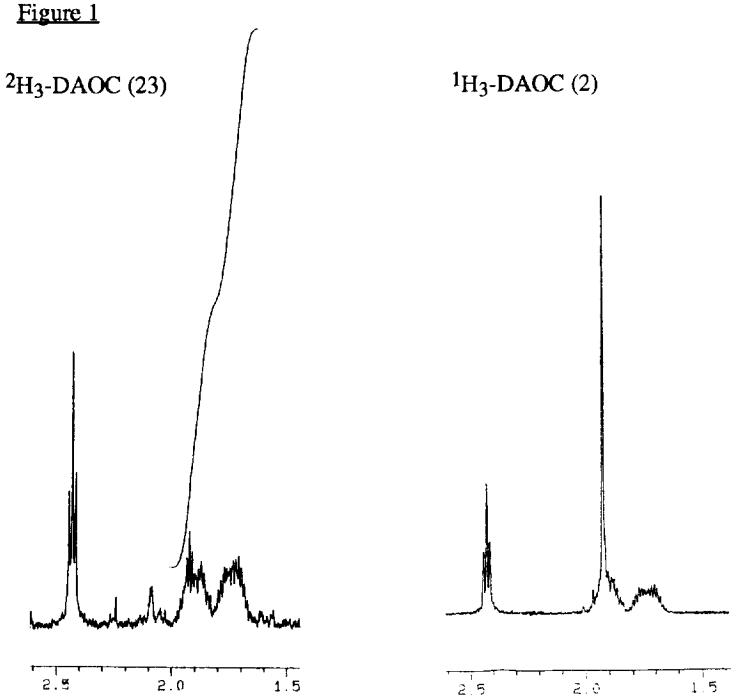
Ring Expansion of (2R) [2'-$^2H_3$] penicillin N (8) by DAOCS

(2R) [2'-$^2H_3$] pen N (8) was incubated with DAOCS, and the resulting DAOC was purified by HPLC, and analysed by 500MHz ^{1}H-nmr. The ring expansion was observed to be stereospecific, proceeding to give DAOC (23) with deuterium labelling only in the exocyclic methyl group (scheme 6, fig. 1).



Scheme 6

Figure 1



Thus DAOC was produced with the same stereochemical outcome as the equivalent transformation carried out by DAOC/ DACS. This result is consistent with a common mechanism for the ring expansion of pen N to DAOC in the DAOCS and DAOC/DACS enzymes: in both cases, the active site of the enzyme presumably binds the penicillin in such a way that the 2β-CH_3 group is closest to an active iron-oxo species, which mediates β-methyl C-H cleavage and subsequent ring expansion.

In summary, we have described a new synthesis of (2R,3S) [4-$^2H_3$]-valine, and this has been applied to observe a stereospecific ring expansion of Pen N to DAOC *via* deacetoxycephalosporin C synthase (DAOCS) from *Streptomyces clavuligerus*.

Experimental Section

Melting points were determined using a Büchi 510 capillary melting point apparatus, and are quoted uncorrected; optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20°C with a pathlength of 1 dm; concentrations (c) are reported in g/100 ml. Microanalyses were performed within the Dyson Perrins Laboratory. Infra-red spectra were recorded on a Perkin-Elmer 1750 FT spectrometer (absorptions are quoted in cm^{-1}). NMR spectra were recorded using Varian Gemini 200 and Bruker AM500 spectrometers; chemical shifts (δ_H) are quoted in parts per million (ppm) and are referenced to residual

protonated solvent resonances; coupling constants (*J*) were recorded to the nearest 0.5 Hz. ^{13}C NMR spectra were recorded using a Varian Gemini 200 operating at 50.3 MHz, a Bruker AM250 operating at 62.9 MHz, or a Bruker AM500 operating at 125.8 MHz; carbon multiplicities were determined by operating the spectrometers in DEPT mode. Deuterium magnetic resonance spectra (^2H NMR) were recorded at 38.3 MHz using a Bruker AM250 spectrometer, and were recorded in protiated solvents, referenced internally to added deuterated solvent. NMR calibration of aqueous samples was achieved by dissolving samples in D_2O (0.5 ml) containing 3-(trimethylsilyl)-propionoic-2,2,3,3-d_4 acid sodium salt (tsp) (0.29 mM) and comparing the integration of resonances due to tsp and those due to sample.

Mass spectra were recorded on the following instruments: V.G. Micromass ZAB 1F (FAB/CI/DCI); V.G.Masslab 20-250 (CI/DCI); V.G. BIO-Q (electrospray).

Thin layer chromatography was performed on Merck kieselgel DC-Alufolien $60F_{254}$ 0.2 mm precoated plates, visualisation was by quenching of uv fluorescence, or 5% w/v dodecamolybdophosphoric acid in ethanol. Flash chromatography was carried out on Baker silica gel (30-60 μm). Ion exchange was carried out on Dowex 50W-X8(H) resin which was charged using 2N HCl (aq.). High performance liquid chromatography (HPLC) was performed using two Gilson 303 pumps, a Rheodyne 7125 injector, a Gilson holochrome variable wavelength detector set at 220 nm, and a column packed with Hypersil ODS (250 x 10 mm diameter).

All solvents were distilled before use; THF was distilled from sodium benzophenone ketyl under nitrogen; CH_2Cl_2 and CH_3CN were distilled from CaH_2 under argon, and stored over activated 4Å molecular sieves; toluene was dried over sodium wire and "degassed" by passing argon through it for 30-60 minutes; BuLi was titrated according to the method of Lipton[24]; diphenyldiazomethane was prepared by oxidation of benzophenone hydrazone using HgO[25]; *tert-* butyl trichloroacetimidate was prepared by reaction of *tert-* butanol with trichloroacetonitrile[22]. Reactions were carried out at room temperature, under an atmosphere of argon, unless otherwise stated.

Incubations were performed on a New Brunswick Scientific G24 environmental incubation shaker under an atmosphere of normal air, centrifugation was performed using a Beckman J2-21 centrifuge with a JA-20 rotor.

(3S, 4R)-*N*-(*tert-* Butyldimethylsilyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylic acid (11): Butyl lithium (2.20 ml of a 2.5 M solution in hexane, 5.5 mmol, 1.0 eq.) was added to a solution of freshly distilled diisopropylamine (0.77 ml, 0.56 g, 5.5 mmol, 1.0 eq.) in anhydrous THF (10 ml) at 0°C under an atmosphere of argon and the solution was stirred for 10 minutes. This solution was then added dropwise to a solution of (4R)-*N*-(*tert-* butyldimethylsilyl)-azetidin-2-one-4-carboxylic acid[21] (10) (0.628 g, 2.74 mmol, 0.5 eq.) in anhydrous THF (10 ml) at 0°C under an atmosphere of argon and the solution was stirred for a further 10 minutes. Trideutero-methyl iodide (CD_3I) (0.50 ml, 8.0 mmol, 1.5eq) was added and the solution stirred for a further 15 minutes. The reaction was then quenched by the addition of a 1:1 mixture of 1N aqueous $KHSO_4$ and ethyl acetate (120 ml). The phases were separated and the aqueous layer was extracted with a further 2 x 100 ml ethyl acetate and all the ethyl acetate layers were combined, washed with water (100 ml), and sat. brine (100 ml), dried over sodium sulphate, and the ethyl acetate was removed *in vacuo* to give the title compound as a white solid (0.631 g, 94%). Recrystallisation from diethyl ether / petroleum ether (b.p. 40-60°C) gave an analytical sample as colourless needles; m.p. 112-115°C; $[\alpha]_D^{20}$ +35.6 (c 1.3,

$CHCl_3$); Found: C 53.67, H 8.42, N 5.48; $C_{11}H_{18}D_3NO_3Si$ requires C 53.62, H 8.59, N 5.69%; ν_{max} ($CHCl_3$) 2 958 (w), 2 862 (w), 2 362 (w), 1 755 (s), 1 726 (m), 1 257 (m), 1 167 (m), 1 111 (m), 843 (m); δ_H (200 MHz, $CDCl_3$) 0.15 (3H, s, $-CH_3$), 0.32 (3H, s, $-CH_3$), 0.98 (9H, s, $(CH_3)_3C$-), 3.30-3.36 (1H, m, $-CHCD_3$), 3.73 (1H, d, *J* 2.5 Hz, -C<u>H</u>-CH-CD_3); δ_C (50.3 MHz, $CDCl_3$) -6.6 and -6.1 (2 x CH_3), 18.4 ((<u>C</u>$H_3)_3$C-), 26.0 ($(CH_3)_3$<u>C</u>-), 52.3 and 56.7 (2 x -CH), 176.3 and 176.6 (2 x -CO); δ_D (250 MHz, $CHCl_3$) 1.42 (s, $-CHCD_3$); *m/z* (CI (NH_3)) 264 (MNH_4^+, 61%), 248 (22), 247 (MH^+, 100), 219 (47), 180 (28), 61 (34).

(3<u>S</u>, 4<u>R</u>) *tert-* Butyl *N*-(*tert-* butyldimethylsilyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylate (13): Boron trifluoride etherate (15 µl) was added to a stirred solution of (3<u>S</u>, 4<u>R</u>)-*N*-(*tert-* butyldimethylsilyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylic acid (11) (0.193 g, 0.78 mmol) in anhydrous dichloromethane / cyclohexane 1:1 (4 ml) at 0°C under an atmosphere of argon. A solution of *tert-* butyltrichloroacetimidate (0.327 g, 1.6 mmol, 2 eq.) in anhydrous cyclohexane (2 ml) was then added rapidly. The reaction was allowed to warm to room temperature and stirred for a further 30 minutes with the formation of a white suspension. It was then quenched by the addition of solid $NaHCO_3$ (0.25 g) and the solvents were evaporated *in vacuo*. The resulting solid material was triturated repeatedly with petroleum ether (b.p. 40-60°C) (50 ml) and the petroleum solution filtered through celite and the petrol evaporated *in vacuo* to give a colourless oil. This was purified by flash chromatography, eluting with 15% diethyl ether / petroleum ether (b.p. 30-40°C) (100 ml) then 25% diethyl ether / petroleum ether (b.p. 30-40°C) (100 ml) to give the title compound as a colourless oil, which solidified on standing at -15°C (0.205 g, 87%); R_f 0.1 (15% diethyl ether / petroleum ether (b.p. 30-40°C)); $[\alpha]_D^{20}$ +31.3 (c 1.0, $CHCl_3$); Found: C 59.31, H 9.72, N 5.05; $C_{15}H_{26}D_3NO_3Si$ requires C 59.56, H 9.66, N 4.63%; ν_{max} ($CHCl_3$) 2 932 (w), 2 850 (w), 1 741 (s), 1 471 (w), 1 370 (m), 1 304 (m), 1 257 (m), 1 158 (m), 1 097 (w), 843 (m); δ_H (200 MHz, $CDCl_3$) 0.11 (3H, s, $-Si-CH_3$), 0.32 (3H, s, $-Si-CH_3$), 0.97 (9H, s, $(CH_3)_3CSi$-), 1.48 (9H, s, $(CH_3)_3CO$-), 3.16 (1H, m, $-CHCD_3$), 3.56 (1H, d, *J* 2.5 Hz, -C<u>H</u>-$CHCD_3$); δ_C (50.3 MHz, $CDCl_3$) -6.6 and -6.0 (2 x $-Si-CH_3$), 18.4 ((<u>C</u>$H_3)_3$CSi-), 26.1 ($(CH_3)_3$<u>C</u>Si-), 27.8 ((<u>C</u>$H_3)_3$CO-), 52.2 (-<u>C</u>H-CD_3), 57.7 (-<u>C</u>H-CH-CD_3), 83.0 ($(CH_3)_3$<u>C</u>O-), 171.5 (-<u>C</u>O_2C-), 175.4 (-CO-N-); δ_D (250 MHz, $CHCl_3$) 1.36 (s, $-CHCD_3$); *m/z* (CI (NH_3)) 304 (26%), 303 (MH^+, 100).

(3<u>S</u>, 4<u>R</u>) *tert-* Butyl *N*-(*tert-* butoxycarbonyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylate (14): 4-(Dimethylamino)-pyridine (DMAP) (0.07 g) was added to a solution of (3<u>S</u>, 4<u>R</u>) *tert-* butyl *N*-(*tert-* butyldimethylsilyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylate (13) (0.658 g, 2.2 mmol) and di-*tert-* butyl dicarbonate (1.0 g, 4.6 mmol) in anhydrous acetonitrile (30 ml) and the solution was stirred for 15 hours at room temperature. The acetonitrile was evaporated *in vacuo* and the resulting oil purified by flash chromatography eluting with 25% diethyl ether / petroleum ether (b.p. 30-40°C) to give the title compound as a colourless oil, which became a semi-solid on standing (0.512 g, 82%); R_f 0.2 (25% diethyl ether / petroleum ether (b.p. 30-40°C)); $[\alpha]_D^{20}$ +25.7 (c 2.0, $CHCl_3$); Found: C 58.10, H 8.27, N 4.95; $C_{14}H_{20}D_3NO_5$ requires C 58.31, H 8.04, N 4.86%; ν_{max} ($CHCl_3$) 3 030 (m), 3 025 (m), 1 816 (s), 1 727 (s), 1 500 (w), 1 458 (w), 1 371 (s), 1 340 (s), 1 151 (s), 1 124 (m), 1 065 (m); δ_H (200 MHz, $CDCl_3$) 1.48 and 1.50 (2 x 9H, 2 x s, 2 x $(CH_3)_3C$-), 3.15 (1H, m, -C<u>H</u>CD_3), 3.91 (1H, d, *J* 3.0 Hz, -C<u>H</u>-CH-CD_3); δ_C (50.3 MHz, $CDCl_3$) 27.8 (2 x (<u>C</u>$H_3)_3$C-), 49.7 (-<u>C</u>H-CD_3), 58.1 (-<u>C</u>H-CH-CD_3), 82.7 and 83.6

(2 x $(CH_3)_3\underline{C}$-), 155.2 (-N-$\underline{C}O_2C$-), 171.4 (-CH$\underline{C}O_2$-), 175.6 (-CH$\underline{C}$ON-); δ_D (250 MHz, $CHCl_3$) 1.41 (s, -$CHCD_3$); *m/z* (CI (NH_3)) 306 (MNH_4^+, 30%), 250 (30), 206 (100), 189 (24), 150 (39).

(2R, 3S) *tert*- Butyl 2-*N*-(*tert*- butoxycarbonylamino)-3-($^2H_3$-methyl)-4-hydroxy-butanoate (15): Sodium borohydride (0.035 g, 0.9 mmol, 3 eq.) was added in small portions to a stirred solution of (3S, 4R) *tert*- butyl *N*-(*tert*- butoxycarbonyl)-3-($^2H_3$-methyl)-azetidin-2-one-4-carboxylate (14) (0.086 g, 0.3 mmol) in methanol (2 ml) at 0°C. The solution was stirred for 10 minutes before the reaction was quenched by the addition of silica gel (2 ml) and methanol (5 ml). The methanol was evaporated *in vacuo* and the silica repeatedly triturated with ethyl acetate (25 ml). The ethyl acetate solution was filtered, and concentrated *in vacuo* to give the title compound as a colourless oil, which became a semi-solid on standing (0.087 g, 100%); R_f 0.2 (25% ethyl acetate / petroleum ether (b.p. 30-40°C)); $[\alpha]_D^{20}$ -7.7 (c 1.3, $CHCl_3$); Found: C 57.38, H 9.46, N 4.78; $C_{14}H_{24}D_3NO_5$ requires C 57.51, H 9.31, N 4.79%.; ν_{max} ($CHCl_3$) 3 417 (br, w), 2 980 (m), 1 717 (br, s), 1 505 (s), 1 370 (s), 1 249 (m), 1 155 (s), 1 072 (w); δ_H (200 MHz, $CDCl_3$) 1.47 and 1.50 (2 x 9H, 2 x s, 2 x $(CH_3)_3$C-), 1.96 (1H, m, -$CHCD_3$), 2.56-2.62 (1H, br, m, -OH), 3.49-3.75 (2H, m, -C$\underline{H}_2$OH), 4.16 (1H, dd, *J* 8.0, 1.5 Hz, -C$\underline{H}$-$CHCD_3$-), 5.32 (1H, d, *J* 8.0 Hz, -NH); δ_C (50.3 MHz, $CDCl_3$) 27.9 and 28.2 (2 x $(\underline{C}H_3)_3$C-), 38.8 (-$\underline{C}$H-CD_3), 56.2 (-NH-CH-), 64.2 (-CH_2OH), 80.0 and 82.2 (2 x $(CH_3)_3\underline{C}$-), 156.5 (-NH-$\underline{C}O_2$C-), 171.9 (-CH-$\underline{C}O_2$C-); δ_D (250 MHz, $CHCl_3$) 1.01 (s, -$CHCD_3$); *m/z* (CI (NH_3)) 293 (MH^+, 35%), 236 (28), 219 (31), 193 (44), 180 (100), 163 (50), 119 (40), 91 (61).

(2R, 3S) *tert*- Butyl 2-*N*-(*tert*- butoxycarbonylamino)-3-($^2H_3$-methyl)-4-(phenylseleno)-butanoate (16): Tributyl phosphine (0.13 ml, 0.106 g, 0.52 mmol) was added to a stirred solution of (2R, 3S) *tert*- butyl 2-*N*-(*tert*- butoxycarbonylamino)-3-($^2H_3$-methyl)-4-hydroxy-butanoate (15) (0.078 g, 0.27 mmol) and *N*-(phenylseleno)-phthalimide (0.162 g, 0.54 mmol, 2 eq.) in anhydrous dichloromethane (2 ml) under an atmosphere of argon and the solution was stirred for 90 minutes at room temperature. The dichloromethane was evaporated *in vacuo*, and the resulting oil was purified by flash chromatography, eluting with 5% diethyl ether / petroleum ether (b.p. 30-40°C) to give the title compound as a colourless oil (0.074 g, 64%); R_f 0.2 (15% diethyl ether / petroleum ether (b.p. 30-40°C)); $[\alpha]_D^{20}$ -9.7 (c 1.7, $CHCl_3$); Found: C 55.32, H 7.41, N 3.18; $C_{20}H_{28}D_3NO_4Se$ requires C 55.68, H 7.24, N 3.25%; ν_{max} ($CHCl_3$) 2 979 (m), 1 714 (s), 1 499 (m), 1 369 (m), 1 154 (s), 1 111 (m); δ_H (200 MHz, $CDCl_3$) 1.42 (18 H, s, 2 x $(CH_3)_3$C-), 2.12-2.29 (1H, m, -$CHCD_3$), 2.64-2.75 and 2.98-3.07 (2H, $\underline{AB}$-X, *J* 4.5, 11.0, 12.0 Hz, -C$\underline{H}_2$CH-), 4.29-4.35 (1H, m, -NH-C$\underline{H}$-), 5.11 (1H, d, *J* 8.0 Hz, -N$\underline{H}$-CH-), 7.23-7.28 (3H, m, Ph (*m*- and *p*-), 7.51-7.56 (2H, m, Ph (*o*-)); δ_C (50.3 MHz, $CDCl_3$) 27.9 and 28.2 (2 x $(\underline{C}H_3)_3$C-), 31.4 (-$\underline{C}$HCD$_3$), 37.2 (-$\underline{C}H_2$), 58.0 (-$\underline{C}$H-CH-CD_3), 79.8 and 82.3 (2 x $(CH_3)_3\underline{C}$-), 127.3-133.4 (aromatic -CH)), 155.7 (-NH-$\underline{C}O_2$C-), 171.1 (-CH-$\underline{C}O_2$C-); δ_D (250 MHz, $CHCl_3$) 1.07 (s, -$CHCD_3$); *m/z* (CI (NH_3)) 433 (MH^+,(^{80}Se) 5%), 377 (15), 338 (42), 219 (28), 163 (61), 78 (48), 73 (100).

(2R, 3S)-*N*-(*tert*- Butoxycarbonyl)-(4-$^2H_3$)-valine *tert*- butyl ester (17): Degassed anhydrous toluene (10 ml) was added to (2R, 3S) *tert*- butyl 2-*N*-(*tert*- butoxycarbonylamino)-3-($^2H_3$-methyl)-4-(phenylseleno)-butanoate (16) (0.394 g, 0.91 mmol) and triphenyl tin hydride (1.290 g, 3.68 mmol) under an atmosphere of argon, and the reaction mixture was heated at reflux for 15 hours, resulting in the

formation of a grey suspension. The toluene was evaporated *in vacuo* and the resulting oil dissolved in ethyl acetate (50 ml), washed with water (50ml, 25 ml), dried over sodium sulphate and the ethyl acetate evaporated *in vacuo* to give a colourless oil. This was purified by flash chromatography, eluting with petroleum ether (b.p. 30-40°C) (1L) then 5% diethyl ether / petroleum ether (b.p. 30-40°C) to give the title compound as a colourless oil, slightly contaminated with tin residues (0.200 g); R_f 0.2 (10% diethyl ether / petroleum ether (b.p. 30-40°C)); ν_{max} ($CHCl_3$) 2 980 (w), 1 710 (s), 1 500 (m), 1 368 (s), 1 155 (s); δ_H (200 MHz, $CDCl_3$) 0.89 (3H, d, *J* 7.0 Hz, -CH-C<u>H</u>$_3$), 1.46 and 1.48 (2 x 9H, 2 x s, 2 x $(CH_3)_3$C-), 2.04-2.15 (1H, m, -C<u>H</u>CD_3), 4.11 (1H, dd, *J* 4.5, 9.0 Hz, -NH-C<u>H</u>-CH-), 5.03 (1H, d, *J* 9.0 Hz, -NH); δ_C (50.3 MHz, $CDCl_3$) 17.2 (<u>C</u>H_3CH-), 27.9 and 28.2 (2 x (<u>C</u>H_3)$_3$C-), 31.2 (-<u>C</u>H-CD_3), 58.8 (-NH<u>C</u>H-), 79.5 and 81.7 (2 x $(CH_3)_3$<u>C</u>-), 155.7 (-NH<u>C</u>O_2C-), 171.5 (-CH<u>C</u>O_2C-); δ_D (250 MHz, $CHCl_3$) 0.95 (s, -CHCD_3); *m/z* (CI (NH_3) 277 (MH^+, 8%), 221 (28), 182 (80), 177 (44), 165 (19), 121 (17), 75 (100).

(2<u>R</u>, 3<u>S</u>)-(4-$^2H_3$)-Valine (18): *p*-Toluene sulphonic acid hydrate (0.138 g) and crude (2<u>R</u>, 3<u>S</u>)-*N*-(*tert*-butoxycarbonyl)-(4-$^2H_3$)-valine *tert*- butyl ester (17) (0.200 g) were dissolved in ethanol / diethyl ether (1:1) (10 ml) and the solution was stirred at room temperature for 3 hours. The solvents were removed *in vacuo* and the resulting material purified by ion- exchange chromatography using Dowex 50W- X8 resin (100-200 mesh), washing with water then eluting with 2N NH_4OH. The fractions which were stained purple by ninhydrin were evaporated *in vacuo* and freeze-dried to give the title compound as a white solid (0.071 g, 65% from (16)); m.p. > 250°C; $[\alpha]_D^{20}$ -25 (c 0.6, 6N HCl), ($[\alpha]_D^{20}$ of fully protiated <u>D</u>-valine -27 (c 3.4, 6N HCl)); ν_{max} (KBr disc) 3 368 (br, m), 2 935 (m), 1 717 (s), 1 453 (m), 1 315 (w), 1 275 (s), 1 176 (w), 1 115 (m), 1 071 (w), 1 027 (w), 714 (s); δ_H (200 MHz, D_2O) 0.74 (3H, d, *J* 7.0 Hz, -CH-C<u>H</u>$_3$), 1.97-2.04 (1H, m, -C<u>H</u>CH_3), 3.36 (1H, d, *J* 4.5 Hz, -C<u>H</u>CH-CD_3); δ_C (62.5 MHz, D_2O) 17.3 (-CH_3), 29.5 (β-CH-), 61.2 (α-CH-), 174.8 (-CO_2^-); δ_D (250 MHz, H_2O) 0.85 (s, -CHCD_3); *m/z* (DCI (NH_3)) 122 (11%), 121 (MH^+, 100), 75 (31).

(2<u>R</u>, 3<u>S</u>)-(4-$^2H_3$)-Valine benzhydryl ester (20): Diphenyldiazomethane (0.115 g, 0.6 mmol, 1.5 eq.) was added to a stirred solution of (2<u>R</u>, 3<u>S</u>)-(4-$^2H_3$)-valine (18) (0.048 g, 0.4 mmol) and *p*-toluene sulphonic acid hydrate (0.084 g, 0.44 mmol, 1.1 eq.) in acetonitrile / water 2:1 (5 ml) at room temperature. The purple solution was rapidly decolourised and it was then stirred for a further 1 hour. Ethyl acetate (5 ml) was added and the solution extracted with 0.5N HCl (2 x 5 ml). The aqueous layers were combined, basified with sat. $NaHCO_3$, extracted with ethyl acetate (5 x 10 ml) and these ethyl acetate layers were combined, dried over sodium sulphate and concentrated *in vacuo* to give the title compound as a white solid (0.05 g, 44%); R_f 0.2 (50% ethyl acetate / petroleum ether (b.p. 30-40°C)); ν_{max} ($CHCl_3$) 3 070 (w), 2 962 (w), 2 364 (w), 2 222 (w), 1 738 (s), 1 602 (m), 1 452 (m), 1 383 (w), 1 310 (w), 973 (m); δ_H (200 MHz, $CDCl_3$) 0.82 (3H, d, *J* 3.0 Hz, -CH-C<u>H</u>$_3$), 1.58 (2H, br, NH_2), 2.09-2.15(1H, m, -C<u>H</u>CH_3), 3.41 (1H, d, *J* 5.0 Hz, -C<u>H</u>-CH-CH_3), 6.93 (1H, s, Ph_2CH-), 7.22-7.48 (10H, m, aromatic -CH); *m/z* (FAB) 287 (MH^+, 24%), 167 (100).

[(*N*-*p*-Methoxybenzyloxycarbonyl)-α-*p*-methoxybenzyl-δ-((<u>R</u>)-α-aminoadipoyl)]-*S*-*p*-methoxybenzyl-(<u>S</u>)-cysteinyl-(2<u>R</u>,3<u>S</u>)-[4-$^2H_3$]-valine benzhydryl ester (22): (2<u>R</u>, 3<u>S</u>)-(4-$^2H_3$)-Valine benzhydryl ester (20) (0.038 g, 0.13 mmol), [(*N*-*p*-methoxybenzyloxycarbonyl)-α-*p*-methoxybenzyl- δ-((<u>R</u>)-α-aminoadipoyl)]-

S-*p*-methoxybenzyl-(S)-cysteine (21) (0.076 g, 0.11 mmol) and 2-ethoxy-N-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (0.035 g, 0.14 mmol) were dissolved in anhydrous dichloromethane (3 ml) under an atmosphere of argon, and the solution was stirred at room temperature for 15 hours. The dichloromethane was evaporated *in vacuo* and the resulting oil dissolved in ethyl acetate (20 ml) and washed with sat. $NaHCO_3$ (15 ml), 1N HCl (15 ml), and brine (15 ml). The ethyl acetate solution was dried over sodium sulphate and the ethyl acetate evaporated *in vacuo* to give a colourless oil. This was purified by flash chromatography, eluting with 30% ethyl acetate / petroleum ether (b.p. 30-40°C) (100 ml) then 40% ethyl acetate / petroleum ether (b.p. 30-40°C) to give the title compound as a colourless oil (0.036 g, 34%); R_f 0.3 (50% ethyl acetate / petroleum ether (b.p. 30-40°C)); ν_{max} ($CHCl_3$) 2 962 (w), 2 254 (s), 1 795 (w), 1 728 (s), 1 466 (m), 1 383 (w), 1 303 (w), 1 250 (s), 1 175 (s), 1 098 (m), 1 035 (s); δ_H (500 MHz, $CDCl_3$) 0.77 (3H, d, *J* 7.0 Hz, -CH-C$\underline{H}_3$), 1.58 (2H, m, γ-H's of α-AAA), 1.66 (1H, m, β-H of α-AAA), 1.81 (1H, m, β-H of α-AAA), 2.04-2.10 (1H, m, β-H of valine), 2.16-2.25 (2H, m, δ-H's of α-AAA), 2.64-2.69 and 2.82-2.86 (2H, AB-X, *J* 6.0, 7.0, 14.0 Hz, β-H's of cysteine), 3.72, 3.76, 3.79 (3 x 3H, s, 3 x -OC$\underline{H}_3$), 3.80 (2H, s, -SC$\underline{H}_2$Ar), 4.35-4.36 (1H, m, α-H of α-AAA), 4.48-4.52 (1H, q, *J* 7.0 Hz, AB-X, α-H of cysteine), 4.63-4.65 (1H, dd, *J* 4.5, 9.0 Hz, α-H of valine), 5.02 and 5.09 (2 x 2H, s, 2 x -OC$\underline{H}_2$Ar), 5.44 (1H, d, *J* 8.0 Hz, -NH), 6.22 (1H, d, *J* 7.0 Hz, -NH), 6.77 (1H, d, *J* 9.0 Hz, -NH), 6.82-6.90 and 7.22-7.34 (aromatic CH); δ_C (62.5 MHz, $CDCl_3$) 17.2 (-CH_3 of valine), 21.3 (-CH_2-γ-C of α-AAA), 31.0 (-CH, β-C of valine), 31.9 (-CH_2-, β-C of α-AAA), 33.2 (-CH_2-, β-C of cysteine), 35.4 (-CH_2-, δ-C of α-AAA), 35.9 (-SCH_2Ar), 52.2, 53.6 and 57.3 (3 x -CH, 3 x α-H), 55.3 (3 x -O-CH_3), 66.8 and 67.0 (2 x -OCH_2Ar), 78.0 (-$\underline{C}HPh_2$), 113.9-114.1, 127.0-130.2, and 139.4-139.6 (aromatic C), 156.1 (-$NHCO_2$-), 170.2, 170.6, 172.1, 172.5 (2 x -$CH\underline{C}O_2$- and 2 x -CH($\underline{C}$O)N-); *m/z* (FAB) 938 (7%), 937 (MH^+, 53), 894 (28), 893 (100), 167 (73), 121 (100).

δ-((R)-α-aminoadipoyl)-(S)-cysteinyl-(2R,3S)-[4-$^2H_3$]-valine (7): Anisole (0.4 ml) was added to [(*N*-*p*-methoxybenzyloxycarbonyl)-α-*p*-methoxybenzyl-δ-((R)-α-aminoadipoyl)]-*S*-*p*-methoxybenzyl-(S)-cysteinyl-(2R,3S)-[4-$^2H_3$]-valine benzhydryl ester (22) (0.036 g, 0.038 mmol) under an atmosphere of argon, then trifluoroacetic acid (4 ml) was added and the flask lowered into an oil-bath preheated to 110°C, and heated at reflux for 30 minutes. The reaction mixture was cooled to room temperature, the solvents were evaporated *in vacuo* and the residue dissolved in toluene (2 ml) which was then evaporated *in vacuo* to remove traces of trifluoroacetic acid. Ethyl acetate (10 ml) was added to the resulting oil, forming a white suspension, which was dissolved by the addition of water (10 ml). The ethyl acetate layer was discarded and the resulting aqueous solution washed with a further 10 ml ethyl acetate then freeze dried to give a white solid containing the title compound (0.020 g); δ_H (500 MHz, D_2O) 0.83 (3H, d, *J* 7.0 Hz, -CHC$\underline{H}_3$), 1.56-1.71 (2H, m, γ-H's of α-AAA), 1.77-1.91 (2H, m, -β-H's of α-AAA), 2.06-2.12 (1H, m, -C$\underline{H}$CH$_3$), 2.31 (2H, t, *J* 7.0 Hz, δ-H's of α-AAA), 2.76-2.86 (2H, AB-X, *J* 6.0, 7.0, 14.0 Hz, β-H's of cysteine), 3.87-3.90 (1H, m, α-H of α-AAA), 4.17 (1H, d, *J* 6.0 Hz, α-H of valine), 4.45-4.47 (1H, m, AB-X, α-H of cysteine) *m/z* (electrospray) 369 (12%), 368 (32), 367 (MH^+,100), 349 (7).

(2R) [2'-$^2H_3$] penicillin N (8): Partially purified isopenicillin N Synthase[23] (3.0 ml, 24 IU) in aqueous TRIS-HCl buffer (50 mM, pH 7.4) was exchanged into ammonium bicarbonate buffer (3.5 ml of a 50 mM aqueous solution at pH 7.8) on a pre-equilibrated Sephadex column (Pharmacia, PD-10).

δ-((R)-α-Aminoadipoyl)-(S)-cysteinyl-(2R,3S)-[4-$^2H_3$]-valine (7) (7 mg, by tsp analysis) was dissolved in water (500 μl), and to this solution was added ammonium bicarbonate (5.4 ml of a 50 mM aqueous solution), dithiothreitol (200 μl of a 100 mM aqueous solution), L-ascorbate (200-μl of a 50 mM aqueous solution at pH 7.7), and $FeSO_4$ (200 μl of a 5 mM aqueous solution). The resulting solution was added to the solution of enzyme, divided into 5 aliquots, and incubated at 27°C and 250 rpm for 10 minutes. A further 20 μl of dithiothreitol solution was then added to each aliquot, and the incubation continued for 40 minutes. The enzyme was then denatured by the addition of acetone (5 ml to each aliquot), the precipitated protein removed by centrifugation (10 krpm, 2 min, 0°C), and the supernatant freeze dried to give the crude title compound (2.2 mg by tsp analysis, 31%); δ_H (500 MHz, D_2O, HOD suppressed) (partial) 1.59 (3H, s, α-CH_3), 4.49 and 5.58 (2H, 2 x d, *J* 4.0 Hz, β-lactam H's). There was no signal at δ 1.53, indicating the absence of (2S) [2'-$^2H_3$] penicillin N.

Ring expansion of (2R) [2'-$^2H_3$] penicillin N (8) by DAOCS: A cofactor solution was prepared containing $FeSO_4$ (1.5 mg, 1 mM), α-ketoglutarate (21 mg, 14.4 mM), L-ascorbate (17.6 mg, 10 mM), dithiothreitol (30.8 mg, 20 mM) and ammonium sulphate (0.33 g, 0.25 M) in distilled water (10 ml), and the pH was adjusted to 7.5 by the addition of 1M NaOH. A solution of deacetoxycephalosporin C synthase[13] (4 ml, 1.26 IU) in TRIS-HCl buffer (50 mM aqueous solution at pH 7.4) was pre-incubated with cofactor solution (400 μl) for 5 minutes at 27°C and 250 rpm. (2R) [2'-$^2H_3$] Penicillin N (8) (2.2 mg by tsp analysis) was added in water (1.6 ml), and the resulting solution was divided into 2 aliquots, and incubated at 27°C and 250 rpm for 2 hours, after which time the protein was denatured by the addition of acetone (8 ml) to each of the aliquots. The precipitated protein was removed by centrifugation (10 krpm, 2 min, 0°C), and the supernatant freeze dried. Purification of the crude incubation mixture by HPLC (Gilson system, solvent 10 mM aqueous ammonium bicarbonate, flow rate 4 ml / min.) gave exocyclic $^2H_3$-deacetoxycephalosporin C (23) (retention time 9 minutes); δ_H (500 MHz, D_2O, HOD suppressed) 1.67-1.95 (4H, 2 x m, β-and γ-H's of α-AAA), 2.41 (2H, t, *J* 7.0 Hz, δ-H's of α-AAA), 3.25 and 3.59 (obscured) (2 x 1H, d, *J* 18.0 Hz, -SCH_2-), 3.75 (1H, t, *J* 6.0 Hz, α-H of α-AAA), 5.08 and 5.56 (2 x 1H, d, *J* 4.0 Hz, β-lactam H's, fig. 1.); δ_D (500 MHz, H_2O) 1.94 (s, -CD_3).

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