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Isopenicillin N Synthase: A New Mode Of Reactivity

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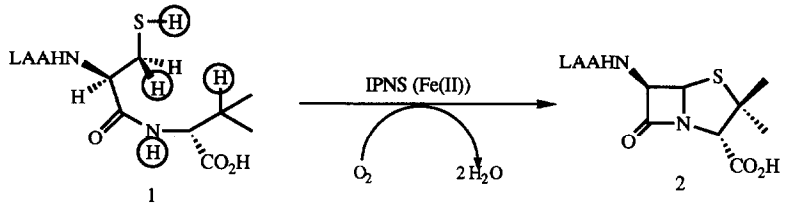
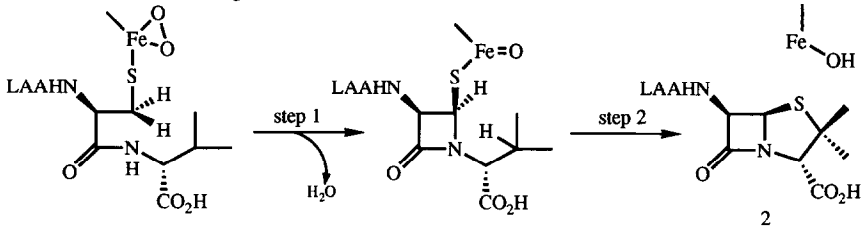
Abstract: Incubation of *L*-δ-(α-aminoadipoyl)-*L*-(3,3-difluorohomocysteinyl)-*D*-valine [an analogue of the natural substrate: *L*-δ-(α-aminoadipoyl)-*L*-(cysteinyl)-*D*-valine] with isopenicillin N synthase (IPNS) resulted in the production of a thiocarboxylic acid, *i.e.* both equivalents of the dioxygen cosubstrate were utilised to oxidise a single carbon of the cysteinyl analogue. This result and others are rationalised in terms of mechanistic proposals for the first ring closure by IPNS. The synthesis of *L*-difluorohomocysteine and related structures, *via* 3,3-difluoro-β-lactams, is described.



1 2

Scheme 1, LAA = δ-(*L*-α-aminoadipoyl)

Isopenicillin N synthase (IPNS) catalyses the desaturative bicyclisation of the tripeptide *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV, 1) into isopenicillin N (2) (Scheme 1).[1] Evidence, based on kinetic studies, indicates that the reaction is stepwise and that initial formation of the β-lactam ring precedes the cyclisation of the thiazolidine ring.[2] The hypothesis that we have developed for this two step ring closure is shown in Scheme 2, wherein a covalent link (3) is formed between the thiol of ACV (1) and the ferrous iron dioxygen complex in the active site.[1] Subsequently spectroscopic studies have provided direct evidence for the formation of such a bond on binding of ACV (1) to IPNS in the presence of NO.[3] The second key element in our hypothesis is the formation of the ferryl-oxo intermediate (4), which is instrumental in the closure of the thiazolidine ring.[1]

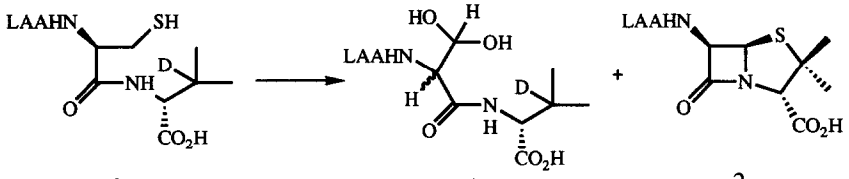


3 Intermediate (4) 2

Scheme 2

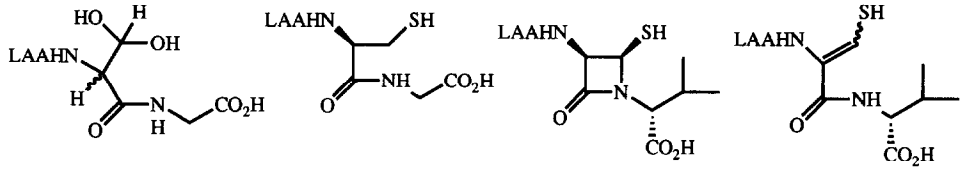
In order to investigate the nature of the intermediate we used the large V_{max} isotope effect on the closure of the second ring to encourage release of the intermediate from the enzyme. Thus,

incubation of the deuterated tripeptide (5) with IPNS resulted in an isotope induced branching of the reaction and the isolation and characterisation of a shunt metabolite (6) (Scheme 3).[4a] An analogous shunt metabolite (7) was also formed on incubation of the AC-glycine analogue (8), which cannot complete the second ring closure, with IPNS.[4b]



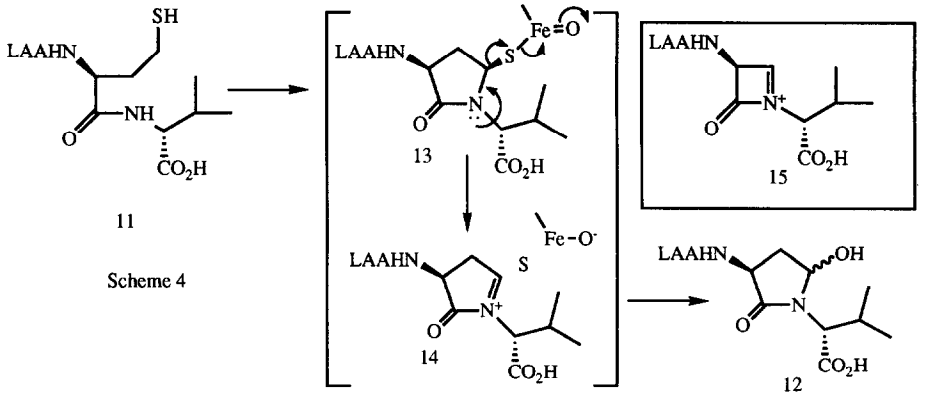
5 Scheme 3 6 2

The β-lactam thiol (9) was shown to decompose to the ene-thiol (10) under the conditions of the incubation, implying a 'free' β-lactam thiol is not a precursor of (6). When the incubation of (8) was carried out in the presence of $NaBT_4$, starting material was recovered in which there was incorporation of tritium into the β-position of cysteine, presumably resulting from the interception of an enzyme bound species, possibly analogous to (4), by the reducing agent.



7 8 9 10

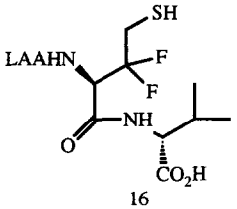
Further support for the involvement of a ferryl-oxo intermediate was obtained by using an analogue of ACV (1), in which the cysteinyl residue was replaced with homocysteine. When (11) was incubated with IPNS, the sole products observed were the epimeric monocyclic γ-lactams (12) (Scheme 4).[5] Incubation under an atmosphere of $^{18}O_2$ resulted in partial incorporation of ^{18}O label into the hydroxyl group of (12), indicative that the quenching hydroxyl was attached to the iron centre, derived from the reduction of dioxygen. The loss of hydrogen from the γ-carbon of (11), was as for the natural substrate ACV (1), shown to be stereospecific.



11 Scheme 4 13 14 15 12

We reasoned that the lack of bicyclic products from the homocysteinyl substrate resulted from the relatively rapid collapse of the γ-lactam intermediate (13) to an imminium species (14) (Scheme 4), analogous to the β-lactam species (15) which was proposed as an intermediate in the formation of (6).[4b] The apparently complete formation of the cation (14) from (13) as compared to the partial formation of (15) may reflect the higher strain energy of the four membered ion (15) compared to the five membered ring analogue (14).

Thus, in contrast to the major natural pathway [(1) to (2)], in the conversions of either (11) to (12) or (5) to (6), only one half of the oxidising potential of the dioxygen molecule is realised in the isolated product. Thus, we proposed that in the case of the formation of (6) and (12), collapse to the intermediate acyl imminium ions [(15) or (14) respectively], resulted in the production of atomic sulphur (Scheme 4). If indeed a cation is involved in the sulphur fragmentation process in the conversion of (11) to (12), then the introduction of strongly electron withdrawing substituents on the adjacent carbon should hinder this process. It was hypothesised that this may result in both oxidising equivalents of the dioxygen cosubstrate being utilised in the oxidation of the tripeptide, resulting in the formation of a bicyclic material or a product of the same oxidation state. In this paper the synthesis of the difluorohomocysteine peptide (16) is described, which was designed to achieve this goal *via* its incubation with IPNS.



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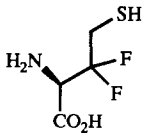

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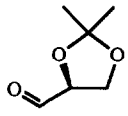

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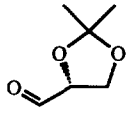

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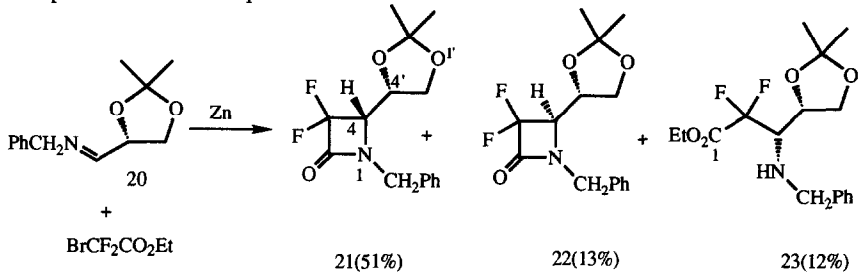
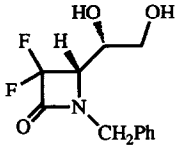
In order to obtain the desired tripeptide (16) a synthesis of 3,3-difluoro-*L*-homocysteine (17) was required. Although some attention has been focussed on the synthesis of fluorinated amino acids, methods for the preparation of β,β-difluoro amino acids are scarce.[6-9] However, a report by Kobayashi *et al*[10] outlining the diastereoselective preparation of a series of difluoroazetidinones led us to an appropriate difluorinated carbon skeleton which could be manipulated to the requisite amino acid.



Scheme 5

Our projected synthesis required *S*-glyceraldehyde acetonide (18)[11] as the starting material in order to obtain the *L*-configured amino acid. To assess the viability of the proposed chemistry *R*-glyceraldehyde acetonide (19) was initially used as it is readily available from inexpensive D-mannitol[12,13]. Thus, Reformatsky reaction of the benzylimine (20) [obtained quantitatively by reaction of the aldehyde (19) with benzylamine] with ethyl bromodifluoroacetate gave the azetidinones (21) and (22) in the ratio of *anti* (21):*syn* (22), *ca.* 4:1 (64%) (Scheme 5). Also isolated

was the ethyl ester (23) (12%). The acetonides [(21) and (22)] were cleaved to their respective diols (24) (87%) and (25) (63%), followed by oxidative cleavage and esterification[14] to the t-butyl esters (26) (85%) and (27) (88%). A two stage reduction of the β-lactam ring of ester (26) firstly with DIBAL to the aldehyde oxidation level then with sodium borohydride afforded the alcohol (28) in moderate overall yield (48%). Over-reduction with the use of excess DIBAL in the first step led to the formation of the amino diol (29) (70%). [Azetidinone (21) was also reduced with lithium aluminium hydride to the amino alcohol (30) (73%).]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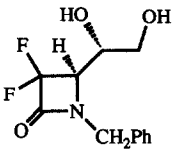
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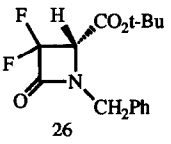
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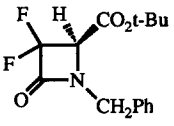
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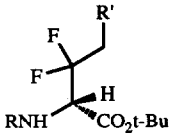
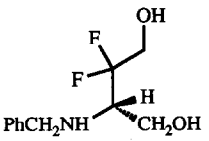

28, R = CH_2Ph, R' = OH
31, R = $CO_2{}^tBu$, R' = OH
32, R = $CO_2{}^tBu$, R' = OSO_2CH_3
34, R = $CO_2{}^tBu$, R' = $SCOCH_3$
35, R = $CO_2{}^tBu$, R' = SCH_2Ph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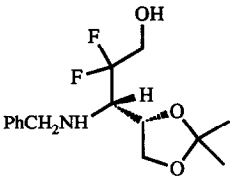
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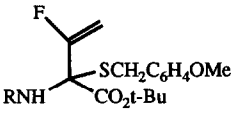
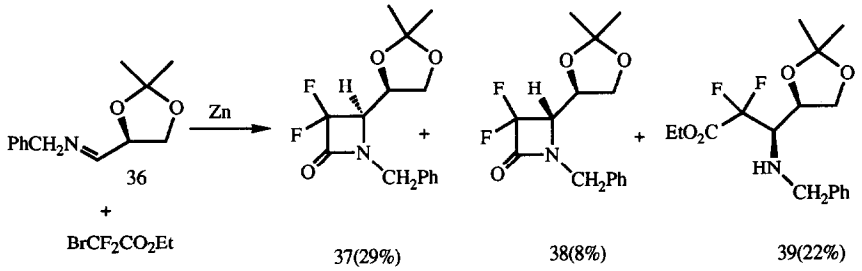

33

Hydrogenolysis of (28) gave the free amine, which was protected using di-t-butyl dicarbonate to give the N-BOC protected *D*-homoserine (31) (74%). Initial attempts to introduce sulphur *via* nucleophilic substitution resulted in elimination of HF. For example, reaction of mesylate (32), obtained quantitatively from alcohol (31), with *p*-methoxybenzyl thiolate gave unreacted starting material (52%) and an elimination product tentatively assigned as the unsaturated derivative (33) (37%), presumably formed *via* elimination of HF and subsequent S_N2' displacement of the mesyl group by the sulphur nucleophile. Successful introduction of sulphur was achieved via conversion of alcohol (31) to its triflate followed by *in situ* treatment with potassium thioacetate to give the thioacetate (34) (76%).[15] Mild alkaline hydrolysis and subsequent *in situ* alkylation afforded the S-benzyl derivative (35) (57%).


36

+

$BrCF_2CO_2Et$

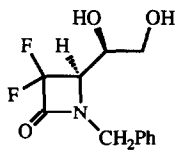
37(29%)

38(8%)

39(22%)

Scheme 6

Having proved the viability of the proposed route with the synthesis of a suitably protected difluoro *D*-homocysteine (35) attention was now focussed on the preparation of the *L*-configured difluoro homocysteine. Reaction of ethyl bromodifluoroacetate with benzylimine (36) under Reformatsky conditions gave the azetidinones (37) (29%) and (38) (8%) (3.6:1) and the ethyl ester (39) (22%) (Scheme 6). This ester (39) could be cyclised in high yield (93%) to azetidinone (37) by treatment with t-butyl magnesium chloride.



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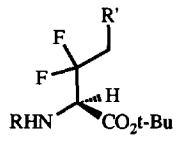
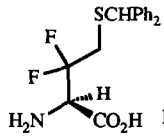


41, R = CH_2Ph, R' = OH
42, R = $CO_2{}^tBu$, R' = OH
43, R = $CO_2{}^tBu$, R' = $SCOCH_3$
44, R = $CO_2{}^tBu$, R' = SH
45, R = $CO_2{}^tBu$, R' = $S)_2$
47, R = $CO_2{}^tBu$, R' = $SCHPh_2$



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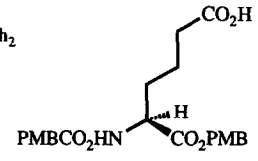

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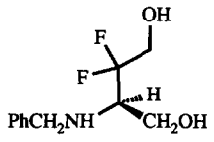

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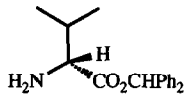

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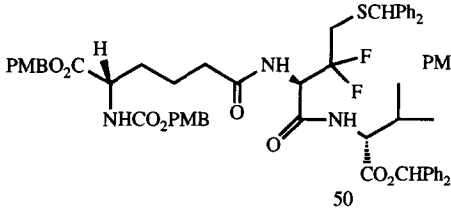
PMB = *p*-methoxybenzyl

Cleavage of the acetonide moiety in (37) to diol (40) (86%) was followed by oxidative cleavage and esterification to ester (27) (88%) identical to that formed previously from diol (25). Double hydride reduction to alcohol (41) (65%) and conversion to the BOC protected amino group afforded protected difluoro-*L*-homoserine (42) (87%). Triflate formation and displacement with potasium thioacetate gave thioacetate (43) in high yield (89%). Hydrolysis with dilute base gave thiol (44) in good yield (which could be converted to the disulfide form (45) quantitatively with iodine in dimethylsulphoxide). Acid deprotection of (44) to amino acid (17) (91%) was followed by alkylation of the sulfur to afford S-benzhydryl β,β-difluoro-*L*-homocysteine (46) (64%) [characterised as its N-BOC, benzhydryl ester derivative (47)]. The amino acid (46) was coupled, using standard methods,[16] with di-p-methoxybenzyl protected *L*-δ-(α-aminoadipic) acid (48) at the N-terminus and *D*-valine benzhydryl ester (49) at the C-terminus to give the fully protected tripeptide (50) (41%). Deprotection in refluxing trifluoroacetic acid containing anisole and conversion to the disufide with oxygen gas was followed by h.p.l.c. purification to yield the desired tripeptide (16).



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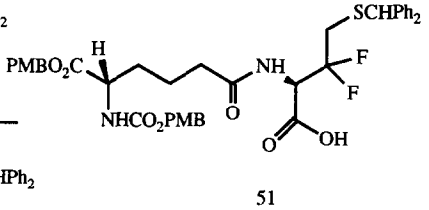

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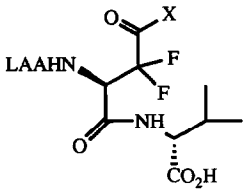
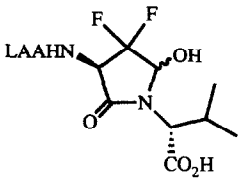
The tripeptide (16) was incubated with IPNS,[17] and the resultant crude mixture was examined by ^1H n.m.r. (500MHz) after protein precipitation. Analysis of the region between 5 and 6ppm indicated the generation of two new products in a ratio of *ca.* 5:1 [major: 5.62(dd, *J*14.5, 11.5 Hz) and minor: 5.33(dd, *J*14.0, 12.5Hz). These materials were purified by reverse phase h.p.l.c. and assigned as the thiocarboxylic (52) and carboxylic (53) acids on the basis of their electrospray mass spectra and ^1H and ^{19}F n.m.r. spectra.



52, X = SH
53, X = OH



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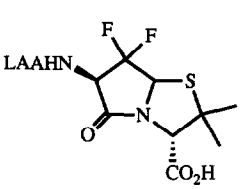

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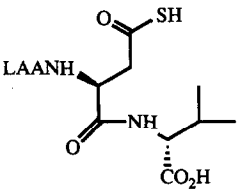
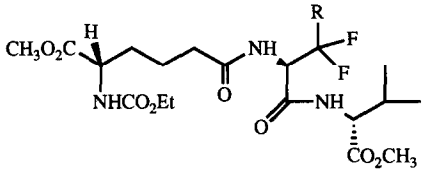


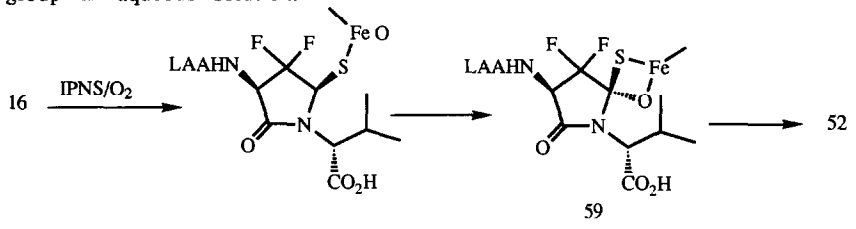
58

The thiocarboxylic acid (52) was shown [by ^{19}F and ^{1}H n.m.r. (500MHz) and mass spectrometry] to decompose to the carboxylic acid (53) upon standing in D_2O at pH 7.5, consistent with the former (52) being the sole enzymatic product, which slowly converts to the latter (53). It was anticipated that the bicyclic lactam (55) or the hydroxy lactams (54) may have been enzymatic products of the reaction, but despite a careful study we have been unable to find any evidence for their formation. Derivatisation of (52) and (53) yielded the tri-esters (56) and (57) respectively which were analysed by DCI mass spectrometry.



56, R = CO_2CH_3
57, R = $COSCH_3$

The incubation of (16) with IPNS under an atmosphere of $^{18}O_2$ gas led to the isolation of (52) with >90% incorporation of a single ^{18}O label. This level is consistent with the level of incorporation observed in the formation of hydroxylated bicyclic lactams with IPNS, but is significantly higher than that observed into the monocyclic hydroxy lactams (12). In the latter case it is likely that the lower levels observed are due to the relatively facile exchange of the hydroxyl group in aqueous solution.

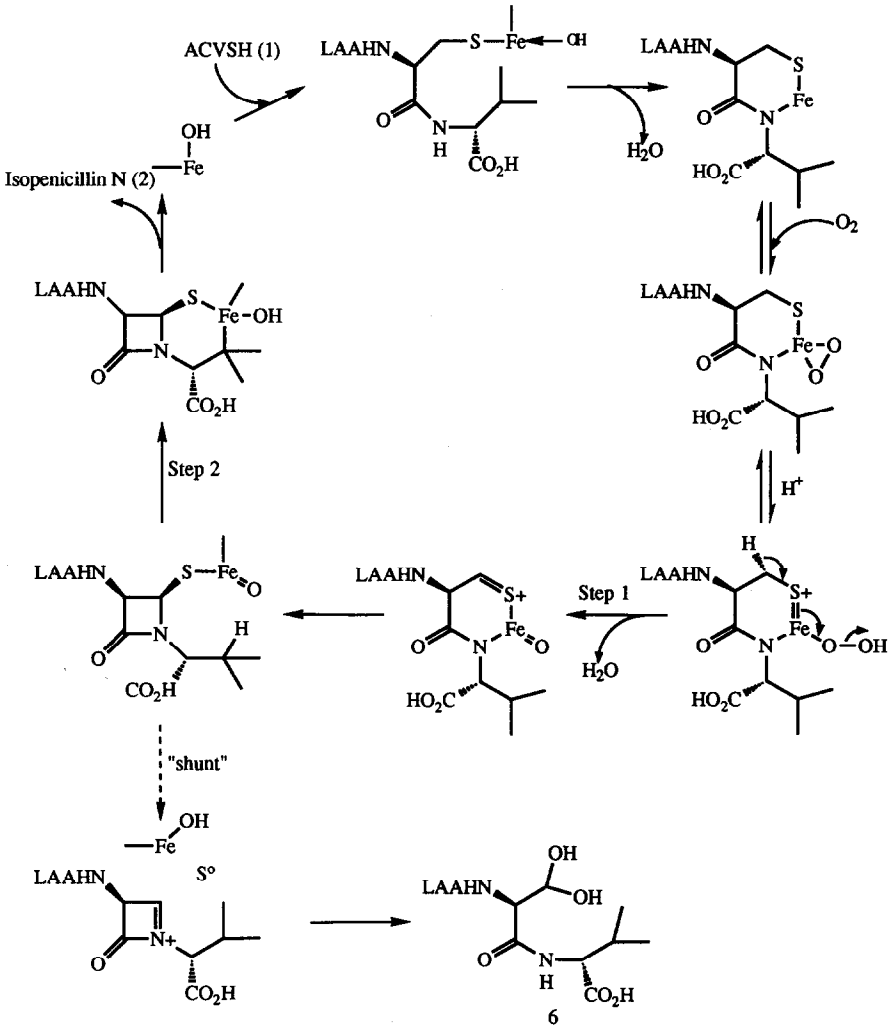


Scheme 7

The results of the incubation of tripeptide (16) prompted us to re-examine [by h.p.l.c. and n.m.r.] the incubation of the protiated homocysteinyl tripeptide (11) with IPNS to look for the generation of a thiocarboxylic acid (58). We were unable to detect any (58) and thus it would appear (within experimental error), that the introduction of two fluorine atoms into the homocysteinyl residue completely biases the reaction pathway away from the production of monocyclic lactams, such as (12), towards the thiocarboxylic acid (52). This is in accord with our previous mechanism since the presence of the two fluorine atoms hinders fragmentation of the ring, and permits the iron-oxo species to oxidise the remaining hydrogen giving after ring opening of (59), the thiocarboxylic acid (52) (Scheme 7). In the case of the protiated material

(11), the collapse of the intermediate is much faster and results in the formation of the monocyclic lactams (12) (Scheme 4).

These experiments demonstrate the power of organic chemistry to manipulate the mode of reactivity of oxygenase catalysis, *via* the introduction of appropriate functionalities. Furthermore these experiments indirectly support our notion of the direct attachment of the thiol functionality of ACV (1) to the iron-dioxygen centre in the conversion to isopenicillin N (2). Our working hypothesis for the reaction cycle is shown in Scheme 8, in which both isotopically sensitive steps and possible modes for the formation of the shunt metabolite are indicated.[18]



Scheme 8

Acknowledgements: we thank the S. E. R. C. and Eli Lilly and Co. Indianapolis for support of this work, Dr R.T. Aplin for mass spectral analyses and Mr J. Pitt and Mr J. W. Keeping for the preparation of IPNS.

Experimental

Standard synthetic procedures were followed. Melting points were determined using a Gallenkamp capillary melting point apparatus and are uncorrected. Infrared spectra were recorded as thin films or $CHCl_3$ solutions on a Perkin-Elmer 1750 FT-IR spectrometer. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. ^1H NMR and ^{13}C NMR spectra were recorded on Varian Gemini-200 or Bruker AM-500 spectrometers. ^{19}F NMR spectra were recorded on a Bruker AM-250 spectrometer. Routine electron impact (EI), chemical ionisation (CI) and desorption chemical ionisation (DCI) mass spectra were run on a VG Masslab 20-250 quadrupole mass spectrometer. GCMS spectra were run using NH_3 CI on a VG Masslab Trio-1 benchtop GCMS quadrupole mass spectrometer. FAB mass spectra were run on a VG Analytical ZAB 1F double focussing mass spectrometer. Electrospray mass spectra were run using 1:1 MeOH/H_2O 1% HOAc at concentrations typically of 50 pmol μl^{-1} on a VG Biotech VG Bio-Q mass spectrometer. Microanalyses were determined on a Carlo-Erba Model 1106 CHNOS elemental analyser. Analytical TLC was performed on commercial Merck silica gel 60 F_{254} aluminium backed plates. Flash chromatography was performed using Merck silica gel 60. Radial chromatography was performed using a Harrison Research Chromatotron 7924 preparative centrifugal thin layer chromatograph. Silica gel 60 PF_{254} (Merck 7749) plates of 1, 2 and 4 mm thickness were used with recommended loadings of 0.30, 0.75 and 1.5 g respectively. HPLC was carried out using dual Waters 6000A pumps, a Rheodyne 7125 injector and a Waters 441 detector. Petrol refers to the fraction of petroleum ether boiling between 40-60°C.

Reformatsky reaction on benzylimine (20)

The benzylimine (20) of (*R*)-glyceraldehyde acetonide (19) (9.4g, 43 mmol) and ethyl bromodifluoroacetate (7.0 ml, 55 mmol) together in dry THF (40 ml) were added dropwise over 0.5 h to a suspension of activated zinc[12] (3.0g, 46 mmol) in refluxing dry THF (40 ml). The mixture was refluxed for a further 1 h followed by cooling to room temperature and the addition of sodium hydrogen sulfate (0.5 M, 30 ml). The aqueous layer was washed with ether (3x) and the combined organic layers washed with H_2O, dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification by column chromatography (20% Et_2O/petrol) provided crystalline (4*S*, 4'*S*)-azetidinone (21) (6.56g, 51%), (4*R*, 4'*S*)-azetidinone (22) (1.69g, 13%) as an oil and ethyl (3*S*,4*S*)-3-benzylamino-2,2-difluoro-4,5-*O*-isopropylidenepentanoate (23) (1.78g, 12%) also as an oil.

(21): mp 71°; $[\alpha]_D$ -91.0 (c1.20, $CHCl_3$); (Found: C, 60.59; H, 6.02; N, 4.46%. $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); ν_{max}: 1795, 1318, 1207, 1154, 1135, 1102, 1057, 1030, 849 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.33 (6H, s, Me), 3.74 (1H, td, *J* 8.3, 2.4 Hz, H-4), 4.15, 3.71 (2H, AB part of ABX system, J_{AB} 9.0, J_{AX} 6.7, J_{BX} 5.1 Hz, CH_2O), 4.23 (1H, m, H-4'), 4.91, 4.29(J_{LR} 2.1 Hz) (2H, ABq, *J* 14.7 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 24.7q, 26.3q, 45.3t, 66.1td (J_{CF} 23 Hz), 74.7d, 110.6s, 120.1ts (J_{CF} 289 Hz), 128.4d, 128.8d, 129.0d, 134.2s, 160.1ts (J_{CF} 31 Hz). δ_F (235 MHz, $CDCl_3$): -117.2, -125.1 (ABq, *J* 234 Hz); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 46), 101 (39), 91 (44).

(22): $[\alpha]_D$ +57.7 (c1.20, $CHCl_3$); (Found: C, 60.65; H, 6.02; N, 4.55%. $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); ν_{max}: 1795, 1209, 1158, 1067, cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.34, 1.47 (2x3H, s, Me), 3.73 (1H, ddd, *J* 8.6, 6.4, 0.9 Hz, H-4), 4.03, 3.93 (2H, AB part of ABXY system, J_{AB} 8.2, J_{AX} 5.0, J_{AY} 3.0, J_{BX} 6.6, J_{BY} 1.4 Hz, CH_2O), 4.30 (1H, td, *J* 6.4, 5.0 Hz, H-4'), 4.83, 4.26(J_{LR} 1.9 Hz) (2H, ABq, *J* 15.3 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 24.1q, 26.2q, 45.5t, 64.8t, 64.8td (J_{CF} 24 Hz), 72.6d, 109.6s, 120.6ts (J_{CF}290 Hz), 128.4d, 128.6d, 129.3d, 133.8s, 160.9ts (J_{CF} 30 Hz); δ_F (235 MHz, $CDCl_3$): -117.1, -124.7 (ABq, *J* 234 Hz); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 57), 132 (21), 101 (38), 91 (44).

(23): $[\alpha]_D$ -12.5(c1.07, $CHCl_3$); (Found: C, 59.80; H, 6.78; N, 3.86%. $C_{17}H_{23}F_2NO_4$ requires C, 59.46; H, 6.75; N, 4.08%); ν_{max}: 1776, 1218, 1105, 1059 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.33 (3H, t, *J* 7.2 Hz, CH_2CH_3), 1.37, 1.41 (2x3H, s, Me), 3.24 (1H, ddd, *J* 14.3, 7.9, 5.7 Hz, H-3), 3.98 (2H, s, CH_2Ph), 4.11, 3.85 (2H, AB part of ABX system, J_{AB} J_{BX} 7.7, J_{AX} 7.4 Hz, CH_2O), 4.27-4.44 (3H, m, H-4, CH_2CH_3), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 13.7q, 25.2q, 26.2q, 52.5t, 61.1td (J_{CF} 23 Hz), 63.0t, 67.4t, 73.5d, 109.4s, 116.4ts

(J_{CF} 258 Hz), 127.2d, 128.3d, 128.4d, 140.1s, 163.8ts (J_{CF} 32 Hz); δ_F (235 MHz, $CDCl_3$): -107.3, -117.3 (ABq, J 259 Hz); m/z CI(NH_3): 344 (MH^+, 100%).

(3S,4S)-3-Benzylamino-2,2-difluoro-4,5-O-isopropylidenepentan-1-ol (30)
Difluoro β-lactam (21) (0.42g, 1.41 mmol) was dissolved in dry Et_2O (30 ml) and excess lithium aluminium hydride (0.10g) added. After refluxing for 20 min excess reagent was destroyed by the careful addition of H_2O. Acidification with 10% HCl was followed by extraction with Et_2O (3x). The aqueous layer was basified with NaOH (1.0M), extracted with Et_2O (3x) and the organic phase washed with H_2O, dried over $MgSO_4$ and the solvent evaporated to give amino alcohol (30) (0.31g, 73%): $[\alpha]_D$ -6.9 (c0.91, $CHCl_3$); (Found: C, 59.66; H, 7.04; N, 4.31%. $C_{15}H_{21}F_2NO_3$ requires C, 59.78; H, 7.02; N, 4.65%); ν_{max}: 3470-3330, 1251, 1217, 1157, 1132, 1072, 910, 848 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.36, 1.42 (2x3H, s, Me), 3.05 (1H, td, J 9.8, 5.0 Hz, H-3), 3.92 (2H, m, H-1), 4.07, 3.67 (2H, AB part of ABX system, J_{AB} J_{AX} J_{BX} 7.6 Hz, H-5). 4.03, 3.89 (2H, ABq, J 12.9 Hz, CH_2Ph), 4.36 (1H, q, J 6.0 Hz, H-4), 7.2-7.4 (5H, m, Ar); δ_H (50 MHz, $CDCl_3$): 25.0q, 26.1q, 52.9t, 61.7td (J_{CF} 26 Hz), 63.2tt (J_{CF} 31 Hz), 67.4t, 73.4d, 109.6s, 121.6ts (J_{CF} 250 Hz), 127.6d, 128.7d, 128.8d, 139.3s; δ_F (235 MHz, $CDCl_3$): -107.3, -114.3 (ABq, J 287 Hz); m/z CI(NH_3): 302(MH^+, 100%), 200(24), 91(18).

(4S)-1-Benzyl-3,3-difluoro-4-[(1'S)-1',2'-dihydroxyethyl]azetidin-2-one (24)
The acetonide (21) (4.11g, 13.8 mmol) and p-toluenesulfonic acid (3.0g) were dissolved in MeOH/H_2O (10:1, 55 ml) and the solution stirred at 55° for 2.5 h. Dilution with H_2O was followed by Et_2O extraction (3x). The combined extracts were washed with H_2O, dried ($MgSO_4$) and the solvent removed. Radial chromatography (80% Et_2O/petrol) provided crystalline diol (24) (3.10g, 87%): mp 62°; $[\alpha]_D$ -76.4 (c1.22, $CHCl_3$); (Found: C, 56.04; H, 5.36; N, 5.06%. $C_{12}H_{13}F_2NO_3$ requires C, 56.03; H, 5.09; N, 5.45%); ν_{max}: 3435, 1778, 1206, 1128, 1068, 1032 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 2.91, 3.33 (2x1H, br s, exchangeable(D_2O), OH), 3.68, 3.53 (2H, AB part of ABX system, J_{AB} 11.4, J_{AX} 3.2, J_{BX} 5.6 Hz, CH_2OH), 3.87 (1H, td, J 8.0, 1.9 Hz, H-4), 3.93 (1H, m, $CHOH$), 4.86, 4.42(J_{LR} 1.6 Hz) (2H, ABq, J 14.8 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 45.8t, 63.3t, 65.1td (J_{CF} 23 Hz), 70.6d, 119.9ts (J_{CF} 289 Hz), 128.4d, 128.5d, 129.2d, 134.4s, 161.2ts (J_{CF} 30 Hz); δ_F (235 MHz, $CDCl_3$): -117.5, -125.4 (ABq, J 234 Hz); m/z DCI(NH_3): 275 (MNH_4^+, 20%), 258 (MH^+, 71), 108 (40), 91 (100).

(4S)-1-Benzyl-4-(t-butyloxycarbonyl)-3,3-difluoroazetidin-2-one (26)
To the diol (24) (2.26g, 8.8 mmol) in acetone (20 ml) cooled to 0° was added a solution of chromium (VI) oxide (4.1g, 41 mmol) and periodic acid (18.7g, 82 mmol) in H_2O (80 ml). After stirring at 0° for 20 min the reaction was diluted with H_2O and Et_2O extracted (3x). The combined organic extracts were washed with H_2O, dried over $MgSO_4$ and the solvent removed *in vacuo*. The crude oil was dissolved in CH_2Cl_2 (20 ml), cooled to 0° and cyclohexane (40 ml) added. Boron trifluoride etherate (3 drops) was added followed by *t*-butyl trichloroacetimidate (4.52g, 19.6 mmol) in cyclohexane (20 ml) dropwise. After stirring at 0° for 20 min solid $NaHCO_3$ was added and stirring continued for 10 min. Filtration through silica (10% Et_2O/petrol) and subsequent radial chromatography (20% Et_2O/petrol) provided ester (26) (2.22g, 85%) as a clear oil: $[\alpha]_D$ +2.8 (c0.95, $CHCl_3$); (Found: C, 60.32; H, 5.85; N, 5.02%; $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); ν_{max}: 1801, 1746, 1307, 1232, 1195, 1158, 1141, 1109, 1062, 974, 898, 834, 793 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.49 (9H, s, Bu^t), 4.15 (1H, dd, J 6.8, 2.8 Hz, H-4), 4.98, 4.29(J_{LR} 2.1 Hz) (2H, ABq, J 14.8 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 27.7q, 45.1t, 64.5td (J_{CF} 25 Hz), 84.5s, 119.7ts (J_{CF} 291 Hz), 128.7d, 129.3d, 133.1s, 160.0ts (J_{CF} 30 Hz), 164.1s; δ_F (235 MHz, $CDCl_3$): -115.3, -121.3 (ABq, J 225 Hz); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 10), 108 (10), 91 (25).

t-Butyl (2S)-2-benzylamino-3,3-difluoro-4-hydroxybutanoate (28) and (3S)-3-benzylamino-2,2-difluorobutane-1,4-diol (29)
DIBAL (1.0 M, 10 ml) was added to β-lactam (26) (2.22g, 7.5 mmol) in dry Et_2O (50 ml) at -78° and the solution stirred under N_2 for 30 min. H_2O was added and the mixture extracted with Et_2O (3x). The combined extracts were washed with H_2O, dried ($MgSO_4$) and the solvent evaporated. The

resultant crude oil was dissolved in dry EtOH (30 ml) and sodium borohydride (0.30g, 7.9 mmol) added. The reaction was stirred at 0° under N_2 for 45 min. Workup as above was followed by radial chromatography (20% Et_2O/petrol) to give alcohol (28) (1.08g, 48%): $[\alpha]_D$ +37.2 (c1.11, $CHCl_3$), (Found: C, 59.65; H, 6.88; N, 4.38%. $C_{15}H_{21}F_2NO_3$ requires C, 59.78; H, 7.02; N, 4.65%). ν_{max}: 3500-3200, 1734, 1256, 1154, 1075 cm^{-1}. δ_H (500 MHz, $CDCl_3$): 1.50 (9H, s, Bu^t), 3.86, 3.65 (2H, ABq, *J* 12.8 Hz, CH_2Ph), 3.62 (1H, dd, *J* 20.7, 4.9 Hz, H-2), 3.77-3.94 (2H, m, H-4); δ_C (50 MHz, $CDCl_3$): 27.8q, 52.3t, 63.2td (J_{CF} 26 Hz), 64.0tt (J_{CF} 33 Hz), 83.2s, 119.8ts (J_{CF} 251 Hz), 127.8d, 128.6d, 128.8d, 138.4s, 168.4s; δ_F (235 MHz, $CDCl_3$): -111.6, -120.4 (ABq, *J* 258 Hz); m/z CI(NH_3): 302 (MH^+, 100%), 246 (92), 200 (42), 106 (40), 91 (96).

Analogous procedure but with a larger excess DIBAL, thus β-lactam (26) (0.93g, 3.1 mmol) and DIBAL (1.0 M, 10 ml) resulted in the formation of diol (29) (0.51g, 70%): mp 66°; $[\alpha]_D$ +13.3 (c0.97, $CHCl_3$); (Found: C, 57.09; H, 6.67; N, 6.13%. $C_{11}H_{15}F_2NO_2$ requires C, 57.13; H, 6.54; N, 6.06%); ν_{max}: 3500-3100, 1074, 911 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 2.95 (2H, br s, exchangeable (D_2O), OH), 3.09 (1H, ddt, *J* 14.1, 9.7, 4.7 Hz, H-3), 3.75-3.89 (4H, m, H-1,4), 3.91 (2H, s, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 52.0t, 58.3t, 60.6td (J_{CF} 25 Hz), 62.6tt (J_{CF} 32 Hz), 122.2ts (J_{CF} 248 Hz), 127.7d, 128.4d, 128.8d, 139.2s; δ_F (235 MHz, $CDCl_3$): -111.7, -116.4 (ABq, *J* 261 Hz); m/z CI(NH_3): 232 (MH^+, 100%), 200 (25), 150 (16), 91 (46).

t-Butyl (2S)-2-(t-butyloxycarbonylamino)-3,3-difluoro-4-hydroxybutanoate (31)
Benzylamine (28) (0.69g, 2.3 mmol) was dissolved in EtOH (20 ml) and 10% palladium on charcoal (0.20g) added. The suspension was stirred vigorously under an atmosphere of H_2 for 3 h. After filtration through celite washing with Et_2O the solvent was removed and the crude amine dissolved in dioxan/H_2O (1:1, 20 ml). Di-*t*-butyl dicarbonate (0.55g, 2.5 mmol) and $KHCO_3$ (0.25g, 2.5 mmol) were added and the solution stirred under Ar at room temperature overnight. After dilution with H_2O and Et_2O extraction (3x) the combined organic extracts were washed with brine, dried over $MgSO_4$ and the solvent removed. Radial chromatography (20% Et_2O/petrol provided white crystalline protected difluoro *D*-homoserine (31) (0.53g, 74%): mp 74°, $[\alpha]_D$ -39.6 (c0.93, $CHCl_3$); (Found: C, 50.47; H, 7.79; N, 4.26%. $C_{13}H_{23}F_2NO_5$ requires C, 50.15; H, 7.45; N, 4.50%). ν_{max}: 3400, 1737, 1253, 1157, 1074, 1055 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.42, 1.47 (2x9H, s, Bu^t), 3.64-3.77 (2H, m, H-4), 4.02 (1H, br s, exchangeable(D_2O), OH), 4.65 (1H, ddd, *J* 22.3, 8.1, 4.0 Hz, H-2), 5.62 (1H, d, *J* 7.9 Hz, NH); δ_C (50 MHz, $CDCl_3$): 27.6q, 27.9q, 54.4ddd (J_{CF} 32, 24 Hz), 61.7ddt (J_{CF} 36, 27 Hz), 81.6s, 84.0s, 120.5ts (J_{CF} 251 Hz), 156.6s, 166.0s; δ_F (235 MHz, $CDCl_3$): -114.0, -121.4 (ABq, *J* 255 Hz); m/z CI(NH_3): 329 (MNH_4^+, 11%), 312 (MH^+, 41), 273 (53), 254 (43), 217 (100), 212 (46), 156 (24), 91 (40), 57 (34).

t-Butyl (2S)-2-(t-butyloxycarbonylamino)-3,3-difluoro-4-methanesulfonyloxy-butanoate (32)
To alcohol (31) (0.37g, 1.2 mmol) in CH_2Cl_2 (30 ml) under N_2 was added triethylamine (0.20 ml, 1.4 mmol) followed by methanesulfonyl chloride (0.11 ml, 1.4 mmol) and the solution stirred at room temperature overnight. After dilution with H_2O and Et_2O extraction (3x), the combined extracts were washed with 10% aqueous HCl and H_2O then dried ($MgSO_4$) and the solvent removed. Radial chromatography (30% Et_2O/petrol) gave the title compound (32) (0.45g, 97%): mp 70°; $[\alpha]_D$ -18.6 (c1.33, $CHCl_3$); (Found: C, 43.57; H, 6.84; N, 3.45%. $C_{14}H_{25}F_2NO_7S$ requires C, 43.18; H, 6.47; N, 3.60%). ν_{max}: 3380, 1719, 1505, 1396, 1370, 1175, 1156, 1030, 973, 839 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.44, 1.49 (2x9H, s, Bu^t), 3.11 (3H, s, OMs), 4.48 (2H, 6 lines, *J* 12.6 Hz, H-4), 4.72 (1H, br q, *J* 11.1 Hz, H-2), 5.35 (1H, br d, *J* 7.9 Hz, NH); δ_C (50 MHz, $CDCl_3$): 27.6q, 28.0q, 37.9q, 55.5td (J_{CF} 26 Hz), 66.0tt (J_{CF} 31 Hz), 81.1s, 84.5s, 118.1ts (J_{CF} 253 Hz), 155.1s, 165.2s. δ_F -113.6, -115.3 (ABq, *J* 260 Hz). m/z CI(NH_3): 407 (MNH_4^+, 12%), 351 (59), 295 (100), 169 (26), 57 (23).

t-Butyl 2-(t-butyloxycarbonylamino)-3-fluoro-2-(p-methoxybenzylthio) but-3-enoate (33)
To mesylate (32) (0.079g, 0.20 mmol) in dry THF (10 ml) cooled to 0° was added sodium *p*-methoxybenzyl thiolate (0.042g, 0.24 mmol) while stirring. After 15 min the solution was

concentrated, the residue taken up in Et_2O and washed with 10% HCl then H_2O. The solution was dried over $MgSO_4$ and the solvent evaporated. Radial chromatography (20% Et_2O/petrol) provided unreacted starting material (32) (41mg, 52%) and a product tentatively assigned as the unsaturated derivative (33 (0.032g, 37%): ν_{max}: 3410, 1723, 1611, 1513, 1482, 1288, 1252, 1157 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.44, 1.49 (2x9H, s, Bu^t), 3.78, 3.71 (2H, ABq, J 12.1 Hz, SCH_2Ar), 3.78 (3H, s, OMe), 4.97-5.10 (2H, m, $CF{=}CH_2$), 5.83 (1H, s, NH), 6.81, 7.20 (2x2H, d, J 8.7 Hz, Ar); δ_C (125 MHz, $CDCl_3$): 27.5q, 28.2q, 34.0t, 55.2q, 67.4ds (J_{CF} 29 Hz), 80.7s, 84.2s, 94.8dt (J_{CF} 19 Hz), 114.1d, 127.9s, 130.3d, 152.4s, 158.9s, 159.2ds (J_{CF} 261 Hz), 166.3s; δ_F (235 MHz, $CDCl_3$): -107.1, s; m/z DCI(NH_3): 428 (MH^+, 17%), 389 (21), 333 (36), 274 (34), 235 (21), 181 (23), 242 (100), 113 (47), 121 (92), 119 (35).

t-Butyl (2S)-4-acetylthio-2-(t-butyloxycarbonylamino)-3,3-difluorobutanoate (34)
To the alcohol (31) (0.30g, 0.96 mmol) in CH_2Cl_2 (3 ml) at 0° under N_2 was added pyridine (0.17 ml, 2.19 mmol) then triflic anhydride (0.18 ml, 1.07 mmol) and the yellow-orange reaction stirred for 15 min. The mixture was diluted with H_2O, washed with CH_2Cl_2 (3x), the combined extracts dried over $MgSO_4$ and the solvent evaporated *in vacuo*. The crude residue was dissolved in DMF (3 ml) and cooled to 0°. Potassium thioacetate (0.123g, 1.07 mmol) was added and the reaction stirred for 10 min. Dilution with H_2O was followed by Et_2O extraction (3x). The combined organic extracts were washed with brine, dried ($MgSO_4$) and the solvent removed. Purification was achieved via radial chromatography (20% Et_2O/petrol) to give the thioacetate (34) (0.27g, 76%): mp 63°; $[\alpha]_D$ -37.3 (c1.02, $CHCl_3$); (Found: C, 48.70; H, 6.90; N, 4.04%. $C_{15}H_{25}F_2NO_5S$ requires C, 48.76; H, 6.82; N, 3.79%); ν_{max}: 3433, 1714, 1504, 1154 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.39, 1.44 (2x9H, s, Bu^t), 2.32 (3H, s, CH_3CO), 3.60-3.88 (2H, m, H-4), 4.63 (1H, br q, J 11.6 Hz, H-2), 5.39 (1H, br d, J 9.0 Hz, NH). δ_C (50 MHz, $CDCl_3$): 27.7q, 28.1q, 30.1q, 32.1tt (J_{CF} 27 Hz), 57.2td (J_{CF} 27 Hz), 80.9s, 84.1s, 119.9ts (J_{CF} 251 Hz), 155.3s, 165.9s, 193.1s; δ_F -105.7, -106.8 (ABq, J 248 Hz); m/z CI(NH_3): 387 (MNH_4^+, 32%), 370 (MH^+, 57), 331 (44), 314 (33), 275 (100).

t-Butyl (2S)-4-benzylthio-2-(t-butyloxycarbonylamino)-3,3-difluorobutanoate (35)
To the thioacetate (34) (0.100g, 0.27 mmol) in MeOH (3 ml) was added NaOH (0.2 M, 1.5 ml) and benzyl chloride (0.035 ml, 0.030 mmol). After stirring for 15 min at room temperature the reaction was diluted with H_2O and Et_2O extracted (3x). The combined extracts were washed with 10% HCl then brine, dried over $MgSO_4$ and the solvent removed *in vacuo*. Radial chromatography (10% Et_2O/petrol) provided the S-benzyl derivative (35) (0.064g, 57%): mp 74°; $[\alpha]_D$ -34.6 (c0.91, $CHCl_3$); (Found: C, 57.34; H, 7.17; N. 3.45%. $C_{20}H_{29}F_2NO_4S$ requires C, 57.53; H, 7.00; N. 3.35%); ν_{max}: 3370, 1723, 1498, 1252, 1155, 1059, 1038, 1019 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.45, 1.47 (2x9H, s, Bu^t), 2.86 (2H, t, J 15.6 Hz, H-4), 3.81 (2H, s, CH_2Ph), 4.90 (1H, br q, J 11.5 Hz, H-2), 5.32 (1H, br d, J 8.9 Hz, NH), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 27.7q, 28.1q, 33.7tt (J_{CF} 26 Hz), 36.8t, 56.9td (J_{CF} 28 Hz), 80.7s, 83.8s, 122.1ts (J_{CF} 251 Hz), 127.5d, 128.8d, 129.4d, 137.4s, 155.2s, 166.3s; δ_F (235 MHz, $CDCl_3$): -104.8, -105.5 (ABq, J 248 Hz); m/z CI(NH_3): 435 (MNH_4^+, 6%), 418 (MH^+, 14), 379 (21), 362 (22), 323 (100), 306 (21), 196 (20), 91 (56).

(4R)-1-Benzyl-3,3-difluoro-4-[(1'S)-1',2'-dihydroxyethyl]azetidin-2-one (25)
Acetonide (22) (1.59g, 5.3 mmol) was treated with *p*-toluene sulfonic acid (1.1g) according to the method outlined for the conversion of acetonide (21) to diol (24). Purification using radial chromatography (80% Et_2O/petrol) gave diol (25) (0.87g, 63%): mp 68°; $[\alpha]_D$ +28.0 (c1.06, $CHCl_3$); (Found: C, 56.01; H, 4.95; N, 5.39%. $C_{12}H_{13}F_2NO_3$ requires C, 56.03; H, 5.09; N, 5.45%); ν_{max}: 3410, 1779, 1323, 1206, 1128, 1058, 884 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 3.32 (2H, br s, OH), 3.66, 3.54 (2H, AB part of ABX system, J_{AB} 11.7, J_{AX} 3.0, J_{BX} 6.1 Hz, CH_2OH), 3.84-3.93 (2H, m, H-1',4), 4.79, 4.21(J_{LR} 1.4 Hz) (2H, ABq, J 15.2 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 45.3t, 63.0t, 65.4td (J_{CF} 23 Hz), 68.6d, 120.9ts (J_{CF} 286 Hz), 128.3d, 128.7d, 129.4d, 133.8s, 161.7ts (J_{CF} 30 Hz); δ_F (235 MHz, $CDCl_3$): -116.0, -123.6 (ABq, J 232 Hz); m/z CI(NH_3): 275 (MNH_4^+, 100%), 258 (MH^+, 55), 108 (20), 91 (60).

(4R)-1-Benzyl-4-(t-butyloxycarbonyl)-3,3-difluoroazetidin-2-one (27), from diol (25)
Using the method outlined for the conversion of diol (24) to ester (26), diol (25) (1.46g, 5.67 mmol) was treated with chromium (VI) oxide (2.80g, 28 mmol) and periodic acid (12.8g, 56 mmol) then *t*-butyl trichloroacetimidate (2.87g, 12.5 mmol). The crude product was purified by radial chromatography (20% Et_2O/petrol) to give ester (27) (1.49g, 88%): $[\alpha]_D$ -1.9 (c1.17, $CHCl_3$); (Found: C, 60.45; H, 5.83; N, 4.36%. $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); ν_{max}: 1801, 1746, 1307, 1233, 1195, 1159, 1109, 1063, 974, 898, 834, 793 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.47 (9H, s, Bu^t), 4.16 (1H, dd, *J* 6.8, 2.7 Hz, H-4), 4.94, 4.28 (2H, ABq, *J* 14.8 Hz, CH_2Ph), 7.2-7.4 (5H, m, Ar); δ_C (50 MHz, $CDCl_3$): 27.7q, 45.1t, 64.5td (J_{CF} 25 Hz), 84.4s, 119.8ts (J_{CF} 294 Hz), 128.7d, 129.3d, 133.1s, 159.8ts (J_{CF} 31 Hz), 164.0s. δ_F (235 MHz, $CDCl_3$): -115.4, -121.4 (ABq, *J* 225 Hz); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 11), 259 (15), 241 (26), 108 (16), 91 (51).

Reformatsky reaction on benzylimine (36)
The benzylimine (36) of (S)-glyceraldehyde acetonide (18) (4.51g, 20.5 mmol) was treated with ethyl bromodifluoroacetate (4.0 ml, 31.3 mmol) and activated zinc (1.44g, 22.0 mmol) according to the method outlined for benzylimine (20). Column chromatography (10 to 20% Et_2O/petrol) gave (4*R*,4'*R*)-azetidinone (37) (1.75g, 29%), (4*S*, 4'*R*)-azetidinone (38) (0.49g, 8%) and ethyl (3*R*,4*R*)-3-benzylamino-2,2-difluoro-4,5-*O*-isopropylidenepentanoate (39) (1.56g, 22%).
(37): mp 72°; $[\alpha]_D$ 103.6 (c1.09, $CHCl_3$); (Found: C, 60.65; H, 5.95; N, 4.85%. $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); Analogous IR, ^1H, ^{13}C and ^{19}F NMR data to compound (21); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 43).
(38): $[\alpha]_D$ -63.1 (c1.26, $CHCl_3$); (Found: C, 60.36; H, 6.01; N, 4.48%. $C_{15}H_{17}F_2NO_3$ requires C, 60.60; H, 5.76; N, 4.71%); Identical IR, ^1H, ^{13}C and ^{19}F NMR to compound (22); m/z CI(NH_3): 315 (MNH_4^+, 100%), 298 (MH^+, 61).
(39): $[\alpha]_D$ +17.1 (c1.22, $CHCl_3$); (Found: C, 59.46; H, 6.77; N, 3.94%. $C_{17}H_{23}F_2NO_4$ requires C, 59.46; H, 6.75; N, 4.08%); Identical IR, ^1H, ^{13}C and ^{19}F NMR to compound (23); m/z CI(NH_3): 344 (MNH_4^+, 100%), 242 (23), 240 (23), 150 (27), 108 (64), 91 (45).

(4R)-1-benzyl-3,3-difluoro-4-[(4'R)-2',2'-dimethyl-1',3'-dioxolan]-azetidin-2-one (37), via cyclisation of (39)
To the ester (39) (2.51g, 7.3 mmol) in dry Et_2O (50 ml) cooled to 0°C was added t-butyl magnesium chloride (2 M, 4.75 ml) dropwise while stirring under N_2. After 30 min the cooling bath was removed and stirring continued overnight. Saturated aqueous NH_4Cl (30 ml) was added and stirring continued for 15 min. Dilution with H_2O was followed by Et_2O extraction (3x). The combined extracts were washed with brine, dried over $MgSO_4$ and the solvent evaporated to give clean β-lactam (37) (2.02g, 93%), with analogous IR and NMR data to that previously obtained.

(4R)-1-Benzyl-3,3-difluoro-4-[(1'R)-1',2'-dihydroxyethyl]azetidin-2-one (40)
According to the method outlined for the conversion of acetonide (21) to diol (24), acetonide (37) (1.50g, 5.0 mmol) was treated with p-toluene sulfonic acid (1.0g) in MeOH:H_2O (10:1, 33 ml) at 55°for 2 h. Purification by radial chromatography (80% Et_2O/petrol) provided diol (40) (1.11g, 86%): mp 64°; $[\alpha]_D$ +75.9 (c1.25, $CHCl_3$); (Found: C, 55.89; H, 4.99; N, 5.31%. $C_{12}H_{13}F_2NO_3$ requires C, 56.03; H, 5.09; N, 5.45%); analogous IR, ^1H, ^{13}C and ^{19}F NMR data to compound (24); m/z CI(NH_3): 275 (MNH_4^+, 9%), 258 (MH^+, 7), 241 (52), 108 (100), 106 (80).

(4R)-1-Benzyl-4-(t-butyloxycarbonyl)-3,3-difluoroazetidin-2-one (27), from diol (40)
The diol (40) (1.46g, 5.7 mmol) was oxidised with chromium (VI) oxide (2.80g, 28 mmol) and periodic acid (12.8g, 56 mmol) then esterified with *t*-butyl trichloroacetimidate (2.87g, 12.5 mmol) according to the procedure for diol (24). Radial chromatography (20% Et_2O/petrol) gave *t*-butyl ester (27) (1.49g, 88%), identical by IR and NMR to that previously obtained.

t-Butyl (2R)-2-benzylamino-3,3-difluoro-4-hydroxybutanoate (41) and (3R)-3-benzylamino-2,2-difluorobutane-1,4-diol (60)
β-Lactam (27) (0.58g, 1.95 mmol) was reacted with DIBAL (1.0 M, 2.15 ml) and subsequently with sodium borohydride (0.11g, 2.90 mmol) according to the procedure for the reduction of β-lactam (26). The residue was purified to give alcohol (41) (0.38g, 65%); $[\alpha]_D$ -37.9 (c1.08, $CHCl_3$); (Found: C, 59.71; H, 6.82; N, 4.44%. $C_{15}H_{21}F_2NO_3$ requires C, 59.78; H, 7.02; N, 4.65%) with analogous IR, ^1H, ^{13}C and ^{19}F NMR data to compound (28); m/z CI(NH_3): 302 (MNH_4^+, 2%), 228 (15), 225 (31), 208 (100), 164 (31), 108 (43).

When excess DIBAL was used, thus β-lactam (27) (1.06g, 3.56 mmol) and DIBAL (1.0 M, 8.0 ml), alcohol (41) (0.14g, 13%) was isolated after radial chromatography (20% Et_2O/petrol) along with diol (60) (0.32g, 39%); mp 66°; $[\alpha]_D$ -13.4 (c1.22, $CHCl_3$); (Found: C, 57.42; H, 6.76; N, 6.37%. $C_{11}H_{15}F_2NO_2$ requires C, 57.13; H, 6.54; N, 6.06%); Identical IR, ^1H, ^{13}C and ^{19}F NMR to compound (29); m/z CI(NH_3): 232 (MH^+, 100%), 200 (27), 174 (29), 91 (78).

t-Butyl (2R)-2-(t-butyloxycarbonylamino)-3,3-difluoro-4-hydroxybutanoate (42)
The benzylamine (41) (1.98g, 6.57 mmol) was hydrogenolysed (10% Pd-C, 1.0g) then treated with di-*t*-butyl dicarbonate (1.58g, 7.24 mmol) and $KHCO_3$ (0.72g, 7.24 mmol) according to the procedure for the conversion of (28) to (31). Radial chromatography (20% Et_2O/petrol) afforded alcohol (42) (1.78g, 87%) as a white solid: mp 77°; $[\alpha]_D$ +34.3 (c1.25, $CHCl_3$); (Found: C, 50.23; H, 7.35; N, 4.06%. $C_{13}H_{23}F_2NO_5$ requires C, 50.15; H, 7.45; N, 4.50%); analogous IR, ^1H, ^{13}C and ^{19}F NMR data to compound (31); m/z DCI(NH_3): 329 (MNH_4^+, 10%), 312 (MH^+, 38), 273 (19), 256 (38), 217 (43), 212 (21), 200 (15), 156 (25), 110 (14), 91 (38), 57 (100).

t-Butyl (2R)-4-acetylthio-2-(t-butyloxycarbonylamino)-3,3-difluorobutanoate (43)
Using the method employed for the conversion of alcohol (31) to thioacetate (34), alcohol (42) (0.37g, 1.19 mmol) was treated with pyridine (0.21 ml, 2.64 mmol) and triflic anhydride (0.22 ml, 1.32 mmol) then potassium thioacetate (0.15g, 1.31 mmol). Workup followed by radial chromatography (15% Et_2O/petrol) yielded thioacetate (43) (0.39g, 89%); mp 60°; $[\alpha]_D$ +36.3 (c1.22, $CHCl_3$); (Found: C, 48.63; H, 6.75; N, 3.72%. $C_{15}H_{25}F_2NO_5S$ requires C, 48.76; H, 6.82; N, 3.79%); analogous IR, ^1H, ^{13}C and ^{19}F NMR data to compound (34); m/z CI(NH_3): 387 (MNH_4^+, 17%), 370 (MH^+, 29), 331 (25), 314 (20), 275 (100), 214 (20), 168 (21), 149 (21), 74 (38), 57 (36).

t-Butyl (2R)-2-(t-butyloxycarbonylamino)-3,3-difluoro-4-mercaptobutanoate (44)
To the thioacetate (43) (0.55g, 1.49 mmol) in MeOH (10 ml) at 0° was added precooled NaOH (0.2 M, 8.1 ml) and the solution stirred for 5 min. Dilution with H_2O and acidification with 10% HCl was followed by Et_2O extraction (3x). The combined extracts were washed with H_2O, dried over $MgSO_4$ and the solvent evaporated. The crude residue was purified by radial chromatography (10% Et_2O/petrol) to give the title compound (44) (0.38g, 78%) as white crystals: mp 74°; $[\alpha]_D$ +28.2 (c1.01, $CHCl_3$); (Found: C, 47.87; H, 7.38; N, 4.58%. $C_{13}H_{23}F_2NO_4S$ requires C, 47.69; H, 7.08; N, 4.28%); ν_{max}: 3430, 3367, 1723, 1504, 1253, 1156, 1069, 1047 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.42, 1.46 (2x9H, s, Bu^t), 1.87 (1H, br t, *J* 8.3 Hz, SH), 2.97 (2H, m, CH_2S), 4.86 (1H, m, H-2), 5.34 (1H, br d, *J* 9 Hz, NH); δ_C (50 MHz, $CDCl_3$): 27.7q, 28.0q, 28.2tt (J_{CF} 29 Hz), 56.0 td (J_{CF} 27 Hz), 80.8s, 83.8s, 120.6ts (J_{CF} 250 Hz), 155.3s, 166.1s. δ_F (235 MHz, $CDCl_3$): -108.1, -109.0 (ABq, *J* 245 Hz); m/z CI(NH_3): 345 (MNH_4^+, 11%), 328 (MH^+, 36), 289 (70), 272 (49), 233 (100), 228 (58), 172 (71), 74 (88), 58 (87).
Conversion to the disulfide (45) was achieved by dissolving thiol (44) (0.100g, 0.30 mmol) in DMSO (2 ml) and adding a catalytic amount of iodine. After stirring at room temperature for 3 h H_2O was added and the mixture extracted with Et_2O (3x). The combined extracts were washed with brine, dried over $MgSO_4$ and the solvent removed *in vacuo*. Radial chromatography (20% Et_2O/petrol) followed to give the disulfide (45) (98mg, 98%): δH (500 MHz, $CDCl_3$): 1.45, 1.49 (2x9H, s, But), 3.37 (2H, m, CH_2S), 4.74 (1H, br q, *J* 11.6 Hz, H-2), 5.35 (1H, br d, *J* 8.7 Hz, NH). δ_C (50 MHz, $CDCl_3$): 27.8q, 28.1q, 44.8tt (J_{CF} 26 Hz), 57.2td (J_{CF} 26 Hz), 80.9s, 84.1s, 120.5ts (J_{CF} 251 Hz), 155.3s, 166.0s.

S-Benzhydryl-β,β-difluoro-L-homocysteine (46)
To the thiol (44) (0.38g, 1.03 mmol) in dioxane (10 ml) was added HCl (6 M, 10 ml) and the solution stirred at 50° overnight. The solvent was removed *in vacuo* and the residue taken up in H_2O. After washing with Et_2O (2x) the aqueous layer was freeze dried to give difluoro L-homocysteine (12) (0.27g, 91%) as a white solid. The amino acid (17) (0.21g, 1.01 mmol) and diphenylmethanol (0.21g, 1.14 mmol) were dissolved in trifluoroacetic acid (5 ml) and the solution stirred under Ar at room temperature for 1 h. After evaporation of the trifluoroacetic acid, Et_2O (10 ml) was added then 10% aq. NaOAc until pH 5-6. After cooling to 0° the solid was filtered washing with cold Et_2O and H_2O. The solid residue was dried overnight to give the title compound (46) (0.22g, 64%).

S-Benzhydryl-N-(t-butyloxycarbonyl)-β,β-difluoro-L-homocysteine benzhydryl ester (47)
S-Benzhydryl difluoro homocysteine (46) (0.041g, 0.12 mmol) was added to H_2O (5 ml). A solution of di-*t*-butyl dicarbonate (0.026g, 0.12 mmol) in dioxane (5 ml) was added followed by enough triethylamine to effect solution. The reaction was stirred at room temperature overnight. After dilution with H_2O and acidification with 10% HCl the mixture was extracted with Et_2O (3x). The combined extracts were washed with brine, dried ($MgSO_4$) and the solvent evaporated. The crude residue was dissolved in MeCN (10 ml) and excess diphenyldiazomethane (0.1g) in MeCN (5 ml) was added. The solution was stirred at room temperature for 30 min. Excess reagent was destroyed by the addition of HOAc (1 ml) then the solvent removed *in vacuo*. Recrystallisation of the residue from Et_2O/petrol provided the fully protected amino acid (47) (0.029g, 39%): mp 116°; $[\alpha]_D$ +11.1 (c0.4, $CHCl_3$); (Found: C, 69.44; H, 6.26; N, 2.41%. $C_{35}H_{35}F_2NO_4S$ requires C, 69.63; H, 5.84; N. 2.32%); ν_{max}: 3411, 1747, 1720, 1496, 1452, 1256, 1161, 1017, 981 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 1.43 (9H, s, Bu^t), 2.80 (2H, t, *J* 15.4 Hz, H-4), 5.18 (1H, br q, H-2), 5.34 (1H, br d, NH), 5.37 (1H, s, SC*H*Ph_2), 6.93 (1H, s, OC*H*Ph_2), 7.24-7.42 (20H, m, Ar); δ_C (125 MHz, $CDCl_3$): 28.2q, 34.9tt (J_{CF} 27 Hz), 54.4d, 56.7td (J_{CF} 27 Hz), 79.2d, 80.9s, 121.7ts (J_{CF} 250 Hz), 127.1d, 127.1d, 127.5d, 128.2d, 128.6d, 130.0s, 132.3s, 139.1s, 140.2s, 154.8s, 166.3s; δ_F (235 MHz, $CDCl_3$): -103.5, -104.2 (ABq, *J* 249 Hz). m/z (FAB): 626 (MNa^+, 2%), 167 (100), 57 (20).

N-p-Methoxybenzyloxycarbonyl-α-p-methoxybenzyl ester L-δ-(α-aminoadipoyl)-S-benzhydryl-β,β-difluoro-L-homocysteinyl-D-valine benzhydryl ester (50)
Triethylamine (0.048 ml, 0.49 mmol) was added to a solution of diprotected amino adipic acid (48) (0.20g, 0.45 mmol) cooled to -10° under Ar. After stirring for 15 min isobutyl chloroformate (0.064 ml, 0.49 mmol) was introduced, the cooling bath removed and stirring continued for 30 min. S-Benzhydryl difluoro-L-homocysteine (46) (0.13g, 0.38 mmol) in H_2O (5 ml), containing enough triethylamine to effect solution, was added to the reaction and the solution stirred at 0° for 1 h then room temperature for 1.5 h. After the addition of H_2O and Et_2O extraction (2x) the aqueous layer was acidified (10% HCl) then extracted with EtOAc (2x). The combined EtOAc extracts were washed with brine, dried ($MgSO_4$) and the solvent evaporated to give dipeptide (51) (0.096g, 32%) which was used without further purification. Dipeptide (51) (0.096g, 0.12 mmol) together with D-valine benzhydyl ester (49) (0.039g, 0.14 mmol) and EEDQ (0.034g, 0.14 mmol) in dry CH_2Cl_2 (2 ml) was stirred with anhydrous Na_2SO_4 (0.05g) at room temperature under Ar overnight. After evaporation to dryness the residue was dissolved in EtOAc, washed successively with saturated aqueous $NaHCO_3$, 10% HCl and brine then dried ($MgSO_4$) and the solvent removed *in vacuo*. Recrystallisation from CH_2Cl_2/petrol provided the protected tripeptide (50) (0.053g, 41%): mp 129-130°; $[\alpha]_D$ +0.4 (c0.5, $CHCl_3$); (Found: C, 67.90; H, 5.92; N, 4.16%. $C_{58}H_{61}F_2N_3O_{10}S$ requires C, 67.62; H, 5.97; N, 4.08%); ν_{max}: 3428, 1734, 1680, 1525, 1511, 1254, 1183 cm^{-1}; δ_H (500 MHz, $CDCl_3$): 0.78, 0.89 (2x3H, d, *J* 6.9 Hz, CH*Me*$_2$), 1.55-1.68 (4H, 2xm, C*H*$_2$C*H*$_2$CH$_2$CO), 2.07-2.29 (3H, 2xm, CH$_2$CO, C*H*Me$_2$), 2.85 (2H, t, *J* 16.4 Hz, CH$_2$S), 3.78 (6H, s, OMe), 4.35 (1H, m, Hα-AA), 4.70 (1H, dd, *J* 8.7, 4.3 Hz, Hα-Val), 5.02 (2H, ABq, *J* 11.8 Hz, OCH$_2$), 5.09 (2H, s, OCH$_2$), 5.15 (1H, dt, *J* 18, 9 Hz, Hα-Cys), 5.36 (1H, s, SC*H*Ph_2), 5.41 (1H, d, *J* 7.8 Hz, NH), 6.57 (1H, d, *J* 8.4 Hz, NH), 6.90 (1H, s, OC*H*Ph_2), 6.85-6.87 and 7.21-7.41 (28H, m, Ar); δ_C (125 MHz, $CDCl_3$): 17.2q, 18.9q, 21.0t, 31.4d, 31.9t, 35.0tt (J_{CF} 27 Hz), 35.1t, 53.5d, 54.5d, 55.2td (J_{CF} 28 Hz), 55.2q, 55.2q, 57.6d, 66.8t, 67.0t, 78.2d, 113.9d, 114.0d, 121.9ts (J_{CF} 248 Hz), 127.0d, 127.4d, 127.5d, 128.0d, 128.2d, 128.4d, 128.5d, 128.6d, 129.9d, 130.1d,

139.3s, 139.5s, 140.3s, 140.3s, 156.1s, 159.6s, 159.8s, 165.1s, 170.2s, 172.0s, 172.5s. δ_F (235 MHz, $CDCl_3$): -102.8, -105.1 (ABq, *J* 249 Hz); m/z (FAB): 1029 (MH^+).

L-δ-(α-Aminoadipoyl)-L-(3,3-difluorohomocystinyl)-D-valine (16)
To the protected tripeptide (50) (57 mg, 0.055 mmol) was added anisole (0.5 ml) then trifluoroacetic acid (5.0 ml) and the solution refluxed under Ar for 30 min. The trifluoroacetic acid was evaporated removing the final traces by azeotroping with toluene (2x) and the residue was partitioned between EtOAc and H_2O. The aqueous layer was basified to pH 9-10 with saturated aqueous $NaHCO_3$ and O_2 bubbled through for 4h. Purification by reverse phase HPLC (ODS, 30% MeOH/ 25 mM NH_4HCO_3) and freeze drying yielded tripepetide (16) in the disulphide form (11 mg, 38%): δ_H (500 MHz, D_2O): 0.87, 0.91 (2x3H, d, *J* 8.6 Hz, CH_3), 1.65-1.73 and 1.82-1.85 (2x2H, m, $CH_2CH_2CH_2CO$), 2.12-2.17 (1H, m, $CH(CH_3)_2$), 2.43 (2H, t, *J* 7.2 Hz, CH_2CO), 3.46 (2H, t, *J* 16.2, CH_2S), 3.71 (1H, t, *J* 5.8 Hz, Hα-AA), 4.12 (1H, d, *J* 5.5 Hz, Hα-Val), 5.20 (1H, dd, *J* 17.3, 7.7 Hz, $CHCF_2$); δ_F (235 MHz, D_2O): -104.5, -105.9 (ABq, *J* 250 Hz); m/z (electrospray): 825 (MH^+, 45%), 413 ($0.5MH_2^{2+}$, 100).

Incubation of δ-(L-α-aminoadipoyl)-L-(3,3-difluorohomocystinyl)-D-valine (16) with IPNS
IPNS in Tris buffer (30 IU/ml, 1 ml) was exchanged with aqueous NH_4HCO_3 (50 mM, 3.5 ml) on a Sephadex G-25 gel filtration column. This solution was added to a mixture of ascorbic acid (5 mM, 0.1 ml), dithiothreitol (100 mM, 0.1 ml), iron (II) sulfate (5 mM, 0.05 ml) and tripeptide (16) (1.5 mg), and the volume made up to 5.0 ml by the addition of NH_4HCO_3 (50 mM) buffer. The mixture was divided into two portions and shaken (250 rpm) at 28° for 15 min. Further dithiothreitol (100 mM, 0.05 ml) was added to each and shaking continued for 45 min. After protein precipitation by the addition of acetone and centrifugation the supernatant was decanted. The solvent was removed and the residue freeze dried. Purification by reverse phase HPLC (ODS, NH_4HCO_3 25 mM) gave the thiocarboxylic (52) and carboxylic (53) acids in the ratio (52):(53), >5:1.
(52): δ_H (500 MHz, D_2O): 0.89, 0.93 (2x3H, d, *J* 6.8 Hz, CH_3), 1.65-1.78 and 1.82-1.94 (2x2H, m, $CH_2CH_2CH_2CO$), 2.12-2.18 (1H, m, $CHMe_2$), 2.43 (2H, t, *J* 7.1 Hz, CH_2CO). 3.74 (1H, t, *J* 6.2 Hz, Hα-AA), 4.12 (1H, d, *J* 5.2 Hz, Hα-Val), 5.62 (1H, dd, *J* 14.3, 11.6 Hz, $CHCF_2$); δ_F (235 MHz, D_2O): -106.4, -107.8 (ABq, *J* 244 Hz); m/z (electrospray): 450 (MNa^+, 27%), 430 (4), 429 (20)428 (MH^+, 100).
(53): δ_H (500 MHz, D_2O): 0.88, 0.91 (2x3H, d, *J* 6.8 Hz, CH_3), 1.65-1.76 and 1.80-1.94 (2x2H, m, $CH_2CH_2CH_2CO$), 2.10-2.16 (1H, m, $CHMe_2$), 2.43 (2H, t, *J* 7.6 Hz, CH_2CO). 3.72 (1H, t, *J* 5.8 Hz, Hα-AA), 4.11 (1H, d, *J* 5.7 Hz, Hα-Val), 5.33 (1H, dd, *J* 14.0, 12.7 Hz, $CHCF_2$); δ_F (235 MHz, D_2O): -111.6, -113.0 (ABq, *J* 250 Hz); m/z (electrospray): 434 (MNa^+, 29%), 412 (MH^+, 100).
The same incubation carried out under an atmosphere of $^{18}O_2$ gas afforded, after HPLC purification, the thiocarboxylic acid (52); identical by ^1H and ^{19}F NMR to the previous sample, m/z (electrospray): 452 (MNa^+, 21%), 435 (5), 432 (6), 431 (27), 430 (MH^+, 100) [450 and 428 (<5)]..

Derivatisation of carboxylic acid (53)
To a solution of acid (53) in H_2O (2 ml) was added saturated aqueous $NaHCO_3$ (ca. 0.10 ml) to give a pH around 8-9. Diethyl pyrocarbonate (0.150 ml) was added and the mixture stirred at room temperature for 2 h. The solution was extracted with CH_2Cl_2 (3x2 ml) then acidified to pH 1 with 10% HCl. The aqueous layer was extracted with EtOAc (3x5 ml), dried over $MgSO_4$ and filtered. To this solution was added freshly prepared ethereal diazomethane solution and the reaction stirred at room temperature for 30 min. N_2 was bubbled through until the yellow colour disappeared and the solvent removed *in vacuo* to afford a complex mixture by ^1H NMR. The presence of the *N*-ethoxycarbonyl trimethyl ester derivative (56) was indicated by the mass spectrum; m/z DCI(NH_3): 529 (6%), 527 (38), 526 (MH^+, 100),480 (42), 230 (44), 167 (85), 121 (100).

Derivatisation of thiocarboxylic acid (52)
Derivatisation of thioacid (52), from the $^{18}O_2$ experiment, according to the above procedure for acid (53) gave a complex mixture by ^1H NMR. The mass spectrum showed the *N*-ethoxycarbonyl trimethyl ester derivative (57); m/z DCI(NH_3): 545 (28%), 544 (MH^+, 40), 442 (80) 426 (77), 412 (80), 225 (100).

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