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# ISOLATION OF DIHYDROCLAVAMINIC ACID, AN INTERMEDIATE IN THE BIOSYNTHESIS OF CLAVULANIC ACID

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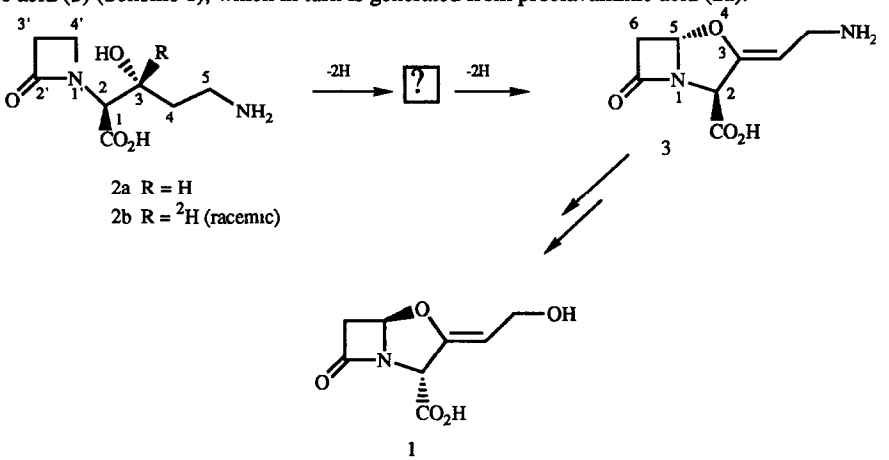
Abstract: A primary isotope effect was utilised in an *in vitro* study to allow the isolation and characterisation of an intermediate between proclavaminic acid and clavaminic acid, in clavulanic acid biosynthesis.[1]

Clavulanic acid (1), a medicinally important bicyclic β-lactam produced by *Streptomyces clavuligerus*, is a potent inhibitor of β-lactamases from both Gram-positive and Gram-negative organisms and also possesses weak antibacterial activity. *In vivo* studies involving the feeding of labelled precursors have established that the carbons of the beta-lactam ring are derived from glycerol[2] and those of the C-5 unit from L-ornithine[3]. Subsequent *in vitro* studies have demonstrated that the biosynthesis of clavulanic acid (1) proceeds *via* clavaminic acid (3) (Scheme 1), which in turn is generated from proclavaminic acid (2a).[4,5]




2a R = H
2b R = $^2$H (racemic)

3

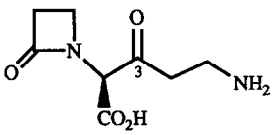
1

**Scheme 1**

The four electron oxidative cyclisation of proclavaminic acid (2a) to clavaminic acid (3) has been demonstrated to be carried out by an α-ketoglutarate and ferrous dependent oxygenase, clavaminic acid synthase (CAS).[4,5] Thus this enzyme has similar requirements to other oxygenases involved in penicillin and cephalosporin

biosynthesis.[6] Townsend et al have established that the oxygen of the 3-OH of proclavaminic acid (2a) gives rise to the oxygen of the oxazolidine ring of (3),[7] and that the ring closure goes with retention of configuration at the C-4' position of (2a).[8]

In the conversion of proclavaminic acid (2a) to clavaminic acid (3) two distinct chemical events must occur, closure of the oxazolidine ring and desaturation to form the exocyclic double bond, presumably each event being accompanied by the concomitant turnover of at least one equivalent of α-ketoglutarate and dioxygen to succinate and carbon dioxide.[4,9] Thus we considered the possible existence of a stable enzyme free intermediate between (2a) and (3), such as the 3-keto compound (4) or the bicyclic clavam (5a). This paper describes in full detail the experiments by which we determined the latter, dihydroclavaminic acid (5a), to be an intermediate between proclavaminic acid (2a) and clavaminic acid (3), in *in vitro* studies.



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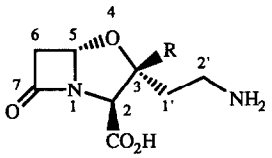
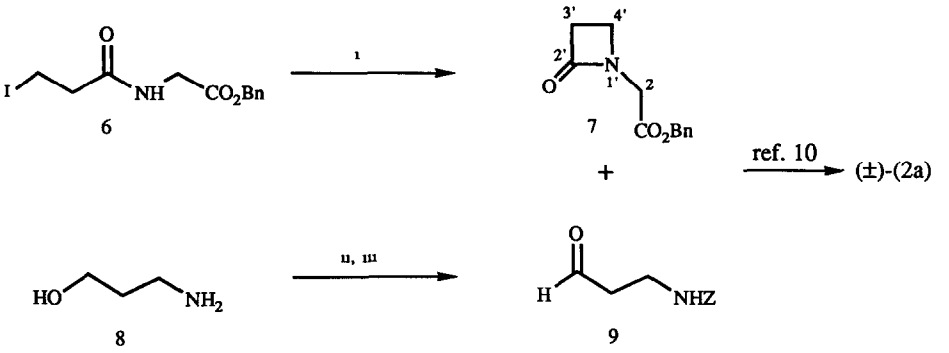


5a R = H
5b R = $^{2}$H
5c = Enantiomer of 5a

Racemic proclavaminic acid (2a) used in this study was synthesised in accord with the reported route[10], as outlined in Scheme 2 ; modifications are described in the experimental section.

Initially we carried out a careful study by $^{1}$H NMR (500MHz) of the incubation of racemic proclavaminic acid (2a) with partially purified CAS from *Streptomyces clavuligerus*.[11] In addition to the signals corresponding to clavaminic acid (3) there was a minor resonance at *ca* 5.4ppm, approximately 5-10% of the intensity of the clavaminic acid resonances, which occurred as an apparent doublet (J 2.5Hz) and thus was reminiscent of the C-5 proton of related dihydroclavulanates.[12] (Figure 1a). Attempts were made to purify this minor product by h.p.l.c. ( at pH 7-8), however, on the small scale (less than 1mg. of (2a)) on which we were working it proved impossible to obtain a sample of sufficent purity for full characterisation. We reasoned that if dihydroclavaminic acid (5a) was indeed an intermediate in the conversion of (2a) to (3), incubation of a proclavaminic acid deuteriated at C-3, *i.e.* (2b), should result in the operation of a primary isotope effect, slowing down the conversion of a deuteriated intermediate (5b) to (3), thus allowing (5b) to accumulate. Recently we used an analogous strategy to bias the production of a shunt metabolite during the enzymatic ring expansion of penicillin N to cephalosporins.[13]




**Scheme 2.** *Reagents and conditions:* Z= $PhCH_2OCO$-; i: NaH, DMF, -10°C; ii: Z-Cl, $CH_2Cl_2$, $Et_3N$; iii: $(COCl)_2$, DMSO, $Et_3N$, $CH_2Cl_2$, -50°C.

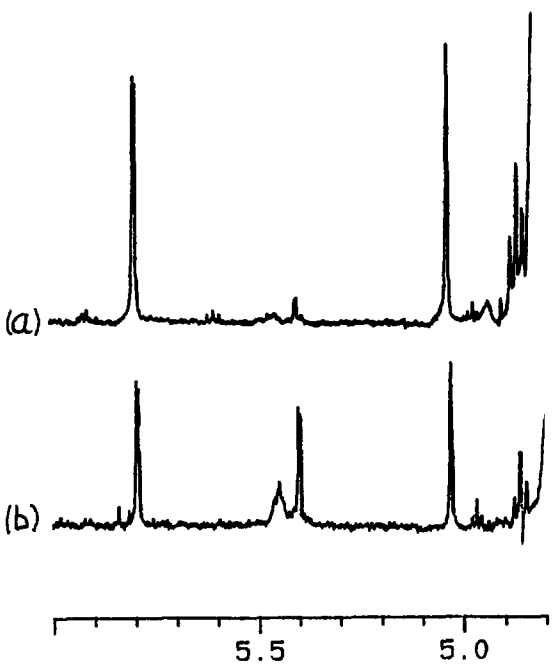
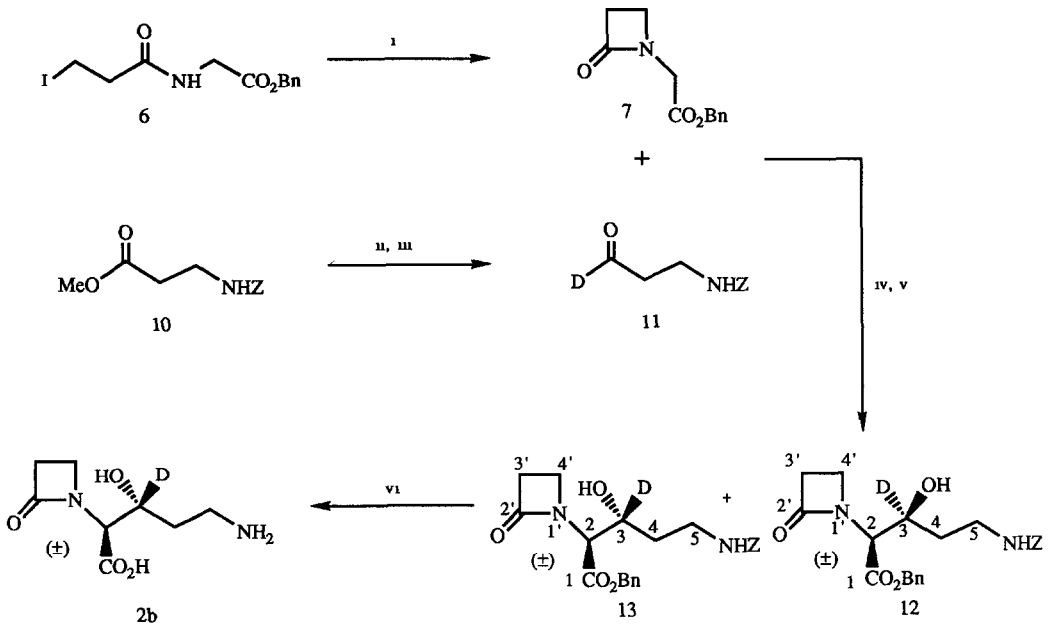



Figure 1 (a) 500 MHz $^1$H-NMR Spectrum of incubation of racemic 2a ;
(b) 500 MHz $^1$H-NMR Spectrum of incubation of racemic 2b.

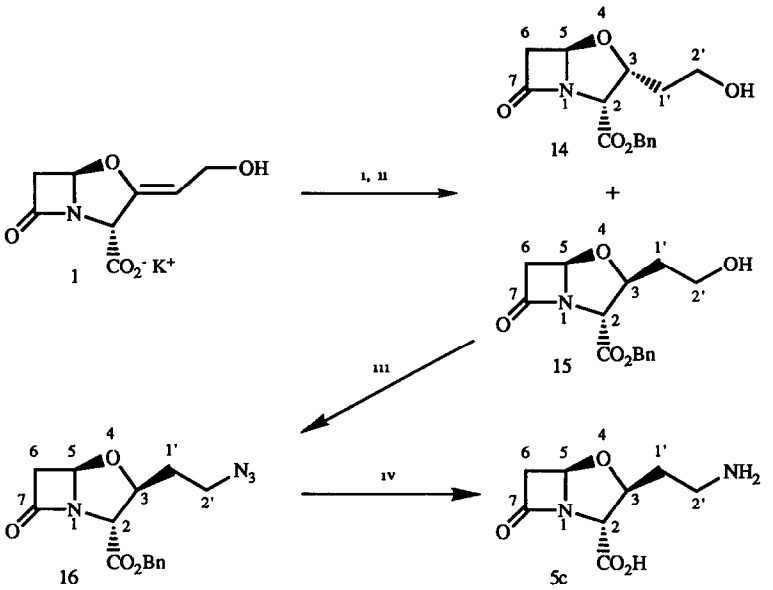
Racemic deuteriated proclavaminic acid (2b) was synthesised in a similar fashion to the fully protiated material using the deuteriated aldehyde (11) derived from methyl N-benzyloxycarbonyl β-alaninate (10) (scheme 3). This was then incubated with partially purified CAS as before. $^1$H NMR examination of the products revealed a large enhancement of the signal at *ca* 5.4ppm (Figure 1b). The component responsible for this signal was

isolated by reverse phase h.p.l.c. and 500MHz $^1$H NMR revealed it to have a spectrum consistent with that for the dihydroclavaminic acid (5b).



**Scheme 3.** *Reagents and conditions:* Z= $PhCH_2OCO$-; i: NaH, DMF, -10°C; ii: $LiAlD_4$, THF, 0°C; iii: $(COCl)_2$, DMSO, $Et_3N$, $CH_2Cl_2$, -50°C; iv: $(TMS)_2NLi$, THF, -78°C, then AcOH; v: DBN, $CH_2Cl_2$, then separate diastereomers by flash chromatography; vi: $H_2$, Pd/C 10%, EtOH/$H_2O$.

To confirm this we undertook a synthesis of (5c), the enantiomer of (5a), from clavulanic acid (Scheme 4). Initially the double bond of potassium clavulanate was reduced by catalytic hydrogenation, and subsequent benzylation yielded the two alcohols (14) and (15) which were separated by preparative HPLC. The alcohol moiety of (15) was substituted for azide to give (16) via a Mitsunobu type process involving diphenyl phosphoryl azide.[14] Catalytic hydrogenation of (16), furnished the desired enantiomeric dihydroclavaminic acid (5c). The relative stereochemistries of the two alcohols (14) and (15) were assigned using nuclear Overhauser effect (n.O.e) experiments and by comparison to literature data.[15] For (15) irradiation of 3-H gave an n.O.e. (5%) to 5-H, whereas irradiation of 1'-H gave no n.O.e. to 5-H. For (14) irradiation of 3-H gave no n.O.e to 5-H, whereas irradiation of 1'-H gave an n.O.e. (3%) to 5-H. We also performed n.O.e. experiments on the amino acid (5c) for which irradiation of 3-H gave an n.O.e. (8%) to 5-H, whereas irradiation of 1'-H gave no n.O.e. to 5-H, thus confirming the relative stereochemistry of (5c) to be as depicted.

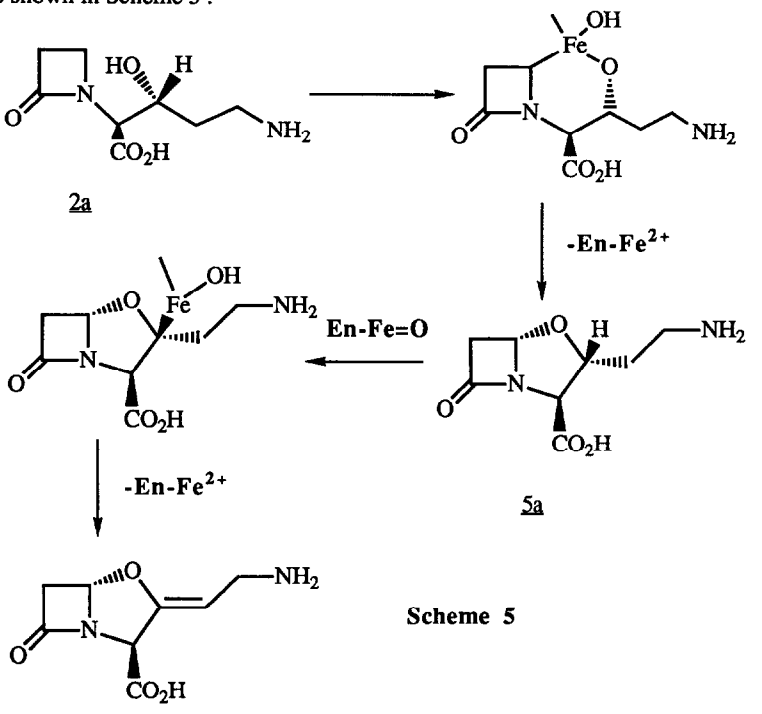


**Scheme 4.** *Reagents and conditions*: i: $H_2$ , 10% Pd on C, pH 6.5; ii: $PhCH_2Br$, DMF, ratio 14:15, 4:1; iii: $Ph_3P$, DEAD, diphenylphosphoryl azide, THF; iv: $H_2$, Pd-C (5%), $EtOH/H_2O$ 1:1.

$^1H$ NMR spectral comparison of the synthetic clavam (5c) with the biologically derived sample (5b) showed them to be identical except for the effects of the latter being deuteriated at C-3. This was also confirmed by doping a sample of the biological material with the synthetic prior to NMR analysis.

A pure sample of (5b) was re-incubated with a highly purified sample of CAS and shown to be efficiently converted to clavaminic acid (3), confirming it to be true intermediate in the conversion of (2) to (3) rather than a shunt metabolite. Thus, in the conversion of (2) to (5) CAS appears to perform an oxidative cyclisation/ desaturation process, rather than the hydroxylation reactions which are more usually associated with this class of oxygenase.[16] This process [(2) to (5)] is analogous to the ring expansion of penicillin N to deacetoxycephalosporin C which is also catalysed by an α-ketoglutarate dependent oxygenase.[6] In common with some other α-ketoglutarate dependent oxygenases such as deacetoxy/deacetyl cephalosporin C synthase (DOAC/DAC synthase) from *Cephalosporium acremonium* [6] and thymine hydroylase,[17] CAS appears to be able to catalyse efficiently the oxidation of sequential intermediates in a biosynthetic pathway. Recently Townsend *et al* have published a purification protocol and partial characterisation of CAS.[9] Based upon kinetic studies they propose that two stepwise oxidations of proclavaminic acid occur, a proposal consistent with our results. They conclude, in contrast to our own *in vitro* results, that there is not a "substantial release of an intermediate" from CAS. It is possible that this discrepancy may be explained by the existence of more than one form of CAS, in a similar fashion to the cephalosporin biosynthesis pathway in *S.clavuligerus* in which two forms of DAOC/DAC synthase both catalyse sequential oxidative steps with different but complimentary specific activities.[18] Further investigations are in progress. A mechanistic hypothesis for the operation of CAS, involving sequential

oxidations, carried out by a ferryl iron species, generated by reaction of CAS with α-ketoglutarate and dioxygen, is shown in Scheme 5 .




**Scheme 5**

## Experimental

Infrared (IR) spectra were recorded on a Perkin-Elmer 681 spectrometer with only selected absorptions being reported. Absorbances are quoted with relative intensities; s = strong, m = medium, and w = weak. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM-500 and Varian Gemini-200 spectrometers. Spectral data recorded using $CDCl_3$ as solvent are reported with chemical shifts quoted in parts per million (δ p.p.m.) using the residual solvent peak as an internal reference. Spectral data recorded using $D_2O$ as solvent are reported with chemical shifts quoted in parts per million (δ p.p.m.) using sodium 3-trimethylsilyl tetradeuteriopropionate (TSP) as an internal reference. Coupling constants (J) are quoted to the nearest 0.5Hz. $^{13}C$ spectra were run using DEPT editing. Mass spectra were recorded on a V. G. Micromass ZAB 1F (DCI), a V. G. 20-250 (DCI/CI/FAB$^+$), a V. G. TRIO 1 (GCMS) spectrometers or a V. G. Micromass Electrospray spectrometer. Peaks are quoted with percentage relative intensities in brackets. Flash chromatography was accomplished on silica gel using Sorbsil™ C60. Thin layer chromatography was performed on aluminium sheets pre-coated with Merck silica gel 60 $F_{254}$, plates being visualised with UV (254nm) or 10%w/v ammonium molybate in 2M sulphuric acid, followed by heat. HPLC of synthetic compounds was carried out on a Gilson 303 HPLC using a capped $SiO_2$ semi-preparative column (normal phase) or a Hypersil semi-

preparative column (octadecylsilane reverse phase). Detection was via a Holochrome U.V. detector at 245 or 220nm respectively. All solvents were distilled before use; tetrahydrofuran (THF) from sodium/benzophenone ketyl, acetonitrile, dimethylsulphoxide (DMSO), and dichloromethane (DCM) from calcium hydride. Dimethylformamide (DMF) was obtained anhydrous from the Aldrich Chemical Company. Triethylamine and 1,5-diazabicyclo[4,3,0]non-5-ene (DBN) were distilled from calcium hydride before use. All other reagents were used as obtained from commercial sources.

**Benzyl 3-iodopropionamidoacetate (6)**

Benzyl 3-bromopropionamidoacetate[10] (3.0g, 10mmol) was dissolved in acetone (50ml) and stirred in the dark with NaI (5.25g, 35mmol). After 24 h the solution was poured into water and extracted three times with EtOAc. The organic layer was separated and washed with sodium thiosulphate solution (5% aq.). The organic phase was separated, dried ($MgSO_4$), and the solvent removed *in vacuo*. The residue was recrystallised from $CH_2Cl_2$:Hexane to give (6) as a white solid (3.05g 88%), which was used directly. $^1H$ NMR (200MHz, $CDCl_3$) δ: 2.81 (2H, t, J=7Hz, C$\underline{H_2}$-CON); 3.38 (2H, t, J=7Hz, I-C$\underline{H_2}$), 4.12 (2H, d, J=5Hz, N-C$\underline{H_2}$), 5.21 (2H, s, C$\underline{H_2}$Ph), 6.22 (1H, br.s, N-$\underline{H}$), 7.37 (5H, s, Ph). $^{13}C$ NMR (50.4MHz, $CDCl_3$) δ: -2.6 ($CH_2$); 40.0 ($CH_2$), 41.4 ($CH_2$), 67.3 ($CH_2$), 128.6 (CH), 128.8 (CH), 135.2 (C), 169.9 (C), 171.0 (C). MS (m/e): 348 ($[MH]^+$, 10); 220 (21), 108 (88), 91 (100). IR ($CHCl_3$) $\nu_{max}$ ($cm^{-1}$): 700 (m), 950 (w), 1200 (s), 1395 (m), 1520 (s), 1680 (s), 1745 (s), 3020 (m), 3440 (m).

**Benzyl (2-oxoazetidin-1-yl)acetate (7)**

Benzyl 3-iodopropionamidoacetate (6) (500mg, 1.44mmol) in dry DMF (20ml) was added dropwise over 3 h to a suspension of NaH (60% dispersion in oil, 63mg, 1.44mmol) in DMF at -10°C. After the addition was complete the stirring was continued for a futher 3 h. The solvent was removed *in vacuo*, the residue dissolved in EtOAc, and washed with $H_2SO_4$ (0.5M) followed by sodium thiosulphate solution (5% aq.). The organic phase was separated, dried ($MgSO_4$), and the solvent removed *in vacuo*. The residue was purified by flash chromatography (silica, EtOAc:Hexane, 2:3) to give the β-lactam (7) as a colourless oil (114mg, 36%). $^1H$ NMR (200MHz, $CDCl_3$) δ: 3.03 (2H, t, J=4Hz, 2xH-3'), 3.42 (2H, t, J=4Hz, 2xH-4'), 4.01 (2H, s, H-2), 5.18 (2H, s, C$\underline{H_2}$Ph), 7.37 (5H, s, Ph). $^{13}C$ NMR (50.4MHz, $CDCl_3$) δ: 37.6 ($CH_2$), 39.9 ($CH_2$), 43.0 ($CH_2$), 67.2 ($CH_2$), 128.6 (CH), 128.7 (CH), 128.8 (CH), 135.2 (C), 168.2 (C), 168.4 (C). MS (m/e): 220 ($[MH]^+$, 100), 178 (23), 108 (13), 91 (13). IR ($CHCl_3$) $\nu_{max}$ ($cm^{-1}$): 700 (m), 965 (w), 1130 (w), 1195 (s), 1410 (s), 1745 (s), 3010 (m). This sample gave identical NMR data to a sample prepared by an analogous route.[10]

**N-Benzyloxycarbonyl-3-aminopropionaldehyde (9)[10]**

Oxalyl chloride (0.92ml, 10.5mmol) was dissolved in $CH_2Cl_2$ (16ml) and cooled to -50°C. Dry DMSO (1.49ml, 21.1mmol) in $CH_2Cl_2$ (8ml) was then added. After 2 mins at -50°C N-benzyloxycarbonyl-3-amino

propan-1-ol[19] (2.0g, 9.57mmol) in $CH_2Cl_2$ (20ml) was added dropwise over 10 mins, keeping the temperature below -50°C at all times. After the addition was complete the reaction was stirred for 15 mins before triethylamine (6.7ml, 47.9mmol) was added. After 5 mins the reaction was allowed to warm to ambient temperature. The mixture was diluted with $CH_2Cl_2$ and washed with water. The organic phase was separated, dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was purified by flash chromatography (silica, EtOAc:Hexane, 3:2) to give (9) as a white solid (1.55g, 78%) on removal of the solvent. $^1H$ NMR (200MHz, $CDCl_3$) δ: 2.75 (2H, t, J=6Hz, C<u>H</u>$_2$-CO), 3.50 (2H, d of t, J=6, 6Hz, C<u>H</u>$_2$-N), 5.10 (2H, s, Ph-C<u>H</u>$_2$), 5.21 (1H, br.s, N-H), 7.35 (5H, s, Ph), 9.80 (1H, s, CO<u>H</u>). $^{13}C$ NMR (50.4MHz, $CDCl_3$) δ: 34.3 ($CH_2$), 43.9 ($CH_2$), 66.6 ($CH_2$), 128.1 (CH), 128.7 (CH), 136.5 (C), 201.5 (CH). MS (m/e): 208 ($[MH]^+$, 39); 108 (100), 91 (57). IR ($CHCl_3$) $\nu_{max}$ ($cm^{-1}$): 700 (w), 1030 (w), 1140 (w), 1230 (m), 1515 (s), 1720 (s), 3450 (m).

**Racemic Proclavaminic acid (2a)**

Racemic *threo*-benzyl-N$^5$-benzyloxycarbonylamino-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (205mg, 0.48mmol) [prepared from (9) and (7) as previously described[10]] was dissolved in EtOH:$H_2O$ (20ml, 7:3) and stirred over Pd-C (10%, 100mg) under a positive pressure of hydrogen. After 3.5 h the mixture was filtered through celite and the solvent removed *in vacuo* to give racemic proclavaminic acid (2a) as a white solid (95mg, 98%). $^1H$ NMR (500MHz, $D_2O$) δ: 1.80-1.94 (2H, m, 2xH-4); 3.03 (2H, t, J=4Hz, 2xH-3'); 3.12-3.22 (2H, m, 2xH-5); 3.51-3.55 and 3.58-3.62 (2H, 2 x m, 2xH-4'); 4.09 (1H, d,J=5.5Hz, H-2); 4.20-4.24 (1H, m, H-3). MS (m/e electrospray) 203 ($[MH]^+$). The data reported here were consistent with those obtained from a sample prepared previously.[10]

**N-Benzyloxycarbonyl-1,1-di-[$^2$H]-3-aminopropan-1-ol**

Lithium aluminium deuteride (1.0g, 24mmol), was suspended in dry THF (180ml) and cooled to -5°C. Methyl N-(benzyloxycarbonyl)-β-alaninate[20] (7.5g, 32mmol) in THF (180ml) was then added dropwise keeping the temperature below 0°C. After the addition was complete the mixture was stirred at 0°C for a further 50 mins. The mixture was then cautiously poured into dilute $H_2SO_4$ (0.5M) and then extracted with ether. The organic layer was then separated and washed with brine. The organic layer was separated, dried ($MgSO_4$), and the solvent removed *in vacuo*. The residue was purified by flash chromatography (silica, EtOAc:Hexane, 3:2) to give the title compound as a colourless oil (4.50, 68%), which was used directly. $^1H$ NMR (200MHz, $CDCl_3$) δ: 1.68 (2H, t, J=7Hz, $CH_2$-C<u>H</u>$_2$-$CD_2$), 2.81 (1H, br.s, O<u>H</u>), 3.30 (2H, m, C<u>H</u>$_2$-N), 5.10 (2H, s, PhC<u>H</u>$_2$), overlayed on 5.12 (1H, br.s, N-H), 7.34 (5H, s, Ph). $^{13}C$ NMR (50.4MHz, $CDCl_3$) δ: 32.3 ($CH_2$); 37.7 ($CH_2$), 66.7 ($CH_2$), 128.2 (CH), 128.7 (CH), 136.6 (C), 157.5 (C). MS (m/e): 212 ($[MH]^+$, 2); 207 (9), 121 (17), 104 (100), 91 (7). IR ($CHCl_3$) $\nu_{max}$ ($cm^{-1}$): 700 (m), 1020 (w), 1140 (m), 1255 (s), 1520 (s), 1710 (s), 3010 (m), 3460 (m).

**N-Benzyloxycarbonyl-1-[$^2$H]-3-aminopropionaldehyde (11)**

Oxalyl chloride (0.39ml, 4.48mmol) was dissolved in $CH_2Cl_2$ (10ml) and cooled to -50°C. Dry DMSO (0.64ml, 8.97mmol) in $CH_2Cl_2$ (5ml) was then added. After 2 mins at -50°C N-benzyloxycarbonyl 1,1-di-[$^2$H]-3-amino propan-1-ol (0.85g, 4.1mmol) in $CH_2Cl_2$ (10ml) was added dropwise over 10 mins, keeping the temperature below -50°C at all times. After the addition was complete the reaction was stirred for 15 mins before triethylamine (2.75ml, 19.8mmol) was added. After 5 mins the reaction was allowed to warm to ambient temperature. The mixture was diluted with $CH_2Cl_2$ and washed with water. The organic phase was separated, dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was purified by flash chromatography (silica, EtOAc:Hexane, 3:2) to give (11) as a white solid (510mg, 60%) on removal of the solvent. $^1$H NMR (200MHz, $CDCl_3$) δ: 2.75 (2H, t, J=6Hz, C<u>H</u>$_2$-CO), 3.50 (2H, t, J=6Hz, C<u>H</u>$_2$-N), 5.10 (2H, s, Ph-C<u>H</u>$_2$), 5.21 (1H, br.s, N-H); 7.35 (5H, s, Ph). $^{13}$C NMR (50.4MHz, $CDCl_3$) δ: 34.3 ($CH_2$); 43.9 ($CH_2$), 66.6 ($CH_2$), 128.1 (CH); 128.7 (CH), 136.5 (C). MS (m/e): 209 ([MH]$^+$, 35), 108 (100), 91 (43). IR ($CHCl_3$) $\nu_{max}$ (cm$^{-1}$): 700 (m), 1030 (m), 1140 (m), 1230 (s), 1515 (s), 1720 (s), 3450 (m).

**Racemic-*threo*-benzyl-N$^5$-benzyloxycarbonylamino-3-[$^2$H]-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (13)**

Racemic *threo*-benzyl-N$^5$-benzyloxycarbonylamino-3-deutero-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate was prepared from (11) (485 mg) in 54% yield by the published procedure[10]. For (13) $^1$H NMR (200MHz, $CDCl_3$) δ: 1.6-1.8 (2H, t, J=7Hz, 2xH-4), 2.95-3.05 (2H, m, 2xH-3'), 3.2-3.53 (4H, m, 2xH-4' and 2xH-5), 4.15 (1H, s, H-2), 4.50 (1H, s, O<u>H</u>), 5.10 and 5.18 (4H, 2xs, urethane C<u>H</u>$_2$Ph and ester C<u>H</u>$_2$Ph), 5.28 (1H, s, N<u>H</u>), 7.37 (10H, s, Ph). $^{13}$C NMR (125.8MHz, $CDCl_3$) δ: 34.2 ($CH_2$), 36.2 ($CH_2$), 37.6 ($CH_2$), 40.3 ($CH_2$), 61.7 (CH), 66.8 ($CH_2$), 67.4 ($CH_2$), 128.2 (CH), 128.3 (CH), 128.5 (CH), 128.7 (CH), 128.8 (CH), 135.3 (C), 136.5 (C), 157.4 (C), 168.8 (C), 169.6 (C). MS (m/e): 428 ([MH]$^+$, 75); 384 (10); 276 (13), 237 (10), 220 (63), 209 (54), 108 (37), 91 (100). IR ($CHCl_3$) $\nu_{max}$ (cm$^{-1}$): 700 (m), 1220 (m), 1410 (m), 1515 (s), 1730 (s), 3000 (m), 3460 (m).

**Racemic-3-[$^2$H]-proclavaminic acid (2b)**

*Threo*-benzyl-N$^5$-benzyloxycarbonylamino-3-[$^2$H]-3-hydroxy-2-(2-oxoazetidin-1-yl)valerate (13) (100mg, 0.23mmol) was dissolved in EtOH:$H_2O$ (20ml, 7:3) and stirred over Pd-C (10%, 57mg) under a positive pressure of hydrogen. After 3.5 h the mixture was filtered through celite and the solvent removed *in vacuo* to give racemic 3-[$^2$H]-proclavaminic acid (2b) as a white solid (40mg, 84%). $^1$H NMR (500MHz, $D_2O$): as for (2a) except 4.09 (1H, s, H-2) and H-3 absent. MS (m/e electrospray): 204 ([MH]$^+$, 100%), 203, 2%). The ratio of peaks at 204 and 203 indicated a deuterium incorporation of greater than 98%.

**(2*R*, 3*R*, 5*R*)- and (2*R*, 3*S*, 5*R*)-Benzyl-1-aza-3-(2'-hydroxyethyl)-4-oxa-7-oxobicyclo[3,2,0]heptane-2-carboxylate (14) and (15)**

The title compounds were prepared by a modification of the method of A.G Brown *et al*[15]. A solution of potassium clavulanate (11g, 46.6 mmol) in 0.1 M aqueous phosphate solution (100 ml) adjusted to pH 6.5 with sodium hydroxide was hydrogenated at ambient temperature and pressure with 10% Pd on carbon for 7 hours.

The catalyst was filtered off and washed, and the combined filtrates were evaporated to a small volume and treated with benzyl bromide (5 ml, 42 mmol) in DMF (80 ml) overnight. The reaction mixture was diluted with ethyl acetate ( 200 ml) and washed three times with a saturated brine solution, and once with a saturated aqueous $NaHCO_3$ solution. The organic layer was cooled to -70 $^0$C and ozone was passed through the solution until light blue (2-3 hours), when it was replaced by nitrogen and allowed to warm to room temperature. Chromatography of the residues of the evaporated mixture on silica gel in ethyl acetate:hexane (1:1) gave the title compound as a mixture of epimers (4.49g, 33%). A sample of the mixture of (15) and (14) (about 1:4) was separated by HPLC (hexane:$CH_2Cl_2$:EtOH, 6:3:0.2). Under these conditions, the minor epimer (15) eluted first, and the major (14) eluted second.

For (14) $^1$H NMR (200MHz, $CDCl_3$) δ: 1.6-1.8 (2H, m, 2xH-1'), 1.97 (1H, br.s, OH), 2.92 (1H, d, J=16Hz, H-6), 3.46 (1H, d of d, J=2.5, 16Hz, H-6), 3.72 (2H, m, 2xH-2'), 4.51-4.68 (1H, m, H-3), 4.71 (1H, d, J=7Hz, H-2), 5.19 (2H, s, $CH_2$Ph), 5.54 (1H, d, J=2.5Hz, H-5), 7.37 (5H, s, Ph). $^{13}$C NMR (125.8MHz, $CDCl_3$) δ: 32.6 ($CH_2$), 45.6 ($CH_2$), 60.0 ($CH_2$), 62.0 (CH), 67.5 ($CH_2$), 81.9 (CH), 85.3 (CH), 128.8 (CH), 134.9 (C), 167.8 (C), 176.0 (C). MS (m/e): 292 ([MH]$^+$, 33), 250 (75), 201 (100), 159 (30), 142 (50). IR (thin film) $\nu_{max}$ (cm$^{-1}$): 700 (m), 750 (m) 1005 (s), 1045 (m), 1190 (s), 1285 (m), 1660 (s), 1740 (s), 1785 (s), 2950 (m), 3440 (m). Acc. Mass measurement: 291.1113 (M$^+$), $C_{15}H_{17}NO_5$ requires 291.1107. N.O.e, data: Irradiation of H-1' gave n.O.e.s of 24% to H-2', 4% to H-2, 10% to H-3 and 3% to H-5. Irradiation of H-2 gave an n.O.e. of 7% to H-3. Irradiation of H-3 gave n.O.e.s of 8% to H-1', 4% to H-2' and 11% to H-2. Irradiation of H-5 gave an n.O.e. of 8% to H-6.

For (15)[15]: $^1$H NMR (300MHz, $CDCl_3$) δ: 2.0-2.07 (2H, m, 2xH-1'), 3.31 (1H, d, J=16Hz, H-6), 3.34 (1H, d of d, J=2.5, 16Hz, H-6), 3.71-3.86 (2H, m, 2xH-2'), 4.31 (1H, d, J=7Hz, H-2), 4.49 (1H, m, H-3), 5.18 (2H, s, $CH_2$Ph), 5.33 (1H, d, J=2.5Hz, H-5), 7.37 (5H, s, Ph). $^{13}$C NMR (125.8MHz, $CDCl_3$) δ: 36.7 ($CH_2$), 44.6 ($CH_2$), 59.6 ($CH_2$), 64.6 (CH), 67.5 ($CH_2$), 85.3 (CH), 86.0 (CH), 128.3 (CH), 128.6 (CH), 128.7 (CH), 135.0 (C), 169.1 (C), 176.0 (C). MS (m/e): 292 ([MH]$^+$, 100), 250 (75), 201 (8), 192 (13). IR (thin film) $\nu_{max}$ (cm$^{-1}$): 695 (m), 745 (m), 1005 (w), 1050 (m), 1190 (s), 1660 (s), 1740 (s), 1790 (s), 2950 (m), 3490 (m). Acc. Mass measurement 291.1116 (M$^+$), $C_{15}H_{17}NO_5$ requires 291.1107. N.O.e. data, Irradiation of H-1' gave n.O.e.s of 14% to H-2', 15% to H-2 and 12% to H-3. Irradiation of H-2 gave n.O.e.'s of 7% to H-1', 3% to H-2' and 2% to H-3. Irradiation of H-3 gave n.O.e.s of 9% to H-1', 3% to H-2', 2% to H-2 and 5% to H-5. Irradiation of H-5 gave n.O.e.s of 5% to H-3 and 8% to H-6.

### (2*R*, 3*S*, 5*R*)-Benzyl-1-aza-3-(2'-azidoethyl)-4-oxa-7-oxobicyclo[3,2,0]heptane-2-carboxylate (16)

(2*R*, 3*S*, 5*R*)-Benzyl 1-aza-3-(2'-hydroxyethyl)-4-oxa-7-oxobicyclo[3,2,0]heptane-2-carboxylate (15) (74mg, 0.25mmol) was dissolved in dry THF (5ml) with $PPh_3$ (106mg, 0.41mmol) and cooled to 0$^o$C. Diethyl azodicarboxylate (65ml,0.41mmol) was added and the mixture stirred at 0$^o$C for 5mins before diphenylphosphoryl azide (66ml,0.31mmol) was added. The solution was allowed to warm to ambient temperature and stirred for a further 36h. The solvent was removed *in vacuo* and the residue purified by HPLC ($CH_2Cl_2$:hexane:EtOH, 3:6:0.1) to give the title compound as a colourless oil (38mg, 47%). $^1$H NMR (200MHz, $CDCl_3$) δ: 1.9-2.2 (2H, m, 2xH-1'), 2.92 (1H, d, J=16Hz, H-6), 3.33 (1H, d of d, J=3Hz, 16Hz, H-6), 3.40-3.51 (2H, m, 2xH-2'), 4.22 (1H, d, J=7Hz, H-2), 4.4 (1H, m, H-3), 5.21 (2H, s, $CH_2$Ph), 5.34

(1H, d, J=3Hz, H-5), 7.38 (5H, s, Ph). $^{13}C$ NMR (125.8MHz, $CDCl_3$) δ: 33.5 ($CH_2$), 44.6 ($CH_2$), 47.8 ($CH_2$), 64.4 (CH), 67.6 ($CH_2$), 84.6 (CH), 85.4 (CH), 128.4 (CH), 128.8 (CH), 134.8 (C), 168.7 (C), 176.2 (C). MS (m/e): 317 ($[MH]^+$, 3), 293 (100), 276 (57), 265 (48), 248 (29), 167 (17). IR (thin film) $\nu_{max}$ ($cm^{-1}$): 700 (m), 1010 (m), 1185 (m), 1270 (s), 1490 (m), 1750 (s), 1790 (s), 2105 (s), 3020 (m).

**(2*R*, 3*S*, 5*R*)-1-Aza-3-(2'-aminoethyl)-4-oxa-7-oxobicyclo[3,2,0]heptane-2-carboxylate (5c)**

(2*R*, 3*S*, 5*R*)-Benzyl 1-aza-3-(2'-azidoethyl)-4-oxa-7-oxobicyclo[3,2,0]heptane-2-carboxylate (16) (15mg, 0.05mmol) was dissolved in EtOH:$H_2O$ (2ml,1:1) and stirred over 5%Pd-C (10mg) under a positive pressure of hydrogen for 2h. The mixture was filtered through celite and the solvent removed *in vacuo*. The residue was purified by reverse phase HPLC ($H_2O$) and the desired fractions dried by freeze drying to give (5c) as a white solid (7mg, 74%). $^1H$ NMR (500MHz, $D_2O$) δ: 2.09-2.14 (1H, m, H-1a'), 2.28-2.31 (1H, m, H-1b'), 2.96 (1H, d, J=16.5Hz, H-6a), 3.21 (2H, t, J=7Hz, 2xH-2'), 3.44 (1H, d of d, J=2.5, 16.5Hz, H-6b), 4.14 (1H, d, J=6.5Hz, H-2), 4.38-4.51 (1H, m, H-3), 5.40 (1H, d, J=2.5Hz, H-5). MS (m/e), 201 ([MH]+, 71), 159 (20), 131 (21). The spectral data *reported* here are identical to those for (5b) except for the lack of deuterium at C-3. N.O.e, data, Irradiation of H-1a' gave n.O.e.s of 27% to H-1b', 7% to H-2', 6% to H-2, and 10% to H-3. Irradiation of H-1b' gave n.O.e.s of 24% to H-1b', 5% to H-2', 10% to H-2, and 2.5% to H-3. Irradiation of H-2 gave n.O.e.s of 2.5% to H-1a', 4% to H-1b' and 3% to H-3. Irradiation of H-3 gave n.O.e.s of 5% to H-1a', 2% to H-1b', 6% to H-2', 2% to H-2 and 8% to H-5. Irradiation of H-5 gave n.O.e.s of 3% to H-2', 7% to H-3, 6% to H-6a and 2% to H-6b.

**Incubation Experiments**

Incubation mixtures contained DTT (1mM), α-ketoglutarate (5mM), $FeSO_4$ (1mM), clavaminic acid synthase (1.2mg/ml) and the substrate (2.5mM) in 8 mM $NH_4HCO_3$, $MgSO_4$, KCl, and 0.8 mM DTT pH 7.5, in a final volume of 2ml. Mixtures were incubated at 27°C in an orbital incubator, shaking at 250 r.p.m.. After 20 mins $FeSO_4$ and DTT (50μl of a 10 times stock solution, in buffer at pH 7.5) were added. After a further 25 mins the reaction was stopped by addition of acetone (5ml). After centrifugation the acetone was removed *in vacuo*. The resulting aqueous solution was titrated to pH 5.0 with 0.01% formic acid in water, followed by freeze drying. Crude mixtures were prepared for NMR analysis by dissolving in $D_2O$ with TSP as reference. Purification of incubation products was achieved utilising a Bondapak amine column (250 x 7mm), eluting with 0.015M formic acid in 95% water, 5% methanol at a flowrate of 1.5ml/min. Detection was via a U.V. detector at 218nm. Retention times for proclavaminic acid (2a/b), dihydroclavaminic acid (5a/b), and clavaminic acid (3) were 5.5-6.0 min, 6.5 min, and 6.8-7.1 min respectively.

(5b). $^1H$ NMR (500 MHz, $D_2O$) δ: 2.05-2.15(1H, m, H-1a'), 2.28-2.35 (1H, m, H-1b'), 2.96 (1H, d, J=16.5 Hz, H-6a), 3.2 (2H, t, J=7 Hz, 2x H-2'), 3.4 (1H, d of d, J=2.5, 16.5 Hz, H-6b), 4.1 (1H, s, H-2), 5.45 (1H, d, J=2.5 Hz, H-5). Addition of 5c gave enhancement of all peaks, in addition to the production of a multiplet at 4.38-4.51.MS (m/e electrospray) 201 ($[MH]^+$, 100%).

(3 )$^1$H NMR (500MHz, $D_2O$) δ: 3.15 (1H, d, J=17 Hz, H-6), 3.60 (1H, dd, J=2.5, 17 Hz, H-6), 3.65-3.76 (2H, AB part of ABX multiplet, $CH_2$-N), 4.86 (1H, t, J=7.5 Hz, C=CH), 5.03 (1H, br.s, H-2), 5.80 (1H, d, J=2.5 Hz, H-5). MS (m/e electrospray) 199 ([MH]$^+$, 100%).

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