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Chemo-Enzymatic Synthesis of Bicyclic γ-Lactams Using Clavaminic Acid Synthase

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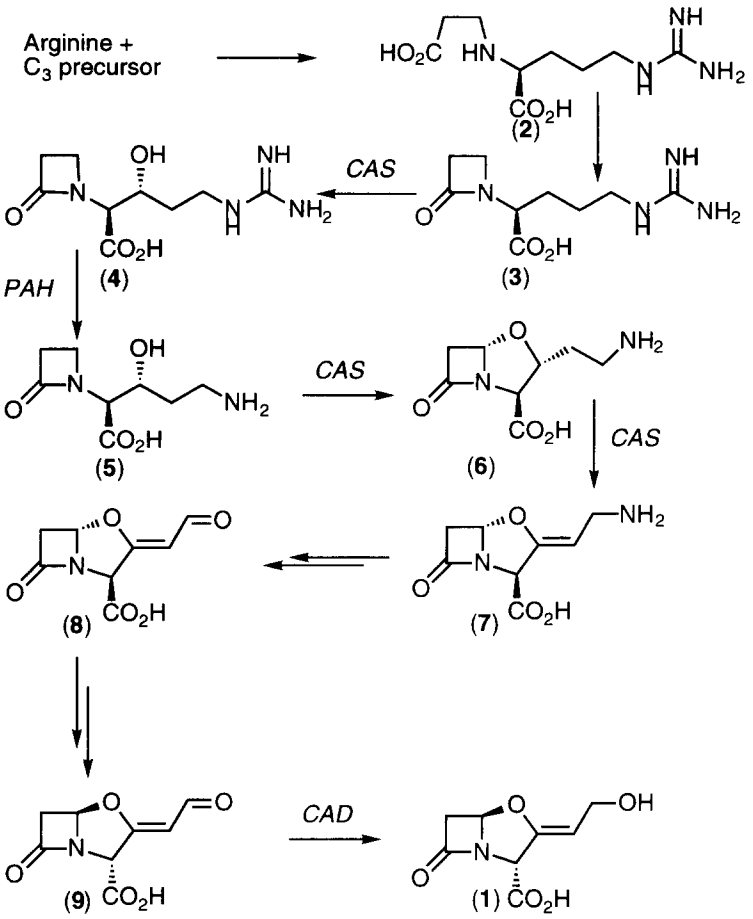
Abstract: Incubation of the γ-lactam analogue of proclavaminic acid, (±)-*threo*-5-amino-3-hydroxy-2-(1'-aza-2'-oxocyclopentyl)-pentanoic acid, led to production of two bicyclic γ-lactam products. © 1997 Elsevier Science Ltd.

Clavulanic acid (**1**) is an important inhibitor of serine β-lactamases, which are largely responsible for bacterial resistance to penicillins.[1] Biosynthetic studies on clavulanic acid (**1**) have shown that the carbons of its β-lactam ring are derived from a primary metabolite with three carbon atoms, such as pyruvate, lactate, glycerol or glycerate[2], whilst its remaining carbons are derived from arginine.[3a] Scheme 1 summarises studies on the clavulanic acid (**1**) biosynthesis pathway, within which the non-haem dioxygenase clavaminic acid synthase (CAS) plays an unprecedented trifunctional role. The early stages of the pathway are still unclear, but it seems that (**2**) or a derivative of it, is cyclised to form the first β-lactam intermediate (**3**).[3] (**2**) is formed from arginine and a 3-C pool metabolite, such as pyruvate, lactate, or glycerate. *In vitro* studies have shown that CAS catalyses the hydroxylation of intermediate (**3**) to give the direct precursor (**4**) of proclavaminic acid (**5**).[4] The process has been shown to be highly stereoselective occurring with retention of stereochemistry.[4b] Proclavaminic acid amidinohydrolase (PAH) catalyses the hydrolysis of (**4**) into proclavaminic acid (**5**) and urea.[3c] Active PAH has been purified[3c], cloned and over-produced in *Escherichia coli* as a fusion protein.[5] CAS also catalyses the cyclisation and desaturation of proclavaminic acid (**5**) to give clavaminic acid (**7**) *via* dihydroclavaminic acid (**6**).[6,7] The desaturation of (**6**) occurs with *syn*-elimination of the requisite hydrogens.[8] Clavaminic acid (**7**) is then proposed to be oxidised to the aldehyde (**8**) (although this intermediate has not been isolated) in a process involving dioxygen. During or subsequent to this step (**8**), or another intermediate, must also undergo stereochemical inversions at the (2S, 5S) centres to give (2R, 5R)-aldehyde (**9**), which on reduction gives clavulanic acid (**1**). This latter step is catalysed by the NADPH-dependent enzyme clavulanic acid dehydrogenase (CAD), which has been isolated from *S. clavuligerus*.[9]



Scheme 1: biosynthesis of clavulanic acid (**1**), PAH = proclavaminic acid amidinohydrolase; CAS = clavaminic acid synthase; CAD = clavulanic acid dehydrogenase

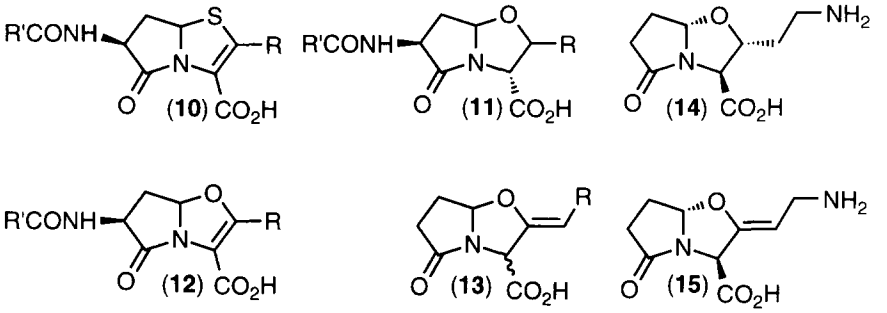
In *S. clavuligerus* CAS exists as two isoenzymes[10], whilst only one isoenzyme has been isolated from the valclavam producer *S. antibioticus*.[11] Both isoenzymes of CAS from *S. clavuligerus* have been cloned and over-expressed in *E. coli* by Marsh *et al.*[12a,12b] while one of the isoenzymes of CAS was independently cloned and over-expressed by Lawlor *et al.*[12c]

Clavaminic acid synthase (CAS) is a member of the iron (II), α-ketoglutarate-dependent dioxygenase family. The second CAS reaction [*i.e.* the conversion of (**5**) to (**7**) via (**6**)] bears a resemblance to the IPNS desaturative ring closure of tripeptide ACV to isopenicillin N during penicillin biosynthesis. IPNS, like CAS, has a requirement for iron (II) and dioxygen but does not require α-ketoglutarate as a co-substrate. However, there is no obvious primary sequence similarity between CAS[12a] and the enzymes of penicillin or cephalosporin biosynthesis.[13]

The mechanism of IPNS has been extensively and productively investigated by the incubation of substrate analogues and the physical characterisation of the subsequently formed products. The studies demonstrate that

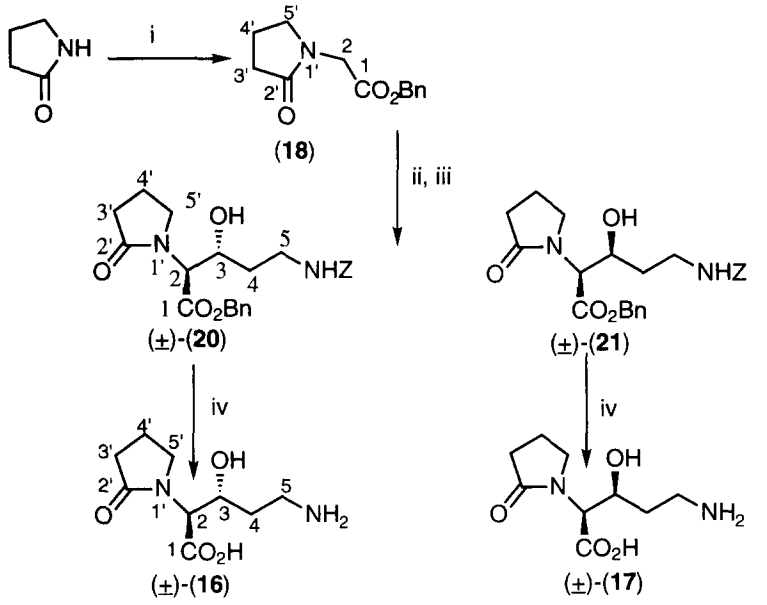
IPNS has lax substrate and product selectivities and have suggested that IPNS may be used as a 'synthetic machine' for the preparation of otherwise inaccessible or difficult to prepare bicyclic lactams. Thus IPNS has been used to prepare a large number of β-lactams including penams, cephams and β-lactams fused to a seven membered ring.[14] However, with one exception, it has not been possible, to prepare bicyclic structures which do not contain a β-lactam using IPNS. In particular, attempts to prepare bicyclic [3.3.0] γ-lactams with IPNS have failed, instead leading to the isolation of monocyclic, acyclic or in one case the production of a bicyclic γ-lactam fused to a six membered disulphide ring.[15]

We[16] and others[17] have been interested in preparing bicyclic γ-lactam analogues of β-lactams since they may show increased resistance towards hydrolysis by β-lactamase enzymes (Scheme 2).[18a] The observation that the bicyclic analogue of the penam (**10**) show weak antibacterial activity[16a,17a] and that the oxygen analogue (**11**) does not display[18a,19a] antibacterial activity, has led us to investigate the synthesis of the γ-lactam oxapenems (**12**).[19] We reasoned the increased ring strain in (**12**) compared to (**10**) may render the γ-lactam ring more reactive and thereby increase the antibiotic potency. Synthetic efforts to make (**12**) or a γ-lactam analogue (**13**) of clavulanic acid (**1**) have also been made, but are problematic, since it is difficult to find conditions to effect the synthesis of the strained bicyclic γ-lactam structure and under which the desired products are stable. Herein, we report details of the chemo-enzymatic synthesis of (**14**) and (**15**) in which CAS was used to effect the key cyclisation and desaturation steps.[20] Although (**14**) and (**15**) do not have the requisite stereochemistry to act as β-lactamase inhibitors/anti-bacterials, their preparation demonstrates that such compounds are stable enough to be isolated and characterised.



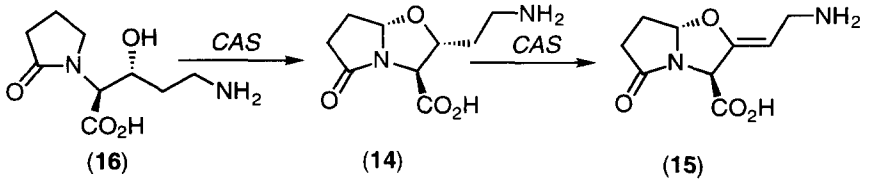
Scheme 2: Synthesis of γ-lactams

Syntheses of the required γ-lactam analogues of proclavaminic acid, (**16**) and its *erythro*-diastereomer (**17**), were carried out by extension of previously reported methodology used for the preparation of proclavaminic acid (Scheme 3).[7b, 20-21] Thus, the pyrollidone (**18**) was synthesised[20, 21] and reacted with the required aldehyde (**19**) [used for the preparation of proclavaminic acid (**4**)[7b]] to give a mixture of the racemic *threo*- and *erythro*-aldol reaction products, (**20**) and (**21**). Diastereoisomers were separated by chromatography, and deprotected by catalytic hydrogenation to give the required racemic substrate analogues (**16**) and (**17**). The relative configurations of (**16**) and (**17**) were assigned by comparison of their 500 MHz ^{1}H NMR spectra to those of the known spectra of *threo*-proclavaminic acid (**5**) and its *erythro*-diastereoisomer.



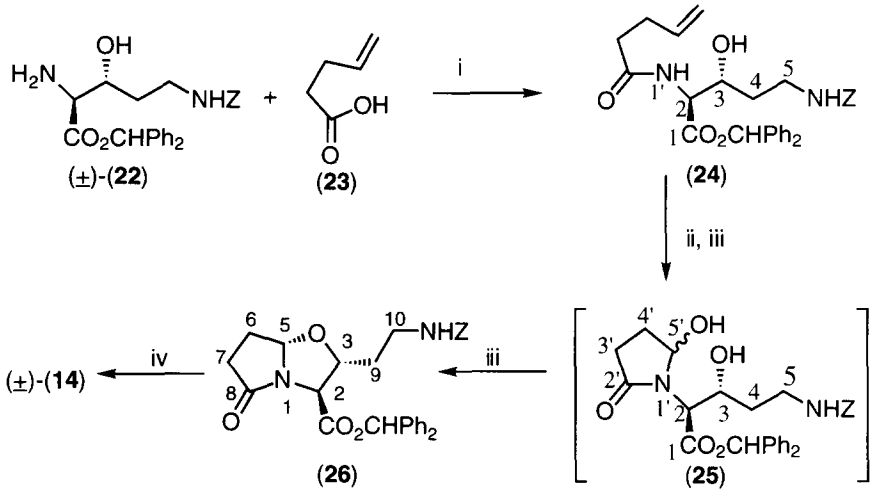
Scheme 3: Synthesis of γ-lactam substrates (**16**) and (**17**). Z = $PhCH_2OCO$, Bn = $PhCH_2$. Reagents: i). NaH, DMF, $BrCH_2CO_2Bn$; ii). $(Me_3Si)_2NLi$, THF, -78°C; iii). $OHC(CH_2)_2NHZ$ (**19**), then H_3O^+; iv). H_2, 10% Pd/C, EtOH-H_2O (7:3)

The analogues, (**16**) and (**17**), were initially incubated with a mixture of CAS isoenzymes derived from *S. clavuligerus* (*ca.* 0.5 I.U.)[7b] and the crude reaction mixture analysed by ^1H NMR (500 MHz) spectroscopy. Two new signals were observed in the crude incubation mixture of the *threo*-analogue (**16**) with CAS at 5.66 and 5.25 p.p.m., which were reminiscent of those observed with γ-lactam oxapenams.[19a] These suggested the presence of the γ-lactam analogues of dihydroclavaminic acid and clavaminic acid, (**14**) and (**15**). These new products were purified by anion-exchange and reverse phase HPLC, and characterised by ^1H NMR (500 MHz) and electrospray ionisation mass spectrometry. The relative stereochemistry of (**14**) was shown to be same as that for dihydroclavaminic acid (**7**) by n.O.e. NMR spectroscopy: *i.e.* irradiation at 5.25 p.p.m. (H-5) gave enhancements at 4.10-4.15 (H-3, 8%). The analyses confirmed that the products were the hoped for γ-lactam analogues of dihydroclavaminic acid (**14**) and clavaminic acid (**15**) (Scheme 4). Incubation of the *erythro*-isomer (**17**) with CAS under identical conditions to above failed to give any observable products by ^1H NMR (500 MHz) spectroscopic or anion-exchange HPLC analyses.



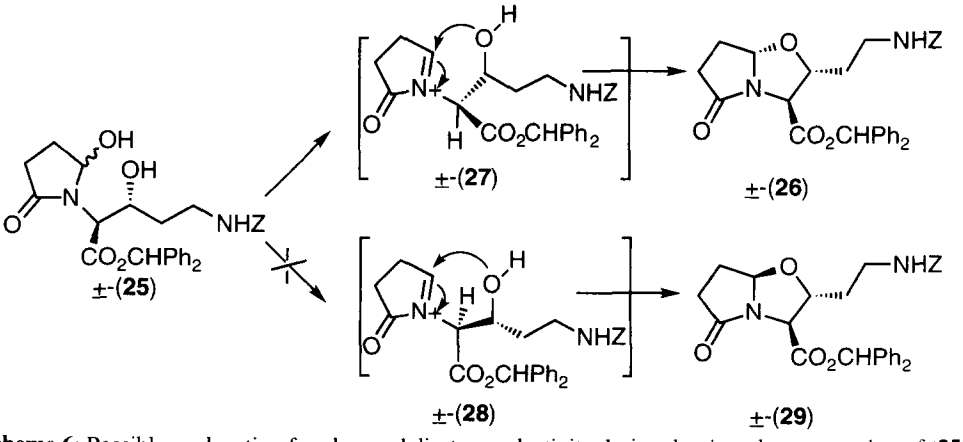
Scheme 4: The conversion of (**16**) to (**14**) and (**15**)

In order to support the spectral assignments of the enzymatic products, racemic (**14**) was synthesised, using an extension of methodology previously used for the synthesis of the γ-lactam analogues of oxapenams (Scheme 5).[19a] Thus, Z-protected 3-hydroxyornithine (**22**)[4c,22] was coupled with pent-4-enoic acid (**23**). Oxidative cleavage of the alkene (**24**) produced the corresponding aldehyde, which spontaneously cyclised to form the epimeric alcohols (**25**). A single (racemic) bicyclic γ-lactam (**26**) was obtained on treatment with acid, which was deprotected to give racemic bicyclic γ-lactam (**14**), which was identical by HPLC and ^{1}H NMR to (**14**) obtained from the incubation of (**16**) with CAS.



Scheme 5: Synthesis of the saturated γ-lactam intermediate (**14**). Z = $PhCH_2OCO$; Reagents: i). 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, CH_2Cl_2; ii). $NaIO_4$, OsO_4 (cat.), THF, H_2O (3:1); iii). CH_2Cl_2, CF_3CO_2H (cat.); iv). H_2, Pd/C, EtOH / H_2O (3:7)

It is noteworthy that the acid-catalysed cyclisation of (**25**) resulted in the isolation of only one [*i.e.* the (2S,



Scheme 6: Possible explanation for observed diastereoselectivity during the ring-closure reaction of (**25**)

5S) and (2R, 5R)] pair of the two possible racemic diastereoisomers of (**26**). There was no evidence for the presence of the alternative diastereomeric pair of products. These results may reflect allylic or A-strain manifested during the ring-closure reaction (Scheme 6),[23] that is ring closure of (**25**) may preferentially occur *via* cyclisation of the racemic acyl iminium ion (**27**). Assuming the reaction is under kinetic control, the stereochemistry at C-5 of the product is controlled by approach of the hydroxyl group in the ring closure reaction. An A-strain analysis suggests that a low-energy conformation is one in which the carbonyl group and the C-2 hydrogen are eclipsed. The alternative conformer for cyclisation, (**28**), which gives rise to the alternative racemic diastereoisomer (**29**), has the bulky protected carboxyl group eclipsing the carbonyl group and is thus less favourable. Similar results were observed during the racemisation of the aldehyde derivative of clavulanic acid (**9**), which gave only one of the two possible diastereoisomers.[9]

Incubation of synthetic (**14**) with CAS derived from recombinant *E. coli* cells (corresponding to CAS or CS isozyme 2[12a,12b]) led to a *ca.* 35% conversion to (**15**), based on integration of the bridgehead hydrogens of (**14**) and (**15**) in the NMR (500 MHz) spectrum. A similar quantity of CAS effected only *ca.* 9% conversion of proclavaminic acid (**5**) to (**6**) under the same incubation conditions. This implies that the bicyclic γ-lactam (**14**) is a better substrate than proclavaminic acid (**5**). The residual starting material (**14**) was recovered from the crude incubation mixture of racemic (**14**) by anion-exchange HPLC. Incubation of the recovered (**14**) with CAS derived from recombinant *E. coli* cells under near identical incubation conditions as before gave only a traces of conversion, suggesting that only one enantiomer of (**14**) [presumably the (2S, 5S) isomer[24]] is a substrate for the CAS cyclisation and desaturation reaction.

It appears that, as in the case of the β-lactam substrates[7], the saturated bicyclic intermediate (**14**) is a comparable substrate for CAS to the monocyclic substrate (**16**). However, it is relatively easy to isolate the saturated bicyclic intermediate (**14**) from the incubation of (**16**) with CAS derived from *S. clavuligerus*. Isolation of dihydroclavaminic acid (**6**) from incubations carried out under similar conditions was extremely difficult, and it was initially necessary to resort to the use of a primary isotope effect.[7] *In vitro* biological activity tests on (**14**) and (**16**) failed to show any antibacterial activity, as would be anticipated from the (2S, 5S) stereochemistry of these two clavams.[25] This is the first example of the production of a 'semi-synthetic' clavam analogue using an enzyme *in vitro*. CAS, like IPNS, may have a relatively lax specificity towards substrates for the cyclisation and desaturation reactions and this may provide a convenient route to novel products for biological testing.

Experimental

The general experimental protocols used were as previously reported.[7b, 21]

2-(1'-Aza-2'-oxocyclopentylethanoate benzyl ester (18)

Sodium hydride (1.15g, 60% disp, 28.8 mmol) was suspended in dry DMF (50mL). Pyrollidone (2.5 mL, 26.3mmol) in DMF (10mL) was then added and the mixture refluxed with a catalytic amount of tetrabutylammonium bromide for 30 minutes. The mixture was then cooled and a solution of 2-bromoethanoate benzyl ester (4.16mL, 26.3mmol) was added and the mixture refluxed for 1.5 hours. The reaction was cooled, poured into brine, extracted with ethyl acetate, dried over $MgSO_4$ and the solvent removed *in vacuo* to give a brown oil. Purification by flash chromatography [silica, ethyl acetate:hexane (4:1)] gave (**18**) (4.34g, 71%) as a yellow oil:γ_{max}(nujol): 1680(s) (amide C=O), 1750(s) (ester C=O), 3010(m) (C_6H_5-) (cm^{-1}); ^1H NMR (200 MHz, $CDCl_3$) δ_H 2.05 (2H, m, H-4'), 2.4 (2H, t, J=5.5Hz, H-3'), 3.45 (2H, t, J=8Hz, H-5'), 4.1 (2H, s, H-2), 5.15 (2H, s, $C\underline{H}_2C_6H_5$), 7.35 (5H, s, C_6H_5-); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ_C 17.9 (C-4'), 30.2 (C-5'), 44.0 (C-2'),

47.6 (C-2), 66.9 ($C\underline{H}_2C_6H_5$), 128.2-128.5 ($\underline{C}_6H_5$-),135.2 (*ipso*-carbon)168.5 (C-1), 175.5 (amide); m/z [CI (NH_3)] 234 (MH^+, 100%).

*threo-(±)-5-Benzyloxycarbonylamino-3-hydroxy-2(1'-aza-2'-oxocyclopentyl)pentanoate benzyl ester (**20**) and erythro-(±)-5-benzyloxycarbonylamino-3-hydroxy-2(1'-aza-2'-oxocyclopentyl) pentanoate benzyl ester (**21**)*

(**18**) (500 mg, 2.16mmol) was dissolved in dry THF (20mL) and cooled to -78 °C. Lithium bis(trimethylsilylamide) (1M in hexane, 2.5mL, 2.5mmol) was added whilst the temperature was maintained below -60 °C. The mixture was cooled to -78 °C and stirred for 15 minutes. A solution of aldehyde (**19**) (520mg, 2.51mmol,[7b,21]) in THF (10mL) was added dropwise and the resulting mixture was stirred for 17 minutes. Acetic acid (0.5mL) was then added and the resulting mixture poured into sulphuric acid (0.5 M). The aqueous mixture was extracted with ethyl acetate, evaporated to dryness and purified by flash chromatography [silica, hexane:ethyl acetate:ethanol (30:20:1)] to give the required (*threo*) diastereoisomer (**20**) (30%, 284mg): ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.45-1.7 (2H, m, H-4), 2.0 (2H, m, H-4'), 2.35 (2H, t, J=8Hz, H-3'), 3.1-3.65 (2 x 2H, m, H-5 and H-5'), 4.35 (1H, m, H-3), 4.6 (1H, bs, H-2), 5.0 and 5.2 (4H, s, 2 x $C_6H_5C\underline{H}_2$-), 5.4 (1H, bs, NH), 7.35 (10 H, s, 2 x $C_6\underline{H}_5$-); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 18.5 (C-4), 30.5, 33.0, 37.5, 47.7 (4 x $\underline{C}H_2$), 60.1 (C-2), 66.7 and 67.1 (2 x COO$\underline{C}H_2$-), 68.6 (C-3), 127.0-128.7 (2 x C_6H_5-), 135.5 and 136.6 (2 x *ipso*-carbons), 157.5 (N$\underline{C}$OO-), 169.2 ($\underline{C}O_2CH_2C_6H_5$), 177.4 ($CH_2\underline{C}ON$); m/z [CI ($NH_3$)] 91 ($C_6H_5CH_2^+$, 40%), 234 (40%) 441 ($MH^+$, 15%), and the *erythro* diastereoisomer (**21**) (10%, 76 mg): ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 1.60-1.85 (2H, m, H-4), 2.0 (2H, m, H-4'), 2.35 (2H, t, J=8Hz, H-3'), 3.2-3.6 (2 x 2H, m, H-5 and H-5'), 4.35 (1H, m, H-3), 4.6 (1H, bs, H-2), 5.0 and 5.2 (4H, s, 2 x $C_6H_5CH_2$-), 5.4 (1H, bs, NH), 7.30 (10 H, s, 2 x $C_6\underline{H}_5$-); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 18.4 (C4), 30.6 (C-5), 33.6 (C-4'), 37.8 (C-3'), 46.8 (C-5'), 60.4 (C-2), 66.6 and 67.2 (2 x COO$\underline{C}H_2$-), 68.7 (C-3), 128.0-128.7 (2 x C_6H_5-), 135.4 and 136.7 (2 x *ipso*-carbons), 169.7 ($\underline{C}O_2CH_2C_6H_5$), 176.5 ($CH_2\underline{C}ON$); m/z [CI ($NH_3$)] 91 ($C_6H_5CH_2$+, 63%), 234 (100%) 441 (MH^+, 12%).

*threo-(±)-5-Amino-3-hydroxy-2(1'-aza-2'-oxocyclopentyl)pentanoic acid (**16**) and erythro-(±)-5-amino-3-hydroxy-2(1'-aza-2'-oxocyclopentyl)pentanoic acid (**17**)*

(**20**) (227mg, 0.516mmol) was dissolved in ethanol:H_2O (7:3, 30mL) and stirred over palladium (10% on charcoal, 150mg) under hydrogen for 4 hours. The catalyst was removed by filtration through Celite®, the ethanol evaporated *in vacuo*, and the aqueous residue freeze-dried to give the (**16**) (67mg, 60%): ^{1}H NMR (200 MHz, D_2O) δ_H 1.4-1.7 (2H, m, H-4), 1.85 (2H, m, H-5), 2.25 (2H, m, H-4'), 2.9 (2H, t, J=8Hz, H-3'), 3.2 (2H, t, J=8Hz, H-5'), 3.85-3.95 (1H, m, H-3), 4.05 (1H, *ca.* d, J=8 Hz, H-2); m/z (electrospray) 217 (MH^+, 100%).

For (**17**): deprotection of *erythro* isomer (**21**) (76mg, 0.051mmol) was carried out as above to give (**17**) (60%, 22.5mg): ^{1}H NMR (200 MHz, D_2O) δ_H 1.5-1.7 (2H, m, H-4), 1.80 (2H, m, H-5), 2.35 (2H, m, H-4'), 2.9 (2H, t, J=8Hz, H-3'), 3.2 (2H, t, J=8Hz, H-5'), 3.90-4.00 (1H, m, H-3), 4.10 (1H, *ca.* d, J=8Hz, H-2); m/z (electrospray) 217 (MH^+, 100%).

*(±)-threo-5-N-Benzyloxycarbonyl-2-N-(pent-4'-ene-1'-oyl)-3-hydroxyornithine diphenylmethyl ester (**24**)*

A mixture of crude (±)-*threo*-5-*N*-benzyloxycarbonyl-3-hydroxyornithine diphenylmethyl ester (**22**) (1.30g, 2.90mmol),[4c,22] pent-4-enoic acid (**23**) (0.279g, 2.91mmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (0.719g, 2.91mmol) was stirred in dry dichloromethane (25mL) at room temperature for 14 hours. The solution was washed with 1M sodium hydrogen carbonate (2 x 25mL), 1M hydrochloric acid (2 x 25mL) and brine (25mL). The organic phase was separated, dried over $MgSO_4$ and the solvent removed *in vacuo*. The residue was purified by flash chromatography [silica, 40-60 petroleum ether:ethyl acetate (1:1)] to give (**24**) (1.20g, 78%): m.p.

112 - 113°C; R_f 0.25 [silica, 40-60 petroleum ether:ethyl acetate, (1:1)]; [Found: C, 70.25%; H, 6.74%; N, 5.21%. $C_{31}H_{34}N_2O_6$ requires: C, 70.17%; H, 6.46%; N, 5.28%]. γ_{max}(nujol): 1279(m), 1456(s), 1536(s), 1657(s), 1683(s), 1753(s), 3309(s) (cm^{-1}); ^1H NMR (200 MHz, $CDCl_3$) δ_H 1.55-1.69 (2H, m, 2 x H-4), 2.40 (4H, s, H-2', H-3'), 3.06-3.25 (1H, m, H-5), 3.43-3.65 (1H, m, H-5), 3.98 (1H, d, J=4.0Hz, OH), 4.22 -4.38 (1H, m, H-3), 4.77 (1H, dd, J=12.0Hz, 1.5Hz, H-2), 4.98-5.19 (4H, m, C$\underline{H}_2C_6H_5$, H-5'), 5.70-5.93 (1H, m, H-4'), 6.38 (1H, d, J=12.0Hz, NH-1'), 6.91 [1H, s, C$\underline{H}$(C_6H_5)2], 7.25-7.43 [15H, m, 3 x $C_6\underline{H}_5$-]; ^{13}C NMR (50.3MHz, $CDCl_3$) δ_C 30.2(CH_2), 34.9 (CH_2), 35.7 (CH_2), 38.1 (C-5), 57.6 (C-3), 69.9 ($\underline{C}H_2C_6H_5$), 70.4(C-2), 78.8 [$\underline{C}$H(C_6H_5)2], 115.8 (C-5'), 127.7, 127.8, 128.8, 129.0, 129.6, 129.7 [3 x C_6H_5-], 138.4 (*ipso*-carbon), 138.7 (C-4'), 141.6 (*ipso*-carbon), 158.2, 171.2, 174.1 (3 x C=O); m/z: 531($[MH]^+$,15%), 437(7), 167(100), 91(61).

*(2S,3R,5S) and (2R,3S,5R)-1-Aza-3-[9-β-benzyloxycarbonylaminoethyl)]-8-oxo-4-oxabicyclo [3,3,0] octane-2-carboxylic acid diphenylmethyl ester (**26**)*

A crystal of osmium tetroxide was added to a solution of (**24**) (200mg, 0.38mmol) in THF:water (3:1, 8mL). After standing for 5 minutes the dark solution was rapidly stirred and sodium periodate (242mg, 1.13mmol) was added over 15 minutes. The solvent was removed *in vacuo* after 2 hours and the residue partitioned between water (25mL) and ethyl acetate (25mL). The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was dissolved in dry dichloromethane (10mL) and a catalytic quantity of CF_3CO_2H (*ca.* 5μL) was added followed by 3Å molecular sieves. After stirring for 18 hours the mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was purified by flash chromatography [silica, 40-60 petroleum ether:ethyl acetate (1:1)] to give (**26**) (80mg, 41%) as an oil. R_f 0.7 [silica, 40-60 petroleum ether:ethyl acetate (3:7)]. ^1H NMR (500MHz, $CDCl_3$) δ_H 1.85-1.94 (1H, m, H-9), 2.06-2.16 (2H, m, H-9, H-6), 2.37 - 2.45 (1H, m, H-6), 2.53 (1H, ddd, J=18.0Hz, 10.5Hz, 5.0Hz, H-7), 2.70 (1H, ddd, J=18.0Hz, 11.0Hz, 7.0Hz, H-7), 3.23-3.30 (1H, m, H-10), 3.35-3.41 (1H, m, H-10), 4.06-4.10 (1H, m, H-3), 4.35 (1H, d, J=5.5Hz, H-2), 4.96 (1H, br. s, NH), 5.11 (2H, s, C$\underline{H}_2C_6H_5$), 5.21 (1H, d, J=5.0Hz, H-5), 6.93 [1H, s, C$\underline{H}$(C_6H_5)2], 7.26-7.42 (15H, m, 3 x $C_6\underline{H}_5$-); ^{13}C NMR (125MHz, $CDCl_3$) δ_C 23.77 (C-6), 30.62 (C-7), 34.35 (C-9), 38.19 (C-10), 61.46 (C-2), 66.68 ($\underline{C}H_2C_6H_5$), 78.34 [$\underline{C}$H(C_6H_5)2], 80.98 (C-3), 92.91 (C-5), 127.0, 127.0, 128.1, 128.2, 128.3, 128.5, 128.6, 128.7 [3 x $\underline{C}_6H_5$-], 136.6, 139.2, 139.4 (3 x *ipso*-carbons), 156.2, 168.8, 179.0 (3 x C=O); m/z: 515($[MH]^+$,0.5%), 503(8), 167(100), 91(28).

*(2S,3R,5S) and (2R,3S,5R)-1-Aza-3-[9-β-(aminoethyl)]-8-oxo-4-oxabicyclo[3,3,0]octane-2-carboxylic acid (**14**)*

A solution of (**26**) (25mg, 0.049mmol) in 30% aqueous ethanol (6mL) was stirred with 10% Pd/C (*ca.* 5 mg) for 2 hours under a positive pressure of hydrogen at room temperature. The mixture was filtered through Celite®, the filtrate poured into water (20mL) and washed with ether (2 x 20mL). The aqueous layer was separated and freeze dried to leave a glass-like solid (8 mg, 77%); ^1H NMR (500MHz, D_2O) δ_H 2.07-2.14 (2H, m, H-6, H-9), 2.25-2.31 (1H, m, H-9), 2.48-2.55 (1H, m, H-6), 2.62 (1H, ddd, J=17.5Hz, 10.5Hz, 4.0Hz, H-7), 2.83 (1H, ddd, J=17.5Hz, 10.0Hz, 7.5Hz, H-7), 3.20 (2H, t, J=7.0Hz, 2 x H-10), 4.07 (1H, d, J=6.5Hz, H-2), 4.30-4.34 (1H, m, H-3), 5.38 (1H, dd, J=6.5Hz, 3.0Hz, H-5); m/z: 215($[MH]^+$,20%), 197(65), 179(64), 115(100), 69(100). This sample was identical to biosynthetic (**14**) by HPLC and ^1H NMR (500 MHz) analysis.

Incubation experiments

Incubations of substrate analogues with CAS were carried out by the previously reported method.[7b] The substrate analogue (2 mg) was incubated at 27 °C and 250 r.p.m. with CAS purified from *S. clavuligerus* SC2 (*ca.* 0.5 IU)

or derived from recombinant *E. coli* cells (*ca.* 0.2 IU).[12c] One international unit (I.U.) of CAS activity is defined as the amount of enzyme required to convert 1 μmol of proclavaminic acid (**5**) to clavaminic acid (**6**) in one minute under the defined conditions.[12c] The reaction was quenched and the crude incubation mixture was examined by ^{1}H NMR (500 MHz) spectroscopy. Products were isolated by HPLC [Bondapak amine® (250 x 7 mm), 0.015 M HCO_2H in 5% (v/v) aqueous methanol at 1.5 mL min^{-1}, λ = 218 nm].

For (**14**): A 3% conversion of (**16**) to (**14**) was observed with CAS from *S. clavuligerus* SC2. Purification by HPLC as above, with a retention volume of 8.7-9.75mL (29 μg): ^{1}H NMR (500MHz, D_2O, referenced to TSP) δ_H 1.74-1.93 (2H, m, H-9, H-6), 2.02-2.09 (1H, m, H-9), 2.24-2.32 (1H, m, H-6), 2.34-2.40 (1H, m, H-7), 2.57-2.75 (1H, m, H-7), 3.05 (2H, *ca.* t, J=7 Hz, 2 x H-10), 3.92 (1H, d, J=6.5 Hz, H-2), 4.10-4.15 (1H, m, H-3), 5.25 (1H, dd, J=6.5, 3 Hz, H-5); m/z (electrospray) 215 (MH^+, 100%). A 2D COSY correlation spectrum was consistent with the assigned structure. Stereochemical assignments were made by n.O.e. experiment. Thus, irradiation at 5.25 (H-5) gave enhancements at 4.10-4.15 (8%, H-3) and 2.24-2.32 (H-6, 9%); irradiation at 4.10-4.15 (H-3) gave enhancements at 5.25 (H-5, 8%), 3.05 (H-10, 4%), 2.02-2.09 (H-9, 4%), 1.74-1.93 (H-9 and H-6, 3%); irradiation at 3.92 (H-2) gave enhancements at 3.05 (H-10, 4%), 2.02-2.09 (H-9, 4%) and 1.74-1.93 (H-6 and H-9, 6%).

For (**15**): A 5% conversion of (**16**) to (**15**) was observed with CAS purified from *S. clavuligerus* SC2. Purification as above (retention volume = 10.5-11.1 mL) was followed by reverse phase HPLC [ODS (250 x 7 mm), 25 mM aqueous NH_4HCO_3, 1.5 mL min^{-1}, λ = 218 nm] with a retention volume of 6.5-8.0mL (61 μg): ^{1}H NMR (500 MHz, D_2O, referenced to TSP) δ_H 2.06-2.11 (1H, m, H-6), 2.42-2.73 (2H, m, H-6, H-7), 2.7 (1H, dd, J=16Hz, 8 Hz, H-7), 3.46-3.58 (2H, m, 2 x H-10), 4.75 (1H, dt, J=7Hz, 1 Hz, H-9), 4.86 (1H, s, H-2), 5.66 (1H, dd, J=6.5Hz, 3 Hz, H-5); m/z (electrospray) 213 (MH^+, 100%). A 2D COSY correlation spectra was consistent with the assigned structure.

Synthetic (**14**) was incubated with CAS derived from recombinant *E. coli* cells (*ca.* 0.2 IU). Analysis of the products by ^{1}H NMR (500MHz) spectroscopy showed that a *ca.* 35% conversion to (**15**) had occurred. Isolation of residual (**14**) and the product (**15**) was performed by HPLC as above. The spectroscopic and physical properties of (**15**) were identical within the limits of experimental error to those reported above.

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