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STUDIES ON THE SUBSTRATE SPECIFICITY OF CLAVAMINIC ACID SYNTHASE AND ASSOCIATED ENZYMES

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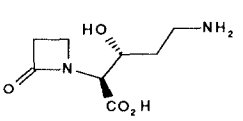
Abstract Fifteen structural analogues of proclavaminic acid have been synthesised and incubated with enzyme preparations from *Streptomyces clavuligerus* in order to test the substrate specificity of the enzymes responsible for converting monocyclic β-lactams to bicyclic β-lactams.

Recent reports have described the involvement of proclavaminic acid (1) and clavaminic acid (2) in the biosynthesis of clavulanic acid (3) in cells of *Streptomyces clavuligerus*[1,2]. Clavaminic acid synthase (CAS), the enzyme responsible for the conversion of proclavaminic acid (1) to clavaminic acid (2), has also been described and has been used in cell free systems to synthesise clavaminic acid (2) from proclavaminic acid (1)[1].

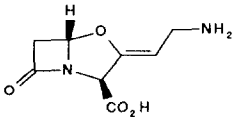
CAS is a Fe^{2+} ion dependent dioxygenase which gives stoichiometric conversion of proclavaminic acid to clavaminic acid in the presence of two equivalents of α-ketoglutarate. The enzyme has been shown to be stereoselective for the naturally occurring (2S,3R) enantiomer of (1) producing clavaminic acid with the (3S,5S) stereochemistry[3].

Clavams other than clavaminic and clavulanic acids have been produced by cultures of *S.clavuligerus*[4]. This may be explained either by a relaxed substrate specificity of CAS, or by the existence of other cyclising enzymes with a similar activity but different selectivities. There also exists the question of whether clavulanic acid could be biosynthesised by more than one pathway, for example *via* alkene (5) or by direct cyclisation of the alcohol (13a, $Y=CH_2OH$) or aldehyde (13b, Y=CHO). The existence of one or more enzymes capable of converting a monocyclic β-lactam to a bicyclic clavam structure with absolute stereospecificity adumbrates interesting synthetic opportunities. A recent report[5] has

demonstrated that CAS can accept a monocyclic γ-lactam substrate to produce a bicyclic γ-lactam, but there is no information concerning the substrate specificity in relation to the 5-aminopentanoate part of the substrate. Consequently, the following study comprises the synthesis and incubation of a range of analogues of proclavaminic acid with cell free preparations from *S. clavuligerus*, and the assay for products containing the clavam nucleus.



(1)



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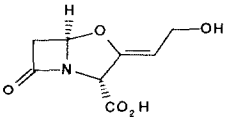
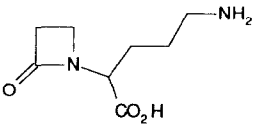
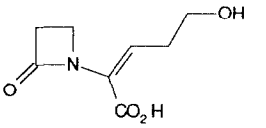


(3)

There are a number of functionalities in proclavaminic acid (1) which could participate in or influence the interactions between the substrate and CAS. These may be necessary for activation (e.g. the 3-hydroxyl) or orientation (e.g. the β-lactam ring). Thus a change in substrate structure might affect the orientation at the active site and hence the required substrate stereochemistry for reaction. Equally a different, but associated, enzyme might display a different stereoselectivity. Consequently mixtures of all stereoisomers of the substrates were tested and not just the stereo-orientation which corresponds to proclavaminic acid. Similarly, a crude cell free preparation of *S. clavuligerus* was employed in the incubations in preference to purified preparations of CAS to ensure the presence of all potentially useful proteins.



(4)



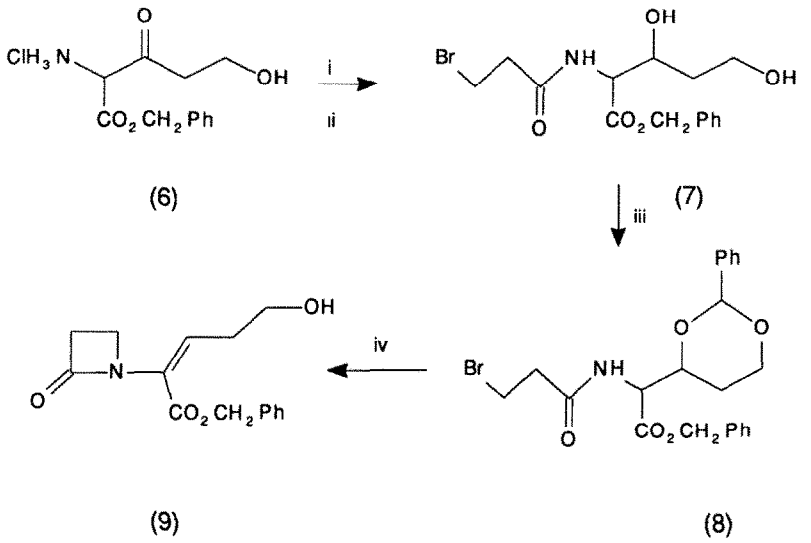
(5)

Two analogues of proclavaminic acid lacking the 3-hydroxyl function (4) and (5) were tested. Dehydroxy proclavaminic acid (4) was prepared as described previously[6] and the α,β-unsaturated structure (5) was obtained as shown in Scheme 1 in which the amine hydrochloride[7] (6) was acylated with 3-bromopropionyl chloride[6] and reduced to give (7). The alcoholic functions were protected to give a mixture of diastereoisomers of the benzylidene acetal (8) from which a single diastereoisomer could be isolated. The mixture of diastereomers was treated with sodium hydride to give the monocyclic β-lactam (9) (34%), in which base catalysed elimination of benzaldehyde had occurred to introduce the double bond. De-esterification to (5) was effected by base catalysed

hydrolysis (NaOH in 10% aqueous THF). Spectroscopic data for these compounds are given in Table 1. Many of the other analogues reported here were synthesised by aldol addition of a suitable aldehyde to benzyl (2-oxoazetidinyl)acetate (10), the adduct being deprotected under hydrogenolytic conditions[6] (Scheme 2). Exceptions to this method of deprotection were in the synthesis of (13k) in which ethyl (2-oxoazetidinyl)acetate had been used and the aldol adduct de-esterified with a molar equivalent of sodium hydroxide solution, and also the syntheses of (13h), (13i) and (13j) where the benzyl esters of the adducts were deprotected at pH 11 with dilute sodium hydroxide solution under conditions similar to those described in reference 6.

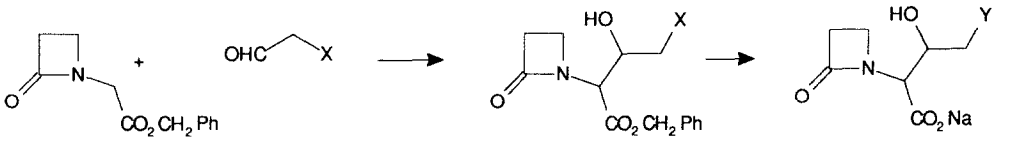
The aldol adducts (12) were isolated in yields ranging from 30-65% and separated from remaining azetidinone starting material by chromatography. Typically the diastereoisomer ratios of adduct were in the range 1:1 to 3:1 and previous experience would suggest that the erythro stereochemistry might predominate[6]. The adduct (12b, X=$CH_2(OH)CH_2OH$) was isolated after acid workup although the acetonide of the corresponding aldehyde (11) was used in the aldol addition. Periodate cleavage of the geminal diol followed by hydrogenolysis yielded the terminal aldehyde (13b).

Scheme 1



Reagents: i 3-bromopropionyl chloride, NaOH; ii $NaBH_4$; iii benzaldehyde, $ZnCl_2$; iv NaH.

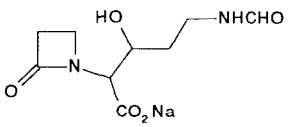
Scheme 2



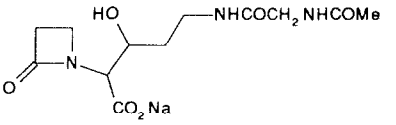
(10) (11) (12) (13)

Table 2 lists ^{1}H nmr data for these aldol adducts, some of which clearly show doubling of signals due to the presence of two diastereoisomers, and for one (12j) both of these were separated on chromatography. Table 3 provides nmr data for test substrates produced in this way.

The above procedures allowed access to proclavaminic acid analogues bearing alternative C5 functionality to the amine as well as two modified C5-N compounds. The N-formyl compound (14) was obtained as a hygroscopic solid by treatment of a mixture of enantiomers of (1) with trichlorophenyl formate[8]. The N-acetylglycyl analogue (15) was produced as described in reference 4a.



(14)



(15)

The test substrates were incubated with cell free preparations from *Streptomyces clavuligerus* under conditions which effected the conversion of proclavaminic acid to clavaminic acid[1], and the production of clavams was sought by hplc assay after imidazole derivatisation[9]. In no case was any evidence of the production of a clavam indicated.

The above results indicate that CAS has a substrate requirement for not only a 3-hydroxyl group, but also exhibits a tight substrate specificity for C-5 analogues of proclavaminic acid. The inability of CAS to utilise C-5 oxygenated analogues of proclavaminic acid also points to a single biosynthetic route to clavulanic acid *via* clavaminic acid. This result is in contrast to the demonstration of a more relaxed specificity of CAS with respect to the lactam moiety of its natural substrate, proclavaminic acid[5], which in turn may give an indication of the functionalities on the substrate which affect, and are involved in, active site binding.

TABLE 1

^{1}H nmr ppm for compounds in Scheme 1

No.	Solvent	nmr spectrum with coupling constants J in Hz
7	$CDCl_3$	1.52-1.90 (2H, m); 2.85 (t, J 6.6) and 2.86 (t, J 6.3) (together 2H); 3.62 (t, J 6.3) and 3.64 (t, J 6.6) (together 2H); 3.80 (t, J 5.0) and 3.89 (t, J 5.0) (together 2H); 4.24 (dt, J 3.0, 9.4) and 4.47 (dt, J 3.4, 8.8) (together 2H); 4.70-4.81 (1H, m); 5.18-5.30 (2H, m); 6.54 (d, J 8.8) and 6.72 (d, J 6.9) (together 1H); 7.36 (5H, s).
8	$CDCl_3$	1.55-1.69 (1H, m); 2.25 (1H, dq, J 5.0, 12.2); 2.65-2.93 (2H, m); 3.53-3.72 (2H, m); 4.92 (1H, dt, J 2.3, 11.9); 4.10-4.21 (1H, m); 4.26 (1H, dd, J 4.0, 11.5); 4.81 (1H, dd, J 3.4, 8.3); 5.21 (2H, s); 5.47 (1H, s); 6.75 (1H, d, J 8.0); 7.20-7.45 (10H, m).
9	$CDCl_3$	2.53 (2H, dt, J 6.5, 8.0); 3.06 (2H, t, J 4.5); 3.70 (2H, t, J 4.5); 3.79 (2H, t, J 6.7); 5.21 (2H, s); 6.83 (1H, t, J 8.1); 7.38 (5H, s).
5	D_2O	2.46 (2H, q, J 6.5); 3.13 (2H, t, J 3.9); 3.69 (2H, t, J 3.8); 3.76 (2H, t, J 6.3); 6.57 (1H, t, J 7.4).

TABLE 2

^{1}H nmr $CDCl_3$ ppm for aldol adducts (12)

No.	X=	nmr spectrum with coupling constants J in Hz
12a	CH_2OCH_2Ph	1.75-2.08 (m, 2H); 2.95 (t, 2H); 3.37+3.42 (2xt, 2H); 3.58-3.76(m, 2H); 4.10-4.35 (d+m, 3H); 4.51(s, 2H); 5.21(s, 2H); 7.33-7.34 (2xs, 10H).
12b	$CH(OH)CH_2OH$	1.40-1.90 (m, 2H); 2.83-3.07 (m, 2H); 3.28-3.62 (m, 4H); 3.80-3.98 (m, 1H); 4.15-4.52 (m, 2H); 5.10-5.28 (m, 2H); 7.20-7.47 (m, 5H).
12c	CH_2OCOEt	1.13 (t, 3H); 1.84-2.04 (m, 2H); 2.32 (q, 2H); 2.98+3.03 (2xt, 2H); 3.29-3.44 (m, 2H); 4.04-4.43 (m, 5H); 5.22 (s, 2H); 7.36 (s, 5H).
12d	$CH_2OCOC_7H_{15}$	0.88 (t, 3H); 1.27 (Br, 8H); 1.60 (m, 2H); 1.81-2.05 (m, 2H); 2.29 (t, 2H); 2.98 (m, 2H); 3.29-3.45 (m, 2H); 4.09 (d, 1H); 4.21-4.34 (m, 4H); 5.23 (s,2H); 7.36 (s, 5H).
12e	$CH_2OCOC_{17}H_{35}$	0.88 (t, 3H); 1.25 (m, 28H); 1.60 (m, 2H); 1.91 (m, 2H); 2.28 (m, 2H); 3.02 (m, 2H); 3.38 (m, 2H); 4.09-4.33 (m, 5H incl. 1 exch.); 5.22 (m, 2H); 7.36 (s, 5H).
12f	$CH_2OCO(CH_2)_2OCH_2Ph$	1.50-2.15 (m, 2H); 2.61 (t, J 6.3, 2H); 2.94 (t, J 4.1) + 2.99-3.10 (m) (together 2H); 3.20-3.50 (m, 2H); 3.73 (t, J 6.3) + 3.74 (t, J 6.3) (together 2H); 4.05 (d, J 3.1) + 4.00-4.50 (m) (together 4H); 4.50 (s) + 4.52 (s) (together 2H); 5.21 (s) + 5.21 (ABq) (together 2H); 7.20-7.50 (m, 10H).
12g	$CH_2OCO(CH_2)_4NHCOPh$	1.40-1.70 (m, 4H); 1.71-2.10 (m, 2H); 2.33 (t, J 6.9, 2H); 2.90-3.07 (m, 2H); 3.17 (q, J 6.5, 2H); 3.25-3.47 (m, 2H); 4.00-4.40 (m, 4H); 4.75-4.95 (br, 1H); 5.09 (s, 2H); 5.21 (ABq, 2H); 7.25-7.44 (m, 10H).
12h	CH_2SPh	1.67-2.06 (m, 2H); 2.90-3.41 (m, 6H); 4.03-4.04 (2xd, 1H); 4.22-4.39 (m, 2H); 5.15-5.27 (m, 2H); 7.13-7.41 (m, 10H).
12i	$\underbrace{CHS(CH_2)_3S}$	1.81-2.25 (m, 4H); 2.81-2.94 (m, 4H); 2.96-3.10 (m, 2H); 3.28-3.43 (m, 2H); 4.04-4.58 (m, 4H); 5.22 (dd, 2H); 7.36 (s, 5H).
12j	$=CH_2$	2.97-3.03 (2xt, 2H); 3.34-3.46 (m, 2H); 3.99 (m, 1H, exch.); 4.31 (d, 1H, J 4.32) 4.64 (m, 1H); 5.16-5.21 (s+m, 2.5H); 5.24+5.31+5.38 (3xm, 1.5H); 5.87-6.00 (m, 1H); 7.36 (s, 5H). and 2.92-3.00 (m, 2H); 3.33-3.45 (m, 2H); 4.16 (d, 1H, J 3.56); 4.34 (d, 1H.exch.); 4.73-4.82 (m, 1H); 5.21-5.26 (s+m, 3H); 5.38-5.44 (2xm, 1H); 5.80-5.93 (m, 1H); 7.37 (s, 5H).

TABLE 3

^{1}H nmr ppm for test compounds (13)

No.	Y=	Solvent	nmr spectrum with coupling constants J in Hz
13a	CH_2OH	D_2O	1.59-1.86 (m, 2H); 2.97 (t, J 4.0, 2H); 3.41-3.53 (m, 1H); 3.53-3.63 (m, 1H); 3.63-3.77 (m, 2H); 4.04 (d, J 4.9) + 4.08-4.26 (m) (together 2H).
13b	CHO	D_2O	1.60-2.00 (m) and 2.75(t, J 7.6) (together 2H); 2.89-3 10 (m, 2H); 3.38-3.75 (m, 2H); 4.02-4.37 (m, 2H); 5.10-5.25 (m) and 9.69(s) (together 1H).
13c	CH_2OCOEt	D_2O	1.08 (t, 3H); 1.74-2.00 (m, 2H); 2.39 (q, 2H); 2.98 (m, 2H); 3.39-3.61 (m, 2H); 4.01-4.24 (m, 4H).
13d	$CH_2OCOC_7H_{15}$	D_2O	0.83 (m, 3H); 1.26 (m, 8H); 1.59 (m, 2H); 1.8-2.0 (m, 2H);2.37 (t, 2H); 2.97 (m, 2H); 3.40-3.65 (m, 2H); 4.05-4.33 (m, 4H).
13e	$CH_2OCOC_{17}H_{35}$	CD_3OD	0.90 (t, 3H); 1.28 (s, 28H); 1.60 (m, 2H); 1.67-1.94 (m, 2H); 2.32 (t, 2H); 2.93 (t, 2H); 3.34-3 62 (m, 2H); 4.04+4.22 (2xm, 4H).
13f	$CH_2OCO(CH_2)_2OH$	D_2O	1.72-2.08 (m, 2H); 2.57-2.79 (m, 2H); 2.90-3.10 (m, 2H); 3.35-3.70 (m, 2H); 3.74-3.95 (m, 2H);4.02-4.40 (m, 4H).
13g	$CH_2OCO(CH_2)_4NH_2$	D_2O	1.55-2.04 (m, 6H); 2.46 (t, J 5.0, 2H); 2.90-3.10 (m, 4H); 3.42-3.54 (m, 1H); 3.54-3.64 (m, 1H); 4.08 (d, J 5.2) + 4.18 (d, J 3.1) + 4.05-4.33 (m) (together 4H).
13h	CH_2SPh	D_2O	1.71-1.88 (m, 2H), 2.63-2.90 (4xm, 2H); 2.94-3.10 (m, 1H); 3.16-3.26 (m, 2H); 3.37-3.43 (m, 1H); 4.02 (d, 1H, J 7.69); 4.11-4.19 (m, 1H); 7.24-7.44 (m, 5H).
13i	$\underbrace{CHS(CH_2)_3S}$	D_2O	1.78-1.97 (m, 4H); 2.70-2.92 (m, 4H); 2.99 (t, J 3.8, 2H); 3.37-3.65 (m, 2H); 4.09-4.32 (m, 3H).
13j	$=CH_2$	D_2O	2.93-2.99 (m, 2H); 3.39-3.64 (m, 2H); 4.26 (d, 1H, J 6.7); 4.58 (dxd, 1H); 5.25-5.40 (m, 2H); 5.84-5.98 (m, 1H).
13k	CH_3	D_2O	1.00 (t, 3H); 1.51-1.79 (m, 2H); 2.83-2.94 (m, 2H); 3.33-3.50 (m, 2H); 3.91-4.14 (m, 2H).

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