Substrate Analogue Studies on Clavaminic Acid Synthase

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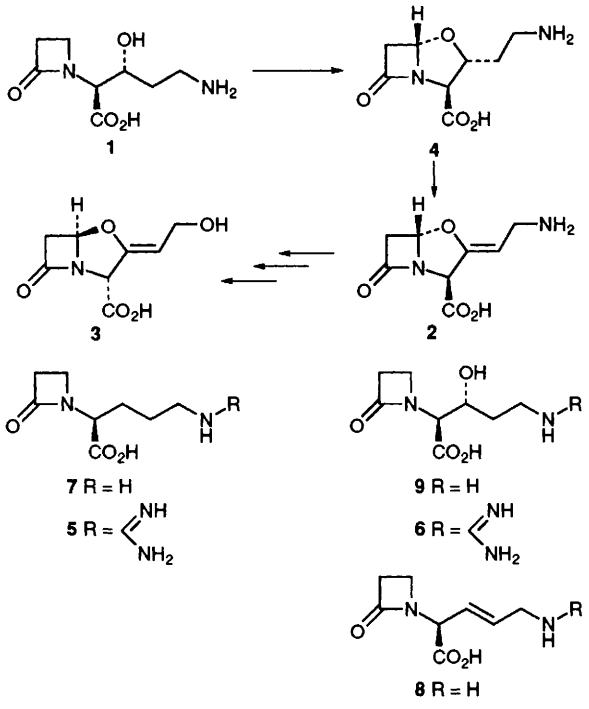
Incubation of *N*-α-acetyl-L-ornithine with clavaminic acid synthase led to the isolation of (*E*)-(2*S*)-5-amino-2-acetamidopent-3-enoic acid and (2*S*)-2-acetamido-5-amino-3-hydroxypentanoic acid, while incubation of *N*-α-acetyl-L-arginine led to the isolation of (2*S*)-2-acetamido-5-guanidino-3-hydroxypentanoic acid.

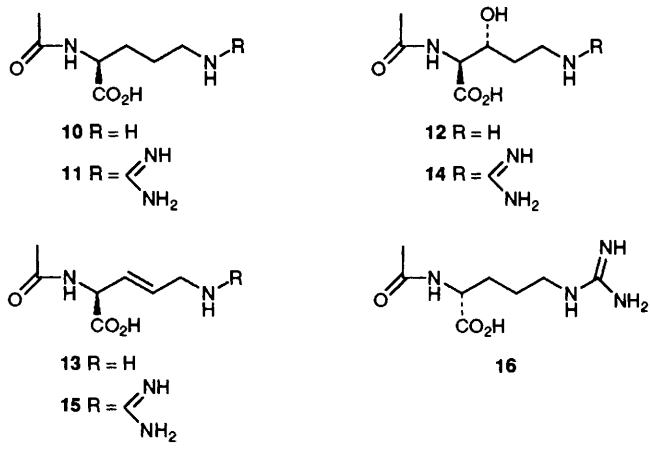
Proclavaminic acid **1** and clavaminic acid **2** are biosynthetic precursors of the β-lactamase inhibitor clavulanic acid **3**, which is produced by *Streptomyces clavuligerus* ATCC 27064.[1–3] The conversion of **1** to **2** is catalysed by the ferrous and α-ketoglutarate dependent oxygenase, clavaminic acid synthase (CAS).[1] The existence of two CAS isozymes has been demonstrated in *S. clavuligerus*.[4] It has been shown that in this conversion CAS catalyses two consecutive oxidations, *i.e.* initial conversion of **1** to dihydroclavaminic acid **4** followed by subsequent desaturation of the latter to give **2**[5] (Scheme 1). It has also been shown that CAS is able to catalyse the conversion of a γ-lactam analogue of proclavaminic acid **1** to the corresponding γ-lactam analogues of **4** and **2**.[6] Recently the guanidino compounds **5** and **6** have been shown to be biosynthetic precursors of **1**[7] and it was demonstrated that CAS has the ability to catalyse the conversion of **5** to **6**, leading



Scheme 1

to speculation[8] that the CAS isozymes[4] may have a trifunctional role in the biosynthetic pathway to **1**. In contrast when **7**, the N^5-amino analogue of **5**, was incubated with CAS it was found the reaction course was biased (>90%) to desaturation to give **8**, rather than alcohol **9**. In order to explore further the binding of substrates to and mechanisms of CAS we have incubated simple analogues of **7** and **5** wherein the β-lactam was replaced by an acetamido group. The results of the incubation of N^2-acetylornithine **10** and N^2-acetylarginine **11** with CAS are reported herein.

Examination of the crude incubation mixture by ^{1}H NMR spectroscopy (500 MHz), resulting from incubation of **10** with CAS,† showed that conversion to the alcohol **12** and the olefin **13** had occurred (**12**:**13** *ca.* 3:1). Purification of the crude mixture by HPLC [Bondapak amine column (250 × 7 mm), 0.015 mol l^{-1} HCO_2H] gave **13** (retention volume: 9.4–10.0 ml); δ_H {500 MHz, D_2O, referenced to sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$]propionate (TSP)} 2.0 (3H, s, CH_3), 2.33 (2H, d, J = 5 Hz, 5-H), 3.50–3.60 (1H, m, 2-H), 5.65–5.75 (1H, m, 4-H) and 5.92–6.04 (1H, m, 3-H); $J_{3H,4H}$ = 15 Hz, indicating the double bond has *E* stereochemistry; *m/z* (electrospray) 173 (MH^+, 100%); and **12** (retention volume: 8.0–9.0 ml). Compound **12** was further purified [reverse phase octadecylsilane column, 0.05% (v/v) aqueous HCO_2H] (retention volume: 5.5–9.0 ml): δ_H (500 MHz, D_2O, TSP reference) 1.75–1.85 (2H, m, 4-H), 2.1 (3H, s, CH_3), 3.15 (2H, *ca.* t, J = 5 Hz, 5-H), 4.19–4.25 (1H, m, 3-H and 4.30 (1H, d, J = 3.5 Hz, 2-H); *m/z* (electrospray) 191 (MH^+, 100%).‡ Thus, it appears that replacing the β-lactam ring with the acetyl group apparently biases CAS activity from predominantly (>10:1) desaturation in the case of the β-lactam substrate **7** to mostly hydroxylation (*ca.* 3:1) in the case of the acetyl substrate **10**. The bias in reactivity obtained by the replacement of the β-lactam ring with the acetyl group, whilst not as significant as replacing the amine group of **7** with the guanyl group of **5**, is still apparent and demonstrates the subtle relationship between the substrate and the oxidising entity generated during the CAS reaction.



In contrast, incubation of *N*-acetyl-L-arginine **11** with preparations of CAS (0.21 IU) derived from recombinant *E. coli* cells resulted in an efficient conversion to **14**. There was no evidence by ^{1}H NMR spectroscopy for the presence of the desaturated product **15**. Purification of the crude incubation mixture by HPLC (reverse phase octadecylsilane column, H_2O) led to the isolation of **14** (retention volume: 4.5 ml): δ_H (500 MHz, D_2O, referenced to TSP): δ_H 1.71–1.95 (2H, m, 4-H), 2.1 (3H, s, CH_3), 3.15–3.40 (2H, m, 5-H), 4.15–4.23 (1H, m, 3-H) and 4.25 (1H, d, J = 3.5 Hz, 2-H); *m/z* (electrospray) 233 (MH^+, 100%).‡ The observed bias to hydroxylation by the replacement of the amino with the guanyl group follows the pattern observed in the β-lactam series.[6] Incubation of *N*-α-acetyl-D-arginine **16** with CAS (2.1 IU) led to little if any production (< 5%) of hydroxylated products. This is consistent with previous results, which indicated that CAS converts substrates with the (2*S*) stereochemistry.[9] No conversion of another potential substrate, *N*-α-benzoyl-L-arginine, was observed by ^{1}H NMR spectroscopy (500 MHz) or HPLC when it was incubated with CAS.

Further incubations of **11** in the presence of $^{18}O_2$ showed that the oxygen of the hydroxy group was mainly (*ca.* 85%) derived from dioxygen. Incubation of **11** with CAS in the presence of $H_2{}^{18}O$, enriched to a final concentration of 47.5 atom %, resulted in a low level of incorporation (*ca.* 8%) of ^{18}O into **14**. This suggests that the majority of the oxygen in the hydroxy group of **14** is derived from dioxygen. For the hydroxylation of *N*-acetyl-L-arginine **11** by the mixture of the two isozymes from *S. clavuligerus* ATCC 27064, preliminary kinetic analyses (using HPLC) gave apparent values for K_m = 0.34 ± 0.02 mmol l^{-1} and V_{max} = 2.5 ± 0.10 μmol min^{-1} mg^{-1}. For the CS2 CAS isozyme purified from recombinant *E. coli* for **11**. K_m = 0.23 ± 0.02 mmol l^{-1}, k_{cat} = 6.4 ± 0.8 s^{-1}, compared with the following values obtained for **5**. K_m = 0.25 ± 0.02 mmol l^{-1}, k_{cat} = 14.7 ± 0.5 s^{-1}.

The above results show that CAS is able to oxidise simple derivatives of ornithine and arginine at unactivated positions. The reaction may be of synthetic utility. In addition we have found that **11** is a convenient replacement for proclavaminic acid **1** substrate in routine assays for CAS activity. Further analogue studies will further define the structural features which are required for binding in the β-lactam binding site of CAS. The oxidation (and especially hydroxylation) of arginine residues, and more generally amino acid derivatives, by 2-oxo acid dependent dioxygenases is a commonly encountered feature in the biosynthesis of secondary metabolites. The hydroxylation of arginine residues at the β-position has been implicated in the biosynthesis of streptothricin F,[10] and at the γ-position in the biosynthesis of the heptapeptide antibiotics, K-582 A and B.[11] It is noteworthy that in the latter case, the L-*threo* isomer is exclusively produced.

† The recombinant CAS used in these and our previously reported studies[4–6] was produced *via* cloning the CAS gene which corresponds to the CS2 gene of the clavulanic acid biosynthesis cluster recently reported by Townsend *et al.*[4] Manuscript in preparation.

‡ The relative stereochemistries of **12** and **14** are inferred from previous studies.[7]

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