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EMERGING EVIDENCE FOR A SHARED BIOSYNTHETIC PATHWAY AMONG CLAVULANIC ACID AND THE STRUCTURALLY DIVERSE CLAVAM METABOLITES

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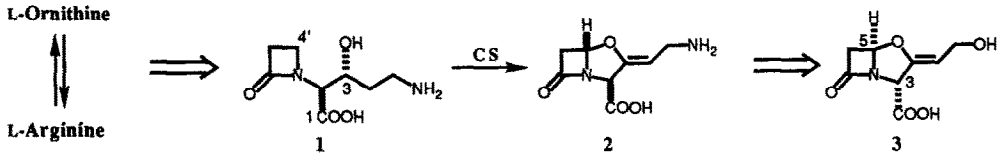
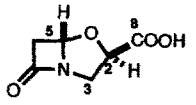
Abstract: [1-^{13}C]-L-Valine and [2,3-$^{13}C_2$]-D,L-proclavaminic acid were administered to *Streptomyces antibioticus* (Tü 1718). ^{13}C-NMR spectroscopic analyses demonstrated intact incorporation of the later into valclavam and 2-hydroxyethyl clavam and the former into valclavam. These results, in conjunction with earlier findings, provide evidence for a common biosynthetic origin among all clavam metabolites.

A striking feature of clavulanic acid (**3**) biosynthesis is that all known biochemical intermediates in the anabolic process are antipodal to the β-lactamase inhibitor itself, *i.e.* the urea cycle amino acids L-ornithine/L-arginine,[1] proclavaminic acid[2,3] (**1**), and clavaminic acid[2] (**2**). While quite a number of clavams have been isolated possessing the 5*S*-ring fusion, only clavulanic acid (and simple *O*-acyl derivatives) is known to have the 5*R*-ring junction. For example, clavams **4**-**7** lack the 3-carboxyl but share a common 2*R*-side chain and co-occur with **3** in *Streptomyces clavuligerus*.[4] Hydroxyethyl clavam[5] (**8**) and valclavam[6] (**9**) have been purified from *S. antibioticus*. Even more complex structures can be found among the clavamycins.[7]



The structure of alanylclavam (**7**) suggests that the terminal amino group of ornithine may be utilized in the formation of the β-lactam ring. In contrast, this nitrogen is provided by the α-amino group of this amino acid in clavulanic acid (**3**).[8] However, regiochemical studies with samples of [^{13}C]-labeled ornithine have shown the *same* orientation upon incorporation of this amino acid into both clavam-2-carboxylate (**4**) and clavulanic acid (**3**).[8] This finding was confirmed and extended by the incorporation of labeled proclavaminic acid (**1**) into **3** and **4**. ^{13}C-NMR spectroscopic analyses revealed identical sites of labeling and comparable efficiencies of incorporation.[8] A common biosynthetic origin between **3** and **4** suggests that **4**, and possibly all the clavams **5**-**9** are similarly constructed in a process involving loss of the C-1 carboxyl of proclavaminic acid. This being so, the carboxyl of alanylclavam (**7**) must derive from a further carbon source, and two additional carbons must account for the formation of valclavam (**9**). We report in this Letter experiments that support this proposal and point to a common biosynthetic origin for *all* clavam metabolites at least up to and including proclavaminic acid (**1**).



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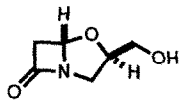

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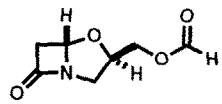

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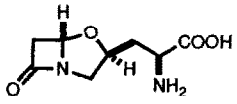

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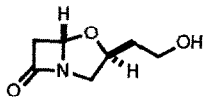

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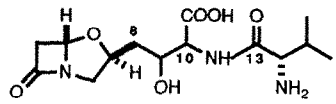

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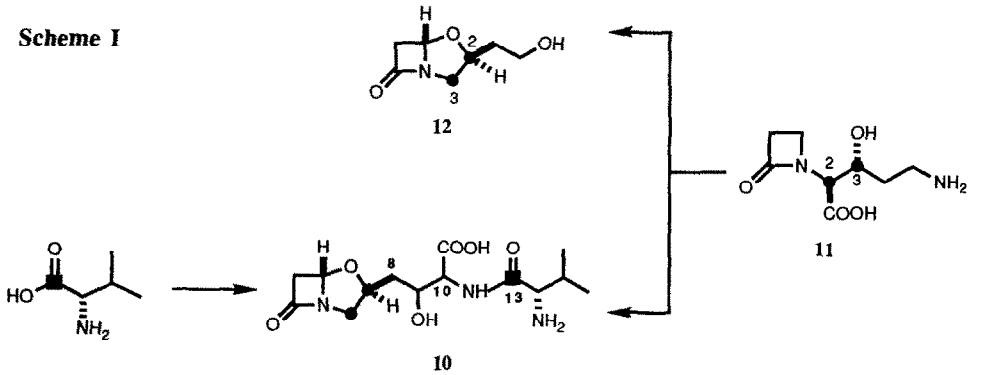
Cultures of *S. antibioticus* (Tübingen 1718) were grown to produce hydroxyethyl clavam (**8**) and valclavam (**9**).[9,10] Seed flasks were inoculated with a spore suspension and incubated (300 rpm, 27 °C) for 48 h as described by Rabenhorst.[9] Ten mL of 48 h seed culture was used to inoculate 1 L of fermentation medium, which was grown at 27 °C with continued shaking. At the onset of clavam production, typically 48 h, 2 mmoles of [1-^{13}C]-L-valine was administered to the growing culture as a sterile solution in water. After an additional 45 h, valclavam (**9**) was isolated from the fermentation medium according to the procedure developed by Rabenhorst modified as follows. The broth was clarified by centrifugation and filtration through Celite, adsorbed onto XAD-4, and valclavam was eluted with 20% aqueous methanol. This fraction was concentrated *in vacuo* and filtered through Sephadex DEAE A-25. Isocratic elution in water of a portion of the lyophilized crude valclavam (390 mg) on a C_{18} HPLC column gave a sample of pure **10** (15 mg). ^{13}C-NMR analysis of the isolated valclavam showed a specific incorporation (5.5%, 168.4 ppm) of label at C-13, consistent with the expected intact incorporation of valine (see Scheme I).

Scheme I



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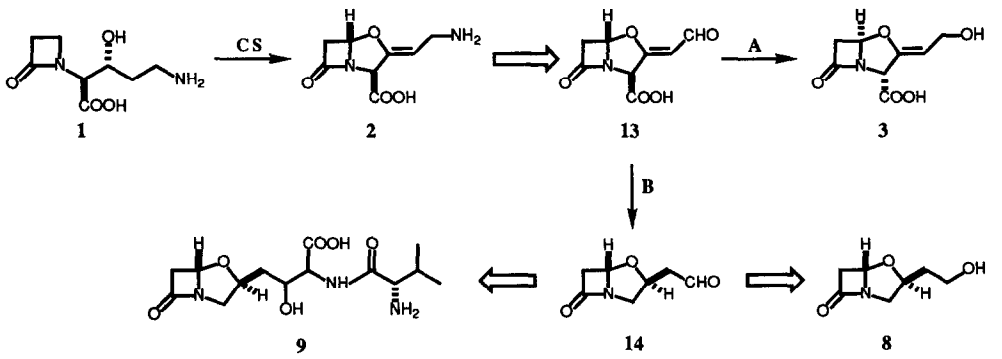
To directly examine the possible biosynthetic parallels between clavulanic acid (**3**) and the clavam metabolites **8** and **9**, [2,3-$^{13}C_2$]-D,L-proclavaminic acid (**11**) was prepared[11] and administered (1.2 mmole, $^1J_{CC}$ = 39.3 Hz) to a 45 h, 2 L fermentation of *S. antibioticus*. Isolation of valclavam (**10**) as before and ^{13}C-NMR analysis showed the specific incorporation of both labels into C-2/3 ($^1J_{CC}$ = 33.0 Hz) at an efficiency of 0.7-0.8%/site. Hydroxyethyl clavam (**12**) was isolated by methanol elution of the XAD-4 column and purified by silica gel chromatography using a step gradient of hexanes:ethyl acetate (1:1) to ethyl acetate. The proton-decoupled ^{13}C-NMR spectrum of **12** (21 mg) revealed an identical pattern and efficiency of label utilization to that seen in valclavam (**10**).

Incorporation of proclavaminic acid into clavams **8** and **9** supports a shared biosynthetic pathway between the stereoisomeric clavam metabolites and clavulanic acid. Previous work has shown that proclavaminic acid is incorporated into **4** produced by *S. clavuligerus*.[8] It is clear that extensive parallels exist among the biosynthetic pathways of these compounds. In the clavulanic acid pathway, clavaminate synthase (CS) catalyzes the oxidative cyclization/desaturation of **1** to yield **2** (Scheme II, Path A).[2a,12] Clavaminate possesses the same stereochemistry at the ring junction (5*S*) uniformly observed in the clavams **4-9**.

In order for clavaminate (**2**) to be transformed into clavulanic acid (**3**), inversions of stereochemistry at C-3 and C-5 are required. It has been proposed that the aldehyde **13** may be the pivotal intermediate in this conversion.[8,13] Formation of the aldehyde **13** through the oxidative deamination of **2**, provides the functionality necessary to rationalize both the "enantiomerization" required to produce **3** as well as the decarboxylation at C-3 to form the clavams. Stereospecific reduction at C-2 of **13** can be envisioned in several ways to provide **14**, from which formation of all the clavams may be rationalized (Scheme II, Path B).

Scheme II



A simple reduction of **14** can be suggested to afford **8**. Baeyer-Villager oxidation of **14** may be proposed to yield **6** directly, from which the formation of **4** and **5** could be readily rationalized. Formation of **7** and **9** is not as straightforward. A net addition of two carbon atoms is necessary to obtain valclavam (**9**) from putative aldehyde **14**. Several pathways can be advanced to account for this overall transformation generating an amine

for amide bond formation with valine. Similarly, alanylclavam (7) could be visualized as a 1-carbon homologation of the hypothetical aldehyde **14** or an oxidative degradation product of a clavam as **9** lacking the amide-linked valine. Further experiments to test the hypotheses outlined in Scheme II will be reported in due course.

Acknowledgements

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