Origin of the β-Lactam Carbons in Clavulanic Acid from an Unusual Thiamine Pyrophosphate-Mediated Reaction

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Communications to the Editor

Three Unusual Reactions Mediate Carbapenem and Carbapenam Biosynthesis

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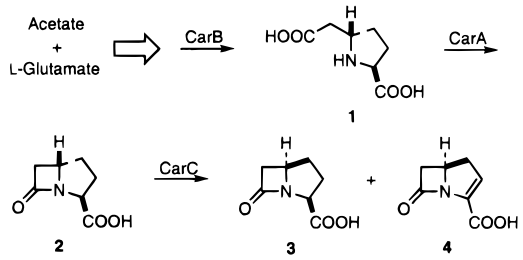
Members of the carbapenem family are important among the β-lactam antibiotics for both their broad spectrum of antibiotic activity and their relative resistance to most clinically encountered β-lactamases.[1] Since the isolation of thienamycin,[2] more than 40 structurally related carbapenems have been identified. The simplest among these is (5*R*)-carbapen-2-em-3-carboxylic acid (**4**).[3] It co-occurs in the Gram-negative bacteria *Serratia marcescens* and *Erwinia carotovora* with two saturated diastereomers, **2** and **3**.[4,5] Unlike **4**, these carbapenams have no antibacterial activity.

Incorporation experiments have established that the bicyclic nuclei of both thienamycin and **2**−**4** are derived from acetate (β-lactam carbons) and glutamate (pyrrolidine ring),[4,6] origins clearly distinct from those to penicillin, cephalosporin,[6,7] and clavulanic acid.[8] Recently, the gene cluster responsible for carbapenem production has been identified in *E. carotovora.* Of the nine open reading frames (ORFs), five, *carA-E*, are thought to be required for the production of **4**, while *carFG* are involved in a poorly understood self-resistance mechanism.[9,10] Transformation of *Escherichia coli* with the entire cluster confered the ability to produce **4**, but genetic disruption of *carD* or *carE* resulted in substantial loss of antibiotic production. Mutation of *carA*, *carB*, or *carC* gave only a carbapenem-negative phenotype.[10]

Previous biochemical and genetic evidence has suggested separately evolved biosynthetic pathways to the four known classes of β-lactam antibiotics.[11] The gene products of *carA* and *carC*, however, show similarities to two enzymes, β-lactam synthetase (β-LS) and clavaminate synthase (CS), respectively, whose roles are well-characterized in the biosynthesis of clavulanic acid.[12,13,14] These observations imply an evolutionary relationship between clavam formation in *Streptomyces clavuligerus* and carbapenam/em biosynthesis. The protein encoded by *carB* reveals similarities to enzymes that interact with acylCoA derivatives, and *carDE* appear to give rise correspondingly to a proline oxidase and a likely allied ferredoxin.[9,15,16] In this work we report the first functional analysis of the carbapenem gene cluster to delineate the sequence of biochemical events to **2**, **3**, and **4** (Scheme 1).

Scheme 1



The *carA*, *carB*, and *carC* genes were cloned from *E. carotovora* genomic DNA by PCR amplification, and each was inserted into the *E. coli* expression vector pET24a (Novagen). The proteins were individually expressed in BL21(DE3)pLysS and found to be soluble (Figure 1B, lanes 2, 4, and 6). The construction of plasmids for their coexpression is shown in Figure 1A. The resulting plasmids, pET24a/*carAB* and pET24a/*carABC*, contained a single T7 RNA polymerase promoter and a single T7 terminator and each gene was preceded by an *E. coli* Shine−Dalgarno sequence for ribosome binding. These plasmids were used individually to transform BL21(DE3)pLysS, and the resulting transformants were examined for overexpression by SDS-PAGE. As shown in lanes 8 and 10 in Figure 1B, coexpression had no adverse effect on the levels of soluble recombinant proteins in either case.

The induction of β-lactamases in *Bacillus lichenformis* (ATCC 14580) by β-lactam antibiotics can be sensitively detected in a colorimetric assay with nitrocefin.[17] Fermentation of BL21 (pET24a/*carABC*) under standard conditions in LB medium showed that the coexpression of these three genes relative to a control led to the low level production of a nitrocefin-positive compound. The titer of this compound, presumed to be **4**, could be enhanced to a level comparable to that of *S. marcescens* itself when a modified medium was used.[4] Once growth had reached $A_{600} = 0.65$, the temperature was reduced to 28 °C and IPTG was added. After 5 h, the supernatant was extracted, and the products were derivatized as their *p*-nitrobenzyl (PNB) esters. The reaction residue was partially purified by silica gel chromatography to remove excess PNB bromide.[4] The crude PNB esters were further purified by HPLC, whereupon ^{1}H NMR analysis

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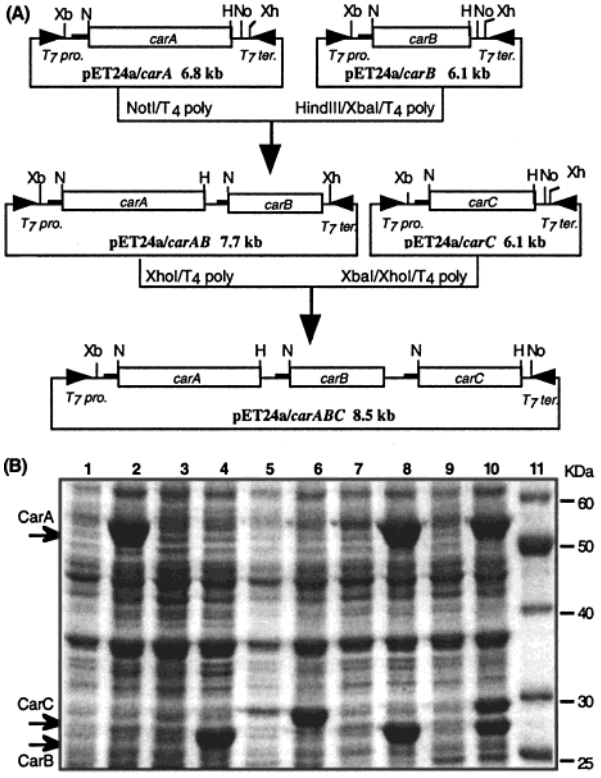


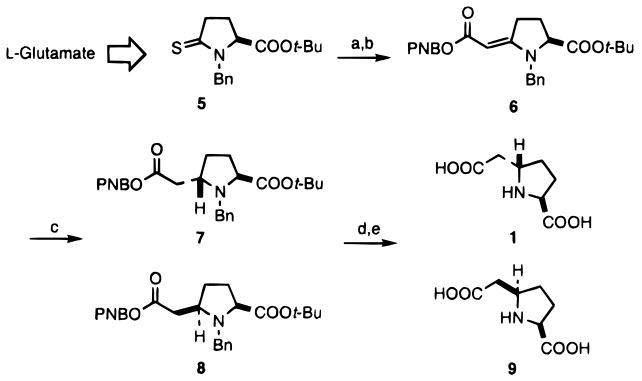
Figure 1. (A) Construction of plasmids pET24a/*carAB* and pET24a/*carABC*. The solid boxes represent ribosome binding sites. H: *Hind*III; N: *Nde*I; No: *Not*I; Xb: *Xba*I; Xh: *Xho*I. (B). SDS-PAGE gel showing soluble proteins. Lane 1, 3, 5, 7, and 9: uninduced. Lane 2, 4, 6, 8, and 10: induced. Lane 11, molecular weight markers. CarA, CarB, and CarC are indicated by arrows. The solid bars represent ribosomal binding sites

showed that BL21 (pET24a/*carABC*) gave **2**, **3**, and **4**, identical to the similarly esterified products isolated from *S. marcescens*.[3,4] These data indicated that *carABC* contain all of the genetic information necessary for the assembly of these compounds from primary metabolic precursors available in *E. coli*.

Employing the conditions optimized for the fermentation of BL21 (pET24a/*carABC*) and subsequent isolation, we turned our attention to determining the possible products generated from BL21 (pET24a/*carAB*). Unexpectedly, ^{1}H NMR analysis showed that **2**, the carbapenam of opposite bridgehead stereochemistry compared to **4**, was the sole β-lactam product of this recombinant strain. In accord with the proposed oxidative role for CarC,[9,18] these findings suggested that this enzyme is responsible for the oxidation of carbapenam **3** to carbapenem **4**. To our surprise, however, the results also indicated that CarC acts as an epimerase inverting the configuration of the ring fusion.

On the basis of this result and the potential role of CarA in closure of the β-lactam ring, we synthesized the proposed product of CarB, amino diacid **1**. As shown in Scheme 2, starting from L-glutamate, thiolactam **5** was prepared according to a previously published procedure in four steps.[19] Eschenmoser coupling[20] of **5** with PNB bromoacetate provided the vinylogous amide **6** in 86% yield. Subsequent reduction with sodium cyanoborohydride/acetic acid yielded the protected amino diacids **7** and **8** in a 1:2 ratio, which were separable by silica gel chromatography. Catalytic hydrogenation, followed by treatment with TFA, yielded the two diastereomeric amino diacids **1** and **9** in pure form.

Scheme 2[a]



[a] Reagents and conditions: (a) PNB bromoacetate, CH_3CN, 40 h. (b) triphenylphosphine, triethylamine, CH_2Cl_2, 20 h. (c) sodium cyanoborohydride, acetic acid, CH_3CN. (d) $Pd(OH)_2/C$, H_2, EtOH. (e) trifluoroacetic acid.

The product of CarB was identified from a fermentation of BL21 (pET24a/*carB*). The supernatant was lyophilized, suspended in 0.2 N acetic acid, and passed through a cation exchange column (Bond Elut SCX, Varian) with aqueous pyridine, followed by HPLC purification. The ^{1}H and ^{13}C NMR spectra of partially purified **1** were coincident with spectra taken of authentic **1** obtained by independent total synthesis (Scheme 2). Further analysis by selective DPFGSE-TOCSY ^{1}H NMR spectroscopy[21] demonstrated that the product of the fermentation was unambiguously a single diastereomer identical to **1**.

In conclusion, we take these findings to support a pathway (Scheme 1) in which **1** is stereospecifically formed by CarB from primary metabolic precursors under the proper choice of fermentation conditions. CarA, homologous to the β-lactam synthetase active in clavulanic acid biosynthesis,[12,13] is then proposed to close this first dedicated intermediate to the significantly more strained carbapenam **2**. Of **2** and **3**, it is noteworthy that the thermodynamically favored *exo*-diastereomer **2** is formed in these two steps limited by the free energy accessible from the hydrolysis of ATP presumed to be coupled in this process[22,23] and the restriction of an L-amino acid precursor. In the most unexpected transformation of this sequence, CarC, a probable non-heme iron oxygenase, carries out both the anticipated desaturation to the carbapenem nucleus of **4**, and the thermodynamically unfavorable epimerization of **2** to **3**. The mechanistic and energetic implications of these observations are the subject of further investigation.

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