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Synthesis of (3*S*,5*R*)-Carbapenam-3-carboxylic Acid and Its Role in Carbapenam Biosynthesis and the Stereo-inversion Problem

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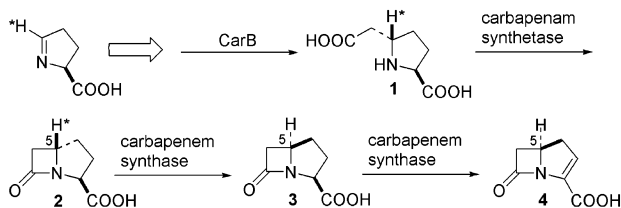
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(5*R*)-Carbapenam-3-carboxylic acid (**4**) is the simplest structurally of over 60 naturally occurring carbapenam β -lactam antibiotics isolated since the discovery of thienamycin,¹ whose gene cluster has been recently identified.² Members of this family and their derivatives are clinically important for their broad spectrum of antibiotic activity and their relative resistance to most clinically encountered β -lactamases.³

We determined that the assembly of all three β -lactams **2**, **3**, and **4** produced by *Erwinia carotovora* (now *Pectobacterium carotovorum*)² and *Serratia* sp. ATCC 39006 is carried out through the action of just three enzymes, CarA (carbapenam synthetase), CarB, and CarC (Scheme 1).^{4–6} This process is accompanied by a remarkable stereochemical inversion at C-5 of **2** to both **3** and **4**, which proceeds by loss of hydrogen label at that stereocenter catalyzed by CarC (carbapenam synthase).⁶

Scheme 1

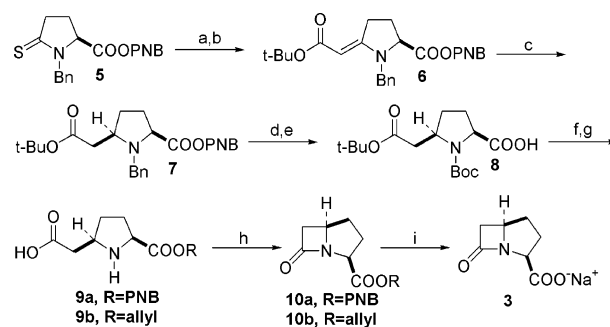


While **3** has always been present in low amounts compared to **2** and **4** during isolation of the β -lactams from fermentation,^{4,7} no direct evidence exists to establish its role as an intermediate, shunt product, or product of the poorly understood self-resistance mechanism.⁸ We report here the first functional analysis of (3*S*,5*R*)-carbapenam carboxylic acid (**3**) as an intermediate in the assembly of **4**. In addition, we address the role of α -ketoglutarate in the epimerization of **2** to **3** and subsequent desaturation to **4**.

Recently⁶ we described the first successful synthesis of an unprotected carbapenam-3-carboxylic acid skeletal system. The instability/lability of the carbapenams is evident in previous attempted syntheses^{9–11} and in the recent report of the carbapenam synthase crystal structure¹² in which **2** was produced enzymically from **1**. Having the sodium salt of **2** in hand, we attempted to duplicate our deprotection strategy in a synthesis of **3**.

As shown in Scheme 2, starting from L-glutamate, thiolactam **5** was prepared according to a previously published procedure in four steps.¹³ Eschenmoser coupling¹⁴ of **5** with *tert*-butylbromo acetate provided the vinylogous amide **6**. Reduction with sodium cyanoborohydride/acetic acid yielded protected (2*S*,5*R*)-amino diacid **7**, which was separated from the (2*S*,5*S*)-amino diacid by silica gel chromatography. Catalytic hydrogenation followed by treatment with (Boc)₂O yielded **8**. Derivatization with *p*-nitrobenzyl bromide, deprotection with trifluoroacetic acid (TFA) gave **9a**, followed by β -lactam cyclization¹⁵ to yield **10a**.

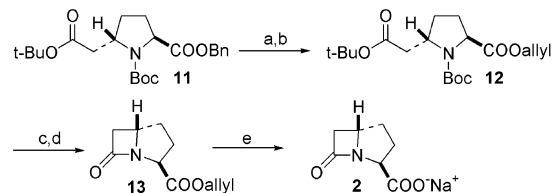
Unfortunately, but in accord with literature reports,^{7,10} hydrogenolysis of **10a** to yield **3** was ineffectual as the protected (3*S*,5*R*)-

Scheme 2^a

^a Reagents and conditions: (a) *tert*-butylbromo acetate, CH₃CN, 40 h. (b) triphenylphosphine, triethylamine, CH₂Cl₂, 20 h. (c) sodium cyanoborohydride, acetic acid, CH₃CN. (d) Pd(OH)₂/C, H₂, EtOH, 48 h. (e) (Boc)₂O, *t*-BuOH/H₂O. (f) RBr, DMF, CsCO₃. (g) TFA. (h) *N*-methyl-2-chloropyridinium triflate, diisopropylethylamine, CH₃CN, 4 h. (i) for **10b**, Pd[PPh₃]₄, *p*-toluenesulfonate sodium salt, MeOH/THF.

carbapenam was unchanged after 15 h using our optimized deprotection conditions⁶ (carboxyl group is *exo*). The absence of reactivity by the epimeric (3*S*,5*S*)-carbapenam (PNB ester is *endo*) is presumably due to the inability of the Pd catalyst to access the ester C–O bond in this hindered bicyclic system.

Recognizing the utility of removing allyl esters in pH sensitive substrates by Pd[PPh₃]₄ catalysts,¹⁶ we turned our attention to the deprotection of (3*S*,5*S*)-carbapenam allyl ester **13** (Scheme 3) as a test case prior to the synthesis of the (3*S*,5*R*)-carbapenam system (Scheme 2). An added advantage in this instance is that the allyl group acts as a two-atom spacer, potentially allowing better interaction with the Pd catalyst during deprotection, compared to the failed hydrogenolysis attempts.

Scheme 3^a

^a Reagents and conditions: (a) NaOH, THF/H₂O. (b) allyl bromide, CsCO₃, DMF. (c) TFA. (d) *N*-methyl-2-chloropyridinium triflate, diisopropylethylamine, CH₃CN, 4 h. (e) Pd[PPh₃]₄, *p*-toluenesulfonate sodium salt, MeOH/THF.

Protected amino-diacid⁶ **11** was treated with base and allyl bromide to yield **12** (Scheme 3). Treatment with TFA, followed by β -lactam cyclization,¹⁵ gave **13**. Standard allyl ester deprotection conditions¹⁶ (i.e., Pd[PPh₃]₄, potassium 2-ethylhexanoate) yielded **2**, but with large amounts of contaminating/side products that could not be removed without causing further product decomposition. Finally, utilizing the Pd[PPh₃]₄/*p*-toluenesulfonate sodium salt

deprotection protocol,¹⁷ the labile **2** was obtained with minimal amounts of decomposition. To complete the synthesis of the (3*S*,5*R*) system, protected acid **8** (Scheme 2) was then treated with allyl bromide, and TFA to give **9b**, which was then cyclized¹⁵ to give β -lactam **10b**. Deprotection of the allyl ester¹⁷ **10b**, gave **3**.

With the two diastereomeric carbapenams **2** and **3** in hand, *carC* cloned from *P. carotovorum* was overexpressed as its N-terminal His₆ tagged protein in *Escherichia coli* and purified under anaerobic conditions by affinity chromatography on a Ni-NTA column using standard procedures.

The ability of purified CarC to catalyze carbapenem synthesis was monitored by paper disk assay on a plate of β -lactam-supersensitive *E. coli* (SC12155) to detect production of hydrolytically unstable **4**.¹⁸ As shown in Figure 1, incubation of **2** and **3** with CarC and added α -ketoglutarate and ascorbate gave clear zones of inhibition (D and C, respectively). Controls with **3** itself (A), or the assay mixture lacking α -ketoglutarate (B), failed to give zones of inhibition in agreement with controls previously performed on **2**.^{5,6} These observations show that **3** is indeed an intermediate in the carbapenem biosynthetic pathway and that α -ketoglutarate is required in the ultimate oxidative desaturation step to the carbapenem nucleus **4**.

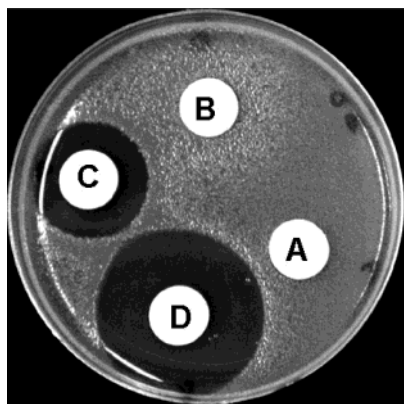


Figure 1. Carbapenem **4** production visualized on a plate of β -lactam supersensitive *E. coli* strain SC12155. All assays were incubated for 60 min at 25 °C in 20 mM phosphate buffer, pH 7.6. (A) Carbapenam **3** sodium salt (2 mM). (B) CarC (1.7 mg/mL), carbapenam **3** sodium salt (2 mM), ascorbate (1 mM). (C) CarC (1.7 mg/mL), carbapenam **3** sodium salt (2 mM), ascorbate (1 mM), α -ketoglutarate (8 mM). (D) CarC (1.7 mg/mL), carbapenam **2** sodium salt (2 mM), ascorbate (1 mM), α -ketoglutarate (8 mM).

These findings were further substantiated by monitoring the CarC reactions by LC/ESI-MS (negative ion mode). Penams **2** and **3**, were incubated with CarC, α -ketoglutarate, and ascorbate for 30 min (controls were run with deactivated enzymes). Both the controls and CarC reaction mixtures showed masses corresponding to the carbapenams (m/z 154 [$M - H$]) and α -ketoglutarate (m/z 145 [$M - H$]). However, the active CarC reaction mixtures of **2** and **3** showed two additional peaks corresponding to the production of carbapenem **4** (m/z 152 [$M - H$]) and succinate (m/z 117 [$M - H$]). These data not only confirm the intermediacy of **3** in the carbapenem biosynthetic pathway, but also provide further evidence that CarC is an α -ketoglutarate-dependent, non-heme iron oxygenase.

CarC requires α -ketoglutarate to convert **3** to **4**, clearly an oxidative process. However, the stereochemical isomerization of **2** to **3** proceeds with no net change in oxidation state. To provide further insight into the isomerization process, and determine whether it is tied to enzyme activation by α -ketoglutarate, we synthesized

2 according to our previous method,⁶ but replacing Superhydride with Superdeuteride, to produce the carbapenem with a deuterium at the C-5 bridgehead position. If CarC is capable of isomerizing **2** to **3** without the use of α -ketoglutarate, then we would observe a mass change from [$C5\text{-}^2H$]-carbapenam **2** (m/z 155 [$M - H$]) to [$C5\text{-}^1H$]-carbapenam **3** (m/z 154 [$M - H$]). No change in mass was observed by ESI-MS analysis of CarC incubated with ascorbate and [5-^2H]-**2** (m/z 155 [$M - H$]). However, upon addition of α -ketoglutarate, production of **4** was observed (confirmed by bioassay and ESI-MS m/z 152 [$M - H$]), accompanied by a small but detectable level of [5-^1H]-**3** (m/z 154 [$M - H$]). Additionally, [5-^2H]-**2** showed a slower rate of consumption in CarC assays as compared to [5-^1H]-**2** as monitored by ESI-MS. This may indicate that the isomerization process of **2** to **3** is the “slow step” in the overall catalytic cycle rather than double bond formation in the conversion of **3** to **4**.

In sum, we take these findings to support the intermediacy of **3** in the overall conversion of **2** to **4** by CarC (Scheme 1). We propose that the contrathermodynamic epimerization of **2** to **3** is coupled at least to the binding of α -ketoglutarate and, while not strictly demonstrated from the data, it is probably coupled to the reduction of molecular oxygen and proceeds by way of radical abstraction at C-5. The presumed Fe(IV)=O species formed in these processes¹⁹ is required to drive the subsequent desaturation process. How the bridgehead hydrogen is replaced in the carbapenam **3** and the stoichiometry and kinetics of reaction are the subjects of future analyses.

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