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

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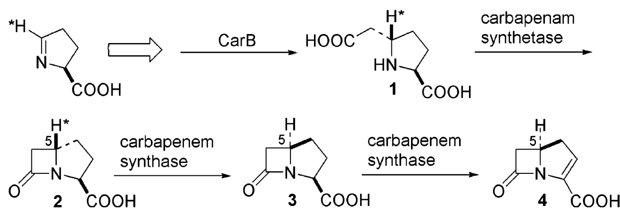
(5*R*)-Carbapenem-3-carboxylic acid (**4**) is the simplest structurally of over 60 naturally occurring carbapenem β-lactam antibiotics isolated since the discovery of thienamycin,[1] whose gene cluster has been recently identified.[2] Members of this family and their derivatives are clinically important 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 (carbapenem synthase).[6]

Scheme 1



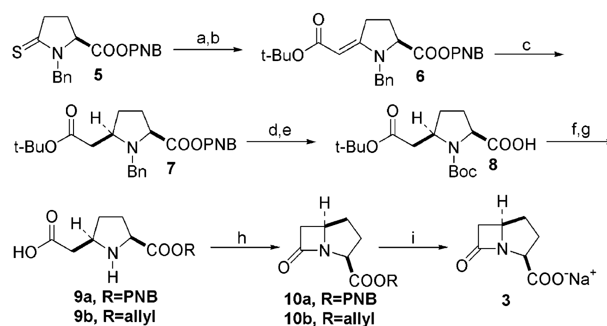
While **3** has always been present in low amounts compared to **2** and **4** during isolation of the β-lactams from fermentation,[4,7] no direct evidence exists to establish its role as an intermediate, shunt product, or product of the poorly understood self-resistance mechanism.[8] We report here 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**.

Recently[6] 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 carbapenem synthase crystal structure[12] 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 coupling[14] 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 $(Boc)_2{=}O$ yielded **8**. Derivatization with *p*-nitrobenzyl bromide, deprotection with trifluoroacetic acid (TFA) gave **9a**, followed by β-lactam cyclization[15] 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*)-carbapenam was unchanged after 15 h using our optimized deprotection conditions[6] (carboxyl group is *exo*). The absence of reactivity by the epimeric (3*S*,5*R*)-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.

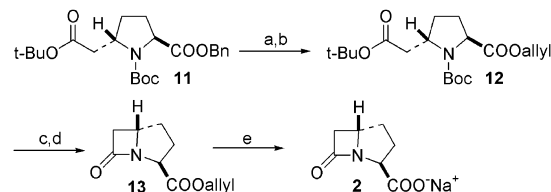
Scheme 2 [a]



[a] Reagents and conditions: (a) *tert*-butylbromo acetate, 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, 48 h. (e) $(Boc)_2{=}O$, *t*-BuOH/H_2O. (f) RBr, DMF, $CsCO_3$. (g) TFA (h) *N*-methyl-2-chloropyridinium triflate, diisopropylethylamine, CH_3CN, 4 h. (i) for **10b**, $Pd[PPh_3]_4$, *p*-toluenesulfinate sodium salt, MeOH/THF.

Recognizing the utility of removing allyl esters in pH sensitive substrates by $Pd[PPh_3]_4$ 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/H_2O. (b) allyl bromide, $CsCO_3$, DMF. (c) TFA. (d) *N*-methyl-2-chloropyridinium triflate, diisopropylethylamine, CH_3CN, 4 h. (e) $Pd[PPh_3]_4$, *p*-toluenesulfinate sodium salt, MeOH/THF.

Protected amino-diacid[6] **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 conditions[16] (i.e., $Pd[PPh_3]_4$, 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 $Pd[PPh_3]_4$/*p*-toluenesulfinate sodium salt

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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 cyclized[15] to give β-lactam **10b**. Deprotection of the allyl ester[17] **10b**, gave **3**.

With the two diastereomeric carbapenams **2** and **3** in hand, *carC* cloned from *P. carotovorum* was overexpressed as its N-terminal His_6 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**.

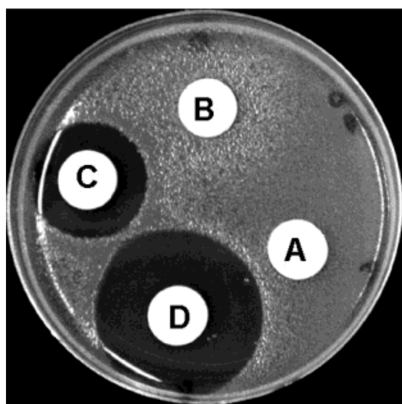

Figure 1. Carbapenem **4** production visualized on a plate of β-lactam supersensitive *E. coli* strain SC12155. All assays were incubated for 60 min at 25 °C in 20 mM phosphate buffer, pH 7.6. (A) Carbapenam **3** sodium salt (2 mM). (B) CarC (1.7 mg/mL), carbapenam **3** sodium salt (2 mM), ascorbate (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 carbapenam 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-^{2}H]-carbapenam **2** (*m/z* 155 [M − H]) to [C5-^{1}H]-carbapenam **3** (*m/z* 154 [M − H]). No change in mass was observed by ESI-MS analysis of CarC incubated with ascorbate and [5-^{2}H]-**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-^{1}H]-**3** (*m/z* 154 [M − H]). Additionally, [5-^{2}H]-**2** showed a slower rate of consumption in CarC assays as compared to [5-^{1}H]-**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 processes[19] 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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