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Non-Heme Iron Oxygenases Generate Natural Structural Diversity in Carbapenem Antibiotics

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Carbapenem antibiotics are of clinical importance because of their high potency, broad spectrum of antimicrobial activity, and resistance to most β-lactamases.[1] Thienamycin (**1**) (Figure 1), the most potent natural member of this family, co-occurs in *Streptomyces cattleya* with four carbapenems that are distinguished by their C-2/C-6 substituents.[2] There are more than 50 known carbapenam/em metabolites, many of which are differentiated only by the oxidation state of their C-2/C-6 substituents. The C-6 ethyl side chain of **1** is derived by C_1-donations from methionine[3,4] and can be methyl, ethyl, or isopropyl, which can be saturated, unsaturated, hydroxylated, or sulfated. Recent work has established that coenzyme A is successively truncated by three enzymes encoded by the thienamycin gene cluster to give the C-2 cysteamine moiety.[5] This side chain can be pantetheine, but is generally cysteamine, which can be N-acetylated or N-propionylated, desaturated and further oxidized to the sulfoxide, or cleaved and oxidized in a stepwise fashion to the sulfonic acid. These carbapenem metabolites comprise a natural combinatorial library whose structural modifications temper the high intrinsic hydrolytic instability of the carbapenem nucleus, as well as affect the antimicrobial spectrum and β-lactamase resistance of each family member.[1] Some of the higher oxidation state carbapenems have either enhanced antibiotic activity or increased β-lactamase resistance, and so, in the broad context of carbapenem biosynthesis, the origin of this oxidative diversity is of particular interest.

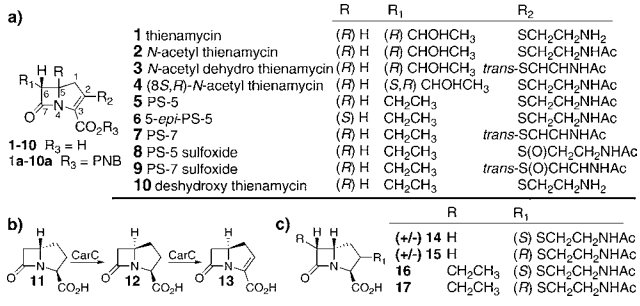

Figure 1. (a) Representative carbapenems. (b) The CarC-catalyzed reaction. (c) Carbapenam thioethers synthesized in this study.

The variability of the carbapenem side chain oxidation state as well as the discovery of a mutant strain[6] of *Streptomyces cattleya* that produced deshydroxy thienamycin (**10**) instead of thienamycin (**1**) led us to believe that the thienamycin gene cluster[7] encoded one or more enzyme(s) capable of oxidizing the C-2/C-6 moieties of carbapenems. Protein sequence analysis of ThnG and ThnQ indicated that each enzyme contained the $Hx(D/E)x_nH$ motif characteristic of nonheme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases, and these seemed promising candidates.[8] However, ThnG and ThnQ are in the same family as CarC encoded by the (5*R*)-carbapenem-3-carboxylate (**13**) gene cluster in *Pectobacterium carotovorum*.[9,10] Despite low homology to CarC, ThnG and ThnQ have been postulated to catalyze steps in thienamycin biosynthesis analogous to the coupled C-5 epimerization and C-2/C-3 desaturation of (2*S*,5*S*)-carbapenam (**11**) to (5*R*)-carbapenem-3-carboxylate (**13**) catalyzed by CarC.[11] To discern their roles in thienamycin biosynthesis, ThnG and ThnQ were analyzed for carbapenem-oxidizing activity, as well as for the ability to catalyze C-5 epimerization and coupled or uncoupled C-2/C-3 desaturation of carbapenams/ems.

The envisioned experiments required carbapenam/ems varying in stereochemical configuration at C-6 as well as C-2/C-6 oxidation state/substitution pattern to serve as substrates and reference standards. Two methods were employed to establish the C-6 substituent and C-5/C-6 configuration by synthesizing precursor azetidinones. The first method provided the *trans* (3*S*,4*R*)-configuration by alkylating the enolate of an azetidinone derived from L-aspartic acid.[12] The second method employed a catalytic asymmetric azetidinone-forming reaction that produced either enantiomer of the *cis* 3,4-disubstituted azetidinones with independent control of the carbapenem C-8 stereocenter.[13] These compounds could be used as precursors of *cis* or *trans* carbapenems. Azetidinones were converted to carbapenems by the Merck method, which allowed various C-2 groups to be introduced.[14,15] The intermediate 2-oxocarbapenams in this route can be reduced and directed to the preparation of carbapenams bearing thioether substituents at C-2 (Figure 1c).[5] Carbapenam thioethers **14**−**17**, (2*S*,5*S*)-carbapenam (**11**), (2*S*,5*R*)-carbapenam (**12**),[16] and 5-*epi*-PS-5 (**6**) were synthesized to test ThnG and ThnQ for coupled or uncoupled carbapenam ring epimerization and desaturation. PS-5 (**5**) was synthesized to test for side chain oxidation activity, because the acetylated cysteaminyl side chain is more stable than the unacetylated deshydroxy thienamycin (**10**). PS-7 (**7**), PS-7 sulfoxide (**9**), PS-5 sulfoxide (**8**), *N*-acetyl thienamycin (**2**), and the diastereomeric mixture (8*S*,*R*)-*N*-acetyl thienamycin (**4**) were synthesized as additional substrates and reference standards.

Thienamycin biosynthetic cluster genes *thnG* and *thnQ* were cloned from genomic DNA and inserted into pET29b each bearing a *C*-terminal His_6-tag. The recombinant proteins were overproduced in *E. coli* Rosetta2(DE3) and purified by Ni-NTA affinity chromatography. *In vitro* reactions in MOPS, pH 7.0, containing $Fe(NH_4)_2(SO_4)_2$, α-KG, ascorbate, the subject carbapenam/em, and either ThnG or ThnQ were incubated and analyzed by HPLC for the formation of new product(s).[17,18] Clear outcomes were observed for both ThnG- and ThnQ-catalyzed reactions with PS-5 (**5**) (Figure 2 and Supporting Information). The products were immediately identifiable as carbapenems by their unique chromophores (λ_{max} = 290−320 nm). ThnQ produced a single new product more polar than **5**, while ThnG produced two products, one with a shorter retention time and one with a longer retention time than that of **5**.

ESI mass spectrometric analysis of the new product (m/z = 313.05) in the ThnQ-catalyzed reaction established that a single oxygen had been incorporated. Its identity was determined by

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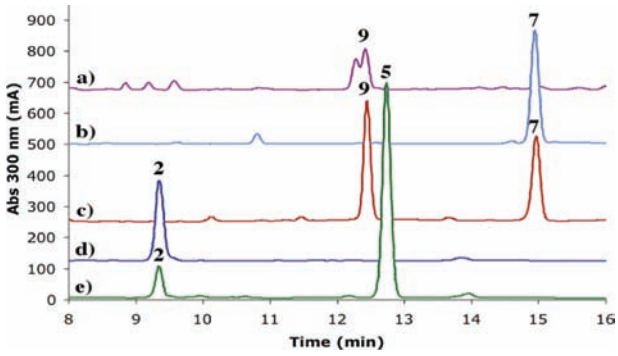

Figure 2. HPLC analysis of ThnG and ThnQ reactions with PS-5 (**5**). (a) PS-7 sulfoxide diasteriomers standard (**9**). (b) PS-7 standard (**7**). (c) ThnG-catalyzed reaction with **5**. (d) *N*-Acetyl thienamycin standard (**2**). (e) ThnQ-catalyzed reaction with **5**.

coinjection with *N*-acetyl thienamycin (**2**) and (8*S*,*R*)-*N*-acetyl thienamycin (**4**) (Figures 2d,e, S3). This HPLC comparison demonstrated that ThnQ stereospecifically hydroxylated PS-5 (**5**) to produce **2**. The chromatographically distinct (8*S*)-*N*-acetyl thienamycin diastereomer was not detected (Figure S4). ESI-MS analysis of the products of the ThnG-catalyzed reaction with **5** indicated that the late eluting product (m/z = 295.05) had an additional degree of unsaturation relative to **5**, and the early eluting product (m/z = 311.15) was both desaturated and oxidized. The carbapenems were identified as PS-7 (**7**) and PS-7 sulfoxide **9** by HPLC comparison to synthetic standards (Figures 2a−c, S5). ThnG was also able convert **7** to its sulfoxide but unable to catalyze desaturation when given PS-5 sulfoxide **8**, indicating that desaturation precedes sulfoxidation (Figure S6).

The oxidative relationships among carbapenems were further demonstrated by conversion of PS-7 (**7**) and *N*-acetyl thienamycin (**2**) into the *S. cattleya* metabolite *N*-acetyl dehydrothienamycin (**3**). Upon reaction with **2**, ThnG produced a less polar product and ESI-MS indicated it contained an additional degree of unsaturation. ThnQ produced a more polar product on reaction with **7**. These new products coeluted under HPLC, had identical masses by ESI-MS, and were assigned the same structure, *N*-acetyl dehydrothienamycin (**3**). Notably no sulfoxide product was observed, consistent with the metabolite profile in *S. cattleya* and suggesting that ThnQ reaction precedes that of ThnG.

HPLC analyses of other carbapenam/ems (**6**, **11**, **12**, **14**−**17**) tested with ThnG and ThnQ did not show appearance of a carbapenem chromophore or other new products. Additionally, *in vitro* reactions with carbapenam/ems (**6**, **11**, **12**, **14**−**17**) employing cell-free extracts harboring ThnG or ThnQ were analyzed with the supersensitive *E. coli* SC 12155 and the Nitrocefin β-lactamase induction assay using *Bacillus licheniformis* ATCC 14580.[19,20] By none of these three sensitive measures could antibiotic production be detected.

These experiments demonstrate that the Fe(II)/α-KG-dependent oxygenases ThnG and ThnQ oxidize the C-2 and C-6 side chains, respectively, of carbapenem substrates. No evidence was found that they catalyze coupled or uncoupled C-5 epimerization and/or C-2/C-3 desaturation in the carbapenam/ems tested. On these bases it appears that the latter two reactions rely on other proteins encoded by the thienamycin gene cluster that are distinct from the non-heme iron oxygenases like CarC, key to the biosynthesis of (5*R*)-carbpenem-3-carboxylate (**13**).[10,11,21] Oxidative modifications of the C-2 and C-6 side chains of carbapenems are major determinants of their antimicrobial spectrum and β-lactamase resistance. These activities strongly suggest that the known carbapenems produced by *S. cattleya* arise from ThnG and ThnQ oxidation of a common biosynthetic precursor and that much of the structural diversity exemplified by this class of antibiotics likely derives from orthologues present in other carbapenem producers. Knowledge of these oxidative relationships will more sharply refine further biosynthetic investigations of this antibiotic family.

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Supporting Information Available: Experimental procedures, HPLC comparisons, and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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