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The Unusual Bifunctional Catalysis of Epimerization and Desaturation by Carbapenem Synthase

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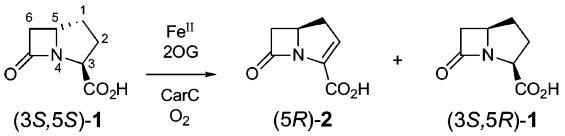
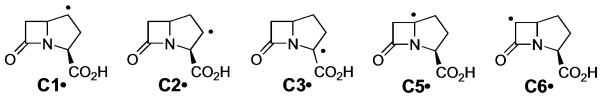
Carbapenems are an important class of antibiotics as they have a broad spectrum of activity and their stability toward serine β-lactamases can provide superior bacteriocidal action over alternatives, such as penicillins and cephalosporins.[1] Although many carbapenems are natural products, for medicinal use they are produced by multistep total synthesis. Studies of carbapenem biosynthesis are of significant interest as they may enable the development of more efficient biochemical production methods. Of the three enzymes involved in the production of the simplest carbapenem ((5*R*)-carbapenem, (5*R*)-**2**), carbapenem synthase (CarC) is particularly interesting.[1b,2] The unusual role of this 2-oxoglutarate-dependent (2OG) nonheme iron (II) oxygenase is to catalyze two distinct processes: C5 epimerization and C2/C3 desaturation of (3*S*,5*S*)-carbapenam ((3*S*,5*S*)-**1**). In addition, the saturated but epimerized product ((3*S*,5*R*)-**1**) is also generated during the transformation[3a] and is also a substrate for CarC.[3b,c]



By analogy with other ferrous- and 2OG-dependent oxygenases, the likely oxidant in CarC is an Fe^{IV}═O intermediate.[4a,b] Such a species can be monotonically reduced via two H atoms to Fe^{III}—OH and $Fe^{II} \leftarrow OH_2$, and such a dehydrogenation is precedented by one of the steps catalyzed by clavaminic acid synthase (CAS) in the biosynthetic pathway leading to the β-lactamase inhibitor clavulanic acid.[4c,d] However, although a sequential loss of H atoms from C2/C3 may well be the operative mechanism when (3*S*,5*R*)-**1** is the substrate, it does not explain the epimerization at C5 during reaction of (3*S*,5*S*)-**1**.

In the current work, we have employed high-level ab initio molecular orbital calculations[5,6] to investigate possible mechanisms for the epimerization step and to examine the unusual bifunctional catalysis by CarC. Geometries of relevant species were optimized with the B3-LYP/6-31G(d) procedure and improved relative energies obtained with G3(MP2)-RAD. Uncharged acids (CO_2H) were chosen in preference to carboxylates in our calculations, since the substrate is likely to be H-bonded to an arginine (R267) or an alternative basic residue.[7]

A recent crystallographic study on CarC complexed with Fe^{II}, 2OG, and (*S*)-*N*-acetylproline ((*S*)-NAP), an analogue of (3*S*,5*S*)-**1**, has provided some insight into substrate binding to CarC.[7] However, it was not possible to unambiguously determine the orientation of (*S*)-NAP in the active site. In combination with QM/MM modeling studies, two likely orientations of (3*S*,5*S*)-**1** in that active site were proposed.[7] One orientation (I) positions C3 and C2 close to the iron center (leading to (Fe)O···H(C) distances of ~4.0 and ~3.8 Å) with C5 exposed to the solvent. In the other orientation (II), it is C5 that is close to the iron ((Fe)O···H(C) ~2.3 Å). On the basis of these results, it was proposed that the first step in the reaction is H abstraction from either C3 or C5. However, because of limitations in the modeling procedure, the possibility of H abstraction from other carbons was not eliminated.[7]



	C1•	C2•	C3•	C5•	C6•
5*S*	43.8	45.9	0.0	56.1	47.3
5*R*	57.3	52.7	0.0	48.7	55.6

Figure 1. Calculated relative energies (kJ mol^{-1}) for radicals derived from (3*S*,5*S*)-**1** and (3*S*,5*R*)-**1**.

Calculations on the radicals derived by H abstraction from C1, C2, C3, C5, and C6 of (3*S*,5*S*)-**1**, and their epimerized (3*S*,5*R*)-**1** forms (Figure 1) show that the C3 radical is significantly the lowest in energy (by 44−57 kJ mol^{-1}).[8] This is not surprising because of the captodative stabilization provided by the adjacent amido and carboxyl groups.[9] On this basis, H abstraction from C3 might be expected to be the preferred process, particularly in orientation I. In related systems, however, it is found that the relative energies of the possible product radicals do not necessarily dictate the regioselectivity of hydrogen transfer, particularly when the abstracting species is an oxygen-centered radical.[10] Thus, our calculations do not preclude initial H abstraction from C1, C2, C5, or C6. Hence, it is of interest to examine epimerization reactions proceeding through each of these carbon-centered radicals.

If reaction proceeds via the C3 radical, (5*S*)-**C3•**, it is conceivable that epimerization at C5 could occur through β-scission of (5*S*)-**C3•** to give the ring-opened radical (5*S*)-**3**, followed by inversion to (5*R*)-**3** and ring closure to (5*R*)-**C3•** (Figure 2).[5] However, the large barrier associated with the initial ring opening (99.2 kJ mol^{-1}) and the unfavorable entropy for ring closure imply that significant catalysis from the enzyme would be required for this pathway to be viable. At this stage, it is not clear what form such catalysis would take.

With the C1, C2, or C6 radicals, our calculations show that the reaction pathways that emerge for epimerization have energy barriers comparable to, or even higher than, that for the C3 ring openings.[6] In two of these cases (C1 and C6), high-energy

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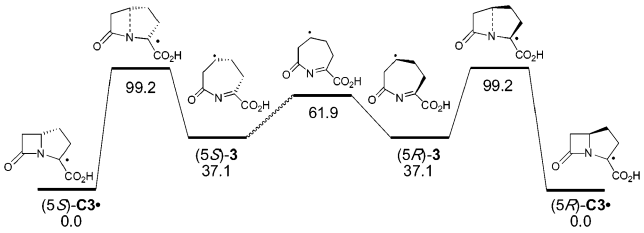


Figure 2. Schematic energy profile (kJ mol^{-1}) for epimerization via the C3 radical (5*S*)-**C3•** derived from (3*S*,5*S*)-**1**.

intramolecular H migrations are also required for subsequent oxidation at C2/C3.[6] Again, in the light of what is known of CarC and related enzymes, such processes appear unlikely.

In the case of (3*S*,5*S*)-**C5•**, however, epimerization to (3*S*,5*R*)-**C5•** is a very straightforward inversion process that is predicted to be exothermic by 7.4 kJ mol^{-1} (Figure 1), with a barrier of just 24.2 kJ mol^{-1}.[5] If (3*S*,5*S*)-**1** is initially bound in orientation II, H abstraction by Fe^{IV}═O at C5 seems particularly likely. In addition, initial abstraction from C5 is consistent with recent labeling experiments,[3a] which indicate that this hydrogen is lost during formation of both (5*R*)-**2** and (3*S*,5*R*)-**1**.

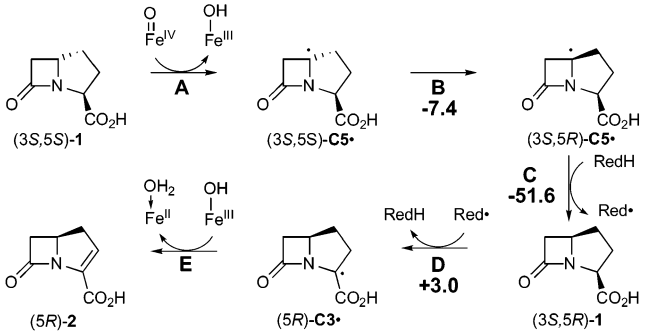
Following epimerization to (3*S*,5*R*)-**C5•**, completion of the catalytic cycle to yield (5*R*)-**2** still requires reduction at C5 and oxidation across C2/C3. Despite the presence of *cis*-oriented H-atoms at C2 and C3, and an overall exothermic reaction in the case of C3, [1,3]-intramolecular H-atom migrations from C3 or C2 to C5 appear unlikely as they are calculated in each case to be associated with a very high barrier (ca. 240 kJ mol^{-1}).[6] Instead, other possibilities for coupling reduction/oxidation at C3 and C5 of (3*S*,5*R*)-**C5•** can be envisioned.

The inherent stability of the C3 radical is likely to contribute to the ensuing oxidation at C2/C3. Indeed, coupling **C5•** to **C3•** can be achieved via protonation/deprotonation at these centers, respectively, through the intermediacy of a radical cation. (5*R*)-**C3•** could then be oxidized at C2 by Fe^{III}−OH, forming the product (5*R*)-**2**. However, such a mechanism does not provide an easy explanation for the observation and processing of (3*S*,5*R*)-**1**.

Alternatively, CarC may directly or indirectly utilize an external H-atom source to exploit the stability of the C3 radical. In this connection, a recent study has shown that the presence of ascorbate influences the trends in the turnover of stereoisomers of 1-carbapenam-3-carboxylate.[3b] For example, the turnover of (3*S*,5*S*)-**1** increased more than 20-fold. Such a large stimulation was not observed for the (3*S*,5*R*) stereoisomer. These results suggest that the efficient biosynthesis of (5*R*)-**2** from (3*S*,5*S*)-**1** may be linked to the presence of ascorbate in vitro, or of other reducing agents, such as mycothiol,[11] in vivo. Although one role of ascorbate in catalysis by 2OG oxygenases appears to be to reduce "unwanted" high oxidation states of iron, the mechanism for this is unclear and need not involve direct interaction of the ascorbate with the iron. Indeed, because the iron in CarC is buried within the active site and shielded by (3*S*,5*S*)-**1** in orientation II, the possibility of an indirect mechanism becomes worthy of attention.

Accordingly, if an equivalent reducing species (RedH) does exist for CarC, the mechanism of Scheme 1 can be envisaged. After epimerization to (3*S*,5*R*)-**C5•** (Step B), in which C5 becomes solvent-accessible, H-atom transfer from RedH to C5 would lead to (3*S*,5*R*)-**1** (Step C), which could then leave as a "shunt" product. We note that the observed loss of label at C5 during the formation of (3*S*,5*R*)-**1**[3a] requires that the C5 hydrogen of this product be derived from a source other than the initial H-atom abstractor. If leakage from the active site does not occur, Red• could abstract an H-atom from C3 (which is also solvent accessible) (Step D),

Scheme 1. Proposed Mechanism of CarC-Catalyzed Carbapenem [(5*R*)-2] Biosynthesis (energies in kJ mol^{-1})



regenerating RedH and priming (5*R*)-**C3•** for immediate oxidation at C2 by the iron species.

Such a mechanism is supported by our calculated bond dissociation energies (BDEs) for the relevant steps in Scheme 1, obtained using ethanethiol as a model for RedH. The BDEs are 355.3 kJ mol^{-1} for ethanethiol, and 406.9 and 358.3 kJ mol^{-1} for C5−H and C3−H of (3*S*,5*R*)-**1**. These indicate that the epimerization and desaturation steps can indeed be effectively coupled, with steps B and C of Scheme 1 each being exothermic by 7.4 and 51.6 kJ mol^{-1}, respectively, and D endothermic by just 3.0 kJ mol^{-1}. Given the uncertainties implicit in our model and in our calculations, it may well be that all three steps are exothermic.

While the mechanism depicted in Scheme 1 is consistent with experimental evidence, the presence of RedH in bacteria has yet to be firmly established. Therefore, theoretical and experimental exploration of other mechanistic possibilities is continuing.

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Supporting Information Available: Details of the calculations, archive entries, and relevant total energies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Livermore, D. M.; Woodford, N. *Curr. Opin. Microbiol.* **2000**, *3*, 489−495. (b) McGowan, S. J.; Bycroft, B. W.; Salmond, G. P. C. *Trends Microbiol.* **1998**, *6*, 203−208. (c) Easton, C. J.; Knowles, J. R. *Biochemistry* **1982**, *21*, 2857−2862.
(2) (a) McGowan, S. J.; Holden, M. T. G.; Bycroft, B. W.; Salmond, G. P. C. *Antonie van Leeuwenhoek* **1999**, *75*, 135−141. (b) Bycroft, B. W.; Chhabra, S. R. *J. Chem. Soc., Chem. Commun.* **1989**, 2325−2326. (c) Sleeman, M. C.; Schofield, C. J. *J. Biol. Chem.* **2004**, *279*, 6730−6736.
(3) (a) Stapon, A.; Li, R.; Townsend, C. A. *J. Am. Chem. Soc.* **2003**, *125*, 8486−8493. (b) Sleeman, M. C.; Smith, P.; Kellam, B.; Chhabra, S. R.; Bycroft, B. W.; Schofield, C. J. *ChemBioChem*. **2004**, *5*, 879−882. (c) Stapon, A.; Li, R.; Townsend, C. A. *J. Am. Chem. Soc.* **2003**, *125*, 15746−15747.
(4) (a) Rohde, J.-U.; In, J.-H.; Lim, M. H.; Brennessel, W. W.; Bukowski, M. R.; Stubna, A.; Münck, E.; Nam, W.; Que, L. *Science* **2003**, *299*, 1037−1039. (b) Price, J. C.; Barr, E. W.; Tirupati, B.; Bollinger, J. M.; Krebs, C. *Biochemistry* **2003**, *42*, 7497−7508. (c) Schofield, C. J.; Zhang, Z. H. *Curr. Opin. Struct. Biol.* **1999**, *9*, 722−731. (d) Baggaley, K. H.; Brown, A. G.; Schofield, C. J. *Nat. Prod. Rep.* **1997**, *14*, 309−333.
(5) For full details, see the Supporting Information.
(6) A full report of these calculations will be published elsewhere.
(7) Clifton, I. J.; Doan, L. X.; Sleeman, M. C.; Topf, M.; Suzuki, H.; Wilmouth, R. C.; Schofield, C. J. *J. Biol. Chem.* **2003**, *278*, 20843−20850.
(8) C3• has bonds that are effectively coplanar, leading to enantiomeric and energetically equivalent (5*S*) and (5*R*) forms.
(9) See, for example: Yu, D.; Rauk, A.; Armstrong, D. A. *J. Am. Chem. Soc.* **1995**, *117*, 1789−1796.
(10) Easton, C. J. *Chem. Rev.* **1997**, *97*, 53−82.
(11) Fahey, R. C. *Annu. Rev. Microbiol.* **2001**, *55*, 333−356.

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