Biosynthesis of Carbapenem Antibiotics: New Carbapenam Substrates for Carbapenem Synthase (CarC)

Mark C. Sleeman,[b] Paul Smith,[a] Barrie Kellam,[a] Siri Ram Chhabra,[a] Barrie W. Bycroft,*[a] and Christopher J. Schofield*[b]

The carbapenem subfamily of β-lactam antibiotics are medicinally useful as they possess a broad spectrum of activity and display resistance to inactivation by "serine" β-lactamases.[1] Unusually for β-lactam antibiotics they are produced by total synthesis and, consequently, their production costs are relatively high compared to those antibiotics produced by fermentation. Gene clusters encoding the enzymes involved in carbapenem production have now been identified,[2–4] and their manipulation may enable an efficient route to carbapenems or useful intermediates. More than ten genes are proposed to be involved in the biosynthesis of the C2- and C6-functionalised carbapenems, such as thienamycin;[3] however, only three proteins, encoded by the *carA*, *carB* and *carC* genes, are directly responsible for the biosynthesis of the simplest carbapenem, (5*R*)-1-carbapen-2-em-3-carboxylate ((5*R*)-carbapenem, **1**, Scheme 1).[2,5]

Carbapenem synthase (CarC) is a ferrous and 2-oxoglutarate-dependent enzyme that catalyses the highly unusual epimerisation and desaturation of the (3*S*,5*S*)-carbapenam **2** to give the (3*S*,5*R*)-carbapenam **3** and the (5*R*)-carbapenem **1**.[6] The conversion of **2** to **1** via **3** is reminiscent of the sequential oxidation and desaturation reactions catalysed by clavaminic acid synthase (another 2-oxoglutarate (2OG) oxygenase) in the biosynthesis of clavulanic acid.[7,8] However in the latter case, each of the reactions encompasses a two-electron oxidation, while with CarC only a single two-electron oxidation is required to go directly from **2** to **1** (Scheme 1). The epimerisation reaction, proposed to be mediated by an Fe^{IV}=O species,[9] has little or no precedent in synthetic chemistry. Furthermore, loss of label from the C5 position[10] in the conversion of **2** to **3** appears to support a direct abstraction process.

The stereochemistry of the antibiotic **1** and the (3*S*,5*R*)-carbapenam **3** from *Pectobacterium carotovora* and *Serratia marcescens* have been unequivocally established.[11] After some confusion, the assignment for the natural carbapenam substrate [(3*S*,5*S*)-carbapenam **2**] has been confirmed.[10,12,13] Studies with CarC have employed either the synthetic (3*S*,5*S*)-carbapenam **2**, or the same stereoisomer produced in situ from a monocyclic (5-carboxymethyl)-proline (CMP, **4**) precursor by coupling of the CarA (carbapenam synthetase) and CarC reactions.[9,10,14] In order to further investigate the mechanism of the unusual transformation of **2** to **1** (Scheme 1), we report here the results of incubating all four possible carbapenam stereoisomers with CarC.

The *trans*-carbapenam (3*S*,5*S*)-**2** and its enantiomer (3*R*,5*R*)-**5** were prepared as reported, by following a route in which the construction of the pyrrolidine ring was achieved by a thermodynamically controlled intramolecular Michael reaction step (Scheme 2a).[12] This route was optimised for the preparation of the *trans*-stereoisomers with a *trans*/*cis* ratio of about 11:1, but is not convenient for the preparation of the *cis* stereoisomers. An alternative route was therefore developed for the *cis* stereoisomers and exemplified for the (3*R*,5*S*) stereoisomer **6** (Scheme 2b). Following preparation of keto-diester **10** from pyroglutamate, the key step was a one-pot *Z* deprotection followed by intramolecular reductive amination[15] (*trans*/*cis*, ca. 1:3).

Optical-rotation measurements of the carbapenam methyl esters were consistent with the production of all four carbapenam stereoisomers: (3*S*,5*S*)-**2**, $[\alpha]_D^{25} = -194.0°$ ($c = 0.30$ in $CHCl_3$), lit. $[\alpha]_D^{24} = -198.5°$ ($c = 0.50$ in $CHCl_3$); (3*R*,5*R*)-**5**, $[\alpha]_D^{21} = +203.0°$ ($c = 0.25$ in $CHCl_3$), lit. $[\alpha]_D^{25} = +198.0°$ ($c = 0.64$ in $CHCl_3$); (3*S*,5*R*)-**3**, $[\alpha]_D^{25} = +123.0°$ ($c = 0.25$ in $CHCl_3$), lit. $[\alpha]_D^{24} = +87.0°$ ($c = 0.41$ in $CHCl_3$); (3*R*,5*S*)-**6**, $[\alpha]_D^{21} = -92.4°$ ($c = 0.30$ in $CHCl_3$), lit. $[\alpha]_D^{24} = -87.7°$ ($c = 0.28$ in $CHCl_3$).[13,16] The observed $[\alpha]_D^{21}$ value for **3** was higher than expected; this suggested contamination of **3** by **5**. ^{1}H NMR analysis indeed revealed the presence of $< 5\%$ of **5**; however, since **5** is a poorer substrate than **3**, the order of reactivity should not be significantly affected.

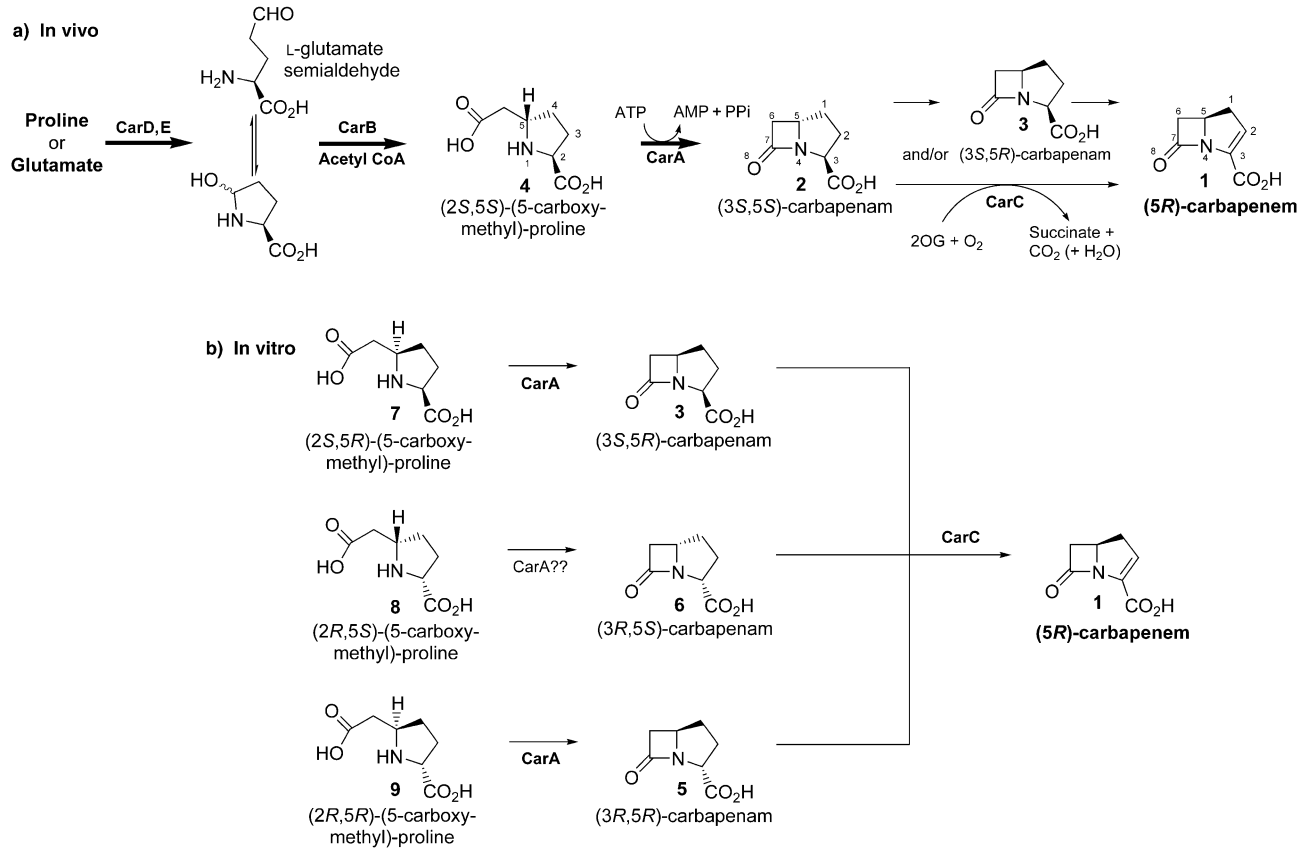
Each of the four stereoisomers was incubated separately with CarC in the presence of Fe^{II} and 2OG. The formation of carbapenem product was assayed for by HPLC/MS (carbapenem 152 Da $[M-H]^-$, carbapenam 154 Da $[M-H]^-$) and bioassay. It was assumed that the production of a zone of antibiosis reflected production of the (5*R*)-carbapenem **1**; precedent suggests it is unlikely that the (5*S*)-carbapenem is bioactive,[17] but this cannot be entirely ruled out.

Care must be taken in comparing results from the stopped assays (the instability and scarcity of the bicyclic β-lactams coupled with the poor activity of CarC rendered continuous assays difficult and precluded analysis of the optical purity of the products). However, the same trend was observed upon repeat analyses and was observed by HPLC monitoring of carbapenem production ($\lambda_{max} = 261$ nm in the absence of ascorbate). The following trends were observed in the presence of ascorbate (1 mM): **2** > **3** > **5** > **6** and in the absence of ascorbate **3** > **5** > **6** > **2** (Table 1). The results for the assigned natural substrate of CarC, (3*S*,5*S*) stereoisomer **2** in the presence of ascorbate, were supportive of previous reports that it is a substrate for CarC. However, the (3*S*,5*R*)-carbapenam **3** was clearly a better in vitro substrate in the absence of ascorbate. Since this stereoisomer is also produced in vivo (Scheme 1), it is pos-

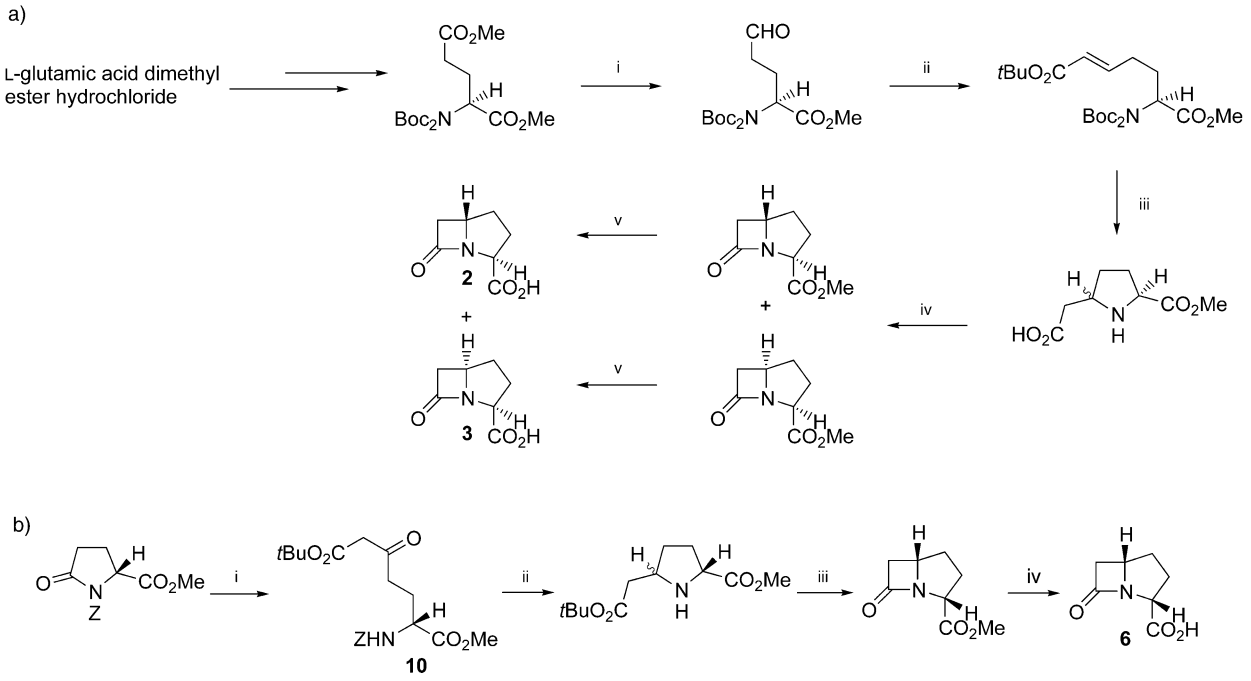
[a] *Dr. P. Smith, Dr. B. Kellam, Dr. S. R. Chhabra, Prof. Dr. B. W. Bycroft*
School of Pharmacy, University of Nottingham
University Park, Nottingham, NG7 2RD (UK)
Fax: (+44) 115-951-5049
E-mail: barrie.bycroft@nottingham.ac.uk

[b] *M. C. Sleeman, Prof. Dr. C. J. Schofield*
Dyson Perrins Laboratory, University of Oxford
South Parks Road, Oxford, OX1 3QY (UK)
Fax: (+44) 186-527-5674
E-mail: christopher.schofield@chem.ox.ac.uk

 DOI: 10.1002/cbic.200300908



Scheme 1. *a) Proposed biosynthesis of (5R)-carbapenem* ***1*** *by CarA, CarB and CarC from primary metabolites. b) Other reactions catalysed by CarA and CarC in vitro.*



Scheme 2. *Synthesis of 1-carbapenam-3-carboxylate stereoisomers. Reagents and conditions: a) Illustrated for the (3S,5S) and (3S,5R) stereoisomers: i) DIBAL-H, Et_2O, −78 °C, 30 min 85 %; ii) Ph_3PCHCO_2tBu, toluene, 110 °C, 12 h, 85 %; iii) HCl/Et_2O, 5 h, then Et_3N, THF, 48 h, 60 %,* trans/cis, ca. *11:1; iv) tris(2,3-dihydro-2-oxobenzoxazol-3-yl)phosphine oxide, Et_3N, MeCN, 24 h, 45 %, diastereomers separated by chromatography; v) PLE immobilised on Eupergit®, phosphate buffer pH 8.0 3.5 h. b) i)* t*BuOAc/lithium diisopropylamide, THF, 30 min, −78 °C, 35 %; ii) 10 % Pd/C, H_2, MeOH, AcOH, 64 psi, 18 h, 48 %,* cis/trans, ca. *3:1; iii) HCl/dioxane, 72 h then tris(2,3-dihydro-2-oxobenzoxazol-3-yl) phosphine oxide, Et_3N, MeCN, 24 h, 20 %, diastereomers separated by chromatography; iv) PLE immobilised on Eupergit®, phosphate puffer pH 8.0, 3.5 h.*

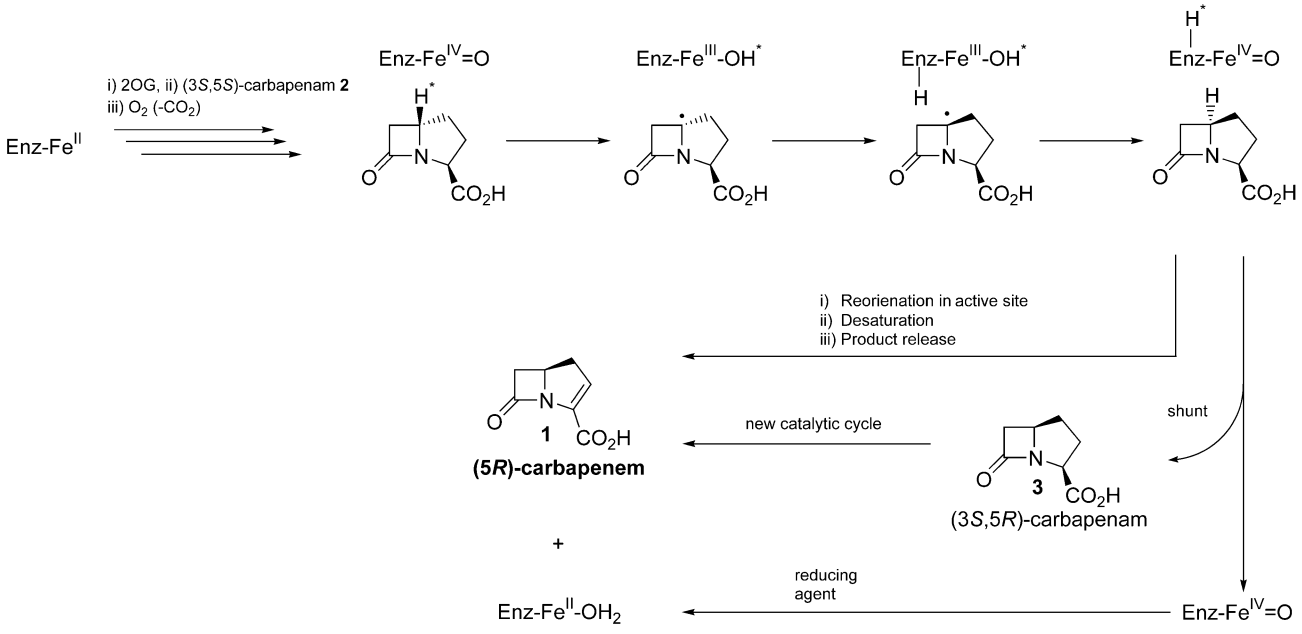
Table 1. *Bioassays of CarC in the presence and absence of ascorbate normalised to that observed for the (3S,5S)-carbapenam in the presence of ascorbate (average of at least two experiments with the same batch of bioassay plates). Standard deviations are shown in brackets.*

	Stereoisomer of 1-carbapenam-3-carboxylate			
	(3*S*,5*S*)-**2**	(3*S*,5*R*)-**3**	(3*R*,5*R*)-**5**	(3*R*,5*S*)-**6**
− ascorbate	2	86 (8.8)	40 (1.5)	7 (6.1)
+ ascorbate	100 (7.5)	76 (7.5)	22 (0)	< 2

sible that **3** could act as an enzyme free intermediate between the (3*S*,5*S*)-carbapenam stereoisomer **2** and the (5*R*)-carbapenem **1** (see below). Both the bioassay and LCMS results indicated that the (3*R*,5*R*)-carbapenam **5**, which possesses analogous stereochemistry at C3 and C5 to the penicillin and clavam antibiotics/β-lactamase inhibitors, was also a substrate for CarC. That the two best substrates under the conditions lacking ascorbate possessed the (5*R*)-stereochemistry is consistent with the unusual epimerisation step being more "difficult" than the desaturation step. Incubation with the (3*R*,5*S*)-substrate **6** revealed it to be a poor substrate; this probably reflects both its status as an unnatural substrate and the necessity for it to undergo epimerisation to produce a bioactive carbapenem. It cannot be ruled out that the low levels of activity seen with this stereoisomer are due to contamination by the (3*S*,5*R*) stereoisomer **3**.

The addition of L-ascorbate to the assay mixture significantly increased turnover of the natural (3*S*,5*S*) stereoisomer **2**. Although the ascorbate may play more than one role in in vitro turnover by 2OG oxygenases (in the case of some enzymes it can replace the "prime substrate" in "uncoupled" reaction cycles) one role for it has been proposed to be rescuing the enzyme from potential oxidation damage due to an "inadvertently" produced Fe^{IV}=O intermediate.[18] Another possible role for ascorbate (or a bacterial equivalent) with CarC is mediating the supply of the hydrogen (possibly from water) to the C5 position of **1** during the epimerisation process. Since the conversion of (3*S*,5*S*)-carbapenam **2** to (5*R*)-carbapenem **1** requires only a single two-electron oxidation, it would seem most efficient for CarC to catalyse epimerisation/desaturation in a single coupled process. Crystallographic analysis of CarC complexed with Fe^{II} and 2OG has indicated that different active-site orientations are required for the desaturation/epimerisation steps.[9] Thus, subsequent to or during epimerisation, it may be that during some catalytic cycles the (3*S*,5*R*) stereoisomer **3**, or an intermediate can "leak" from the active site as a shunt metabolite leaving a reactive Fe^{IV}=O intermediate (Scheme 3). Ascorbate can then serve to reduce this reactive intermediate to Fe^{II}, as proposed for other 2OG oxygenases, and desaturation of the (3*S*,5*R*) stereoisomer **3** can then occur in a new reaction cycle. Ascorbate did not stimulate turnover of the (3*S*,5*R*)-**3** or (3*R*,5*R*)-**5** stereoisomers; perhaps this reflects the fact that they do not need to be epimerised. Under the assay conditions, ascorbate appeared to be a weak inhibitor of the (3*R*,5*R*)-**5** and (3*R*,5*S*)-**6** stereoisomers, possibly due to competition with these unnatural substrates, although some inhibition of the (3*S*,5*R*) stereoisomer **3** was also observed. There is no evidence for ascorbate production in bacteria, but other small-molecule reducing agents have been identified (e.g. mycothiol[19]), and it is reasonable to suppose that bacteria possess a mechanism for reducing protein-bound $Fe^{IV/III}$ to Fe^{II}.

Interestingly CarA, the enzyme prior to CarC in the pathway, has been shown to convert three of the four monocyclic pyrro-



Scheme 3. *Outline of a possible mechanism for the CarC-catalysed epimerisation step. Ascorbate can serve as the reducing agent in vitro. Labelling studies show that the hydrogen that is abstracted from C5 is not returned.[10] In the Scheme, the source of the C5 hydrogen of **1** is shown as coming from the enzyme though its origin and the fate of the hydrogen removed are not known (see text).*

lidine carboxylate stereoisomers to carbapenams (the (2*R*,5*S*) stereoisomer **8** was not tested) in the following order of preference (k_{cat}/K_m ($mM^{-1}s^{-1}$) in parentheses); (2*S*,5*S*)-CMP **4** (1.2 ± 0.1), (2*S*,5*R*)-CMP **7** (0.027 ± 0.003), (2*R*,5*R*)-CMP **9** (0.026 ± 0.001).[14] This order of preference approximately echoes that seen for CarC in the presence of ascorbate. Assuming that the (3*R*,5*R*)- and (3*R*,5*S*)-carbapenams (**5** and **6**), which have not been isolated, do not occur in bacteria (at least in the species so far studied), these observations combined with the present study mean that the stereochemical course of the pathway is ultimately directed by the selectivity of CarB, that is, it is predicted that CarB uses an L(*S*) rather than a D(*R*) amino acid as its preferred substrate.

The observation that the (3*S*,5*S*) stereoisomer **2** is not the only carbapenam substrate for CarC, coupled to the lack of selectivity of CarA, means that it might be possible to construct a carbapenem biosynthesis pathway that does not involve C5 epimerisation via modification of CarB to produce a product with the (5*R*) stereochemistry (corresponding to 5*R* at C5 in the carbapenam).

Experimental Section

CarC was purified as reported previously.[9] Solutions (100 mM) of free carbapenams were prepared from their methyl esters by enzymatic hydrolysis in phosphate buffer (pH 8) by using pig-liver esterase (PLE) on Eupergit® resin.[20]

Bioassay: Assay mixtures were transferred into holed (11 mm diameter) bioassay plates (*E. coli* X580) and incubated at 28 °C overnight. A typical assay mixture consisted of KPi, pH 7.6 (20 mM), 2-oxoglutarate (5 mM), carbapenam (2 mM), CarC (1.6 mg mL^{-1}) and $FeSO_4$ (1 mM). Where L-ascorbate was present, it was at 1 mM. The assay mixture was incubated at 23 °C for 30 min.

HPLC/MS: HPLC/MS was performed by using a Waters high-performance liquid chromatography system connected to a Micro-Mass ZMD mass spectrometer in the negative ion mode. Additional detection with a Waters 966 PDA at 261 nm was employed for assays without ascorbate. Assay mixtures as for the bioassays were mixed with CH_3CN (equal volume), chilled on ice for 5 min and then separated in a centrifuge (17800 *g*) for 5 min before analysis. Controls were carried out under identical conditions, but with CarC omitted. The assay mixture (100 μL) was injected onto a Synergi Polar-RP column (250 × 4.6 mm, Phenomenex) equilibrated in 5% CH_3CN at 1 mL min^{-1}. After 15 min, a gradient to 90% CH_3CN was run over 5 min. These conditions were maintained for 5 min before returning to 5% CH_3CN over 5 min and re-equilibration for 10 min.

Notes added in proof: i) Since submission of this manuscript, an article has been published also describing turnover of (3*S*,5*R*)-carbapenam-3-carboxylic acid by CarC.[21] ii) Malonyl CoA rather than acetyl CoA has been reported to be a substrate for CarB.[22]

Acknowledgements

We thank the Biotechnology and Biological Sciences Research Council (studentships to M.C.S and P.S.), the European Union and Amura (for a CASE award to M.C.S.) for funding.

Keywords: antibiotics · biosynthesis · carbapenem · oxoglutarate · oxygenase

[1] D. M. Livermore, N. Woodford, *Curr. Opin. Microbiol.* **2000**, *3*, 489.
[2] S. J. McGowan, M. Sebaihia, S. O'Leary, K. R. Hardie, P. Williams, G. S. A. B. Stewart, B. W. Bycroft, G. P. C. Salmond, *Mol. Microbiol.* **1997**, *26*, 545.
[3] L. E. Nunez, C. Mendez, A. F. Brana, G. Blanco, J. A. Salas, *Chem. Biol.* **2003**, *10*, 301.
[4] S. Derzelle, E. Duchaud, F. Kunst, A. Danchin, P. Bertin, *Appl. Environ. Microbiol.* **2002**, *68*, 3780.
[5] R. Li, A. Stapon, J. T. Blanchfield, C. A. Townsend, *J. Am. Chem. Soc.* **2000**, *122*, 9296.
[6] Analysis of a mutant strain of *Pectobacterium carotovora* defective in CarC results in the accumulation in the culture medium of only the (3*S*,5*S*)-carbapenam **2** (Bycroft et al., unpublished results).
[7] J. Zhou, W. L. Kelly, B. O. Bachmann, M. Gunsior, C. A. Townsend, E. I. Solomon, *J. Am. Chem. Soc.* **2001**, *123*, 7388.
[8] M. D. Lloyd, K. D. Merritt, V. Lee, T. J. Sewell, B. Wha-Son, J. E. Baldwin, C. J. Schofield, S. W. Elson, K. H. Baggaley, N. H. Nicholson, *Tetrahedron* **1999**, *55*, 10201.
[9] I. J. Clifton, L. X. Doan, M. C. Sleeman, M. Topf, H. Suzuki, R. C. Wilmouth, C. J. Schofield, *J. Biol. Chem.* **2003**, *278*, 20843.
[10] A. Stapon, R. Li, C. A. Townsend, *J. Am. Chem. Soc.* **2003**, *125*, 8486.
[11] B. W. Bycroft, C. Maslen, S. J. Box, A. G. Brown, J. W. Tyler, *J. Chem. Soc. Chem. Commun.* **1987**, 1623.
[12] B. W. Bycroft, S. R. Chhabra, B. Kellam, P. Smith, *Tetrahedron Lett.* **2003**, *44*, 973.
[13] H. Tanaka, H. Sakagami, K. Ogasawara, *Tetrahedron Lett.* **2002**, *43*, 93.
[14] B. Gerratana, A. Stapon, C. A. Townsend, *Biochemistry* **2003**, *42*, 7836.
[15] B. Kellam, Ph. D. thesis, University of Nottingham (UK), **1996**.
[16] A. Avenoza, J. I. Barriobero, J. H. Busto, J. M. Peregrina, *J. Org. Chem.* **2003**, *68*, 2889.
[17] S. M. Schmitt, D. B. R. Johnston, B. G. Christensen, *J. Org. Chem.* **1980**, *45*, 1142.
[18] R. Myllyla, K. Majamaa, V. Gunzler, H. M. Hanauske-Abel, K. I. Kivirikko, *J. Biol. Chem.* **1984**, *259*, 5403.
[19] M. W. Vetting, S. L. Roderick, M. Yu, J. S. Blanchard, *Protein Sci.* **2003**, *12*, 1954.
[20] K. Laumen, E. H. Reimerdes, M. Schneider, H. Gorisch, *Tetrahedron Lett.* **1985**, *26*, 407.
[21] A. Stapon, R. Li, C. A. Townsend, *J. Am. Chem. Soc.* **2003**, *125*, 15746.
[22] M. C. Sleeman, C. J. Schofield, *J. Biol. Chem.* **2004**, *279*, 6730.

Received: December 16, 2003

