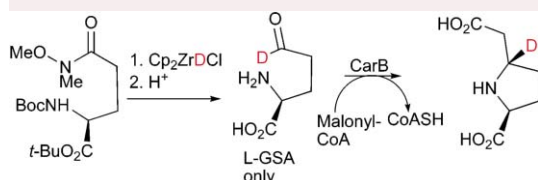


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Synthesis of deuterium labelled L- and D-glutamate semialdehydes and their evaluation as substrates for carboxymethylproline synthase (CarB)—implications for carbapenem biosynthesis

John L. Sorensen, Mark C. Sleeman and Christopher J. Schofield

Syntheses of deuterium labelled L- and D-glutamate semialdehydes is reported. Incubation studies reveal that carboxymethylproline synthase (CarB) condenses L-, but not D-glutamate semialdehyde in the committed step of carbapenem biosynthesis.

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Synthesis of deuterium labelled L- and D-glutamate semialdehydes and their evaluation as substrates for carboxymethylproline synthase (CarB)—implications for carbapenem biosynthesis

John L. Sorensen, Mark C. Sleeman and Christopher J. Schofield*

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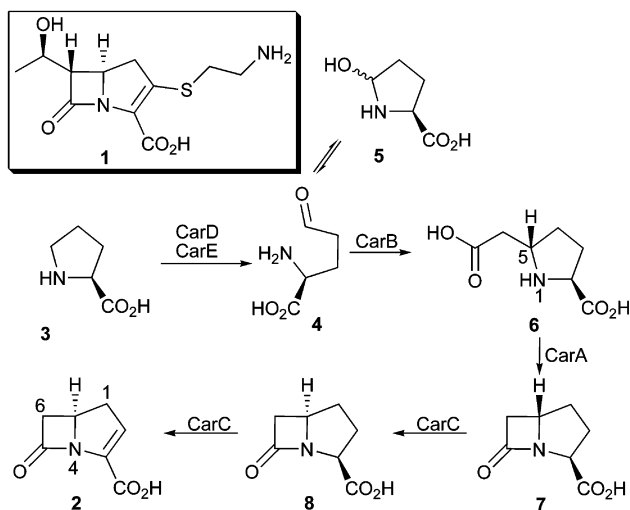
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Carboxymethylproline synthase was shown to condense L-glutamate semialdehyde with malonyl-coenzyme A to produce (2*S*,5*S*)-carboxymethylproline, while incubation of D-glutamate semialdehyde results only in uncoupled turnover of malonyl-CoA.

The carbapenem antibiotics are clinically useful as they have a broad range of activity and display resistance to serine β -lactamases.¹ Although there are many naturally occurring carbapenems, all the clinically used antibiotics are produced by total synthesis. Consequently there is interest in developing fermentation routes to useful carbapenems or intermediates for their production. The gene cluster for the biosynthesis of thienamycin (**1**, Scheme 1) in *Streptomyces cattleya* probably involves at least 11 enzymes.² In contrast the biosynthesis of the simplest carbapenem, (5*R*)-carbapenem (**2**), involves only 5 enzymes that catalyze the transformation of L-proline (**3**) into **2**, and thus serves as an excellent model for the biosynthesis of the nucleus.³

CarD and CarE enzymes are proposed to catalyze the oxidation of L-proline (**3**) to give L-glutamate semialdehyde (**4**) (L-GSA).⁴ The first unique step, condensation of **4** (and/or **5**) with malonyl-CoA to give (2*S*,5*S*)-carboxymethylproline (**6**), is catalyzed by carboxymethylproline synthase (CarB), a member of the crotonase superfamily of acyl-CoA utilizing enzymes.^{3,5} CarA then catalyzes



Scheme 1 Enzymes involved in carbapenem biosynthesis.

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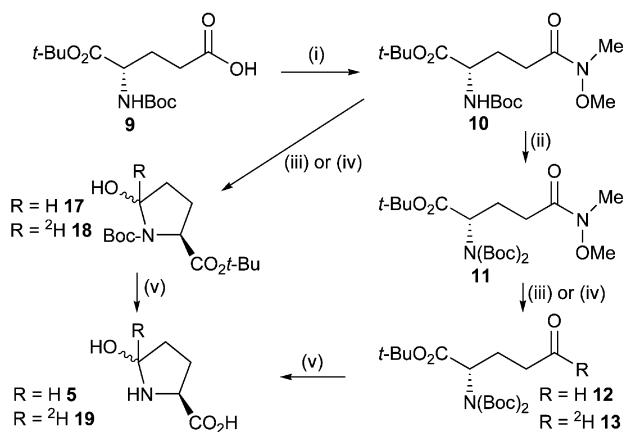
ATP mediated β -lactam formation to give the (3*S*,5*S*)-carbapenam (**7**).⁶ CarC then catalyses the unusual epimerisation and desaturation of **7** to give **2**, likely (at least in part) *via* the (3*S*,5*R*)-carbapenam (**8**).^{4,7–10}

After some uncertainty, recent studies have led to the definition of the stereochemistry of the carbapenem intermediates **7** and **8**.^{8,9} However both CarA and CarC have been reported to accept at least 3 of the 4 possible stereoisomers of their substrates.^{6,10} The only previous study on CarB led to the proposal that it accepted only L-GSA (**4**) as a substrate but this work was carried out with racemic GSA.⁵

Here we report a new method for the synthesis of the L and D enantiomers of both GSA and aspartate semialdehyde (ASA). The results of incubations with CarB reveal that L-, but not D-, GSA is a substrate for CarB.

Previous synthesis of L-GSA (in protected form) relied on the chemoselective transformation of the γ -carboxylate of suitably protected L-glutamic acid into a Weinreb amide (RCON(OMe)Me) followed by a reduction using DIBAL-H¹¹ or a selective reduction of the γ -carboxylate methyl ester with DIBAL-H.¹² A similar approach, *via* the Weinreb amide, has been used for the preparation of L-aspartate semialdehyde.¹³ We sought to avoid the use of DIBAL-H for several reasons including a desire to generate C-5 deuterated substrates (suitably deuterated DIBAL is not commercially available). Based upon a report of the reduction of Weinreb amides to aldehydes by Schwartz's reagent (Cp₂ZrHCl)¹⁴ we examined this reagent as an alternative to DIBAL-H for the synthesis of amino acid semialdehydes. The deuterated version of this reagent (Cp₂Zr²HCl) is commercially available and has also been shown of equal utility in reducing amides.¹⁵

The γ -carboxylate of protected glutamate **9** was first transformed into Weinreb amide **10** (Scheme 2) *via* the activated acid. A previous report^{12a} implied that it may be necessary to add a second Boc group prior to the formation of the aldehyde, therefore in initial work **10** was converted to **11**. Protected glutamate semialdehyde **12** was formed by adding **11** to a suspension of Schwartz reagent in THF at room temperature. Completion of the reaction is indicated by production of a clear solution (the Schwartz reagent is initially insoluble in THF) after *ca.* 15 minutes. The crude reaction mixture can be purified directly by chromatography without the need for work-up. In contrast, the DIBAL-H mediated reaction with **10** required -78 °C for an extended period of time (~ 4 h) and a work up step to remove aluminium by-products before chromatography.¹¹ When the deuterated Schwartz reagent¹⁵ was employed a high level of deuterium



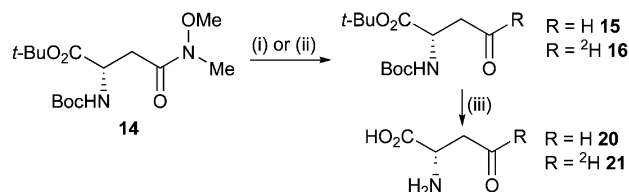
Scheme 2 Reagents and conditions: (i) Py-BOP, NEt₃, then HCl·HN(OCH₃)CH₃, CH₂Cl₂, 80%; (ii) (Boc)₂O, DMAP, CH₃CN, 65%; (iii) Cp₂ZrHCl, 85%; (iv) Cp₂Zr^HHCl, 93% (v) 10% formic acid (aq).

incorporation (> 95% ²H) in the final product (*i.e.* **13**) was observed by ¹H NMR and mass spectrometry.

We employed a similar route to synthesize both protiated and deuterated versions of L- and D- aspartate semialdehyde (ASA). It was not necessary to install a second Boc group on **14** to carry out the Schwartz reduction and free aldehyde **15** was the exclusive isolated product. In a similar manner aldehyde **16** (> 95% ²H) was the product isolated from the reaction employing deuterated Schwartz reagent.

In light of the fact that L-ASA could be synthesized with mono-Boc protection, we investigated if L-GSA could be synthesized in a similar manner. It was thought that the lack of a strong base in the Schwartz reaction could prevent the formation of the mixture of products described for the DIBAL-H reaction.^{12a} Indeed, as in Scheme 2, when **10** was treated with the Schwartz reagent no aldehyde could be detected and the only product of this reaction was *tert*-butyl *N*-Boc-5-hydroxy-L-prolinate (**17**), previously reported as an intermediate in the synthesis of **6**.⁶ Likewise, compound **18** was the exclusive product when deuterated Schwartz reagent was used. While **17** has not been reported as a product of the DIBAL-H reduction of **10**, a product similar to **17** was reported for the reduction of the γ -acid chloride with LiAlH(O^tBu)₃.⁹ The deprotected aldehydes/hemiaminals (*i.e.* **19** or **5** and **20** or **21**) were produced by reaction with formic acid (final concentration 10%) and stored as a 10% formic acid solution at -80 °C.

This is the first report of the application of the organometallic Schwartz reagent to the synthesis of amino acid aldehydes. This reagent rapidly and cleanly converts tertiary amides to the corresponding aldehydes at room temperature and avoids the use of strong base in the reduction. The Schwartz reagent also allows the incorporation of a deuterium isotope into the aldehyde position with relative ease. A deuterated analogue of GSA can be made employing two straightforward chemical transformations from a commercially available precursor using commercially available reagents at each step. These transformations can be used with other amino acids precursors as we have demonstrated by synthesising aspartate semialdehyde with a deuterium label (Scheme 3).

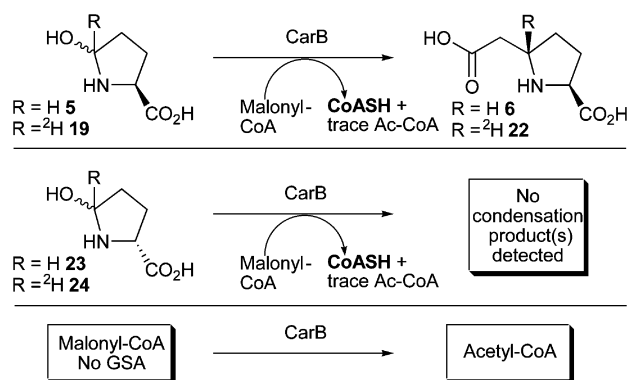


Scheme 3 Reagents and conditions: (i) Cp₂ZrHCl, THF, 40%; (ii) Cp₂Zr^HHCl THF, 73%, (iii) 10% formic acid.

The D- and L-GSA semialdehydes were separately incubated with CarB produced following a modification of the reported procedure,⁵ and the incubation mixtures analysed by LC coupled to an electrospray ionisation mass spectrometer.⁵ In the presence of malonyl-CoA, L-GSA (**5**) was efficiently converted to (2*S*,5*S*)-carboxymethylproline (**6**) with concomitant production of coenzyme A (CoASH) and a trace of acetyl-CoA. In contrast when malonyl-CoA was incubated with CarB in the absence of L-GSA, under the same incubation conditions, complete conversion to *acetyl*-CoA was observed. When C-5 deuterated L-GSA (**19**) was incubated with malonyl-CoA and CarB a clear signal for **22** (> 95% ²H) was observed demonstrating that only exogenously added L-GSA was converted to product **22** and that exchange at C-5 does not occur during the formation of carboxymethylproline (**22**).

When D-GSA (**23**), synthesized as for L-GSA *via* the reduction of *ent*-**10**, was incubated with malonyl-CoA and CarB no trace of any condensation products could be detected. However CoASH and a trace of acetyl-CoA were detected in the assay mixture. When D-GSA (**23**) prepared from the requisite di-Boc protected intermediate (*i.e.* *ent*-**11**) was used in the assay a trace of carboxymethylproline product **6** could be detected, implying partial racemization had occurred, probably due to the increase in relative acidity of the C- α position in the di-Boc intermediate *ent*-**11** compared to the mono-Boc compound *ent*-**10**. Thus synthesis of the aldehydes *via* the mono-Boc derivative is preferred.

Although none of the D-GSA was converted by CarB to form condensation products, this result nonetheless reveals that the hydrolysis of an active site bound acyl-CoA species requires the presence of an amino acid semialdehyde since in the absence of substrate CarB converts malonyl-CoA to acetyl-CoA (Scheme 4).



Scheme 4 Assay conditions. 35 μ L 600 mM TRIS pH 7.5, 8 μ L 0.01 M malonyl-CoA, 5 μ L of 0.01 M GSA (in 10% formic acid) and 2 μ L of CarB (10 mg mL⁻¹ in 20 mM HEPES pH 7.5), incubated at 37 °C for 10 min. then LC-MS.

Preliminary experiments with L-ASA (**20** or **21**) indicate it is not a substrate for CarB.

Thus, we have demonstrated the application of the Schwartz reagent to the synthesis of deprotected amino acid semialdehydes, and shown the utility of this reagent for incorporating a deuterium label at the aldehyde position. The results demonstrate that, unlike CarA and CarC, CarB is selective for a specific stereoisomer of its substrate, (*i.e.* L-GSA) and thus completes the definition of the stereochemistry of the unique intermediates in the biosynthesis of carbapenem **2**.

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Notes and references

1 D. M. Livermore and N. Woodford, *Curr. Opin. Microbiol.*, 2000, **3**, 489; S. J. McGowan, B. W. Bycroft and G. P. C. Salmond, *Trends Microbiol.*, 1998, **6**, 203.

- 2 L. E. Nunez, C. Mendez, A. F. Brana, G. Blanco and J. A. Salas, *Chem. Biol.*, 2003, **10**, 301.
- 3 S. J. McGowan, M. Sebahia, S. O'Leary, K. R. Hardie, P. Williams, G. S. A. B. Stewart, B. W. Bycroft and G. P. C. Salmond, *Mol. Microbiol.*, 1997, **26**, 545.
- 4 A. Stapon, R. Li and C. A. Townsend, *J. Am. Chem. Soc.*, 2003, **125**, 8486.
- 5 M. C. Sleeman and C. J. Schofield, *J. Biol. Chem.*, 2004, **279**, 6730.
- 6 B. Gerratana, A. Stapon and C. A. Townsend, *Biochemistry*, 2003, **42**, 7836.
- 7 I. J. Clifton, L. X. Doan, M. C. Sleeman, M. Topf, H. Suzuki, R. C. Wilmouth and C. J. Schofield, *J. Biol. Chem.*, 2003, **278**, 20843.
- 8 A. Stapon, R. Li and C. A. Townsend, *J. Am. Chem. Soc.*, 2003, **125**, 15746.
- 9 B. W. Bycroft, S. R. Chhabra, B. Kellam and P. Smith, *Tetrahedron Lett.*, 2003, **44**, 973.
- 10 M. C. Sleeman, P. Smith, B. Kellam, S. R. Chhabra, B. W. Bycroft and C. J. Schofield, *ChemBioChem*, 2004, **5**, 879.
- 11 F. Burkhart, M. Hoffmann and H. Kessler, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 1191.
- 12 (a) J. M. Padron, G. Kokotos, T. Martin, T. Markidis, W. A. Gibbons and V. S. Martin, *Tetrahedron: Asymmetry*, 1998, **9**, 3381; (b) A. Sutherland, J. F. Caplan and J. C. Vederas, *Chem. Commun.*, 1999, 555.
- 13 S. J. Roberts, J. C. Morris, R. C. J. Dobson and J. A. Gerrard, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 265.
- 14 J. M. White, A. R. Tunoori and G. I. Georg, *J. Am. Chem. Soc.*, 2000, **122**, 11995.
- 15 J. T. Spletstoser, J. M. White and G. I. Georg, *Tetrahedron Lett.*, 2004, **45**, 2787.