Synthesis of regio- and stereoselectively deuterium-labelled derivatives of L-glutamate semialdehyde for studies on carbapenem biosynthesis†

Christian Ducho,‡ Refaat B. Hamed, Edward T. Batchelar, John L. Sorensen, Barbara Odell and Christopher J. Schofield*

Received 17th February 2009, Accepted 6th April 2009
First published as an Advance Article on the web 11th May 2009
DOI: 10.1039/b903312b

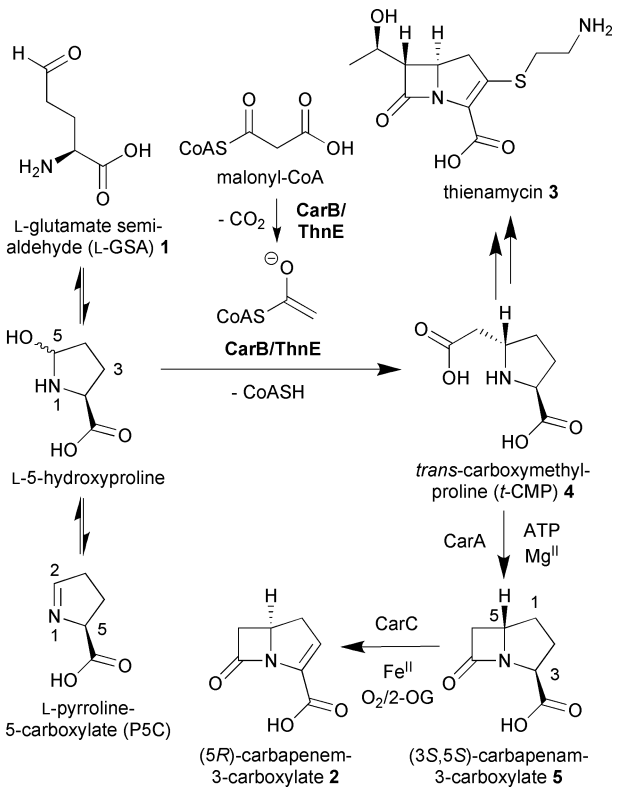
L-Glutamate semialdehyde (L-GSA) is an intermediate in biosynthetic pathways including those leading to the carbapenem antibiotics. We describe studies on asymmetric deuteration or hydrogenation of appropriate didehydro-amino acid precursors for the stereoselective synthesis of C-2- and/or C-3-[^{2}H]-labelled L-GSA suitable for use in mechanistic studies. Regioselective deuterium incorporation into the 5-position of L-GSA was achieved using a labelled form of the Schwartz reagent (Cp_2Zr^2HCl). 4,4-Dideuterated and fully backbone deuterated L-GSAs were prepared. The application of the labelled L-GSA derivatives to biosynthetic studies was exemplified by the chemo-enzymatic preparation of selectively deuterated *trans*-carboxymethylprolines using two different carboxymethylproline synthases (CarB and ThnE), enzymes that catalyse early steps in the biosynthesis of two carbapenems: (5*R*)-carbapenem-3-carboxylate and thienamycin, respectively.

Introduction

The formation of L-glutamate semialdehyde (L-GSA) residues is a major consequence of non-enzymatic oxidative protein damage in cells.[1–3] The amino acid form of L-GSA **1** § is an inhibitor of *Escherichia coli* glucosamine-6-phosphate synthase[4] and cytidine 5′-triphosphate (CTP) synthase.[5] Furthermore, **1** is an intermediate in biosynthetic pathways including those leading to aminolevulinate[6] and the carbapenem antibiotics.

(5*R*)-Carbapenem-3-carboxylate **2** is the simplest member of the carbapenem family of β-lactam antibiotics. Clinically useful carbapenems are prepared by total synthesis, hence, there is interest in developing fermentation-based semisynthetic methodologies for the production of carbapenem antibiotics or intermediates useful for their preparation. The biosynthetic pathways leading to the bicyclic nuclei of the penicillins, cephalosporins and clavulanic acid have been extensively studied (for review see ref. 7), but the pathways to the C-2,C-6 functionalised carbapenems are less well understood.

The essential steps in the biosynthesis of **2** from L-GSA **1** in *Pectobacterium carotovorum* are catalysed directly by three enzymes, CarA–C (Scheme 1).[8,9] However, the biosynthesis of the C-2,C-6 substituted thienamycin **3** in *Streptomyces cattleya* probably involves multiple enzymes.[10–12] Carboxymethylproline synthases (CarB from *P. carotovorum* and ThnE from *S. cattleya*) catalyse the conversion of L-GSA **1** into *trans*-carboxymethylproline (*t*-CMP) **4** using malonyl-CoA as a cosubstrate.[12–16] CarB and ThnE are members of the crotonase superfamily of enzymes,



Scheme 1 Outline of carbapenem biosynthesis.§

University of Oxford, Department of Chemistry, Chemistry Research Laboratory, Mansfield Road, Oxford, United Kingdom OX1 3TA. E-mail: christopher.schofield@chem.ox.ac.uk; Fax: +44 1865 2-75674; Tel: +44 1865 2-75625

† Electronic Supplementary Information (ESI) available: Additional syntheses, determination of absolute configurations and enantiomeric purities of **21** and **22**, representative CarB/ThnE assays and additional spectroscopic data. See http://dx.doi.org/10.1039/b903312b/

‡ Current address: Georg-August-University Göttingen, Department of Chemistry, Institute of Organic and Biomolecular Chemistry, Göttingen, Germany.

§ In solution, L-GSA **1** is observed as an equilibrium between its aldehyde form and the cyclic forms L-5-hydroxyproline and L-pyrroline-5-carboxylate (P5C, Scheme 1). Only the cyclic hydroxyproline form is used in schemes and figures in this paper, though the name L-GSA is used throughout.

many of which stabilise enolate/oxy-anion intermediates in their catalytic mechanisms.[17] CarB and ThnE are unusual amongst crotonases because they catalyse the production of a heterocycle in addition to the decarboxylation of malonyl-CoA and thioester hydrolysis. During the biosynthesis of **2** in *P. carotovorum*, the CarB product, *t*-CMP **4**, then undergoes ATP driven ring closure to give the β-lactam (3*S*,5*S*)-carbapenam-3-carboxylate **5**, in a reaction catalysed by the synthetase CarA.[18] Finally, the 2-oxoglutarate (2-OG)-dependent non-haem Fe(II)-oxygenase CarC catalyses both an unusual epimerisation at the 5-position of the carbapenem skeleton and formation of the C-2,C-3 double bond to give (5*R*)-carbapenem-3-carboxylate **2**.[19–23]

To study the enzyme-catalysed steps in the biosynthesis of **2** and other carbapenems including thienamycin **3**, we required selectively labelled chemical probes, including L-GSA **1**. Various methods for the synthesis of GSA[24–26] and its lower homologue, L-aspartate semialdehyde (L-ASA),[27,28] have been reported. However, methodology for the preparation of selectively labelled GSA derivatives is limited. Previous syntheses of unlabelled protected forms of L-GSA **1** are based on diisopropylaluminium hydride (DIBAL-H) mediated reduction of Weinreb amide[29] or methyl ester derivatives[30,31] of the glutamate 5-carboxylate function. However, suitably deuterated DIBAL is not commercially available.

Here, we describe syntheses of (3*S*)-2,3-[$^2H_2$]-L-GSA **6**, (3*R*)-3-[^2H]-L-GSA **7**, 4,4-[$^2H_2$]-L-GSA **8**, 5-[^2H]-L-GSA **9**, together with that of the fully backbone deuterated [$^2H_6$]-L-GSA **10** (Fig. 1). For the preparation of target compounds **6** and **7**, a synthetic route involving asymmetric deuteration and hydrogenation, respectively, was developed. Use of the commercially available deuterated Schwartz reagent (Cp_2Zr^2HCl) provided access to GSA derivatives **9** and **10**. The *in vitro* conversion of all labelled L-GSA derivatives **6–10** into the corresponding *t*-CMPs in the presence of CarB and/or ThnE exemplifies the potential of **6–10** as reagents for biosynthetic work.

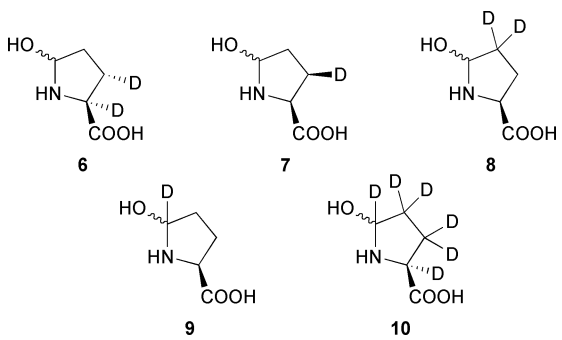


Fig. 1 Target compounds **6–10** (D = ^2H).

Results and discussion

Synthesis of (3*S*)-2,3-[$^2H_2$]-L-GSA 6 and (3*R*)-3-[^2H]-L-GSA 7

L-GSA **1** is a sensitive compound which readily undergoes decomposition, likely including *via* condensation reactions, upon attempted isolation.[25,32,33] We therefore aimed to develop a procedure for the synthesis of the labelled GSA derivatives *via* use of protecting groups that would involve a final deprotection step under acidic conditions to give products that would be immediately used in enzyme incubations.[14,34,35] For the synthesis of C-2- and/or C-3-[^2H]-labelled L-GSA derivatives **6** and **7**, we planned to employ asymmetric hydrogenation and deuteration of α,β-didehydro-amino acid precursors.[36–38] We therefore initially prepared amino acid phosphonate and malonyl aldehyde derivatives in order to obtain protected didehydro-amino acids *via* Horner–Wadsworth–Emmons olefinations (Scheme 2).[37–40]

N-Boc-glycine **11**[41] was converted to the *tert*-butyl ester **12** (75%) using the Eschenmoser method.[42] Subsequent *N*-bromosuccinimide (NBS) mediated bromination gave the sensitive bromo derivative **13**, which was used without further purification in a Michaelis–Arbuzov reaction to yield racemic phosphonate **14** (71% over 2 steps from **12**).

For the synthesis of the sensitive malonyl aldehyde derivatives **15** and **16**, initial attempts employing the method described for the preparation of **15** starting from 1,1,3,3-tetramethoxy-propane[43] resulted in unsatisfactory yields of impure material. Hence, methyl (1,3-dioxolan-2-yl)-acetate **17**, which can be obtained from methyl acrylate in one step,[44] was used as a starting material. Reduction of **17** with DIBAL-H gave aldehyde **15**. For the preparation of [^2H]-labelled aldehyde **16**, a two-step synthesis was employed: treatment of **17** with $LiAl^2H_4$ gave alcohol **18** (63%); subsequent Dess–Martin oxidation[45] gave aldehyde **16**. Both aldehydes **15** and **16** (estimated purities >85% by ^1H NMR) were found to be significantly sensitive, hence they were directly employed in subsequent Horner–Wadsworth–Emmons olefinations without purification.

As anticipated,[46] didehydro-amino acid (***Z*)-19** was isolated as the main product (82%) from the Horner–Wadsworth–Emmons reaction of phosphonate **14** with aldehyde **15** in the presence of the base KO*t*-Bu. (***E*)-19** was isolated as a major by-product (7%). (***Z*)-** and (***E*)-19** could be readily separated *via* column chromatography. Only the (*Z*) isomer was required for the asymmetric deuteration because it is reported that asymmetric hydrogenation or deuteration using rhodium catalysts (*vide infra*) occurs more rapidly, and with significantly better enantioselectivities for (*Z*)-didehydro-amino acids than for the (*E*)-isomers.[47] Horner–Wadsworth–Emmons reaction of phosphonate **14** with deutero-aldehyde **16** gave **20** with a similar product ratio ((***Z*)-20** in 75% yield, (***E*)-20** in 11% yield).

The stereochemistry of the didehydro-amino acids was initially assigned for (***E*)-19** and (***E*)-20** using ^1H NMR gradient 1D nuclear Overhauser enhancement (nOe) spectroscopy (Fig. 2). The small γ-gauche effect ($\Delta\delta = 0.3$ ppm) for the ^{13}C NMR resonance of the ester carbonyl carbon atom (C-1) of (***E*)-19** as compared to (***Z*)-19** was also consistent with the assigned configuration.[48] The

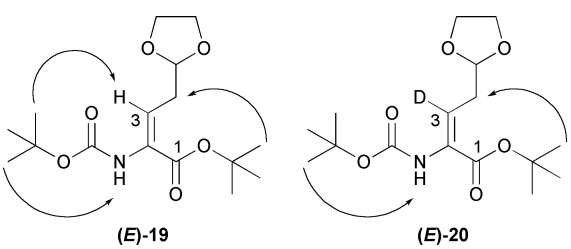
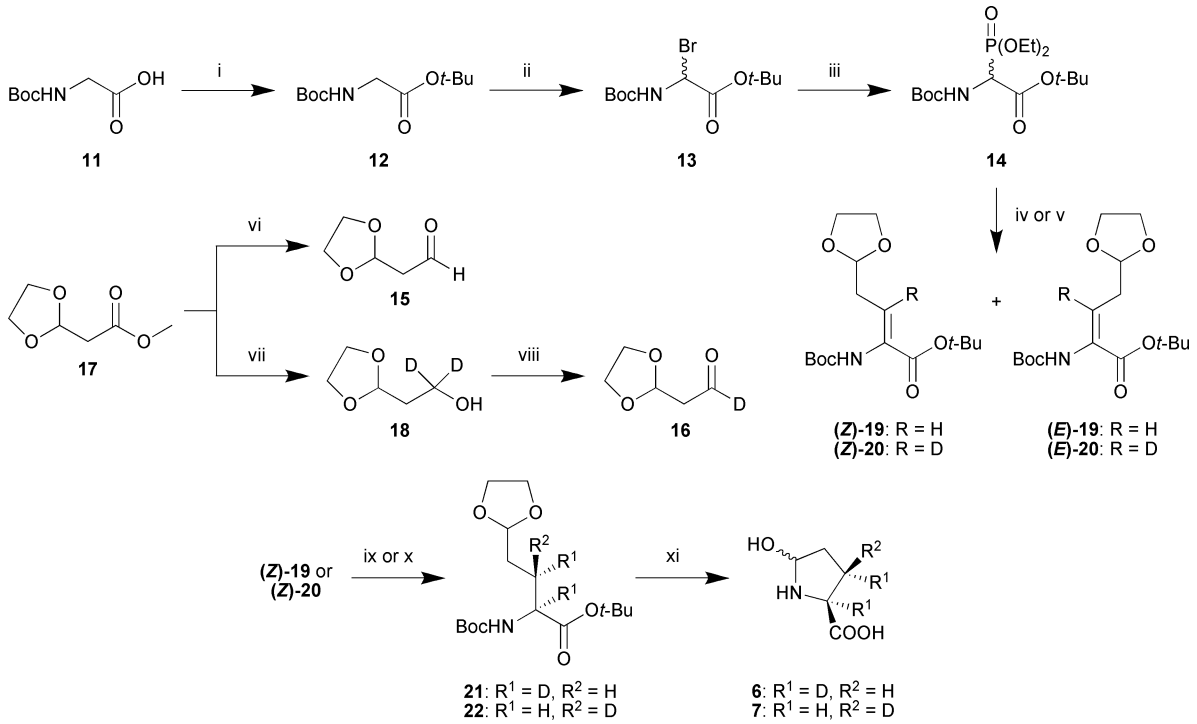


Fig. 2 Observed ^1H NMR nOes for (***E*)-19** and (***E*)-20**.



Scheme 2 Synthesis of L-GSA derivatives **6** and **7** (D = ^{2}H). *Reagents and conditions*: (i) *t*-BuOH, *N*,*N*-DMF-dineopentylacetal, toluene, reflux, 5 h, 75%; (ii) NBS, 2,2′-azobis-(2-methylpropionitrile) (AIBN), CCl_4, reflux, 2 h, no purification; (iii) $P(OEt)_3$, CH_2Cl_2, rt, 2.5 h, 71% over 2 steps from **12**; (iv) (a) KO*t*-Bu, THF, −70 °C, 5 min; (b) **15**, −70 °C to rt, 20 h, 82% (***Z***)**-19** and 7% (***E***)**-19**; (v) (a) KO*t*-Bu, THF, −70 °C, 5 min; (b) **16**, −70 °C to rt, 20 h, 75% (***Z***)**-20** and 11% (***E***)**-20**; (vi) (a) DIBAL-H, THF, −78 °C, 6 h; (b) MeOH–H_2O, −78 °C to rt, no purification; (vii) (a) $LiAl^2H_4$, THF, rt to reflux, 3 h; (b) H^+/H_2O, 63%; (viii) Dess–Martin periodinane, CH_2Cl_2, rt, 3.5 h, no purification; (ix) (***Z***)**-19**, $^2H_2$ (1 bar), (*S*,*S*)-Me-DUPHOS-Rh, MeOH, rt, 6 days, 98% **21** (er > 95 : 5); (x) (***Z***)**-20**, H_2 (1 bar), (*S*,*S*)-Me-DUPHOS-Rh, MeOH, rt, 6 days, 90% **22** (er > 95 : 5); (xi) 10% HCOOH–H_2O, 60 °C, 1 h.

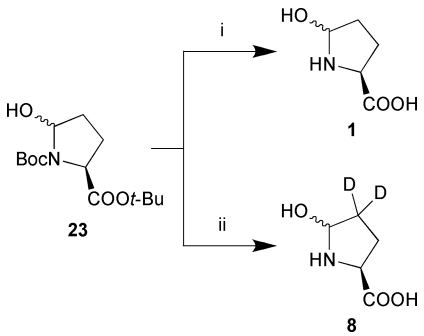
$^3J_{CH}$ coupling constant determined for C-1 in a ^{1}H-coupled ^{13}C NMR experiment on (***E***)**-19** was 12.4 Hz, a value that is typical for ^3J *trans*-C–H-coupling in olefins.[48] Further, two other reported ^{1}H NMR criteria for the stereochemical assignment of protected didehydro-amino acids[49] were fulfilled: (i) the proton at the β-position (H-3) of the double bond of (***Z***)**-19** was shielded with respect to the isomer (***E***)**-19** ($\Delta\delta$ ~ 0.7 ppm), and (ii) the N–H protons of (***Z***)**-19** and (***Z***)**-20** were shielded with respect to the (*E*) isomers ($\Delta\delta$ ~ 0.3 ppm for both **19** and **20**).

Asymmetric deuteration of (***Z***)**-19** and hydrogenation of (***Z***)**-20** was then attempted using the chiral catalyst (+)-1,2-bis-((2*S*,5*S*)-2,5-dimethylphospholano)-benzene-(cyclooctadiene)-rhodium(I) tetrafluoroborate ((*S*,*S*)-Me-DUPHOS-Rh),[50] which is reported to give excellent yields and stereoselectivities for the synthesis of L-amino acids *via* asymmetric hydrogenation of *N*-Cbz-protected didehydro-amino acid *tert*-butyl esters.[51] Application of (*S*,*S*)-Me-DUPHOS-Rh to the synthesis of **21** and **22** from the *N*-Boc-protected didehydro-amino acid *tert*-butyl esters (***Z***)**-19** and (***Z***)**-20** resulted in excellent yields (98% and 90%, respectively) and enantiomeric ratios (>95 : 5, for the determination of enantiomeric ratios and absolute configurations see ESI†). For both **21** and **22**, the incorporation levels of deuterium were found to be extremely high (>98% as determined by high resolution mass spectrometry).

Cleavage of the three protecting groups from **21** and **22** was achieved in one step *via* treatment with 10% aqueous formic acid. Due to the sensitivity of L-GSA derivatives, isolation of **6** and **7** was not carried out, but the acidic solutions were directly used in CarB enzyme assays after buffering (*vide infra*).[14,35] LC-MS analysis of the deprotection reaction mixtures did not indicate the presence of any partially deprotected by-products, but gave peaks corresponding to **6** (m/z = 132 $[M - H]^-$) and **7** (m/z = 131 $[M - H]^-$).

Synthesis of 4,4-[$^2H_2$]-L-GSA 8

For the preparation of 4,4-[$^2H_2$]-L-GSA **8**, unlabelled *N*-Boc-L-GSA *tert*-butyl ester **23** was first prepared according to an established procedure.[52] Deprotection of **23** under the usual conditions (10% aqueous formic acid) gave unlabelled L-GSA **1**, as anticipated (Scheme 3). However, when deprotection of **23** was carried out with deuterated formic acid (^{2}HCOO^{2}H) in ^2H_2O, incorporation of two deuterium atoms (>90% ^{2}H) in the product was detected by LC-MS analyses (data not shown). Because neither C-2- and/or C-3–^{2}H-labelled L-GSA derivatives (*vide supra*) nor 5-[^{2}H]-L-GSA (*vide infra*) displayed any loss of label under the deprotection conditions in aqueous formic acid, H/^{2}H exchange upon deprotection of **23** in deuterated solvent must occur selectively at the C-4 of GSA (Scheme 3). H/^{2}H exchange at C-4 was further demonstrated by the conversion of 4,4-[$^2H_2$]-L-GSA **8** to the 4,4-[$^2H_2$]-*t*-CMP derivative in the presence of malonyl-CoA and CarB, which was detected and purified by LC-MS (m/z = 174 $[M - H]^-$), followed by ^{1}H NMR analyses of the product (*vide infra*, see also ESI†). Deuterium



Scheme 3 Synthesis of L-GSA **1** and deuterated derivative **8** (D = ^{2}H). *Reagents and conditions*: (i) 10% HCOOH–H_2O, 60 °C, 1 h; (ii) 10% ^{2}HCOO^{2}H–^2H_2O, 60 °C, 1 h.

incorporation into the GSA backbone likely occurs *via* enol and/or enamine formation under the acidic conditions of the deprotection step.

Synthesis of 5-[^{2}H]-L-GSA 9 and [$^2H_6$]-L-GSA 10

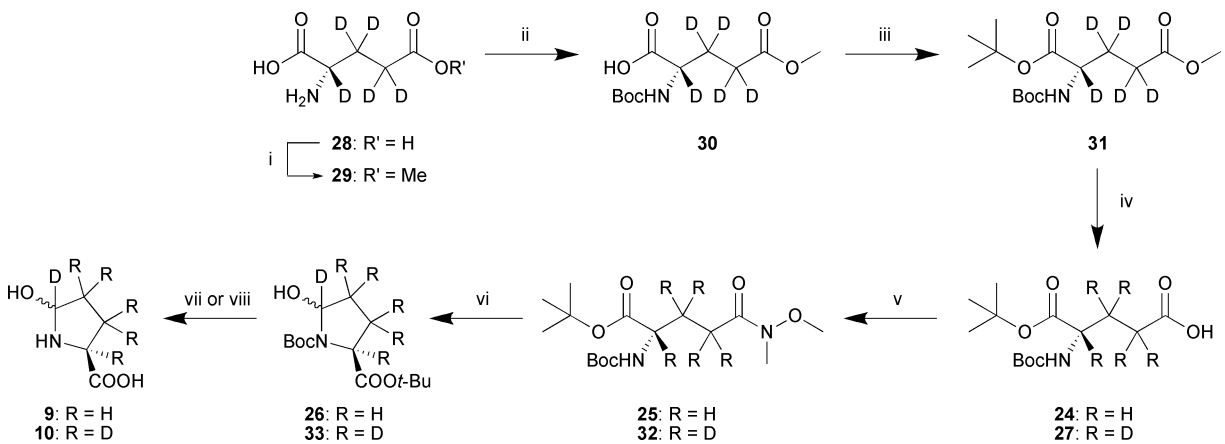
Further labelled derivatives were then synthesised (Scheme 4). In the case of **9**,[53] commercially available 1-(*tert*-butyl) *N*-Boc-L-glutamate (*N*-Boc-L-Glu-O*t*-Bu) **24** was used as a starting material. Weinreb amide **25** was synthesised employing (benzotriazol-1-yloxy)-tripyrrolidino-phosphonium hexafluorophosphate (PyBOP)[54] as coupling agent (81%). Subsequent reduction with the deuterated form of the organometallic Schwartz reagent (bis-(cyclopentadienyl)-zirconium chloride deuteride, Cp_2Zr^2HCl)[55] gave the protected 5-[^{2}H]-L-GSA derivative **26** in 71% yield and with a high level of deuterium incorporation (>95% ^{2}H, MS analysis). Deprotection of **26** under the established conditions gave **9** (Scheme 4).

The synthesis of **10** was carried out in an analogous manner. 1-(*tert*-Butyl) *N*-Boc-[$^2H_5$]-L-glutamate **27** was first prepared from [$^2H_5$]-L-glutamic acid **28**. Selective methyl ester formation in the 5-position was achieved using thionyl chloride in methanol to give **29** in 87% yield,[56] and subsequent *N*-Boc protection furnished **30** (58%). *tert*-Butyl ester formation in the 1-position was carried out employing Boc anhydride and DMAP to activate the carboxylic acid[57] (product **31**, 63%). After methyl ester saponification (81%), **27** was used in our established sequence of Weinreb amide synthesis (product **32**, 82%) followed by Schwartz reduction with Cp_2Zr^2HCl to give *N*-Boc-[$^2H_6$]-L-GSA *tert*-butyl ester **33** in 66% yield for the last step and in an overall yield of 14% over 6 steps from [$^2H_5$]-L-glutamic acid **28**. Finally, deprotection of **33** using deuterated formic acid in ^2H_2O, to maintain the ^{2}H label at the C-4 position (*vide supra*), gave **10** (Scheme 4).

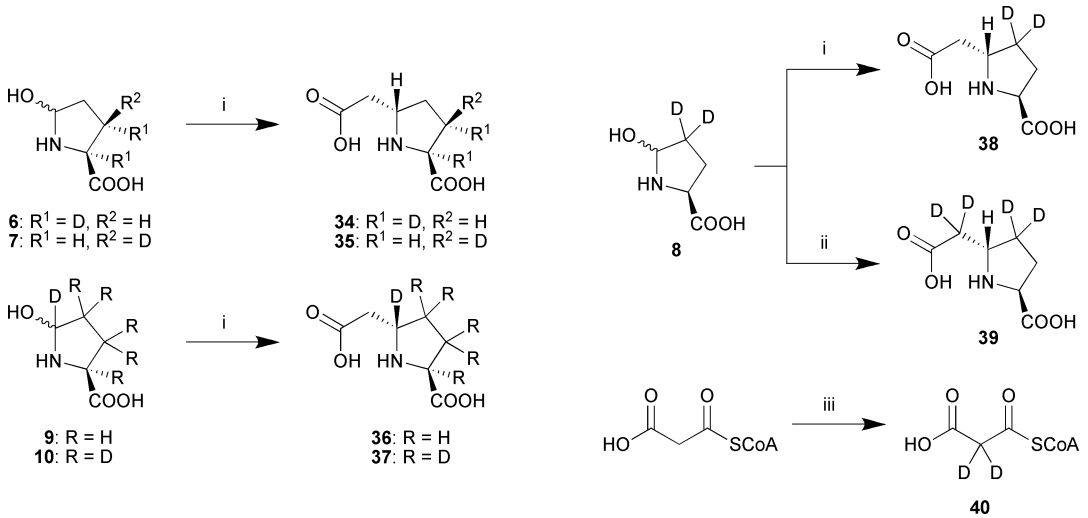
High resolution MS analysis of **33** (m/z = 276.2082 [M – OH]$^+$) revealed an overall level of isotopic purity of ~85% with the remaining ~15% representing the 'mono-backbone-protiated' compound (m/z = 275.2107 [M – OH]$^+$). NMR and MS analyses indicated that most of the deuterium–hydrogen exchange occurred in the *tert*-butyl ester forming reaction. However, other work employing alternative methods for the *tert*-butyl esterification to give **31** (*e.g.* DCC coupling) led to higher final amounts of 'mono-backbone-protiated' **33**. The final isotopic purity obtained was considered sufficient for use of **10** in biosynthetic studies.

Application in biosynthetic studies: preparation of deuterated *t*-CMP derivatives using CarB and ThnE

The deuterated L-GSA derivatives **6**, **7**, **9** and **10** were then employed in *in vitro* assays with CarB. In all cases, conversion into the corresponding *t*-CMP derivatives **34–37** in the presence of malonyl-CoA and CarB (Scheme 5) was demonstrated by LC-MS analyses. In the case of GSA derivatives **6**, **7** and **9**, no unconverted GSA was detected *via* LC-MS. Since D-GSA is not a substrate for CarB,[15,35] these results support the proposal that **6** and **7** (and therefore also precursors **21** and **22**) were prepared with the desired L-stereochemistry in high enantiomeric purities (er > 95 : 5, see also ESI†). L-GSAs **10** and **1** were converted into *t*-CMP products **37** and **4**, respectively, *via* incubation with ThnE and malonyl-CoA in an analogous manner (Scheme 5, Fig. 3, see also ESI†).



Scheme 4 Synthesis of L-GSA derivatives **9** and **10** (D = ^{2}H). *Reagents and conditions*: (i) MeOH, $SOCl_2$, 0 °C, 1 h, 87%; (ii) **29**, $(Boc)_2O$, NEt_3, THF, rt, 20 h, 58%; (iii) *t*-BuOH, $(Boc)_2O$, DMAP, rt, 20 h, 63%; (iv) LiO^2H, ^2H_2O, THF, 0 °C to rt, 20 h, 81%; (v) HNMe(OMe), PyBOP, NEt_3, DCM, rt, 20 h, 81% **25** and 82% **32**; (vi) (a) Cp_2Zr^2HCl, THF, rt, 15 min; (b) ^2H_2O, rt, 5 min, 71% **26** and 66% **33**; (vii) **26**, 10% HCOOH–H_2O, 60 °C, 1 h (for **9**); (viii) **33**, 10% ^{2}HCOO^{2}H–^2H_2O, 60 °C, 1 h (for **10**).



Scheme 5 Preparation of deuterated *t*-CMP derivatives **34–39** using CarB and/or ThnE (D = ^{2}H). *Reagents and conditions*: (i) solution of L-GSA derivative from deprotection reaction, malonyl-CoA, CarB or ThnE (for **37** and **38**), tris-HCl buffer (pH 9.0), 37 °C, 10 min; (ii) solution of **8** from deprotection reaction, [$^2H_2$]-malonyl-CoA **40**, CarB, [$^2H_{11}$]-tris-^{2}HCl buffer in ^2H_2O (p^{2}H 9.0, uncorrected), 37 °C, 10 min; (iii) [$^2H_{11}$]-tris-^{2}HCl buffer in ^2H_2O (p^{2}H 7.5, uncorrected), 37 °C, 18 h.

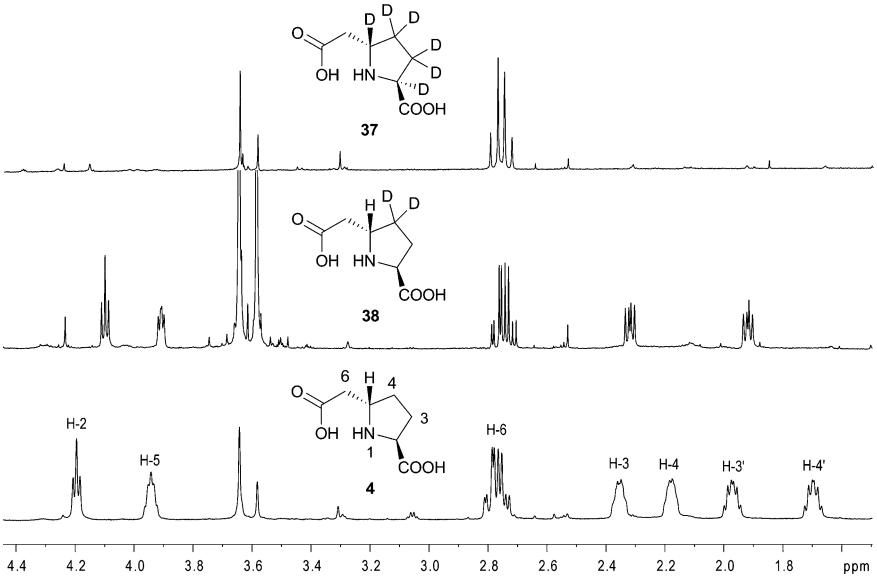


Fig. 3 ^{1}H NMR spectra of the deuterium-labelled *t*-CMP products **37** and **38** as well as of non-labelled *t*-CMP **4** resulting from incubations of the appropriate GSA derivatives with ThnE and malonyl-CoA.

When 4,4-[$^2H_2$]-L-GSA **8** was incubated with malonyl-CoA and CarB or ThnE under identical conditions, 4,4-[$^2H_2$]-*t*-CMP **38** was detected *via* LC-MS and characterised using ^{1}H NMR analyses after semipreparative LC-MS purification (*vide supra*, Scheme 5, Fig. 3, see also ESI†). Similarly, formation of 4,4,6,6-[$^2H_4$]-*t*-CMP **39** was detected upon incubation of **8** with [$^2H_2$]-malonyl-CoA **40** in the presence of CarB in deuterated buffer (**40** prepared *via* incubation of malonyl-CoA in deuterated buffer[58]). Under these conditions, high levels of deuterium incorporation into **39** (>90% ^{2}H) were achieved. In agreement with the proposed outline mechanism for CarB[13–15] and recent mechanistic results for the CarB catalysed reaction,[16] no significant deuterium–hydrogen exchange (loss of deuterium label) occurred in any position of the L-GSA backbone.

Conclusions

In conclusion, we have developed syntheses of regio- and stereoselectively [^{2}H]-labelled L-glutamate semialdehyde (L-GSA) derivatives **6–10**. They were converted into the corresponding *trans*-carboxymethylproline (*t*-CMP) derivatives **34–39** in the presence of CarB or ThnE. The outline mechanism for the CarA catalysed reaction[18] makes deuterium–hydrogen exchange in the *t*-CMP backbone during β-lactam ring formation unlikely. Therefore,

chemo-enzymatically obtained *t*-CMP derivatives **34–39** represent useful chemical probes to study the mechanism of the unusual CarC catalysed reaction yielding carbapenem **2**. In addition, isotope-labelled compounds **34–39** might serve as valuable tools to further elucidate the more complex biosynthetic pathway leading to thienamycin **3**.

Experimental

General methods

Chemicals were from Sigma-Aldrich Co Ltd, Fisher Scientific, Lancaster, Rathburn, Cambridge Isotope Laboratories Inc., Strem and Argo. Reactions involving oxygen and/or moisture sensitive reagents were carried out under an atmosphere of nitrogen or argon using anhydrous solvents. Asymmetric hydrogenation and deuteration was performed in a glove box under an argon atmosphere. Anhydrous solvents were obtained by passing HPLC grade solvents through a column of activated basic alumina (Brockmann 1, standard grade, ~150 mesh) under nitrogen pressure or purchased from commercial suppliers. All other solvents were analytical or HPLC grade. Water was purified using a Millipore Milli-Q system.

Column chromatography was carried out on silica gel 60 (0.040–0.063 mm) under flash conditions or as short column chromatography (fritted funnel) where indicated. TLC was performed on aluminium plates precoated with silica gel 60 F_{254} (Merck). Visualisation of the spots was carried out using $KMnO_4$ staining under heating (staining solution: 1 g $KMnO_4$, 6 g K_2CO_3 and 1.5 mL 5% $NaOH_{(aq)}$ (w/v), all dissolved in 100 mL H_2O).

400 MHz-, 500 MHz- and 700 MHz-^{1}H as well as 101 MHz- and 126 MHz-^{13}C NMR spectra were recorded on Bruker DPX400, AV400, DRX500, AV500 and AV700 spectrometers. ^{13}C NMR spectra are ^{1}H-decoupled unless otherwise stated. 77 MHz-^{2}H (D) NMR spectra were obtained on a Bruker AV500 spectrometer. 162 MHz-^{31}P NMR spectra (^{1}H-decoupled) were measured on a Bruker AV400 spectrometer. All spectra were recorded at room temperature and referenced internally to solvent reference frequencies. Chemical shifts (δ) are quoted in ppm. Coupling constants (J) are reported in Hz to the nearest 0.5 Hz. Assignment of signals was carried out using ^{1}H,^{1}H-COSY and HMQC spectra obtained on the spectrometers mentioned above. The nOe experiments carried out used a standard gradient 1-D NOESY DPFGSE pulse sequence with a mixing time of 1.0 s. Low resolution ESI mass spectrometry was performed on a Fisons Platform spectrometer operating in positive or negative ionisation mode. High resolution (HR) ESI mass spectrometry was carried out on a Micromass LCT spectrometer. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter with a Na source using a 10 cm cell. Melting points (mp) were measured on a Leica Galen III instrument and are not corrected.

Analytical LC-MS experiments were performed on a Micromass/Waters ZMD machine equipped with a Sielc Primesep 100 column (4.6 × 250 mm, particle 5 µm, pore 100 Å). Conditions: solution A H_2O + 0.05% formic acid, solution B MeCN + 0.1% formic acid. From 0 to 5 min A–B 95 : 5 v/v, from 5 to 25 min gradient of 5–70% B, from 25 to 30 min gradient of 70–100% B, from 30 to 45 min 100% B, then from 45 to 60 min A–B 95 : 5 v/v, flow rate 1.0 mL min^{-1}, injection volume 70 µL, ESI-MS detection in the negative ionisation mode. Semipreparative LC-MS was carried out on the same system equipped with a mixed mode Waters Spherisorb column (10 × 250 mm, particle 5 µm). Conditions: column equilibrated with 5% aqueous MeOH, gradient to 10% aqueous MeOH with 0.1% formic acid.

***tert*-Butyl *N*-Boc-(diethylphosphinyl)-glycinate 14.** To a solution of **12**[59] (5.00 g, 21.6 mmol) in degassed CCl_4 (100 mL), NBS (3.84 g, 21.6 mmol) and AIBN (80 mg, 0.49 mmol) were added at rt and the reaction mixture stirred under reflux for 2 h. After cooling to 0 °C and filtration, the filtrate was evaporated under reduced pressure to yield crude *tert*-butyl *N*-Boc-bromoglycinate **13** (8.37 g) as a yellowish oil, which was stored under nitrogen at –20 °C without further characterisation.

Crude **13** (8.37 g) was dissolved in anhydrous CH_2Cl_2 (50 mL), and a solution of triethylphosphite (4.08 mL, 23.8 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise at rt. The reaction mixture was stirred at rt for 2.5 h and the solvent evaporated under reduced pressure. The resultant yellow oil (9.24 g) was purified by column chromatography (petroleum ether–EtOAc, 3 : 2 → 1 : 1) to give **14** (5.65 g, 71% over 2 steps from **12**) as a colourless oil; δ_H (400 MHz; DMSO-d_6) 1.23 (6 H, t, J 7.0, OCH_2CH_3), 1.39 (9 H, s, *t*-Bu), 1.42 (9 H, s, *t*-Bu), 4.02–4.13 (4 H, m, OCH_2CH_3), 4.55 (1 H, dd, J 9.0 and 23.5, NHC*H*) and 7.48 (1 H, d, J 9.0, N*H*CH); δ_C (101 MHz; DMSO-d_6) 16.2 (d, J 5.5), 16.2 (d, J 5.5), 27.5, 28.1, 52.7 (d, J 148.5), 62.6 (d, J 6.5), 62.8 (d, J 6.5), 79.0, 82.0, 155.2 (d, J 8.0) and 165.8; δ_P (162 MHz; DMSO-d_6) 17.42; m/z (HR-ESI$^+$) 390.1649 (M + Na$^+$. $C_{15}H_{30}NNaO_7P$ requires 390.1652).

1-[^{2}H]-2-(1,3-dioxolan-2-yl)-ethanal 16. To a solution of **18** (368 mg, 3.06 mmol) in anhydrous CH_2Cl_2 (5 mL), a solution of the Dess–Martin periodinane (1.95 g, 4.60 mmol) in anhydrous CH_2Cl_2 (30 mL) was slowly added at rt. The reaction mixture was stirred at rt for 3.5 h. The reaction was quenched by addition of saturated $Na_2S_2O_3$ solution (5 mL) and saturated $NaHCO_3$ solution (2 mL) and subsequently stirred at rt for 20 min. The pH was adjusted to ~7 by further addition of saturated $NaHCO_3$ solution and the resulting mixture filtered through a thin pad of silica (CH_2Cl_2). The filtrate was evaporated to give crude **16** (233 mg, estimated purity >85% according to ^{1}H NMR) as a yellowish oil. Due to significant sensitivity of the aldehyde, it was used directly in the subsequent Horner–Wadsworth–Emmons reaction after NMR characterisation; δ_H (400 MHz; $CDCl_3$) 2.78 (2 H, d, J 4.5, $CHCH_2$), 3.88–4.07 (4 H, m, OCH_2CH_2O) and 5.27 (1 H, t, J 4.5, $CHCH_2$); δ_C (101 MHz; $CDCl_3$) 47.3 (t, J 3.5), 65.1, 100.2 and 198.9 (t, J 27.0).

1,1-[$^2H_2$]-2-(1,3-dioxolan-2-yl)-ethanol 18. To a suspension of $LiAl^2H_4$ (405 mg, 9.65 mmol) in anhydrous THF (50 mL), a solution of methyl (1,3-dioxolan-2-yl)-acetate **17**[44] (1.41 g, 9.65 mmol) in anhydrous THF (30 mL) was added dropwise at rt over 35 min. The reaction mixture was stirred at rt for a further 2 h and under reflux for 1 h. After cooling to 0 °C, the reaction was quenched by addition of water (3 mL). After addition of 1 N HCl down to pH 3, the pH was readjusted to ~9 using saturated $NaHCO_3$ solution. Most of the THF was evaporated under reduced pressure, the residue filtered through a thin pad of silica (Et_2O) and the filtrate evaporated under reduced pressure. The resultant colourless oil (1.18 g) was purified by column chromatography (petroleum ether–Et_2O, 1 : 3) to give **18** (727 mg,

63%) as a colourless oil; δ_H (400 MHz; $CDCl_3$) 1.95 (2 H, d, *J* 4.5, $CHCH_2$), 2.40 (1 H, br s, CD_2OH), 3.83–4.05 (4 H, m, OCH_2CH_2O) and 5.02 (1 H, t, *J* 4.5, $CHCH_2$); δ_C (101 MHz; $CDCl_3$) 35.3, 57.7 (quin, *J* 22.0), 64.8 and 103.8; *m*/*z* (HR-ESI$^+$) 143.0648 (M + Na^+. $C_5H_8D_2NaO_3$ requires 143.0648).

tert*-Butyl 2-(*tert*-butyloxycarbonyl-amino)-4-(1,3-dioxolan-2-yl)-but-2-enoate 19.** To a solution of KO*t*-Bu (100 mg, 0.891 mmol) in anhydrous THF (8 mL), a solution of phosphonate **14** (312 mg, 0.849 mmol) in anhydrous THF (8 mL) was added at −70 °C. After 5 min, a solution of crude aldehyde **15**[59] (197 mg) in anhydrous THF (8 mL) was added dropwise at −70 °C over 10 min. The reaction mixture was stirred for 20 h and slowly warmed to rt during this period. The reaction was quenched by addition of MeOH (5 mL) and the solvent evaporated under reduced pressure. The residue was dissolved in EtOAc (50 mL) and water (50 mL). The organic phase was washed with water (1 × 50 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The resultant yellowish oil (310 mg) was purified by column chromatography (hexane–EtOAc, 4 : 1) to give (Z*****)-19** (229 mg, 82%) and (***E*****)-19** (20 mg, 7%) as colourless oils; (***Z*****)-19**: δ_H (500 MHz; C_6D_6) 1.38 (9 H, s, *t*-Bu), 1.40 (9 H, s, *t*-Bu), 2.51–2.64 (2 H, m, $CHCH_2$), 3.11–3.19 (2 H, m, OCH_2CH_2O), 3.30–3.37 (2 H, m, OCH_2CH_2O), 4.68–4.76 (1 H, m, $CHCH_2$) and 6.56–6.72 (2 H, m, C=CH, NH); δ_C (126 MHz; C_6D_6) 28.3, 28.6, 33.5, 65.2, 80.2, 81.4, 103.9, 126.0, 153.6 and 164.3; *m*/*z* (HR-ESI$^+$) 352.1727 (M + Na^+. $C_{16}H_{27}NNaO_6$ requires 352.1731); (***E*****)-19**: δ_H (500 MHz; C_6D_6) 1.26 (9 H, s, *t*-Bu), 1.36 (9 H, s, *t*-Bu), 3.12 (2 H, dd, *J* 4.5 and 7.5, $CHCH_2$), 3.30–3.37 (2 H, m, OCH_2CH_2O), 3.53–3.59 (2 H, m, OCH_2CH_2O), 4.97 (1 H, t, *J* 4.5, $CHCH_2$), 6.96 (1 H, br s, NH) and 7.27–7.36 (1 H, m, C=CH); δ_C (126 MHz; C_6D_6) 28.2, 28.6, 34.4, 65.4, 80.0, 82.8, 104.5, 120.6, 153.4 and 163.9; *m*/*z* (HR-ESI$^+$) 352.1732 (M + Na^+. $C_{16}H_{27}NNaO_6$ requires 352.1731).

tert*-Butyl 2-(*tert*-butyloxycarbonyl-amino)-3-[^{2}H]-4-(1,3-dioxolan-2-yl)-but-2-enoate 20.** To a solution of KO*t*-Bu (89 mg, 0.79 mmol) in anhydrous THF (7 mL), a solution of phosphonate **14** (279 mg, 0.759 mmol) in anhydrous THF (7 mL) was added at −70 °C. After 5 min, a solution of crude deutero-aldehyde **16** (133 mg) in anhydrous THF (7 mL) was added dropwise at −70 °C over 10 min. The reaction mixture was stirred for 20 h and slowly warmed to rt during this period. The reaction was quenched by addition of MeOH (5 mL) and the solvent evaporated under reduced pressure. The residue was dissolved in EtOAc (50 mL) and water (50 mL). The organic phase was washed with water (1 × 50 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The resultant yellow-brown oil (300 mg) was purified by column chromatography (hexane–EtOAc, 4 : 1) to give (Z*****)-20** (189 mg, 75%) and (***E*****)-20** (27 mg, 11%) as colourless oils; (***Z*****)-20**: δ_H (500 MHz; C_6D_6) 1.38 (9 H, s, *t*-Bu), 1.40 (9 H, s, *t*-Bu), 2.47–2.66 (2 H, m, $CHCH_2$), 3.10–3.17 (2 H, m, OCH_2CH_2O), 3.29–3.35 (2 H, m, OCH_2CH_2O), 4.67–4.77 (1 H, m, $CHCH_2$) and 6.68 (1 H, br s, NH); δ_C (126 MHz; C_6D_6) 28.3, 28.6, 33.3, 65.2, 80.2, 81.4, 103.9, 125.6 (t, *J* 24.0), 153.6 and 164.3; δ_D (77 MHz; C_6H_6) 6.59 (s); *m*/*z* (HR-ESI$^+$) 353.1791 (M + Na^+. $C_{16}H_{26}DNNaO_6$ requires 353.1793); (***E*****)-20**: δ_H (500 MHz; C_6D_6) 1.25 (9 H, s, *t*-Bu), 1.36 (9 H, s, *t*-Bu), 3.13 (2 H, d, *J* 4.5, $CHCH_2$), 3.29–3.36 (2 H, m, OCH_2CH_2O), 3.52–3.59 (2 H, m, OCH_2CH_2O), 4.98 (1 H, t, *J* 4.5, $CHCH_2$) and 7.00 (1 H, br s, NH); δ_C (126 MHz; C_6D_6) 28.2, 28.6, 34.3, 65.4, 80.0, 82.8, 104.4, 120.2 (t, *J* 18.0), 153.4 and 163.9; *m*/*z* (HR-ESI$^+$) 353.1793 (M + Na^+. $C_{16}H_{26}DNNaO_6$ requires 353.1793).

tert*-Butyl (2*S*,3*S*)-2-(*tert*-butyloxycarbonyl-amino)-2,3-[$^2H_2$]-4-(1,3-dioxolan-2-yl)-butanoate 21.** A solution of didehydro-amino acid (Z*****)-19** (100 mg, 0.304 mmol) and (*S*,*S*)-Me-DUPHOS-Rh (4.0 mg, 6.6 μmol) in MeOH (10 mL) was stirred under an atmosphere of $^2H_2$ (1 bar) at rt for 6 days. The solvent was evaporated under reduced pressure. The residue was purified by column chromatography (petroleum ether–EtOAc, 4 : 1) to give **21** (99 mg, 98%) as a colourless oil; $[\alpha]_D^{25}$ +6.4 (*c* 1.00 in $CHCl_3$); δ_H (500 MHz; C_6D_6) 1.27 (9 H, s, *t*-Bu), 1.41 (9 H, s, *t*-Bu), 1.69–1.84 (3 H, m, $CHDCH_2$), 3.24–3.31 (2 H, m, OCH_2CH_2O), 3.40–3.47 (2 H, m, OCH_2CH_2O), 4.68–4.74 (1 H, m, $CHCH_2$) and 5.11 (1 H, br s, NH); δ_D (77 MHz; C_6H_6) 1.99 (1 D, s, $CHDCH_2$) and 4.43 (1 D, s, NHC*D*); δ_C (126 MHz; C_6D_6) 27.2 (t, *J* 19.5), 28.2, 28.8, 30.2, 54.2 (t, *J* 22.5), 65.2, 79.5, 81.5, 104.3, 155.9 and 172.3; *m*/*z* (HR-ESI$^+$) 356.2017 (M + Na^+. $C_{16}H_{27}D_2NNaO_6$ requires 356.2013).

tert*-Butyl (2*S*,3*R*)-2-(*tert*-butyloxycarbonyl-amino)-3-[^{2}H]-4-(1,3-dioxolan-2-yl)-butanoate 22.** A solution of didehydro-amino acid (Z*****)-20** (92 mg, 0.28 mmol) and (*S*,*S*)-Me-DUPHOS-Rh (4.0 mg, 6.6 μmol) in MeOH (10 mL) was stirred under an atmosphere of H_2 (1 bar) at rt for 6 days. The solvent was evaporated under reduced pressure. The residue was purified by column chromatography (hexane–EtOAc, 4 : 1) to give **22** (84 mg, 90%) as a colourless oil; $[\alpha]_D^{25}$ +7.2 (*c* 1.00 in $CHCl_3$); δ_H (500 MHz; C_6D_6) 1.28 (9 H, s, *t*-Bu), 1.41 (9 H, s, *t*-Bu), 1.71–1.83 (2 H, m, $CHDCH_2$), 2.01–2.08 (1 H, m, $CHDCH_2$), 3.24–3.31 (2 H, m, OCH_2CH_2O), 3.40–3.47 (2 H, m, OCH_2CH_2O), 4.50 (1 H, dd, *J* 5.5 and 8.0, NHC*H*), 4.71 (1 H, t, *J* 4.5, $CHCH_2$) and 5.11 (1 H, d, *J* 8.0, N*H*CH); δ_D (77 MHz; C_6H_6) 1.73 (s); δ_C (126 MHz; C_6D_6) 27.3 (t, *J* 19.5), 28.2, 28.8, 30.3, 54.5, 65.2, 79.5, 81.5, 104.3, 156.0 and 172.3; *m*/*z* (HR-ESI$^+$) 355.1954 (M + Na^+. $C_{16}H_{28}DNNaO_6$ requires 355.1950).

***tert*-Butyl N^2-Boc-N^5-methoxy-N^5-methyl-L-glutaminate 25.** To a solution of *N*-Boc-L-Glu-O*t*-Bu **24** (267 mg, 0.882 mmol) in anhydrous CH_2Cl_2 (10 mL), PyBOP (459 mg, 0.882 mmol) and NEt_3 (122 μL, 0.882 mmol) were added at rt. After stirring for 20 min at rt, more NEt_3 (245 μL, 1.76 mmol) and *N*,*O*-dimethylhydroxylamine hydrochloride (124 mg, 1.32 mmol) were added and the reaction mixture was stirred at rt for 20 h. More CH_2Cl_2 (20 mL) was added, and the organic solution was washed with 5% $NaHSO_4$ solution (1 × 10 mL), water (1 × 10 mL), saturated $NaHCO_3$ solution (1 × 10 mL), water (2 × 10 mL), brine (1 × 10 mL) and again water (1 × 10 mL). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The resultant crude product was purified by column chromatography (hexane–EtOAc, 4 : 1) to give **25** (246 mg, 81%) as a colourless oil; δ_H (400 MHz; $CDCl_3$) 1.43 (9 H, s, *t*-Bu), 1.44 (9 H, s, *t*-Bu), 1.85–1.94 (1 H, m, CH_2CH_2C=O), 2.08–2.15 (1 H, m, CH_2CH_2C=O), 2.43–2.56 (2 H, m, CH_2CH_2C=O), 3.14 (3 H, s, NCH_3), 3.65 (3 H, s, $NOCH_3$), 4.08–4.19 (1 H, m, NHC*H*) and 5.19 (1 H, br d, *J* 8.0, N*H*CH); δ_C (101 MHz; $CDCl_3$) 24.7, 27.6, 28.0, 28.3, 32.2, 53.7, 61.2, 79.6, 81.9, 155.5, 171.6 and 173.5.

***N*-Boc-5-[^{2}H]-L-GSA *tert*-butyl ester 26.** To a suspension of Cp_2Zr^2HCl (160 mg, 0.618 mmol) in anhydrous THF (2 mL), a

solution of **25** (214 mg, 0.618 mmol) in anhydrous THF (2 mL) was added at rt. The reaction mixture was stirred at rt for 15 min and then quenched by addition of ^2H_2O (50 μL) and further stirring at rt for 5 min. The solvent was evaporated under reduced pressure. The resultant crude product was purified by column chromatography (hexane–EtOAc, 4 : 1) to give **26** (126 mg, 71%) as a colourless oil; δ_H (500 MHz; $CDCl_3$) 1.45–1.51 (18 H, m, 2 × *t*-Bu), 1.78–2.12 (2 H, m, CH_2CH_2CD-OH), 2.21–2.52 (2 H, m, CH_2CH_2CD-OH) and 4.13–4.31 (1 H, m, NC*H*C=O); δ_D (77 MHz; C_6H_6) 5.48 (0.5 D, s, CH_2CH_2CD-OH) and 5.70 (0.5 D, s, CH_2CH_2CD-OH); δ_C (126 MHz; $CDCl_3$) 27.9–28.5, 59.9–61.0, 80.1–81.3, 153.1–154.5 and 171.5–172.8; *m*/*z* (HR-ESI$^+$) 311.1676 (M + Na^+. $C_{14}H_{24}DNNaO_5$ requires 311.1688).

1-(*tert*-Butyl) *N*-Boc-[$^2H_5$]-L-glutamate 27. To a solution of **31** (191 mg, 0.593 mmol) in THF (3 mL), a solution of Li^{2}H monohydrate (27 mg, 0.60 mmol) in ^2H_2O (3 mL) was added dropwise at 0 °C. The reaction mixture was warmed to rt and stirred at rt for 20 h. The organic solvent was evaporated under reduced pressure. The remaining aqueous solution was washed with Et_2O (1 × 7 mL), acidified with 1 N HCl and extracted with Et_2O (3 × 7 mL). The combined organics from this extraction were washed with water (1 × 10 mL), dried over $MgSO_4$ and evaporated under reduced pressure to give **27** (149 mg, 81%) as a colourless oil; $[\alpha]_D^{25}$ +4.1 (*c* 0.87 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 1.44 (9 H, s, *t*-Bu), 1.47 (9 H, s, *t*-Bu), 5.16 (1 H, s, N*H*CD); δ_D (77 MHz; $CHCl_3$) 1.81 (1 D, s, CD_2CD_2C=O), 2.05 (1 D, s, CD_2CD_2C=O), 2.33 (2 D, s, CD_2CD_2C=O) and 4.11 (1 D, s, NHC*D*); δ_C (126 MHz; $CDCl_3$) 26.9–27.7 (m), 28.0, 28.3, 29.1–29.6 (m), 52.3–53.3 (m), 80.1, 82.4, 155.6, 171.3 and 177.9; δ_C (126 MHz; ^{2}H-decoupled; $CHCl_3$) 27.3, 28.0 (q, *J* 127.0), 28.3 (q, *J* 127.0), 29.4, 52.9, 80.1, 82.4, 155.6, 171.3 and 177.7; *m*/*z* (HR – ESI$^-$) 307.1915 (M-H^+. $C_{14}H_{19}D_5NO_6$ requires 307.1923).

5-Methyl-[$^2H_5$]-L-glutamic acid 29. To a solution of [$^2H_5$]-L-glutamic acid **28** (441 mg, 2.90 mmol) in anhydrous MeOH (1.5 mL), $SOCl_2$ (300 μL, 4.11 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and the solvent evaporated under reduced pressure. The resultant yellowish solid was purified by column chromatography (CH_2Cl_2–MeOH, 9 : 1) to give **29** (421 mg, 87%) as a colourless solid; mp 146 °C; $[\alpha]_D^{25}$ +5.1 (*c* 1.15 in H_2O); δ_H (400 MHz; D_2O) 3.68 (3 H, s, OCH_3); δ_D (77 MHz; H_2O) 1.63 (1 D, s, CD_2CD_2C=O), 1.67 (1 D, s, CD_2CD_2C=O), 2.06 (2 D, s, CD_2CD_2C=O) and 3.50 (1 D, s, NH_2CD); δ_C (126 MHz; D_2O) 24.0–24.6 (m), 28.5–29.2 (m), 52.0–52.6 (m), 172.4 and 176.6; δ_C (126 MHz; ^{2}H-decoupled; H_2O) 24.4, 28.9, 52.3, 52.5 (q, *J* 148.0), 172.3 and 176.6; *m*/*z* (HR-ESI$^+$) 167.1077 (M + H^+. $C_6H_7D_5NO_4$ requires 167.1075).

5-Methyl-*N*-Boc-[$^2H_5$]-L-glutamic acid 30. To a solution of **29** (421 mg, 2.53 mmol) in anhydrous THF (8 mL), NEt_3 (1.05 mL, 7.53 mmol) and $(Boc)_2O$ (830 mg, 3.80 mmol) were added at rt. The reaction mixture was stirred at rt for 20 h and the solvent evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (30 mL) and washed with 5% $NaHSO_4$ solution (1 × 10 mL), water (1 × 10 mL) and brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The resultant crude product (645 mg) was purified by column chromatography (hexane–EtOAc, 4 : 1, then EtOAc–HOAc, 49 : 1) to give **30** (394 mg, 58%) as a colourless oil; $[\alpha]_D^{25}$ –0.6 (*c* 0.95 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 1.46 (9 H, s, *t*-Bu), 3.70 (3 H, s, OCH_3) and 5.21 (1 H, s, N*H*CD); δ_D (77 MHz; $CHCl_3$) 2.02 (1 D, s, CD_2CD_2C=O), 2.24 (1 D, s, CD_2CD_2C=O), 2.45 (1 D, s, CD_2CD_2C=O), 2.48 (1 D, s, CD_2CD_2C=O) and 4.35 (1 D, s, NHC*D*); δ_C (126 MHz; $CDCl_3$) 26.3–27.2 (m), 28.3, 29.4–30.1 (m), 51.8, 52.4–53.6 (m), 80.4, 155.7, 173.5 and 175.8; δ_C (126 MHz; ^{2}H-decoupled; $CHCl_3$) 26.7, 28.3 (q, *J* 127.0), 29.4, 51.8 (q, *J* 147.0), 52.5, 80.4, 155.7, 173.4 and 175.7; *m*/*z* (HR-ESI$^-$) 265.1439 (M – H^-. $C_{11}H_{13}D_5NO_6$ requires 265.1453).

1-(*tert*-Butyl) 5-methyl-*N*-Boc-[$^2H_5$]-L-glutamate 31. To a solution of **30** (289 mg, 1.09 mmol) in *t*-BuOH (8 mL), $(Boc)_2O$ (468 mg, 2.15 mmol) and DMAP (39 mg, 0.32 mmol) were added at rt. The reaction mixture was stirred at rt for 20 h and the solvent evaporated under reduced pressure. The resultant crude product was purified by column chromatography (hexane–EtOAc, EtOAc gradient) to give **31** (221 mg, 63%) as a colourless oil; $[\alpha]_D^{25}$ +6.5 (*c* 0.95 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 1.44 (9 H, s, *t*-Bu), 1.47 (9 H, s, *t*-Bu) and 3.68 (3 H, s, OCH_3); δ_D (77 MHz; $CHCl_3$) 1.89 (1 D, s, CD_2CD_2C=O), 2.12 (1 D, s, CD_2CD_2C=O), 2.33 (1 D, s, CD_2CD_2C=O), 2.40 (1 D, s, CD_2CD_2C=O) and 4.17 (1 D, s, NHC*D*); δ_C (126 MHz; $CDCl_3$) 28.0, 28.3, 29.1–29.4 (m), 29.4–29.7 (m), 51.7, 52.9–53.9 (m), 79.8, 82.2, 155.4, 171.3 and 173.3; *m*/*z* (HR-ESI$^+$) 323.2235 (M + H^+. $C_{15}H_{23}D_5NO_6$ requires 323.2225).

***tert*-Butyl-N^2-Boc-N^5-methoxy-N^5-methyl-[$^2H_5$]-L-glutaminate 32.** To a solution of **27** (127 mg, 0.413 mmol) in anhydrous CH_2Cl_2 (5 mL), PyBOP (215 mg, 0.413 mmol) and NEt_3 (57 μL, 0.413 mmol) were added at rt. After stirring for 20 min at rt, more NEt_3 (114 μL, 0.826 mmol) and *N*,*O*-dimethylhydroxylamine hydrochloride (60 mg, 0.62 mmol) were added and the reaction mixture was stirred at rt for 20 h. More CH_2Cl_2 (20 mL) was added, and the organic solution was washed with 5% $NaHSO_4$ solution (1 × 10 mL), water (1 × 10 mL), saturated $NaHCO_3$ solution (1 × 10 mL), water (2 × 10 mL), brine (1 × 10 mL) and water again (1 × 10 mL). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The resultant crude product was purified by column chromatography (hexane–EtOAc, 4 : 1) to give **32** (119 mg, 82%) as a colourless oil; $[\alpha]_D^{25}$ +5.7 (*c* 1.10 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 1.43 (9 H, s, *t*-Bu), 1.46 (9 H, s, *t*-Bu), 3.17 (3 H, s, NCH_3), 3.67 (3 H, s, $NOCH_3$) and 5.18 (1 H, s, N*H*CD); δ_D (77 MHz; $CHCl_3$) 1.91 (1 D, s, CD_2CD_2C=O), 2.12 (1 D, s, CD_2CD_2C=O), 2.46 (1 D, s, CD_2CD_2C=O), 2.52 (1 D, s, CD_2CD_2C=O) and 4.17 (1 D, s, NHC*D*); δ_C (126 MHz; $CDCl_3$) 26.7–27.3 (m), 27.4–27.6 (m), 28.0, 28.3, 32.3, 52.8–54.0 (m), 61.2, 79.6, 81.9, 155.5, 171.6 and 173.7; δ_C (126 MHz; ^{2}H-decoupled; $CHCl_3$) 26.8, 27.5 (q, *J* 127.0), 27.6, 27.9 (q, *J* 127.0), 31.8 (q, *J* 140.0), 52.8, 60.8 (q, *J* 144.0), 79.1, 81.5, 155.1, 171.2 and 173.2; *m*/*z* (HR-ESI$^+$) 352.2497 (M + H^+. $C_{16}H_{26}D_5N_2O_6$ requires 352.2490).

***N*-Boc-[$^2H_6$]-L-GSA *tert*-butyl ester 33.** To a suspension of Cp_2Zr^2HCl (108 mg, 0.417 mmol) in anhydrous THF (3 mL), a solution of **32** (99 mg, 0.28 mmol) in anhydrous THF (3 mL) was added at rt. The reaction mixture was stirred at rt for 15 min and then quenched by addition of ^2H_2O (50 μL) and further stirring at rt for 5 min. The solvent was evaporated under reduced pressure. The resultant crude product was purified by column chromatography (hexane–EtOAc, 4 : 1) to give **33** (54 mg, 66%)

as a colourless oil; δ_H (400 MHz; $CDCl_3$) 1.44 (9 H, s, *t*-Bu) and 1.47 (9 H, s, *t*-Bu); δ_D (77 MHz; $CHCl_3$) 1.77 (1 D, s, CD_2CD_2CD–OH), 2.01 (1 D, s, CD_2CD_2CD–OH), 2.18 (1 D, s, CD_2CD_2CD–OH), 2.45 (1 D, s, CD_2CD_2CD–OH), 4.21 (1 D, s, NC*D*C=O), 4.64 (0.5 D, s, CD_2CD_2CD–OH) and 4.77 (0.5 D, s, CD_2CD_2CD–OH); δ_C (126 MHz; $CDCl_3$) 27.5–27.7 (m), 27.8–27.9 (m), 28.0, 28.2, 60.6–60.7 (m), 76.3–76.5 (m), 80.1, 80.8, 154.5 and 172.4; δ_C (126 MHz; ^{2}H-decoupled; $CHCl_3$) 27.4, 28.0, 28.1 (q, *J* 127.0), 28.3 (q, *J* 127.0), 60.6, 76.7, 80.1, 80.8, 154.5 and 172.5; *m*/*z* (HR-ESI$^+$) 276.2082 (M – OH$^+$. $C_{14}H_{18}D_6NO_4$ requires 276.2076).

Preparation of [^{2}H]-labelled *t*-CMP derivatives 34–39 from L-GSA derivatives 6–10 using CarB or ThnE

Solutions of L-GSA derivatives **6**, **7**, **9** and **10** for direct use in CarB or ThnE reactions were obtained by deprotection of the appropriate precursors as follows: the protected L-GSA derivative (2–5 mg) was dissolved in 10% aqueous formic acid to give a final concentration of ~15 mM, and the reaction mixture was stirred at 60 °C for 1 h. After cooling to rt, the solution was frozen, stored at –80 °C and directly used for CarB or ThnE assays without further purification. L-GSA derivative **8** was obtained in a similar fashion from precursor **23**[52] and 10% deuterated formic acid (^{2}HCOO^{2}H) in ^2H_2O.

Typical CarB and ThnE reactions were carried out in the following manner: to a mixture of 35 µL 600 mM tris-HCl buffer (pH 9.0), 8 µL 10 mM malonyl-CoA solution and 5 µL L-GSA solution in 10% aqueous formic acid (as obtained from the deprotection reactions), 2 µL of CarB or ThnE solution (50 mg mL^{-1} in 50 mM tris-HCl pH 7.5 (for CarB) and 20 mg mL^{-1} in 50 mM tris-HCl pH 7.5 (for ThnE)) were added. For cloning, expression and purification of CarB and ThnE see ref. 12,15. The assay mixture was incubated at 37 °C for 10 min and the enzymatic reaction then quenched by addition of 50 µL MeOH. After cooling on ice for 10 min and centrifugation (5 min at 13 000 rpm), the supernatant was analysed *via* analytical LC-MS. Each assay was performed as a double experiment and with an additional control sample (assay mixture without addition of CarB or ThnE).

For spectroscopic identification of *t*-CMP products **4**, **37** and **38**, the standard ThnE assay was scaled up 10 times. After the usual work-up (quenching with 500 µL of MeOH) and centrifugation, the supernatant was lyophilised. The residue was redissolved in 180 µL H_2O–MeOH 95 : 5 and then subjected to purification *via* semipreparative LC-MS (Waters ZMD machine). Fractions containing the desired products were pooled, lyophilised and subjected to ^{1}H NMR analyses (Bruker AVIII 700 MHz NMR machine with a 5 mm TCI inverse cryoprobe, 1 mm sample tubes, samples dissolved in 6 µL ^2H_2O).

The CarB reaction with **8** leading to **39** was carried out as the standard small-scale assay, but employing 35 µL 600 mM [$^2H_{11}$]-tris-^{2}HCl in ^2H_2O (p^{2}H 9.0, uncorrected), 8 µL of a 10 mM solution of [$^2H_2$]-malonyl-CoA **40** in 100 mM [$^2H_{11}$]-tris-^{2}HCl buffer in ^2H_2O (p^{2}H 7.5, uncorrected, *vide infra*), 5 µL deprotection solution of **8** and 2 µL of a diluted CarB solution (original CarB solution was diluted 100-fold with 100 mM [$^2H_{11}$]-tris-^{2}HCl buffer in ^2H_2O (p^{2}H 7.5, uncorrected)). The solution of [$^2H_2$]-malonyl-CoA **40** was obtained in the following manner: malonyl-CoA (8.9 mg, 10 µmol) was dissolved in 100 mM [$^2H_{11}$]-tris-^{2}HCl buffer in ^2H_2O (p^{2}H 7.5, uncorrected, 1.04 mL). The resulting solution was incubated at 37 °C for 18 h and then directly used in the CarB assay.

Acknowledgements

We thank the BBSRC, the Deutsche Akademie der Naturforscher Leopoldina, Germany (BMBF-LPD 9901/8–137, C. D.), and the Ministry of Higher Education, Egypt (R. B. H.), for funding.

Notes and references

1 I. Oliment and R. L. Levine, *Arch. Biochem. Biophys.*, 1991, **289**, 371.
2 J. R. Requena, C.-C. Chao, R. L. Levine and E. R. Stadtman, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 69.
3 M. Akagawa, D. Sasaki, Y. Kurota and K. Suyama, *Ann. N. Y. Acad. Sci.*, 2005, **1043**, 129.
4 S. L. Bearne and R. Wolfendon, *Biochemistry*, 1995, **34**, 11515.
5 S. L. Bearne, O. Hekmat and J. E. MacDonnell, *Biochem. J.*, 2001, **356**, 223.
6 C. E. Pugh, J. L. Harwood and R. A. John, *J. Biol. Chem.*, 1992, **267**, 1584.
7 N. J. Kershaw, M. E. C. Caines, M. C. Sleeman and C. J. Schofield, *Chem. Commun.*, 2005, **34**, 4251.
8 S. J. McGowan, M. Sebaihia, 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.
9 R. Li, A. Stapon, J. T. Blanchfield and C. A. Townsend, *J. Am. Chem. Soc.*, 2000, **122**, 9296.
10 L. E. Nunez, C. Mendez, A. F. Brana, G. Blanco and J. A. Salas, *Chem. Biol.*, 2003, **10**, 301.
11 M. F. Freeman, K. A. Moshos, M. J. Bodner, R. Li and C. A. Townsend, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 11128.
12 R. B. Hamed, E. T. Batchelar, J. Mećinovic, T. D. W. Claridge and C. J. Schofield, *ChemBioChem*, 2009, **10**, 246.
13 M. C. Sleeman and C. J. Schofield, *J. Biol. Chem.*, 2004, **279**, 6730.
14 B. Gerratana, S. O. Arnett, A. Stapon and C. A. Townsend, *Biochemistry*, 2004, **43**, 15936.
15 M. C. Sleeman, J. L. Sorensen, E. T. Batchelar, M. A. McDonough and C. J. Schofield, *J. Biol. Chem.*, 2005, **280**, 34956.
16 E. T. Batchelar, R. B. Hamed, C. Ducho, T. D. W. Claridge, M. J. Edelmann, B. Kessler and C. J. Schofield, *Angew. Chem.*, 2008, **120**, 9462, (*Angew. Chem., Int. Ed.*, 2008, **47**, 9322).
17 R. B. Hamed, E. T. Batchelar, I. J. Clifton and C. J. Schofield, *Cell. Mol. Life Sci.*, 2008, **65**, 2507.
18 B. Gerratana, A. Stapon and C. A. Townsend, *Biochemistry*, 2003, **42**, 7836.
19 A. Stapon, R. Li and C. A. Townsend, *J. Am. Chem. Soc.*, 2003, **125**, 15746.
20 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.
21 M. C. Sleeman, P. Smith, B. Kellam, S. R. Chhabra, B. W. Bycroft and C. J. Schofield, *ChemBioChem*, 2004, **5**, 879.
22 M. Topf, G. M. Sandala, D. M. Smith, C. J. Schofield, C. J. Easton and L. Radom, *J. Am. Chem. Soc.*, 2004, **126**, 9932.
23 T. Borowski, E. Broclawik, C. J. Schofield and P. E. M. Siegbahn, *J. Comput. Chem.*, 2006, **27**, 740.
24 H. Luesch, D. Hoffmann, J. M. Hevel, J. E. Becker, T. Golakoti and R. E. Moore, *J. Org. Chem.*, 2003, **68**, 83.
25 V. A. Mezl and W. E. Knox, *Anal. Biochem.*, 1976, **74**, 430.
26 R. D. Farrant, V. Walker, G. A. Mills, J. M. Mellor and G. J. Langley, *J. Biol. Chem.*, 2001, **276**, 15107.
27 D. W. Tudor, T. Lewis and D. J. Robins, *Synthesis*, 1993, 1061.
28 C. V. Coulter, J. A. Gerrard, J. A. E. Kraunsoe, D. J. Moore and A. J. Pratt, *Tetrahedron*, 1996, **52**, 7127.
29 F. Burkhart, M. Hoffmann and H. Kessler, *Angew. Chem.*, 1997, **109**, 1240, (*Angew. Chem., Int. Ed.*, 1997, **36**, 1191).
30 J. M. Padron, G. Kokotos, T. Martin, T. Markidis, W. A. Gibbons and V. S. Martin, *Tetrahedron: Asymmetry*, 1998, **9**, 3381.
31 A. Sutherland, J. F. Caplan and J. C. Vederas, *Chem. Commun.*, 1999, 555.
32 C. E. Pugh, J. L. Harwood, T. Lewis, T. Cromartie and R. A. John, *Biochem. Soc. Trans.*, 1991, **19**, 319S.

33 C. E. Pugh, S. P. Nair, J. L. Harwood and R. A. John, *Anal. Biochem.*, 1991, **198**, 43.
34 H. J. Vogel and B. D. Davis, *J. Am. Chem. Soc.*, 1952, **74**, 109.
35 J. L. Sorensen, M. C. Sleeman and C. J. Schofield, *Chem. Commun.*, 2005, 1155.
36 H. J. Kreuzfeld, C. Döbler, U. Schmidt and H. W. Krause, *Amino Acids*, 1996, **11**, 269.
37 J. E. Baldwin, K. D. Merritt and C. J. Schofield, *Tetrahedron Lett.*, 1993, **34**, 3919.
38 J. E. Baldwin, K. D. Merritt, C. J. Schofield, S. W. Elson and K. H. Baggaley, *J. Chem. Soc., Chem. Commun.*, 1993, 1301.
39 U. Schmidt, A. Lieberknecht, U. Schanbacher, T. Beuttler and J. Wild, *Angew. Chem.*, 1982, **94**, 797, (*Angew. Chem., Int. Ed.*, 1982, **21**, 776).
40 U. Schmidt, A. Lieberknecht and J. Wild, *Synthesis*, 1984, 53.
41 R. Houssin, J.-L. Bernier and J.-P. Hénichart, *Synthesis*, 1988, 259.
42 H. Brechbühler, H. Büchi, E. Hatz, J. Schreiber and A. Eschenmoser, *Helv. Chim. Acta*, 1965, **48**, 1746.
43 P. Shi-Qi and E. Winterfeldt, *Liebigs Ann. Chem.*, 1989, 1045.
44 T. Hosokawa, T. Ohta, S. Kanayama and S.-I. Murahashi, *J. Org. Chem.*, 1987, **52**, 1758.
45 D. B. Dess and J. C. Martin, *J. Org. Chem.*, 1983, **48**, 4155.
46 U. Schmidt, H. Griesser, V. Leitenberger, A. Lieberknecht, R. Mangold, R. Meyer and B. Riedl, *Synthesis*, 1992, 487.
47 B. D. Vineyard, W. S. Knowles, M. J. Sabacky, G. L. Bachman and D. J. Wienkauff, *J. Am. Chem. Soc.*, 1977, **99**, 5946.
48 U. Vogeli and W. v. Philipsborn, *Org. Magn. Reson.*, 1975, **7**, 617.
49 R. Mazurkiewicz, A. Kuźnik, M. Grymel and N. Ku,źnik, *Magn. Reson. Chem.*, 2005, **43**, 36.
50 M. J. Burk, *J. Am. Chem. Soc.*, 1991, **113**, 8518.
51 T. Masquelin, E. Broger, K. Müller, R. Schmid and D. Obrecht, *Helv. Chim. Acta*, 1994, **77**, 1395.
52 I. Collado, J. Ezquerra, J. J. Vaquero and C. Pedregal, *Tetrahedron Lett.*, 1994, **35**, 8037.
53 The preparation of **9** has been reported in communication format (see ref. 35).
54 J. Coste, D. Le-Nguyen and B. Castro, *Tetrahedron Lett.*, 1990, **31**, 205.
55 J. T. Spletstoser, J. M. White and G. I. Georg, *Tetrahedron Lett.*, 2004, **45**, 2787.
56 A. R. Katritzky, H. Tao, R. Jiang, K. Suzuki and K. Kirichenko, *J. Org. Chem.*, 2007, **72**, 407.
57 K. Takeda, A. Akiyama, H. Nakamura, S. Takizawa, Y. M. H. Takayanagi and Y. Harigaya, *Synthesis*, 1994, 1063.
58 K. Saito, A. Kawaguchi, Y. Seyama, T. Yamakawa and S. Okuda, *J. Biochem.*, 1981, **90**, 1697.
59 The synthesis of this compound is described in the ESI†.