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# Biosynthesis of the (2*S*,3*R*)-3-Methyl Glutamate Residue of Nonribosomal Lipopeptides

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***Abstract:*** The calcium-dependent antibiotics (CDAs) and daptomycin are therapeutically relevant nonribosomal lipopeptide antibiotics that contain penultimate C-terminal 3-methyl glutamate (3-MeGlu) residues. Comparison with synthetic standards showed that (2*S*,3*R*)-configured 3-MeGlu is present in both CDA and daptomycin. Deletion of a putative methyltransferase gene *glmT* from the *cda* biosynthetic gene cluster abolished the incorporation of 3-MeGlu and resulted in the production of Glu-containing CDA exclusively. However, the 3-MeGlu chemotype could be re-established through feeding synthetic 3-methyl-2-oxoglutarate and (2*S*,3*R*)-3-MeGlu, but not (2*S*,3*S*)-3-MeGlu. This indicates that methylation occurs before peptide assembly, and that the module 10 A-domain of the CDA peptide synthetase is specific for the (2*S*,3*R*)-stereoisomer. Further mechanistic analyses suggest that GlmT catalyzes the SAM-dependent methylation of α-ketoglutarate to give (3*R*)-methyl-2-oxoglutarate, which is transaminated to (2*S*,3*R*)-3-MeGlu. These insights will facilitate future efforts to engineer lipopeptides with modified glutamate residues, which may have improved bioactivity and/or reduced toxicity.

## Introduction

Nonribosomal peptides are complex and diverse secondary metabolites that display a wide range of biological activities and include a number of important therapeutic agents.[1] Their structural complexity stems from the wide range of building blocks, which are utilized in their construction. In addition to proteinogenic amino acids, nonribosomal peptides can contain many unusual amino acids, fatty acids, polyketide moieties and are often glycosylated. By understanding the biosynthetic origins of these building blocks and their mode of assembly, it is envisaged that new methods might be developed for engineering the biosynthesis of new nonribosomal peptides with improved biological activities.[2]

With this in mind, we have been investigating the biosynthesis of the calcium-dependent antibiotics (CDAs) produced by *Streptomyces coelicolor*.[3] CDAs (Figure 1) belong to the nonribosomal lipopeptide group of antibiotics, including daptomycin, which recently became the first new structural class of naturally derived antimicrobial agents to reach the clinic in over 30 years.[4] CDAs and daptomycin, along with the related A54145 lipopeptides,[5] all possess N-terminal fatty acid side chains and share several common amino acids, including acidic residues Asp, Glu, and 3-methylglutamic acid (3-MeGlu), some of which are conserved at the same relative positions in the decapeptide lactone cores. These acidic residues are important for coordinating calcium ions, which are essential for antimicrobial activity. CDAs and A54145 are each produced as mixtures of lipopeptides, possessing either Glu or 3-MeGlu as the penultimate C-terminal residue, which are often difficult to separate. In A54145[5b] and daptomycin,[6] the 3-MeGlu-containing variants are more potent antibiotics than the Glu variants. However, 3-MeGlu-containing peptides have more toxic side effects than the Glu-containing variants.[5b] Therefore, it is highly desirable to develop methods for engineering the biosynthesis of these antibiotics that control the specificity of incorporation for Glu,

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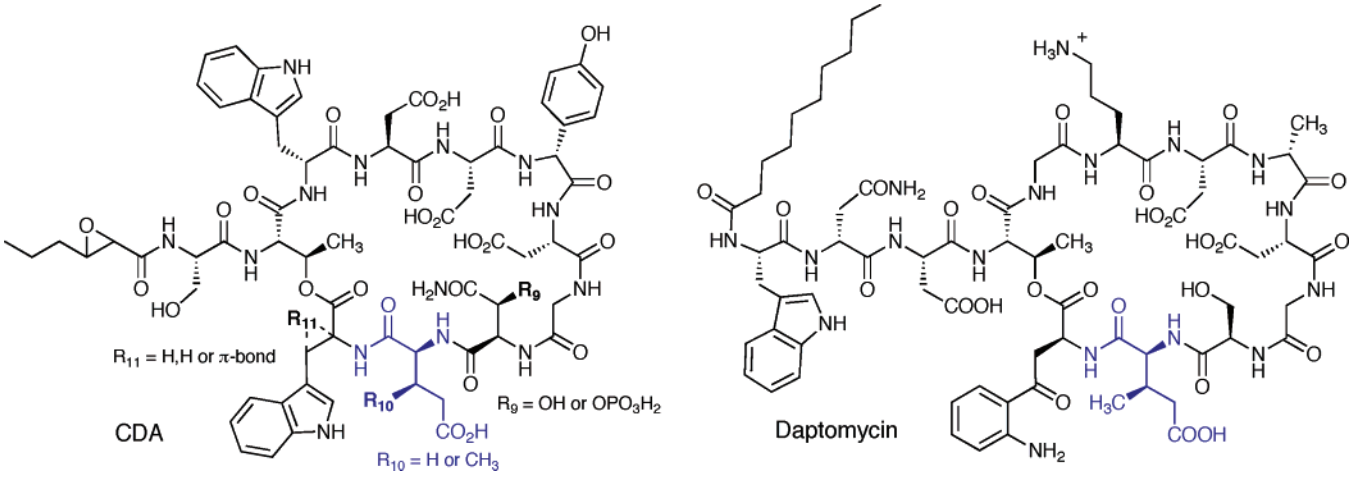


***Figure 1.*** The calcium-dependent antibiotics (CDAs) and daptomycin. The CDA structural variants that are known to be produced by the wild-type *S. coelicolor*[3a] are as follows: CDA1, $R_9 = OPO_3H_2$, $R_{10} = H$; CDA2, $R_9 = OPO_3H_2$, $R_{10} = CH_3$; CDA3, $R_9 = OH$, $R_{10} = H$; CDA4, $R_9 = OH$, $R_{10} = CH_3$. In addition the a-series contains *Z*-Δtrp ($R_{11} = \pi$-bond) and the b-series contains L-Trp ($R_{11}$ = H,H) at position 11.

3-MeGlu, or Glu analogues (including stereoisomers); consequently, new therapeutically relevant lipopeptides with increased activity and reduced toxicity could be biosynthesized. To achieve this, it is first necessary to elucidate the biogenesis of 3-MeGlu, within CDAs, and further explore how the adenylation domain (A-domain) of the nonribosomal peptide synthetase (NRPS) governs the specificity for Glu and 3-MeGlu.

Previously, we identified gene SCO3215 within the *cda* biosynthetic gene cluster, which was tentatively suggested to encode a glutamate-3-methyltransferase (GlmT)[3a] based on the low, but significant, sequence similarity between the SCO3215 gene product and other methyltransferases. Additionally, the amino acid specificity-conferring code[7] for the active site of the CdaPS3 module 10 A-domain is unlike other Glu-activating domains, suggesting that this A-domain may have evolved specificity for 3-MeGlu as well as Glu.[3a] This alternative A-domain specificity suggests that methylation of Glu occurs before peptide assembly. GlmT was thus predicted to catalyze the direct methylation of Glu to give 3-MeGlu.[3a] In a model metabolic flux analysis of CDA production,[8] we also considered the possibility that GlmT may alternatively methylate α-ketoglutarate, leading to 3-methyl-2-oxoglutarate, which is then transaminated to 3-MeGlu.

Upon completion of the work described here, the sequence of the gene clusters of daptomycin from *Streptomyces roseosporus*[4d] and A54145 from *Streptomyces fradiae*[5c] were published, and genes *dptI* and *lptI*, which showed similarity to *glmT*, were identified. Additionally, the amino acid substrate binding pockets of the A-domains responsible for activating 3-MeGlu or Glu in the corresponding penultimate modules of the daptomycin and A54145 NRPS (DptD and LptD) were shown to be very similar in sequence to the module 10 A-domain of CdaPS3. This suggests a common mode of 3-MeGlu biosynthesis and NRPS A-domain activation during the assembly of the three groups of lipopeptides. In this paper, we sought to probe the biosynthetic origins of 3-MeGlu in *S. coelicolor* and further explore the in vivo specificity of the corresponding A-domain of module 10 responsible for incorporation of Glu/3-MeGlu into CDAs.

## Results and Discussion

**Synthesis of (2*S*,3*R*)- and (2*S*,3*S*)-Methyl Glutamic Acid.** Previous work demonstrated that the 3-MeGlu in CDA was the L-isomer,[9] which is consistent with the absence of an epimerization (E) domain within the CdaPS3 module 10.[3a] Furthermore, the stereochemistry of the 3-MeGlu residue of daptomycin was inferred to be the L-*threo*- or (2*S*,3*R*)-stereoisomer through the stereospecificity of two enzymes, glutamine synthase and glutamate decarboxylase.[10] The high protein sequence similarity of the putative methyltransferases (GlmT, DptI, and LptI) and the corresponding active sites of the A-domains for Glu/3-MeGlu from the *cda*, *dpt*, and *lpt* gene clusters, respectively, is strongly suggestive that the 3-MeGlu residues in CDA and the other lipopeptides will possess identical absolute configuration. However, it is well established that enzyme stereospecificity can be completely inverted by as few as two point mutations,[11] or in some cases simply by changing the medium surrounding the enzyme.[12] Therefore, an unambiguous stereocontrolled synthesis of (2*S*,3*R*)- and (2*S*,3*S*)-3-MeGlu diastereoisomers was developed for comparison with the natural 3-MeGlu derived by hydrolytic degradation of CDAs and daptomycin. These synthetic compounds are also potentially valuable for subsequent experiments aimed at dissecting 3-MeGlu biosynthesis and the in vivo specificity of the Glu/3-MeGlu-activating A-domain of CdaPS3. The synthetic route shown in Scheme 1 was designed so that all four possible 3-MeGlu stereoisomers might be prepared with complete stereocontrol, without recourse to separation of diastereoisomers.

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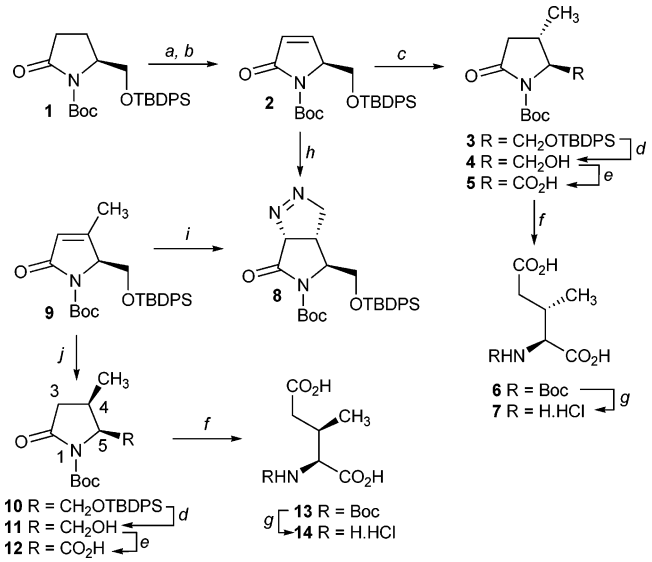
***Scheme 1.*** Synthesis of (2*S*,3*S*)- and (2*S*,3*R*)-3-Methyl Glutamic Acids[a]




[a] Conditions: (a) LDA, PhSeBr; (b) $H_2O_2$, $CH_3CO_2H$; (c) $CH_3Li$, Cu(I)I; (d) TBAF, 20% $CH_3CO_2H$; (e) $NaIO_4$, $RuCl_3$; (f) LiOH; (g) 4 M HCl in 1,4-dioxane; (h) $CH_2N_2$; (i) heat at 120 °C; (j) $H_2$, Pt(IV)$O_2$.

Asymmetric syntheses of 3-MeGlu diastereoisomers have been reported.[13] The first approach utilized an Arndt−Eistert homologation of (2*S*,3*S*)-3-methyl aspartate to generate the (2*S*,3*R*)-3-MeGlu.[13a] However, this synthesis is not easily modified to prepare other stereoisomers or $\beta$-substituted analogues. The second synthetic strategy utilized a Ni(II) homochiral Shiff base of glycine in a diastereoselective conjugate addition of ethyl crotonate, but this approach only produced the (2*S*,3*S*)-3-MeGlu, and in low yield.[13b] During the course of our investigation, a third approach was reported that also incorporated a homochiral glycinyl Schiff base in a conjugate addition with ethyl crotonate to produce both (2*S*,3*R*)- and (2*S*,3*S*)-3-MeGlu stereoisomers; unfortunately, the lack of complete stereocontrol required the tedious separation of diastereoisomers.[13c] It was thus apparent that the development of a new convergent synthesis to all possible stereoisomers of 3-MeGlu was necessary. Ideally, this synthesis should be easily modifiable allowing for the preparation of a variety of $\beta$-substituted Glu analogues.

Our synthetic approach begins with the known lactam **1**,[14] which is derived from L-pyroglutamic acid. Accordingly, $\alpha$-phenylselenation of the lactam **1**, followed by oxidative elimination, via the selenoxide, results in the $\alpha,\beta$-unsaturated lactam **2**, which is treated with lithium dimethyl cuprate to give exclusively the *trans*-$\beta$-methyl lactam **3**.[14] The absolute configuration of **3** was previously confirmed by X-ray crystallography.[14c] Removal of the silyl protecting group and oxidation of the alcohol **4** gave the acid **5**. Hydrolysis of the lactam **5** with aqueous LiOH followed by acidification and extraction into diethyl ether gave the Boc-protected free acid **6**, which was treated with 4 M HCl in dioxane to remove the Boc group and reveal the product (2*S*,3*S*)-methyl glutamic acid as the HCl salt **7**. The optical rotation of the product was identical to that reported previously for the same stereoisomer prepared by alternative routes.[13a,c]

To prepare the opposite (2*S*,3*R*)-methyl glutamic acid, the intermediate $\alpha,\beta$-unsaturated lactam **2** was subjected to a 1,3-dipolar cycloaddition with diazomethane[15] to generate the pyrazoline **8**, as a single diastereoisomer. Thermolysis of the pyrazoline ring[15] under solvent-free conditions gave the $\beta$-methyl-$\alpha,\beta$-unsaturated lactam **9**. Hydrogenation of **9**, at atmospheric pressure, using $PtO_2$ as a catalyst gave the *cis*-$\beta$-methyl lactam **10**. The absolute configuration of **10** was confirmed from NOESY NMR, which showed an NOE between H5 and H4, but no NOE cross-peak was evident between H5 and the $\beta$-methyl protons, which is consistent with the *cis*-stereochemistry as shown. In the case of the corresponding *trans*-$\beta$-methyl lactam **3**, of known absolute configuration, the opposite pattern of NOEs is evident with H5, showing an NOE to the $\beta$-methyl protons but not to H4. Furthermore, in the COSY spectra of *trans*-$\beta$-methyl lactam **3**, there is no cross-peak between H5 and H4, which is consistent with a dihedral angle ($\phi$) close to 90° as indicated from the crystal structure.[14c] On the other hand, the COSY spectra of *cis*-$\beta$-methyl lactam **10** show a clear cross-peak between H4 and H5, due to a smaller dihedral angle between H4 and H5 of ca. 33°, which is predicted from energy minimized models of **10** generated using bond angles and lengths extracted from the crystal structure of the diastereoisomer **3**.[14c] Desilylation of **10** and oxidation of the alcohol **11** gave the acid **12**, which was hydrolyzed and subjected to acidolysis, as described above, to give (2*S*,3*R*)-methyl glutamic acid **14** as the HCl salt, which was identical by optical rotation to the same product produced by independent routes.[13a,c] The advantage of this synthetic route over the others reported[13] is that all four stereoisomers of 3-MeGlu can be produced from the common precursor **2** or its available enantiomer without the need to separate diastereoisomers. Also by adding different alkyl cuprate reagents to **2** it is possible to generate alternative *trans*-$\beta$-alkylated lactams (analogous to **3**). Similarly, synthetic methodology exists which would enable the transformation of **2** into the opposite *cis*-$\beta$-alkylated lactams (analogous to **10**).[16]

**Assignment of the Absolute Stereochemistry of 3-MeGlu in CDA and Daptomycin.** With the synthetic (2*S*,3*S*)- and (2*S*,3*R*)-3-methyl glutamic acids in hand, it was possible to establish the configuration of the natural 3-MeGlu derived from CDA. Accordingly, a pure sample of CDA4a, which is known to contain 3-MeGlu,[3a,9] was hydrolyzed with aqueous 6 M HCl, and the hydrolysate was derivatized with dabsyl chloride,[17] which facilitates improved retention and separation of the amino acid derivatives on reverse phase HPLC. This allowed identification of all the dabsylated amino acids from CDA, except (*Z*)-dehydrotryptophan, which presumably undergoes further hydrolysis to indolepyruvate. Conditions for the separation of the synthetic dabsylated (2*S*,3*S*)- and (2*S*,3*R*)-3-methyl glutamate diastereoisomers on reverse phase were then established. This showed that the (2*S*,3*S*)-dabsyl derivative has a retention time

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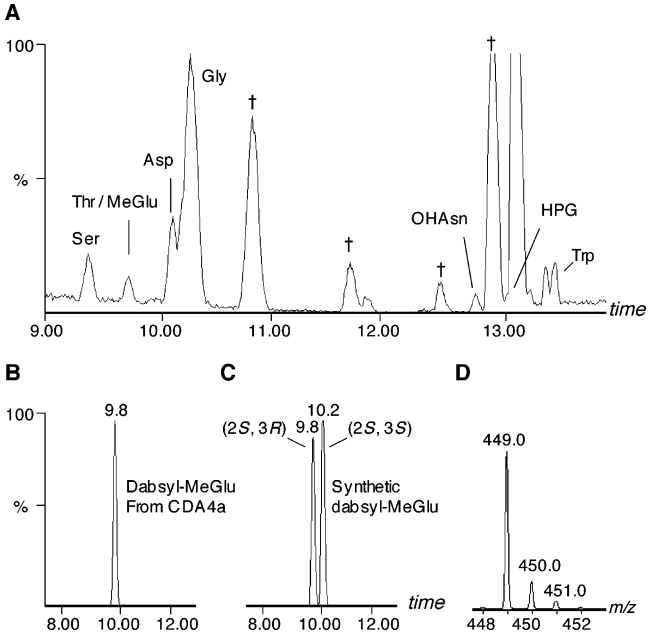


***Figure 2.*** Assignment of the absolute stereochemistry of 3-MeGlu in CDA. (A) LC-MS of the dabsylated amino acid derivatives derived from acidic hydrolysis of CDA4a. (B) Expansion of the mass extracted chromatogram identifies the 3-MeGlu derivative with a retention time of 9.8 min. (C) Extracted mass chromatogram of the synthetic derivatized standards (2*S*,3*S*)- and (2*S*,3*R*)-MeGlu. Spiked mixtures show that the natural 3-MeGlu is the (2*S*,3*R*)-diastereoisomer with retention time of 9.8 min as opposed to the (2*S*,3*S*)-diastereoisomer, which has a longer retention time of 10.2 min. (D) MS of the peak 9.8 min, *m*/*z* 449.0, corresponds to $[M + H]^+$ ion of dabsylated 3-MeGlu. Cross sign is the unassigned degradation side products/impurities.

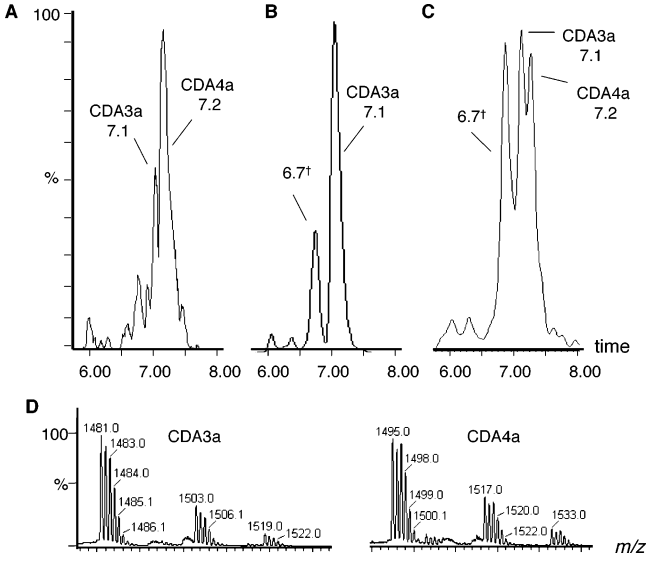


***Figure 3.*** LC-MS analysis of the Δ*glmT* and recomplementation with synthetic precursors. (A) Chromatogram of extracts from the wild-type strain MT1110. MS of the peak at 7.1 and 7.2 min gave *m*/*z* 1480.8 and 1494.8 corresponding to CDA3a and CDA4a $[M + H]^+$ ions, respectively. The corresponding sodiated and potassiated molecular ions are present and consistent with this. (B) LC-MS of the Δ*glmT* mutant showed only (cross sign) CDA3a (7.1 min, *m*/*z* 1481.2 $[M + H]^+$). (C) Chromatogram obtained after the exogenous supply of 300 μg/mL (2*S*,3*R*)-3-MeGlu to the Δ*glmT* mutant. Shows peaks at both 7.1 and 7.2 min consistent with production of CDA3a and CDA4a. (D) The mass spectra for the peaks (at 7.1 and 7.2 min) obtained from feeding (2*S*,3*R*)-3-MeGlu to Δ*glmT* (chromatogram C) show expected molecular ions for CDA3a (1481.0 $[M + H]^+$, 1503.0 $[M + Na]^+$, 1519.0 $[M + K]^+$) and CDA4a (1495.0 $[M + H]^+$, 1517.0 $[M + Na]^+$, 1533.0 $[M + K]^+$). (Cross sign) The peak at 6.7 min in Δ*glmT* (B) and (C), but also evident to a much lower extent in the wild-type, has *m*/*z* 1499.5, which corresponds to the $[M + H]^+$ for a postulated linear form of CDA3a, which is proposed to arise from hydrolysis of the lactone ring or failure of the thioesterase domain to effect complete cyclization. Similar byproducts have been identified during production of daptomycin and other lipopeptides.[4a]

at 10.2 min, with the (2*S*,3*R*)-diastereoisomer at 9.8 min (Figure 2). Comparing the LC-MS chromatographs of the derivatized hydrolysate with that of synthetic standards established that the 3-MeGlu residue in CDA4a has (2*S*,3*R*)-absolute configuration. Similar hydrolysis, derivatization, and LC-MS analysis of daptomycin demonstrated that the same (2*S*,3*R*)-3-MeGlu is also present within daptomycin. This agrees with the earlier assignment that was inferred from the stereospecificity of glutamine synthase and glutamate decarboxylase[10a] and further suggests a common mode of biosynthesis for 3-MeGlu in the *S. coelicolor* and *S. roseosporus* producer strains.

**Deletion of the *glmT* Gene and Recomplementation with Synthetic (2*S*,3*R*)-3-MeGlu.** To probe the biosynthetic origins of (2*S*,3*R*)-3-MeGlu in CDAs, a mutant strain of *S. coelicolor* (Δ*glmT*) was generated, which contained an in-frame deletion within the *glmT* gene. Accordingly, a DNA fragment of ca. 2.0 kb incorporating 396 bp of the downstream end of *glmT* and the adjacent SCO3214 (*trpE2*) gene and a fragment of ca. 1.5 kb including 4 bp of the upstream end of *glmT* and the flanking SCO3216 gene were generated by PCR. These fragments were first ligated into plasmid pMT3000 cloned and then re-isolated from *E. coli*. The resulting 3.5 kb in-frame deletion fragment (missing 615 bp of *glmT*) was digested from pMT3000 and subcloned into the plasmid pMAH[18] (see Supporting Information for more details). Transformation of *S. coelicolor* MT1110[19] protoplasts with the resulting plasmid, followed by homologous recombination and subsequent screening of second crossover recombination events,[3a] resulted in the mutant strain Δ*glmT*. The wild-type MT1110 strain was chosen as a host for these experiments because this strain tends to produce nonphosphorylated CDAs,[3a] which aids LC-MS analysis. In addition, the MT1110 strain is more easily cultivated than the more widely used *S. coelicolor* 2377.

The MT1110 wild-type and Δ*glmT* mutant strains were grown in liquid culture under conditions that favored production of (*Z*)-dehydrotryptophan-containing CDA. The cells were harvested by centrifugation, and the supernatants were analyzed by LC-MS.[3a] This showed that the wild-type MT1110 produces, as the major product, CDA4a that contains 3-MeGlu along with a minor amount of the Glu variant CDA3a (Figure 3). In contrast, the mutant Δ*glmT* produces only the Glu-containing CDA3a, with no MeGlu-containing CDAs evident. Indeed, repeated fermentation and analysis of Δ*glmT* under a variety of conditions clearly reveals the complete absence of any 3-MeGlu-containing CDAs. While this supports the hypothesis that the gene product GlmT is a methyltransferase involved in the biosynthesis of 3-MeGlu residues in CDA, these results do not reveal any information about the timing of the methylation per se. To determine the order of the steps on the pathway, the synthetic (2*S*,3*R*)-3-MeGlu natural stereoisomer was fed in increasing concentrations to the Δ*glmT* mutant during fermenta-

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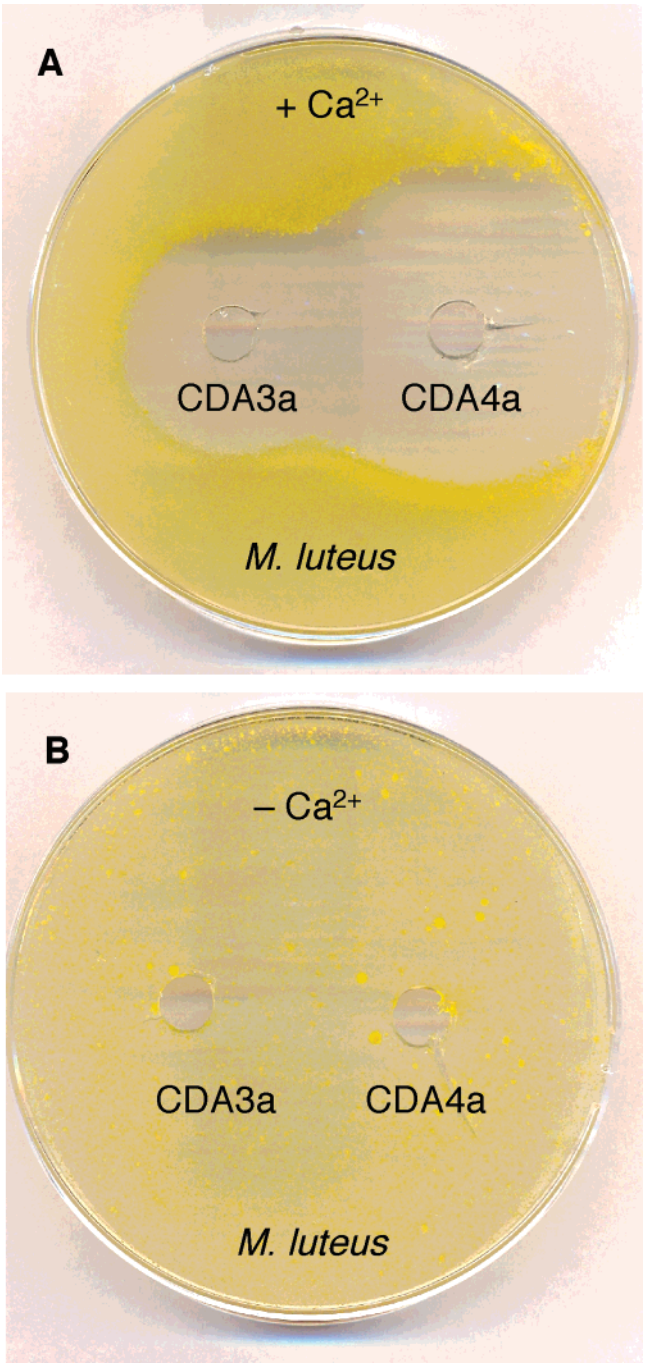
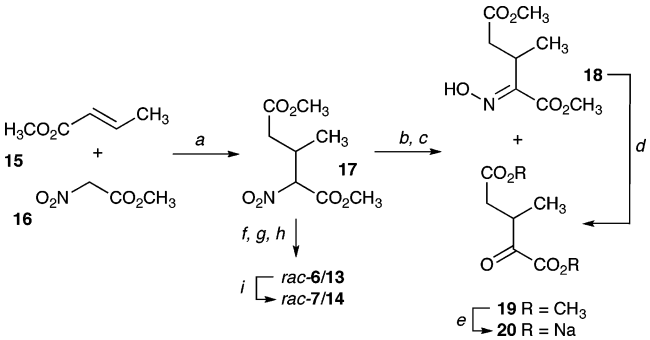



***Figure 4.*** Comparison of the antimicrobial activities of Glu-containing CDA3a and 3-MeGlu-containing CDA4a. Bioassays were carried out as described previously[28] using *M. luteus* as the indicator strain. Equimolar solutions of CDA3a and CDA4a in deionized water (6.0 $\mu$g in 100 $\mu L^{-1}$) were added to the 5 mm left- and right-hand wells, respectively. (A) In the presence of $Ca^{2+}$ (16 mM), zones of inhibitions for CDA3a and CDA4a are ca. 1.5 and 2.5 cm, respectively. (B) In the absence of $Ca^{2+}$, no zones were evident.

tion. LC-MS analysis clearly shows that upon the addition of as little as 50 $\mu$g $mL^{-1}$ of (2*S*,3*R*)-3-MeGlu the mutant Δ*glmT* reverts to wild-type phenotype and produces both MeGlu-containing CDA4a as well as Glu-containing CDA3a (Figure 3). In contrast, the feeding of (2*S*,3*S*)-3-MeGlu to the Δ*glmT* strain, at relatively high total concentration of 300 $\mu$g $mL^{-1}$, had no effect on the production of CDAs; no MeGlu-containing lipopeptides were evident by LC-MS. This indicates that, in addition to Glu, the enzyme CdaPS3 is able to activate and incorporate the natural (2*S*,3*R*)-3-MeGlu, but not the (2*S*,3*S*)-diastereoisomer, which demonstrates that methylation of Glu, or its precursor, occurs before peptide assembly. The ability of the A-domain of CdaPS3 module 10, and to lesser extent the adjacent condensation (C)-domains, to discriminate between the diastereoisomers is unlikely to be fortuitous. Indeed, this selectivity is probably a consequence of the A-domain having evolved specificity for (2*S*,3*R*)-3-MeGlu from an ancestral Glu-activating A-domain.

***Scheme 2.*** Synthesis of 3-Methyl-2-oxoglutarate and Racemic 3-Methyl Glutamic Acids[a]




[a] Conditions: (a) DBU; (b) $CH_3ONa$ in $CH_3OH$; (c) $TiCl_3-CH_3CO_2NH_4$ and aq. HCl;[20] (d) HCl−$CH_3OH$ then HCOH−$H_2O$; (e) aq. NaOH; (f) Raney Ni; (g) $(Boc)_2O$, 4-DMAP, $Et_3N$; (h) LiOH; (i) 4 M HCl in 1,4-dioxane.

By using this knockout strategy and recomplementation with synthetic (2*S*,3*R*)-3-MeGlu, it is now possible to control the functionality of the penultimate residue in CDAs. To demonstrate the importance of this biosynthetic control, we isolated pure samples of CDA3a and CDA4a and subjected them to well-plate bioassay using *Micrococcus luteus* as the indicator strain (Figure 4). The resulting zones of inhibition indicate that, like daptomycin and A54145, the 3-MeGlu-containing CDA4a sample is more active than the Glu variant.

**Recomplementation of Δ*glmT* with Racemic 3-MeGlu Diastereoisomers and 3-Methyl-2-oxoglutarate.** While the results described above show that the GlmT is involved in the biosynthesis of the 3-MeGlu residue in various CDAs, and that the methyl group is introduced before peptide assembly, the nature of the substrate for GlmT is not necessarily apparent. Either L-Glu is methylated directly to (2*S*,3*R*)-3-MeGlu or, alternatively, the precursor α-ketoglutarate is first methylated to (3*R*)-methyl-2-oxoglutarate and then transaminated to (2*S*,3*R*)-3-MeGlu. To explore which of these scenarios is most likely, we synthesized 3-methyl-2-oxoglutarate (Scheme 2). This involved the Michael addition of methyl crotonate **15** with the methyl nitroacetate **16** enolate anion to give **17**. A Neff reaction[20] catalyzed with $TiCl_3$ in an aqueous solution of HCl gave a mixture of the oxime **18** and the ketone **19**. Further selective acidic hydrolysis of the oxime gave more ketone **19**, which was subjected to saponification, resulting in the 3-methyl-2-oxoglutarate **20** as the disodium salt. Also, using the nitro intermediate **17**, all four possible stereoisomers of 3-MeGlu were prepared as a mixture by hydrogenation of the nitro group to the corresponding amine. Boc protection and basic hydrolysis then gave a racemic mixture of **6**/**13**, which was treated with 4

(20) McMurry, J. E.; Melton, J. *J. Org. Chem.* **1973**, *38*, 4367−4373.

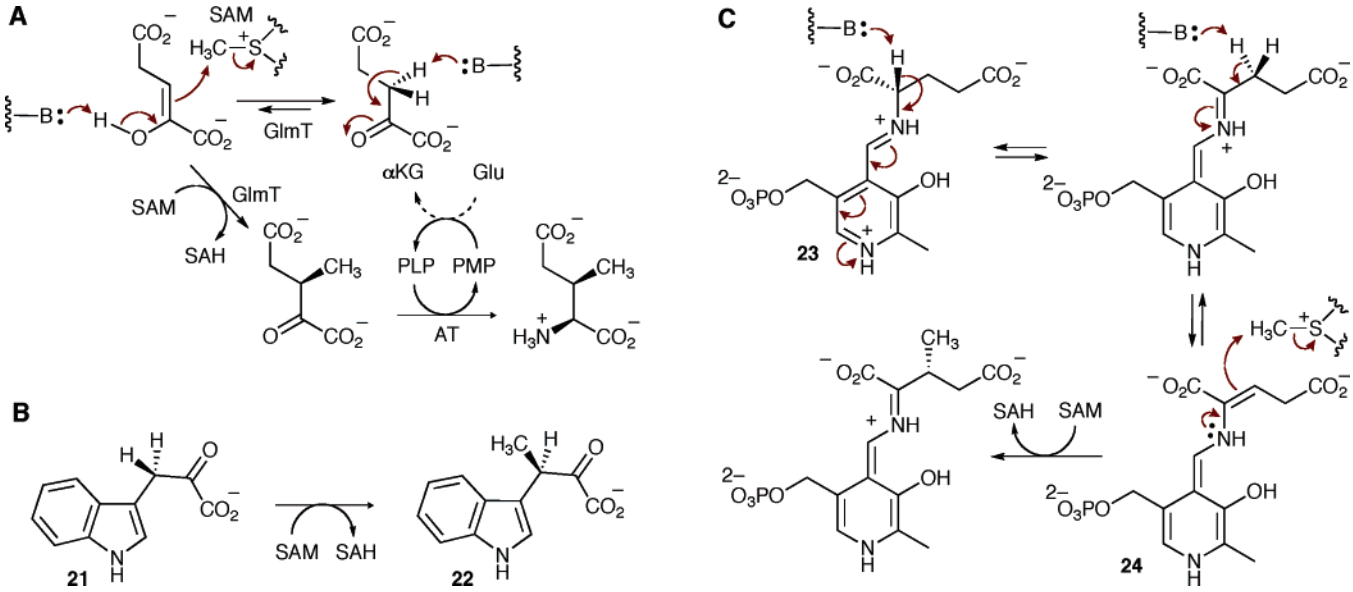



***Figure 5.*** (A) Possible mode of biosynthesis of (2*S*,3*R*)-3-methyl glutamic acid in *S. coelicolor*. (B) (*S*)-adenosylmethionine-dependent methylation of indolepyruvate during indolmycin biosynthesis.[22b] (C) Formation of a Schiff base between Glu and PLP could also facilitate $\beta$-methylation of Glu in CDA biosynthesis. Abbreviations: GlmT, glutamate- or 2-oxoglutarate-3-methyltransferase; AT = putative aminotransferase enzyme or associated activity; SAM, (*S*)-adenosylmethionine; SAH, (*S*)-adenosylhomocysteine.

M HCl in dioxane to give a racemic mixture of 3-MeGlu diastereoisomers (*rac*-**7**/**14**).

The racemic mixture of 3-MeGlu diastereoisomers (*rac*-**7**/**14**) was then fed to the Δ*glmT* mutant strain. As expected, this re-established the production of CDA4a containing 3-MeGlu. However, in addition to the identification of CDA3a containing Glu, a third product with identical molecular weight to CDA4a (*m*/*z* 1495 $[M + H]^+$), but with a slightly different retention time, was repeatedly observed by LC-MS. This third product was not present in either feeding experiments with (2*S*,3*R*)- or (2*S*,3*S*)-3-MeGlu, which suggests that one of the (2*R*)-MeGlu diastereoisomers in the racemic mixture is also incorporated into the CDA pool, likely to include a CDA4a diastereoisomer (*epi*-CDA4a). While the scale of the feeding experiments and difficulty of HPLC separation prevented the isolation and more detailed characterization of the purported new product *epi*-CDA4a, this finding suggests that future mutasynthesis of CDA analogues may be viable with D- as well as L-glutamate analogues.

Following this, 3-methyl-2-oxoglutarate **20** was fed to the Δ*glmT* mutant strain. LC-MS analysis of the culture supernatant, after 6 days growth, revealed that by feeding increasing amounts of **20** (between 0.46 and 2.25 mg $mL^{-1}$) the biosynthesis of 3-MeGlu-containing CDA4a is initiated. The higher concentrations of 3-methyl-2-oxoglutarate **20** compared with that of (2*S*,3*R*)-3-MeGlu required to bring about CDA4a production could be due to competing metabolism or poorer cellular uptake. On the other hand, (2*S*,3*R*)-3-MeGlu could enter the cell via specific glutamate or nonspecific amino acid uptake mechanisms.[21] Despite the higher concentrations of 3-methyl-2-oxoglutarate required to re-establish CDA4a production, these results suggest that 3-methyl-2-oxoglutarate is a possible precursor of the (2*S*,3*R*)-3-MeGlu residue in CDAs. Indeed, analysis of the GlmT protein sequence reveals a putative (*S*)-adenosylmethionine (SAM) binding site and similarity to ubiquinone methyltransferases (UbiE), and other typical SAM-dependent methyltransferases. Most SAM-dependent methylation reactions occur with inversion of configuration at the methyl group, which is consistent with the attack of the SAM methyl group by a nucleophilic substrate in an $S_N2$-like mechanism.[22] The similarity between GlmT and a typical SAM-dependent methyltransferase would therefore suggest that α-ketoglutarate is an electronically more plausible substrate. Clearly the formation of a resonance-stabilized nucleophilic enolate or enol form of α-ketoglutarate in the active site of GlmT is entirely feasible (Figure 5A). A mechanistically similar SAM-dependent methylation of the related α-keto acid, indolepyruvate **21**, to give the $\beta$-methyl-α-keto acid **22** is also known to occur during the biosynthesis of indolmycin (Figure 5B).[22b] On the other hand, the direct SAM-dependent $\beta$-methylation of Glu, by the typical mechanism, would require the formation of a highly unstable $\beta$-anion, which is much less likely. There is, however, a group of unusual methyltransferases from the radical SAM superfamily[23a,b] which utilize methylcoblamin[23c] to transfer methyl groups to non-nucleophilic sites, possibly by a radical mechanism.[23d] Despite this, GlmT shows no sequence similarity with and lacks the conserved CxxxCxxC motif common to the proteins in the radical SAM superfamily.[23a,b] Thus, the evidence presented here, combined with simple mechanistic considerations based on early work,[22,23] suggests that GlmT is likely to catalyze the SAM-dependent methylation of α-ketoglutarate to give (3*R*)-methyl-2-oxoglutarate.

There are several ways in which (3*R*)-methyl-2-oxoglutarate might be subsequently transformed to (2*S*,3*R*)-3-MeGlu. First, a PLP-dependent aminotransferase could transfer the amine

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group from an amino acid donor to (3*R*)-methyl-2-oxoglutarate. Apart from the specific 4-hydroxyphenylglycine aminotransferase (HpgT encoded by SCO3227),[24] there are no other genes in the *cda* biosynthetic gene cluster, which encodes proteins that are similar to known aminotransferases. There are, however, numerous PLP-dependent aminotransferases in *S. coelicolor* which are predicted to transfer the amine group from various amino acid donors to α-ketoglutarate to form L-Glu and various α-keto acids [http://streptomyces.org.uk/]. Any number of these enzymes could potentially transaminate (3*R*)-methyl-2-oxoglutarate. Indeed, from the point of view of economy, it is tempting to suggest that such an aminotransferase activity might utilize L-Glu as the amino donor, which would replenish the supply of α-ketoglutarate for subsequent methylation by GlmT (Figure 5A). A similar cyclic pathway is proposed that utilizes tyrosine as the co-substrate of HpgT in the biosynthesis of 4-hydroxyphenylglycine in CDA and other nonribosomal peptide antibiotics.[24] In addition to this, the primary metabolic NADPH-dependent glutamate synthase (SCO1977, SCO2025, SCO2026) and NADP-specific glutamate dehydrogenase (SCO4683) enzymes in *S. coelicolor*,[25] which normally utilize α-ketoglutarate as a substrate in the biosynthesis of L-Glu, might similarly be responsible for producing (2*S*,3*R*)-3-MeGlu from (3*R*)-methyl-2-oxoglutarate.

Finally, while our results point to GlmT functioning as a 2-oxoglutarate methyltransferase, the possibility that GlmT methylates Glu by first forming a Schiff base with a pyridoxal-5′-phosphate (PLP) or a functionally related pyruvoyl cofactor cannot be discounted. Similar to amino acid decarboxylase and amino acid racemase enzymes, formation of such a Schiff base (e.g., **23**, Figure 5C) followed by tautomerization steps would facilitate SAM-dependent *β*-methylation of a nucleophilic enamine **24** by the standard two-electron mechanism. In this event, the fact that synthetic 3-methyl-2-oxoglutarate re-establishes production of CDA containing 3-MeGlu in the Δ*glmT* mutant could be accounted for through the presence of a promiscuous aminotransferase within *S. coelicolor*.

## Conclusions

Under typical fermentation conditions, the wild-type *S. coelicolor* produces a mixture of Glu and 3-MeGlu-containing CDAs, which are difficult to separate. We have shown that, by deleting the putative methyltransferase encoding gene, *glmT*, from the *cda* biosynthetic gene cluster, the resulting mutant Δ*glmT* produces exclusively Glu-containing CDA. We have also developed a stereocontrolled synthesis of (2*S*,3*R*)- and (2*S*,3*S*)-methylglutamic acids, which showed that 3-MeGlu in CDAs and daptomycin possesses the same (2*S*,3*R*)-absolute configuration. Feeding experiments with the Δ*glmT* strain revealed that the A-domain of CdaPS3 module 10, and probably to a lesser extent the adjacent C-domains, is able to discriminate between the synthetic 3-methylglutamates and only the (2*S*,3*R*)-isomer is incorporated into the CDAs. Using the Δ*glmT* mutant strain and supplementing the medium with synthetic (2*S*,3*R*)-3-methylglutamic acid, it is thus possible to control the distribution of Glu- and MeGlu-containing peptides in *S. coelicolor*. Indeed, a similar approach might be used to control the functionality of penultimate residues in daptomycin and A54145. This is important because we have demonstrated that, like daptomycin and A54145, the presence of a 3-MeGlu rather than Glu as the penultimate C-terminal residue of CDA leads to increased antimicrobial activity.

Interestingly, feeding a racemic mixture of all four 3-MeGlu stereoisomers led to the production of a new product, which is predicted to be an *epi*-CDA4a containing an unnatural D-configured 3-MeGlu residue. We have also synthesized racemic 3-methyl-2-oxoglutarate and showed that addition of this compound to the Δ*glmT* mutant re-establishes the biosynthesis of 3-MeGlu-containing CDA4a. From this analysis and earlier mechanistic studies, we suggest that GlmT is likely to be a SAM-dependent 2-oxoglutarate-3-methyltransferase. The product (3*R*)-methyl-2-oxoglutarate is probably transaminated, or otherwise transformed, to (2*S*,3*R*)-3-MeGlu acid prior to activation and incorporation into CDAs by CdaPS3.

Currently, we are using the synthetic methodology along with stereochemical and biosynthetic knowledge generated here in order to develop new methods for engineering lipopeptides of this type. For example, by feeding other synthetic 3-alkyl Glu and 3-alkyl-2-oxoglutarate analogues to Δ*glmT*,[3a] combined with possible active site modification of the corresponding A-domain,[3b] it should be possible to generate new lipopeptides with a variety of modified glutamates at the important penultimate C-terminal position. It is entirely possible that these new lipopetides could have improved activity, but lower toxicity, than the current MeGlu-containing lipopeptides, including daptomycin, which is now widely used in the clinic.

## Experimental Section

**(5*S*)-3,4-Dihydro-5-*tert*-butyldiphenylsilyloxymethyl-*N*-*tert*-butyloxycarbonylpyrrolidin-2-one (2).** LDA (9.98 mL, 7.14 mmol) was added dropwise over 15 min to (5*S*)-*N*-*tert*-butyloxycarbonyl-5-*tert*-butyldiphenylsilyloxymethylpyrrolid-2-one[14] (2 g, 4.41 mmol) in anhydrous THF (25 mL) at −78 °C. The reaction mixture was stirred at −78 °C for 20 min, allowed to warm to 0 °C for ca. 2 min, and then re-cooled to −78 °C. A solution of phenylselenenyl bromide (1.25 g, 5.29 mmol) in anhydrous THF (5 mL) was added rapidly, and stirring was continued at −78 °C. After 1.5 h, the reaction was allowed to warm to room temperature, water (75 mL) was then added, and the mixture was extracted with $CH_2Cl_2$ (3 × 50 mL). The organic extracts were washed with saturated aqueous $NaHCO_3$ (50 mL) and saturated aqueous NaCl (50 mL) and then dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (80:20 hexane/EtOAc) provided α-phenylselenenyl derivative as a white solid (1.74 g, 60% yield). A portion of this (1.1 g, 1.8 mmol) was dissolved in THF (8 mL) with glacial acetic acid (0.23 mL). After cooling to 0 °C, aqueous hydrogen peroxide (0.95 mL, 27% w/v) was added and the reaction stirred for 30 min. Following this, cold saturated aqueous $NaHCO_3$ (80 mL) was added and the resulting mixture was extracted with diethyl ether (3 × 80 mL). The combined organic extracts were washed with saturated aqueous NaCl (80 mL), dried over $MgSO_4$, filtered, concentrated, and purified by flash chromatography (75:25 hexane/EtOAc) to give **2** (0.7 g, 86%) as a pale yellow solid: $R_f$ 0.15 (80:20 hexane/EtOAc); mp 93−95 °C; $[\alpha]_D = -125.6°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.05 (9H, s, $SiC(CH_3)_3$), 1.46 (9H, s, $CO_2C(CH_3)_3$), 3.85 (1H, dd, $J$ = 6.5, 10.0 Hz, $CH_aH_bO$), 4.14 (1H, dd, $J$ = 3.5, 10.0 Hz, $CH_aH_bO$), 4.66−4.70 (1H, m, $CHCH_2O$), 6.20 (1H, dd, $J$ = 2.0, 6.5 Hz, COCHC*H*), 7.30 (1H, dd, $J$ = 2.0, 6.0 Hz, COC*H*CH), 7.39−7.65 (10H, m, Ar−H); $^{13}$C NMR (100.6 MHz,

(24) (a) Hubbard, B. K.; Thomas, M. G.; Walsh, C. T. *Chem. Biol*. **2000**, *7*, 931−942. (b) Chiu, H.-T.; Hubbard, B. K.; Shah, A. N.; Eide, J.; Fredenburg, R. A.; Walsh, C. T.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8548−8553. (c) Choroba, O. W.; Williams, D. H.; Spencer, J. B. *J. Am. Chem. Soc.* **2000**, *122*, 5389−5390.

(25) Fisher, S. H. *J. Bacteriol*. **1989**, *171*, 2372−2377.

$CDCl_3$) $\delta$ 19.7 (Si*C*$(CH_3)_3$), 27.1 (SiC(*C*$H_3)_3$), 28.4 (OC(*C*$H_3)_3$), 63.4 (CH*C*$H_2$O), 63.9 (C*H*$CH_2$O), 83.3 (O*C*$(CH_3)_3$), 127.8 (CO*C*HCH), 128.2 (Ar−C3/5), 130.4 (Ar−C4), 133.3 (Ar−C1), 135.9 (Ar−C2/6), 149.6 (*C*$O_2$CH), 149.8 (COCH*C*H), 170.0 (*C*$O_2$C$(CH_3)_3$); LRMS-ESI (*m*/*z*): 925 ([2M + Na]$^+$, 100%), 352 ([M − $C_5H_7O_2$]$^+$, 60%), 475 ([M + Na]$^+$, 10%). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calcd for $C_{26}H_{33}NO_4SiNa$, 474.2077. Found, 474.2060. Anal. Calcd for $C_{26}H_{33}NO_4Si$: C, 69.14; H, 7.36; N, 3.10. Found: C, 69.1; H, 7.5; N, 3.0. IR (neat): $\nu$ 1778 (C═O), 1704 (C═O) cm$^{-1}$.

**(4*S*,5*S*)-5-*tert*-Butyldiphenylsilyloxymethyl-*N*-*tert*-butyloxycarbonyl-4-methylpyrrolidin-2-one (3).**[14b] Methyllithium (2.13 mL, 3.41 mmol, 1.6 M solution in diethyl ether) was added to a suspension of Cu(I)I (0.35 g, 1.86 mmol) in anhydrous toluene (20 mL) under $N_2$ at −78 °C and stirred for 30 min. This mixture was allowed to warm to −40 °C with stirring for 30 min and then cooled to −78 °C, resulting in a solution of $(CH_3)_2$CuLi. α,β-Unsaturated lactam **2** (0.70 g, 1.55 mmol) in anhydrous toluene (10 mL) was added to the cuprate solution at −78 °C under $N_2$. The reaction mixture was allowed to warm to −40 °C and stirred for 15 min. Saturated aqueous $NH_4Cl$ (50 mL) was added, and the mixture was extracted with diethyl ether (3 × 50 mL). The combined organic extracts were repeatedly washed with $H_2O$ (until no blue color persisted), dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (100:20 hexane/EtOAc) provided **3** (0.45 g, 62%) as a white solid: $R_f$ 0.40 (70:30 hexane/EtOAc); mp 70−72 °C, $[\alpha]_D = -23.0°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.96 (9H, s, SiC(C*H*$_3)_3$), 1.08 (3H, d, $J$ = 7.0 Hz, CHC*H*$_3$), 1.36 (9H, s, OC(C*H*$_3)_3$), 2.01 (1H, dd, $J$ = 2.0, 17.0 Hz, CO*H*$_a$H$_b$CH), 2.32−2.42 (1H, m, $CH_2$C*H*$CH_3$), 2.91 (1H, dd, $J$ = 8.5, 17.0 Hz, COCH$_a$*H*$_b$CH), 3.66 (1H, m, C*H*$CH_2$O), 3.67 (1H, dd, $J$ = 2.0, 13.0 Hz, CHC*H*$_a$H$_b$O), 3.78 (1H, dd, $J$ = 3.0, 12.0 Hz, CHCH$_a$*H*$_b$O), 7.25−7.37 (10H, m, Ar−H); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 19.5 (Si*C*$(CH_3)_3$), 21.8 (CH*C*$H_3$), 27.2 (SiC(*C*$H_3)_3$), 28.4 (OC-(*C*$H_3)_3$), 28.6 ($CH_2$*C*H$CH_3$), 40.8 (CO*C*$H_2$), 64.8 (CH*C*$H_2$O), 66.6 (*C*H$CH_2$O), 83.1 (O*C*$(CH_3)_3$), 128.2 (Ar−C3/5), 130.2 (Ar−C4), 133.1 (Ar−C1), 133.5 (Ar−C1), 135.9 (Ar−C2/6), 150.4 (N*C*$O_2$), 174.8 (*C*O$CH_2$). LRMS-ESI (*m*/*z*): 957.6 ([2M + Na]$^+$, 100%), 490.2 ([M + Na]$^+$, 9%), 368.2 ([M − $C_5H_7O_2$]$^+$, 60%). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calcd for $C_{27}H_{37}NO_4SiNa$, 490.2390. Found, 490.2390. Anal. Calcd for $C_{27}H_{37}NO_4Si$: C, 69.34; H, 7.97; N, 2.99. Found: C, 69.5; H, 7.9; N, 2.7.

**(4*S*,5*S*)-*N*-*tert*-Butyloxycarbonyl-5-hydroxymethyl-4-methylpyrrolidin-2-one (4).** A solution of tetrabutylammonium fluoride in THF (36 mL, 1.0 M, 35.78 mmol) was added to lactam **3** (2.0 g, 4.28 mmol) in a mixture of THF (2 mL) and 20% aqueous $CH_3CO_2H$ (2.8 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 24 h. EtOAc (50 mL) and aqueous $NH_4Cl$ (50 mL, 20% w/v) were added, and the organic layer was then extracted, dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (50:50 hexane/EtOAc) gave **4** (0.66 g, 67%) as a white solid: $R_f$ 0.19 (50:50 hexane/EtOAc); $[\alpha]_D = -46.2°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.18 (3H, d, $J$ = 7.0 Hz, CHC*H*$_3$), 1.56 (9H, s, OC(C*H*$_3)_3$), 2.05 (1H, dd, $J$ = 3.5, 17.5 Hz, C*H*$_a$H$_b$CHCH$_3$), 2.23−2.28 (1H, m, $CH_2$C*H*$CH_3$), 2.79−2.88 (1H, dd, $J$ = 8.5, 17.5 Hz, CH$_a$*H*$_b$CHCH$_3$), 3.73−3.88 (3H, m, C*H*C*H*$_2$OH); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 20.9 (*C*$H_3$), 28.4 (C(*C*$H_3)_3$), 28.6 (*C*H$CH_3$), 40.4 ($CH_2$*C*H$CH_3$), 64.6 (CH*C*$H_2$OH), 67.2 (*C*H$CH_2$OH), 83.9 (*C*$(CH_3)_3$), 151.6 (N*C*$O_2$), 174.3 (*C*OCH$_2$). LRMS-ESI (*m*/*z*): 481.2 ([2M + Na]$^+$, 100%), 252 ([M + Na]$^+$, 2%). HRMS-ESI (*m*/*z*): [2M + Na]$^+$ calcd for $C_{22}H_{38}N_2O_8Na$, 481.2526. Found, 481.2520.

**(2*S*,3*S*)-*N*-*tert*-Butyloxycarbonyl-3-methylpyroglutamic Acid (5).** $RuCl_3$ (1 mg, 0.005 mmol) was added to a solution of lactam **4** (50.0 mg, 0.22 mmol) in a mixture of $CH_3CN/CCl_4/H_2O$ (2:2:3, 1.5 mL) with $NaIO_4$ (0.146 g, 0.64 mmol). The reaction mixture was stirred for 3 h at room temperature. Saturated aqueous NaCl was added (10 mL), and the mixture was extracted with $CH_2Cl_2$ (3 × 20 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (20:80 hexane/EtOAc with 0.1% $HCO_2H$) gave **5** (49.5 mg, 93%) as a white solid: mp 154−156 °C; $[\alpha]_D = -8.6°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.21 (3H, d, $J$ = 6.5 Hz, CHC*H*$_3$), 1.43 (9H, s, C(C*H*$_3)_3$), 2.12 (1H, dd, $J$ = 4.5, 17.0 Hz, C*H*$_a$H$_b$CHCH$_3$), 2.37−2.47 (1H, m, $CH_2$C*H*$CH_3$), 2.75 (1H, dd, $J$ = 8.5, 17.0 Hz, CH$_a$*H*$_b$CHCH$_3$), 4.15 (1H, d, $J$ = 3.5 Hz, C*H*$CH_2$OH); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 20.8 (*C*$H_3$), 28.2 (C(*C*$H_3)_3$), 30.1 (*C*HCH$_3$), 39.8 (*C*$H_2$CHCH$_3$), 66.0 (*C*H$CO_2$H), 84.4 (*C*$(CH_3)_3$), 149.8 (N*C*$O_2$), 173.0 (*C*O$CH_2$), 176.5 (CH*C*$O_2$H). LRMS-ESI (*m*/*z*): 509.2 ([2M + Na]$^+$, 100%), 266.1 ([M + Na]$^+$, 4%). HRMS-ESI (*m*/*z*): [2M + Na]$^+$ calcd for $C_{22}H_{34}N_2O_{10}$-Na, 509.2111. Found, 509.2104.

**(2*S*,3*S*)-3-Methylglutamic Acid Hydrochloride Salt (7).** LiOH (23.6 mg, 0.56 mmol) in $H_2O$ (0.5 mL) was added to lactam **5** (45.6 mg, 0.19 mmol) in THF (2 mL). The mixture was stirred for 18 h at room temperature and evaporated under reduced pressure. $H_2O$ (5 mL) was added to the residue, and the solution was adjusted to pH 4.0 with $CH_3COOH$ and extracted with diethyl ether (3 × 50 mL). The combined ethereal extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give *N*-Boc-MeGlu **6** (0.041 g, 84%). A fraction of **6** (26.0 mg, 0.010 mmol) was treated with a solution of 4 M HCl in 1,4-dioxane (2 mL), and the mixture was stirred at room temperature under $N_2$ for 18 h. The reaction mixture was then evaporated under reduced pressure, $H_2O$ (10 mL) was added, and the mixture was washed with $CH_2Cl_2$ (3 × 10 mL). The aqueous extract was evaporated under reduced pressure, and the resulting solid was triturated with toluene to give (2*S*,3*S*)-3-MeGlu·HCl **7** (15.2 mg, 94%) as a white hygroscopic solid: $[\alpha]_D = +36.7°$ ($c$ = 1.05, 6 N HCl) lit.[13a,c] = +36.8 ($c$ = 1.03, 6 N HCl); $^1$H NMR (400 MHz, $D_2O$) $\delta$ 0.78 (3H, d, $J$ = 6.5 Hz, C*H*$_3$), 2.23 (1H, dd, $J$ = 7.5, 15.0 Hz, C*H*$_a$H$_b$CO$_2$H), 2.29−2.35 (1H, m, C*H*CH$_3$), 2.41 (1H, dd, $J$ = 5.8, 15.0 Hz, CH$_a$*H*$_b$CO$_2$H), 3.82 (1H, d, $J$ = 2.6 Hz, C*H*$NH_2$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 14.4 (*C*$H_3$), 31.1 (*C*HCH$_3$), 37.5 (*C*$H_2CO_2H$), 57.0 (*C*H$NH_2$), 171.4 ($CH_2$*C*O$_2$H), 176.3 (CH*C*$O_2$H). LRMS-ESI (*m*/*z*): 162.0 ([M + H]$^+$, 100%), 323 ([2M + H]$^+$, 4%). HRMS-ESI (*m*/*z*): [M + H]$^+$ calcd for $C_6H_{12}NO_4$, 162.0766. Found, 162.0768.

**(3*R*,4*R*,5*S*)-5-*tert*-Butyldiphenylsilyloxymethyl-*N*-*tert*-butyloxycarbonyl-3,4-pyrazolinepyrrolidin-2-one (8).** An ethereal solution of diazomethane (ca. 18.7 mmol in 10 mL) was added to the α,β-unsaturated lactam **2** (0.60 g, 1.33 mmol), and the mixture was left to stir at room temperature for 72 h. The solvent was then evaporated under reduced pressure, and the residue was purified by flash chromatography (80:20 hexane/EtOAc) to give **8** (0.45 g, 68%) as a pale yellow solid: $R_f$ 0.21 (80:20 hexane/EtOAc); mp 53−55 °C; $[\alpha]_D = -208.1°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.09 (9H, s, SiC(C*H*$_3)_3$), 1.47 (9H, s, OC(C*H*$_3)_3$), 2.58−2.64 (1H, m, COCHC*H*), 3.67 (1H, dd, $J$ = 2.0, 10.5 Hz, CHC*H*$_a$H$_b$), 3.95 (1H, m, C*H*$CH_2$O), 3.98 (1H, dd, $J$ = 2.5, 10.5 Hz, CHCH$_a$*H*$_b$), 4.49 (1H, ddd, $J$ = 2.5, 5.5, 18.5 Hz, NNCH$_a$*H*$_b$), 5.16 (1H, dd, $J$ = 10.0, 18.5 Hz, NNC*H*$_a$H$_b$), 5.87 (1H, dd, $J$ = 2.5, 9.0 Hz, COC*H*CH), 7.39−7.65 (10H, m, Ar−H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 19.6 (Si*C*$(CH_3)_3$), 27.2 (SiC-(*C*$H_3)_3$), 28.3 ($CO_2$C(*C*$H_3)_3$), 31.1 (COCH*C*H), 63.4 (*C*HCH$_2$O), 64.9 (CH*C*$H_2$O), 83.3 ($CO_2$*C*$(CH_3)_3$), 86.9 (NN*C*$H_2$), 96.7 (CO*C*HCH), 128.4 (Ar−C3/5), 130.5 (Ar−C/4), 132.5 (Ar−C1), 133.0 (Ar−C1), 135.9 (Ar−C2/6), 149.8 (*C*OCHCH), 165.0 (*C*$O_2$C$(CH_3)_3$). LRMS-ESI (*m*/*z*): 516.0 ([M + Na]$^+$, 100%). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calcd for $C_{27}H_{35}N_3O_4SiNa$, 516.2295. Found, 516.2280. Anal. Calcd for $C_{27}H_{35}N_3O_4Si$: C, 65.69; H, 7.15; N, 8.51. Found: C, 65.8; H, 7.3; N, 8.2. IR (neat): $\nu$ 1783 (C═O), 1712 (C═O) cm$^{-1}$.

**(5*S*)-3,4-Dihydro-5-*tert*-butyldiphenylsilyloxymethyl-*N*-*tert*-butyloxycarbonyl-4-methylpyrrolidin-2-one (9).** Pyrazoline **8** (0.40 g, 0.81 mmol) was heated, under solvent-free conditions, at 120 °C for 3 h. The residue was then purified by flash chromatography (80:20 hexane/EtOAc) to give **9** (0.16 g, 42%) as a white solid: $R_f$ 0.29 (80:20 hexane/EtOAc); mp 114−117 °C; $[\alpha]_D = -80.1°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.01 (9H, s, SiC(C*H*$_3)_3$), 1.46 (9H, s, OC(C*H*$_3)_3$),

2.04 (3H, s, CHCC$H_3$), 3.92 (1H, dd, $J$ = 2.0, 11.0 Hz, CHC$H_a$H$_b$O), 4.23 (1H, dd, $J$ = 3.5, 11.0 Hz, CHCH$_a$$H_b$O), 4.42 (1H, br, C$H$CH$_2$O), 5.94 (1H, br, C$H$CCH$_3$), 7.38−7.64 (10H, m, Ar−H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 15.5 (CHC$C$H$_3$), 19.9 (Si$C$(CH$_3$)$_3$), 27.3 (SiC($C$H$_3$)$_3$), 28.7 (OC($C$H$_3$)$_3$), 61.3 (CH$C$H$_2$O), 66.5 ($C$HCH$_2$O), 84.0 (O$C$(CH$_3$)$_3$), 124.1 ($C$HCCH$_3$), 128.5 (Ar−C3/5), 130.5 (Ar−C4), 133.3 (Ar−C1), 133.7 (Ar−C1), 136.2 (Ar−C2/6), 149.9 (CH$C$CH$_3$), 161.1 (N$C$O$_2$), 170.4 ($C$OCHC). LRMS-ESI ($m/z$): 953.0 ([2M + Na]$^+$, 100%), 488 ([M + Na]$^+$, 80%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_{27}H_{35}N_3O_4SiNa$, 488.2233. Found, 488.2216. Anal. Calcd for $C_{27}H_{35}NO_4Si$: C, 69.94; H, 7.58; N, 3.01. Found: C, 69.7; H, 7.6; N, 2.9. IR (neat): $\nu$ 1781 (C═O), 1708 (C═O) cm$^{-1}$.

**(4*R*,5*S*)-5-*tert*-Butyldiphenylsilyloxymethyl-*N*-*tert*-butyloxycarbonyl-4-methylpyrrolidin-2-one (10).** Platinum(IV) oxide (40 mg) was added to a solution of α,β-unsaturated lactam **9** (0.36 g, 0.77 mmol) in EtOAc (30 mL). The reaction mixture was stirred at room temperature under an atmosphere of $H_2$ for 18 h, filtered through Celite, and then evaporated under reduced pressure and purified by flash chromatography (1:1 hexane/EtOAc) to give **10** (0.28 g, 80%) as a white solid: $R_f$ 0.29 (80:20 hexane/EtOAc); mp 126−128 °C; $[\alpha]_D = -25.3°$ ($c$ = 1.0, $CHCl_3$); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 0.96 (9H, s, SiC(C$H_3$)$_3$), 0.18 (3H, dd, $J$ = 5.5, CHC$H_3$), 1.33 (9H, s, OC(C$H_3$)$_3$), 2.40−2.53 (2H, m, C$H_a$H$_b$CHCH$_3$ and CH$_a$H$_b$C$H$CH$_3$), 2.64 (1H, dd, $J$ = 11.0, 15.0 Hz, CH$_a$$H_b$CHCH$_3$), 3.68 (1H, dd, $J$ = 2.5, 12.0 Hz, CHC$H_a$H$_b$O), 3.90−3.92 (2H, m, CHCH$_a$$H_b$O and C$H$CH$_2$O), 7.28−7.60 (10H, m, Ar−H); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.6 (CH$C$H$_3$), 19.4 (Si$C$(CH$_3$)$_3$), 27.2 (SiC($C$H$_3$)$_3$), 28.4 (CO$_2$C($C$H$_3$)$_3$), 30.5 ($C$HCH$_3$), 41.2 ($C$H$_2$CHCH$_3$), 62.4 (CH$C$H$_2$O), 62.4 ($C$HCH$_2$O), 83.8 (O$C$(CH$_3$)$_3$), 128.2 (Ar−C3/5), 130.2 (Ar−C4), 132.7 (Ar−C1), 133.3 (Ar−C1), 136.0 (Ar−C2/6), 150.1 (N$C$O$_2$), 175.3 ($C$OCH$_2$). LRMS-ESI ($m/z$): 957 ([2M + Na]$^+$, 100%), 490 ([M + Na]$^+$, 15%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_{27}H_{37}NO_4SiNa$, 490.2390. Found, 490.2393. Anal. Calcd for $C_{27}H_{37}NO_4Si$: C, 69.34; H, 7.97; N, 2.99. Found: C, 69.5; H, 8.2; N, 3.1. IR (neat): $\nu$ 1784 (C═O), 1710 (C═O) cm$^{-1}$.

**(4*R*,5*S*)-*N*-*tert*-Butyloxycarbonyl-5-hydroxymethyl-4-methylpyrrolidin-2-one (11).** Lactam **10** (100 mg, 0.22 mmol) was deprotected with tetrabutylammonium fluoride as described above (**3** → **4**). Purification by flash chromatography (35:65 hexane/EtOAc) gave **11** (37 mg, 73% yield) as a white solid: $R_f$ 0.17 (50:50 hexane/EtOAc); mp 120−122 °C; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.21 (3H, dd, $J$ = 6.5 Hz, CHC$H_3$), 1.54 (9H, s, C(C$H_3$)$_3$), 2.40−2.61 (3H, m, C$H_2$C$H$CH$_3$), 3.80 (1H, dd, $J$ = 2.5, 11.5 Hz, CHC$H_a$H$_b$OH), 4.02−4.07 (2H, m, C$H$CH$_a$$H_b$OH); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 14.7 (CH$C$H$_3$), 28.5 (C($C$H$_3$)$_3$), 30.4 ($C$HCH$_3$), 40.9 ($C$H$_2$CHCH$_3$), 61.4 (CH$C$H$_2$OH), 62.8 ($C$HCH$_2$OH), 83.5 ($C$(CH$_3$)$_3$), 150.9 (N$C$O$_2$), 175.4 ($C$OCH$_2$). LRMS-ESI ($m/z$): 481 ([2M + Na]$^+$, 100%), 252 ([M + Na]$^+$, 25%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_{11}H_{19}NO_4Na$, 252.1212. Found, 252.1217. IR (neat): $\nu$ 3425 (OH), 1774 (C═O) cm$^{-1}$.

**(2*S*,3*R*)-*N*-*tert*-Butyloxycarbonyl-3-methylpyroglutamic Acid (12).** Alcohol **11** (100 mg, 0.22 mmol) was oxidized with $RuO_4$, and the product was purified as described above (**4** → **5**), resulting in **12** (37 mg, 70% yield) as a white solid: $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.16 (3H, d, $J$ = 6.5 Hz, CHC$H_3$), 1.49 (9H, s, C(C$H_3$)$_3$), 2.38 (1H, dd, $J$ = 11.5, 16.5 Hz, C$H_a$H$_b$CHCH$_3$), 2.56−2.78 (2H, m, CH$_a$$H_b$C$H$CH$_3$), 4.58 (1H, d, $J$ = 8.5 Hz, C$H$CO$_2$H); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 14.1 (CH$C$H$_3$), 26.8 (C($C$H$_3$)$_3$), 27.4 ($C$HCH$_3$), 37.9 ($C$H$_2$CHCH$_3$), 62.3 ($C$HCO$_2$H), 83.0 ($C$(CH$_3$)$_3$), 148.2 (N$C$O$_2$), 172.4 ($C$OCH$_2$), 174.2 (CHC$O_2$H). LRMS-ESI ($m/z$): 509 ([2M + Na]$^+$, 100%), 282 ([M + K]$^+$, 20%), 266 ([M + Na]$^+$, 5%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_{11}H_{17}NO_5Na$, 266.1004. Found, 266.0993. IR (neat): $\nu$ 3215 (OH), 1783 (C═O) cm$^{-1}$.

**(2*S*,3*R*)-*N*-*tert*-Butoxycarbonyl-3-methylglutamic Acid (13).** The lactam **12** (29 mg, 0.12 mmol) was hydrolyzed with LiOH as described above (**5** → **6**) to give **13** (21.5 mg, 69%) as a white solid: $^1$H NMR (300 MHz, $D_2O$) $\delta$ 0.77 (3H, d, $J$ = 7.0 Hz, CHC$H_3$), 1.28 (9H, s, C(C$H_3$)$_3$), 2.16−2.49 (3H, m, C$H_2$CH), 4.15 (1H, d, $J$ = 4.0 Hz, C$H$NH); $^{13}$C NMR (100.6 MHz, $D_2O$) $\delta$ 14.3 (CH$C$H$_3$), 27.8 (C($C$H$_3$)$_3$), 31.9 ($C$HCH$_3$), 37.9 ($C$H$_2$CH), 57.1 ($C$HNH), 81.7 ($C$(CH$_3$)$_3$), 158.2 (NH$C$O), 176.0 and 177.0 (both $C$O$_2$H). LRMS-ESI ($m/z$): 284 ([M + Na]$^+$,100%), 545 ([2M + Na]$^+$, 40%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_{11}H_{19}NO_6Na$, 284.1110. Found, 284.1119. IR (neat): $\nu$ 3321 (OH), 1708 (C═O) cm$^{-1}$.

**(2*S*,3*R*)-3-Methylglutamic Acid Hydrochloride Salt (14).** Acidolysis of **13** (20 mg, 0.076 mmol) as described above (**6** → **7**) gave (2*S*,3*R*)-3-MeGlu·HCl **14** (9.3 mg, 75%) as a hygroscopic white solid: $[\alpha]_D = +22.1°$ ($c$ = 1.01, 6 N HCl) lit.[13a,c] = +22.6 ($c$ = 1.03, 6 N HCl); $^1$H NMR (400 MHz, $D_2O$) $\delta$ 0.93 (3H, d, $J$ = 6.8 Hz, $CH_3$), 2.30−2.35 (1H, m, C$H_a$H$_b$CH), 2.46−2.57 (2H, m, CH$_a$$H_b$C$H$), 3.90 (1H, d, $J$ = 4.2 Hz, C$H$CO$_2$H); $^{13}$C NMR (100.6 MHz, $D_2O$) 14.9 ($CH_3$) 31.0 ($CH_2CH$), 37.5 ($CH_2$COOH), 58.0 (C$H$N) 172.3 ($CH_2CO_2$H), 176.3 (CH$CO_2$H). LRMS-ESI ($m/z$): 162.1 ([M − Cl]$^+$, 38%), 184.1 ([M − HCl + Na]$^+$, 18%). HRMS-ESI ($m/z$): [M − Cl]$^+$ calcd for $C_6H_{12}NO_4$, 162.0761. Found, 162.0760. IR (neat): $\nu$ 2937 (OH), 1718 (C═O) cm$^{-1}$.

**3-Methyl-2-nitropentanedioic Acid Dimethyl Ester (17).** 1,8-Diazabicyclo[5.4.0]undeca-7-ene (DBU) (1.25 mL, 8.39 mmol) was added to a mixture of methyl crotonate **15** (6.23 mL, 58.78 mmol) and methyl nitroacetate **16** (1.0 g, 8.39 mmol) in anhydrous THF (10 mL) under $N_2$ at room temperature and stirred in the absence of light for 72 h. Aqueous HCl (1.2 M, 100 mL) was then added, and the mixture was extracted with diethyl ether (3 × 50 mL), dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (90:10 toluene/EtOAc) gave **17** (1.4 g, 76%) as a colorless oil: $R_f$ 0.13 (toluene); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.09 (3H, d, $J$ = 6.5 Hz, CHC$H_3$), 2.40−2.50 (2H, m, C$H_2$CO$_2$CH$_3$), 2.85−2.98 (1H, m, C$H$CH$_3$), 3.63 (3H, s, CH$_2$CO$_2$C$H_3$), 3.77 (3H, s, CHCO$_2$C$H_3$), 5.24 (1H, dd, $J$ = 6.5, 15.0 Hz, C$H$NO$_2$); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$ 16.4 (CH$C$H$_3$), 31.8 ($C$HCH$_3$), 37.0 (CHCH$_2$CO$_2$CH$_3$), 52.2 (CHCO$_2$$C$H$_3$), 53.8 (CH$_2$CO$_2$$C$H$_3$), 91.0 ($C$HNO$_2$), 164.4 (CH$_2$$C$O$_2$), 171.9 (CH$C$O$_2$). LRMS-ESI ($m/z$): 242.1 ([M + Na]$^+$, 100%). HRMS-ESI ($m/z$): [M + Na]$^+$ calcd for $C_8H_{13}NO_6Na$, 242.0641. Found, 242.0648.

**3-Methyl-2-oxopentanedioic Acid Dimethyl Ester (19).** 2-Nitropentanedioate derivative **17** (1.0 g, 4.28 mmol) was dissolved in methanol (8.57 mL) and treated with $NaOCH_3$ in $CH_3OH$ (0.77 mL, 4.29 mmol, 30% w/v) under $N_2$ at room temperature with stirring. A solution of ammonium acetate (7.93 g, 12.8 mmol) in $H_2O$ (15 mL) was then added to the Ti(III)Cl in 2 M aqueous HCl (8.82 mL, 17.15 mmol, 30% w/v). The resulting $TiCl_3$−$NH_4CH_3CO_2$ solution was then added in one portion to the reaction mixture containing the anion **17**. After stirring for 1.5 h, the reaction mixture was extracted with diethyl ether (4 × 50 mL), and the combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (10:1 toluene/EtOAc) gave the ketone **19** (0.23 g, 26%) and the corresponding oxime **18** (0.35 g, 38%) as oils. The oxime **18** (0.23 g, 1.13 mmol) was then dissolved in methanol (15 mL) with concentrated HCl (5 mL). Aqueous HCOH (10 mL, 37% w/v) was then added, and the mixture was stirred at room temperature for 18 h. $H_2O$ (100 mL) was then added, and the mixture was extracted with diethyl ether (3 × 100 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (10:1 toluene/EtOAc) gave the further ketone **19** (total 410 mg, 51%): $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.22 (3H, d, $J$ = 7.5 Hz, $CH_3$), 2.50−2.56 (1H, dd, $J$ = 5, 16.5 Hz, C$H_a$H$_b$CO$_2$CH$_3$), 2.83−2.89 (1H, dd, $J$ = 9, 16.5 Hz, CHa$H_b$CO$_2$CH$_3$), 3.68−3.7 (1H, m, C$H$CH$_3$), 3.68 (3H, s, CO$_2$C$H_3$), 3.92 (3H, s, COCO$_2$C$H_3$); $^{13}$C NMR (100.6 MHz, $CDCl_3$) $\delta$ 16.3 ($CH_3$), 37.2 ($CH_2$), 38.6 ($C$HCH$_3$), 52.4 (CH$_2$CO$_2$$C$H$_3$), 53.4 (COCO$_2$$C$H$_3$), 161.6 (CH$_2$$C$O$_2$-CH$_3$), 172.6 (CO$C$O$_2$CH$_3$), 196.3 ($C$OCO$_2$CH$_3$). LRMS-EI/CI ($m/z$): 206 ([M + NH$_4$]$^+$, 100%). HRMS-ESI ($m/z$): [M + NH$_4$]$^+$ calcd for $C_8H_{16}NO_5$, 206.1023. Found, 206.1024. IR (neat): $\nu$ 1729 (C═O), 1801 (C═O) cm$^{-1}$.

**(±)-3-Methyl-2-oxoglutarate Disodium Salt (20).** The ketone **19** (100 mg 0.463 mmol) in 1,4-dioxane (5 mL) was treated with aqueous NaOH solution (5 mL, 0.28 M, 1.40 mmol) at room temperature and stirred for 18 h. The mixture was then evaporated under reduced pressure and concentrated in vacuo to provide **20** (72 mg, 77%) as a white solid: $^1$H NMR (400 MHz, $D_2O$) $\delta$ 1.02 (3H, d, $J$ = 7.0 Hz, C$H_3$), 2.06 (1H, dd, $J$ = 8.5, 15.0 Hz, C$H_a$H$_b$CH), 2.45−2.49 (1H, dd, $J$ = 6.0, 15.0 Hz, CH$_a$$H_b$CH), 3.18−3.20 (1H, m, C$H$CH$_3$); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$ 14.8 (*C*H$_3$), 39.6 (*C*H$_2$), 40.3 (*C*H), 171.5 (CH$_2$*C*O$_2$), 181.0 (CO*C*O$_2$), 209.3 (*C*OCO$_2$). LRMS-ESI$^-$ (*m*/*z*): 159.0 ([M − 2Na + H]$^-$, 100%). HRMS-ESI$^-$ (*m*/*z*): [M − 2Na + H]$^-$ calcd for $C_6H_7O_5$, 159.0299. Found, 159.0300.

**Production and Purification of CDA4a and CDA3a:** Sterile SV2[3a] liquid media (5 L) was inoculated with agar plugs of *S. coelicolor* MT1110 from a 3 day R5 plate.[26] The cultures were then incubated at 28 °C and 180 rpm for 5 days. The cells were harvested by centrifugation. The resulting supernatants were acidified to pH 2.0 by the addition of aqueous HCl (0.5 M), and the crude CDA was extracted using Varian C-18 Bond Elute SPE cartridges (2 g). The cartridges were washed with $H_2O$ (120 mL), eluted with $CH_3OH$ (60 mL), and the eluant was evaporated under reduced pressure. Purification of the crude extracts was achieved by repeated preparative and semipreparative reversed phase HPLC: Phenomenex C-18 10 $\mu$m, 250 × 21.2 mm, and 5 $\mu$m, 250 × 10 mm columns; solvent A was $H_2O$ with 0.1% $HCO_2H$, and solvent B was acetonitrile with 0.1% $HCO_2H$. The flow rate was 20 and 5 mL min$^{-1}$, respectively, with a starting gradient of 0% B and 100% A, increasing to 100% B over 30 min and then held for a further 5 min. Separate fractions for CDA3a and CDA4a (retention time = ca. 16.0 and 16.2 min, respectively) were pooled and evaporated under reduced pressure to give pure CDA4a (11 mg L$^{-1}$) and CDA3a (2 mg L$^{-1}$), which were identical to samples isolated and characterized previously.[3a,9]

**Hydrolysis, Derivatization, and LC-MS Analysis of 3-MeGlu from Daptomycin and CDA4a.** Daptomycin or CDA4a (10 mg) was heated at reflux in 6 M aqueous HCl (40 mL) under oxygen-free conditions for 24 h. The aqueous solution was extracted with ethyl acetate (20 mL) and lyophilized under reduced pressure. Dabsyl chloride (15 mM) in acetone (400 $\mu$L) was added to a solution of the amino acid hydrolysate in aqueous $NaHCO_3$ (400 $\mu$L, 0.15 M, pH 9.0).[17] The mixture was heated at 70 °C for 20 min and diluted with EtOH (total 2 mL), centrifuged at 5000 *g* for 5 min, and the supernatant analyzed by LC-MS. Synthetic (2*S*,3*R*)- and (2*S*,3*S*)-3-MeGlu standards (1−2 mg) were similarly dabsylated and analyzed.

LC-MS analysis was carried out on a Micromass LCT orthogonal acceleration time-of-flight mass spectrometer, equipped with an electrospray ionization source run in positive mode (scanning from 100 to 1000 *m*/*z*) combined with a Waters 2790 separation module. Gradient elution was carried out using a reversed phase C-18 column, 3 $\mu$m 150 × 4.6 mm (Phenomenex). Solvent A was $H_2O$ with 1% acetonitrile and 0.1% $HCO_2H$, and solvent B was acetonitrile with 1% $H_2O$ and 0.1% $HCO_2H$. The flow rate was 1 mL min$^{-1}$ with a gradient of 20% B and 80% A, increasing to 70% B over 10 min and then increasing to 100% B over the next minute and then held for a further 4 min.

**Deletion of *glmT* from *S. coelicolor* MT1110.** A standard "double cross over" gene replacement strategy[3a] was used to delete *glmT* (SCO3215) from the chromosome of the *S. coelicolor* MT1110 wild-type strain. A delivery plasmid was generated by cloning into *E. coli* plasmid pMT3000, using host strain *E. coli* XL1 BLUE, two fragments A and B that flank the chromosomal sequence to be deleted. The upstream fragment A was generated by PCR using StE8 cosmid DNA[27] as the template with the forward primer (5′-AAGAATTCTCGATCACCGCGACCCGTGGC, encompassing the nucleotide coordinates 3523243 to 3523263 and an *Eco*RI site underlined) and reverse primer (5′-AATCTAGATCACGGTGGGTCCGCTCGTCT encompassing the nucleotide coordinates 3525205 to 3525224 and a *Xba*I site). The downstream fragment B was similarly generated using the same cosmid as the template with the forward primer (5′-AATCTAGATCACGGTGGGTCCGCTCGTCT, encompassing the nucleotide coordinates 3525840 to 3525860 and a *Xba*I site) and reverse primer (5′-AAGAAGCTTCAGCATCGTGTCCGGTGCCCC, encompassing the nucleotide coordinates 3527324 to 3527344 and a *Hin*DIII site). The cloned deletion fragment A+B was extracted from the plasmid by restriction digestion, with *Bgl*II, purified, and then used in a ligation reaction with *Bam*HI restriction digested pMAH vector[18] (via a *Bam*HI:*Bgl*II ligation). The ligation mix was in turn used to transform *E. coli* XL1 BLUE competent cells from which the pMAH vector containing the deletion construct was re-isolated and similarly used to transform *E. coli* strain ET12567. The resulting nonmethylated plasmid was then used to transform protoplasts of *S. coelicolor* MT1110. Spores were prepared from hygromycin-resistant colonies, and Hyg-sensitive colonies (putative deletants) were identified following two rounds of nonselective subculturing. Total DNA extraction[26] followed by PCR analysis confirmed the loss of ca. 615 bp from within *glmT* (see Supporting Information).

**Recomplementation of *S. coelicolor* Δ*glmT* with 3-Methylglutamates and 3-Methyl-2-oxoglutarate.** Small-scale liquid feeding experiments were carried out in SV2[3a] media (7 mL) inoculated with one 5 mm plug from a 3 day R5 agar plate of Δ*glmT*. The cultures were then incubated at 28 °C, 180 rpm, for 2 days. Filter-sterilized aqueous solutions of substrates **7**, **14**, and *rac*-**7**/**14** were then added to give final concentrations of 50, 100, 200, and 300 $\mu$g mL$^{-1}$ (of each stereoisomer). The substrate 3-methyl-2-oxoglutarate **20** was similarly fed at the following final concentrations of 0.5, 1.06, 1.37, and 2.25 mg mL$^{-1}$. After a total of 6 days fermentation, the cultures were harvested by centrifugation, and the supernatants were adjusted to pH 2.0, extracted on Varian C-18 Bond Elute SPE cartridges (0.5 g), eluted with MeOH (2 mL), and evaporated in vacuo. Extracts were then analyzed by LC-MS (as above except scanning from 700 to 1700 *m*/*z*).

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**Supporting Information Available:** LC-MS data from feeding synthetic precursors along with further results and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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