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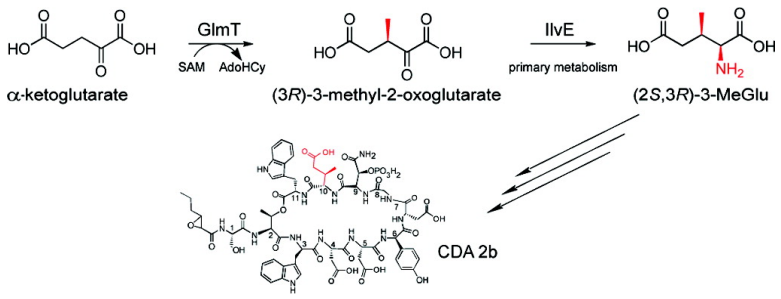

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Stereospecific Enzymatic Transformation of α-Ketoglutarate to (2*S*,3*R*)-3-Methyl Glutamate during Acidic Lipopeptide Biosynthesis

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Abstract: The acidic lipopeptides, including the calcium-dependent antibiotics (CDA), daptomycin, and A54145, are important macrocyclic peptide natural products produced by *Streptomyces* species. All three compounds contain a 3-methyl glutamate (3-MeGlu) as the penultimate C-terminal residue, which is important for bioactivity. Here, biochemical in vitro reconstitution of the 3-MeGlu biosynthetic pathway is presented, using exclusively enzymes from the CDA producer *Streptomyces coelicolor*. It is shown that the predicted 3-MeGlu methyltransferase GlmT and its homologues DptI from the daptomycin producer *Streptomyces roseosporus* and LptI from the A54145 producer *Streptomyces fradiae* do not methylate free glutamic acid, PCP-bound glutamate, or Glu-containing CDA in vitro. Instead, GlmT, DptI, and LptI are *S*-adenosyl methionine (SAM)-dependent α-ketoglutarate methyltransferases that catalyze the stereospecific methylation of α-ketoglutarate (αKG) leading to (3*R*)-3-methyl-2-oxoglutarate. Subsequent enzyme screening identified the branched chain amino acid transaminase IlvE (SCO5523) as an efficient catalyst for the transformation of (3*R*)-3-methyl-2-oxoglutarate into (2*S*,3*R*)-3-MeGlu. Comparison of reversed-phase HPLC retention time of dabsylated 3-MeGlu generated by the coupled enzymatic reaction with dabsylated synthetic standards confirmed complete stereocontrol during enzymatic catalysis. This stereospecific two-step conversion of αKG to (2*S*,3*R*)-3-MeGlu completes our understanding of the biosynthesis and incorporation of β-methylated amino acids into the nonribosomal lipopeptides. Finally, understanding this pathway may provide new possibilities for the production of modified peptides in engineered microbes.

Introduction

A challenging problem in treatment of bacterial infections is the increasing appearance of strains that are resistant against conventional antibiotics. Therefore, the design of new antimicrobial compounds remains an urgent concern. The broad structural diversity of secondary metabolites, particularly those produced by the large multimodular nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), offers a promising source of such new bioactive compounds.[1,2] The origin of this diversity arises from the large pool of available building blocks, which are utilized in the assembly of these natural products. In nonribosomal peptides, these building blocks include proteinogenic and non-proteinogenic amino acids as well as fatty acids. In addition, further modifications, such as glycosylation[3] (vancomycin), O-, N-, or C-methylation[4] (melithiazol, cyclosporine, and yersiniabactin), halogenation[5] (syringomycin E), hydroxylation[6] (mannopeptimycin), and phosphorylation[7,8] (CDA), can also occur prior to, during, or after peptide assembly. Understanding the biosynthetic pathways required for the biosynthesis and the incorporation of such building blocks into these complex natural products is an important issue, not only for uncovering the mechanism of new intriguing enzymatic reactions, but also to facilitate the engineered production of new therapeutic agents.

An important class of antibiotics with significant therapeutic potential are the acidic lipopeptides, including the calcium-dependent antibiotic (CDA),[9] daptomycin,[10−12] and the related

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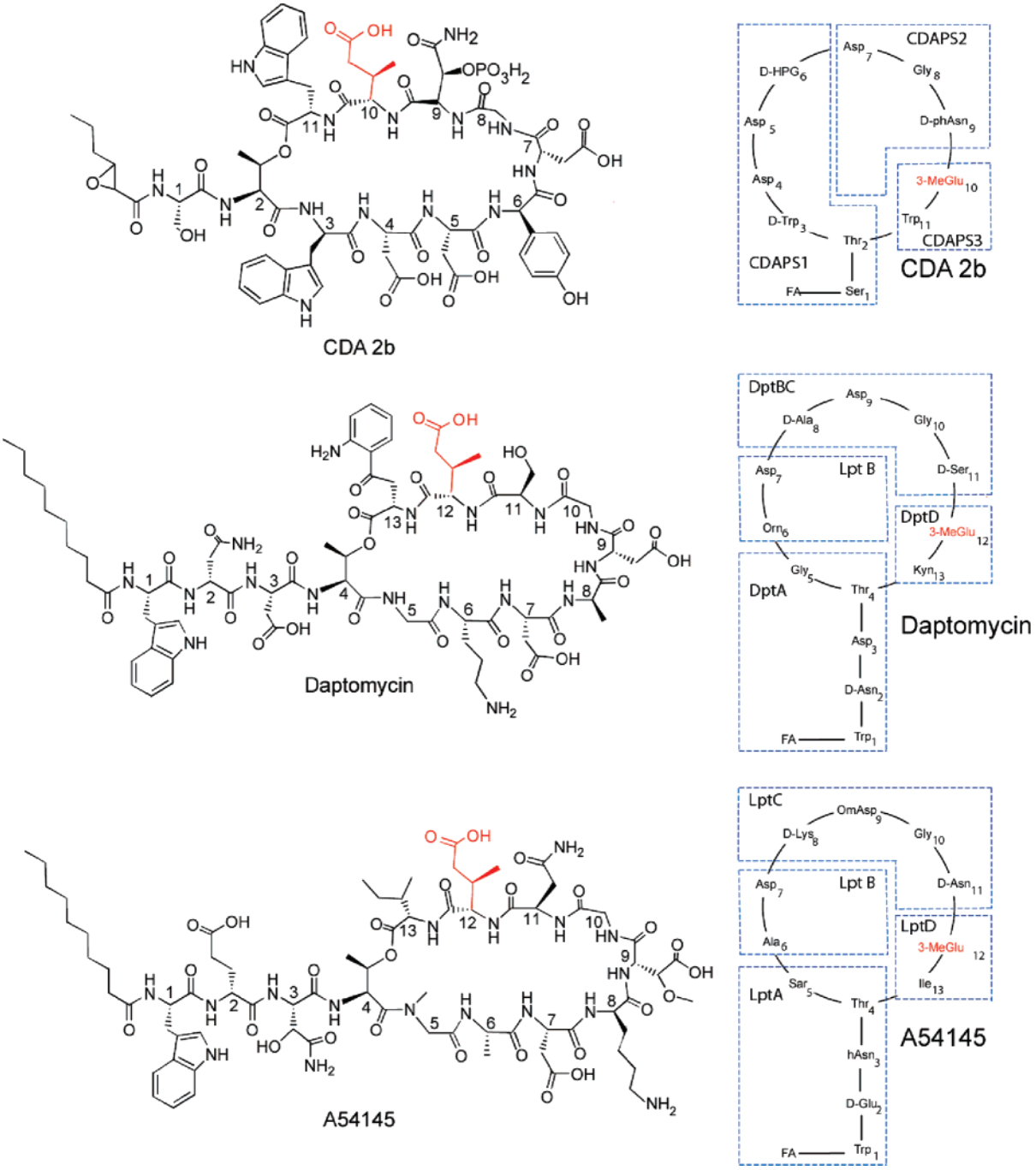

Figure 1. Structures of CDA, daptomycin, and A54145 lipopeptides. The schemes on the right show the module distribution of corresponding peptide synthetases. The 3-MeGlu residue is marked in red. FA = fatty acid, CDAPS1−3 = CDA peptide synthetase 1−3, DptA-D = daptomycin peptide synthetases, LptA-D = A54145 peptide synthetases.

A54145 lipopeptide[13−15] (Figure 1). Remarkably, daptomycin represents the first new structural class of naturally derived antimicrobial agents, which has reached the clinic during the last 30 years. From the structural point of view, CDA, daptomycin, and A54145 are peptidic macrolactones with branched cyclic structures. All of these peptides contain several acidic residues that were shown to be important for the coordination of calcium ions and bioactivity.[10,16]

In addition, the acidic lipopeptides comprise a wide range of N-terminal fatty acid chains and show a high content of non-proteinogenic amino acids. A common structural feature of CDA, daptomycin, and A54145 is the presence of a β-methylated Glu residue at the same relative ring positions in the decapeptide lactone cores. Whereas CDA from *Streptomyces*

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coelicolor and the A54145 lipopeptide from *Streptomyces fradiae* are produced as mixtures of compounds containing 3-MeGlu or Glu,[13,17] fermentative production of daptomycin from *Streptomyces roseosporus* results exclusively in 3-MeGlu containing peptides.[18] For daptomycin and CDA, this residue was shown to be the (2*S*,3*R*)-stereoisomer of 3-MeGlu.[19−21] In all of the acidic lipopeptides tested so far, the 3-MeGlu-containing products were shown to exhibit a higher bioactivity than the Glu-containing analogues.[14,16,18,19,22]

These observations identify this building block as a promising target for engineering acidic lipopetide biosynthesis controlling the incorporation of Glu, 3-MeGlu, or further Glu analogues in search for improved bioactivities. However, from the chemical point of view, the stereospecific functionalization of amino acids at the relatively unreactive β-position is a synthetic challenge. Despite this, there are some reports concerning asymmetric synthesis of 3-MeGlu diastereoisomers,[23−25] and, recently, a convergent multistep synthesis to all possible stereoisomers of 3-MeGlu was published.[19] However, the in vitro utilization of the biosynthetic enzymes responsible for 3-MeGlu formation would enormously facilitate the preparation of 3-MeGlu and other β-functionalized Glu analogues, which are required for engineering new lipopeptide products. For these reasons, it is crucial to understand the biosynthesis of 3-MeGlu and to determine the mechanism of its incorporation into the acidic lipopeptide backbone.

A gene SCO3215 was identified within the CDA biosynthetic gene cluster of *S. coelicolor*, which was suggested to encode a *S*-adenosyl methionine (SAM)-dependent glutamate-3-methyltransferase (GlmT) based on low, but significant, sequence similarity to other methyltransferases.[9] Within the daptomycin and A54145 gene clusters in *S. roseosporus* and *S. fradiae*, there also exist genes *dptI* and *lptI* encoding proteins that show high similarity to GlmT. Fermentation of deletion mutants of *S. coelicolor* (Δ*glmT*) and *S. roseosporus* (Δ*dptGHIJ*) led to production of CDA and daptomycin analogues, respectively, containing exclusively Glu instead of 3-MeGlu.[18,19] Moreover, complementation of the Δ*dptGHIJ* mutant by *dptI* or *glmT* and complementation of Δ*glmT* with synthetic 3-MeGlu restored the biosynthesis of the 3-MeGlu-containing compounds.[18,19] These results show that GlmT, DptI, and presumably also LptI are methyltransferases involved in the biosynthesis of 3-MeGlu residues in CDA, daptomycin, and A54145. However, the substrate and mechanism of the methylation reaction and the nature of the other possible steps in the biosynthetic pathway remained unclear.

In this study, the methyltransferase GlmT from *Streptomyces coelicolor* is biochemically characterized in vitro enabling a complete stereospecific in vitro synthesis of (2*S*,3*R*)-3-MeGlu using *S. coelicolor* enzymes exclusively. Furthermore, we show that DptI and LptI from *S. roseosporus* and *S. fradiae* catalyze the same reaction as GlmT. By testing various possible substrates, we determine the substrate specificity of the methyltransferase. This combined with recent in vivo results[19] provides complete mechanistic details of the individual steps occurring during the biosynthesis and incorporation of the 3-MeGlu residues into the acidic lipopeptide group of antibiotics.

Notably the study presents the first example of enzymes from secondary and primary metabolism cooperating in the biosynthesis of a nonproteinogenic amino acid required for assembly of nonribosomal peptides. It is also possible that similar pathways, utilizing both primary and secondary metabolic enzymes, may exist in the biosynthesis of other β-methylated amino acid building blocks, such as the β-methylated Phe residue of the mannopeptimycins.[26]

Experimental Section

Strains, Culture Media, and General Methods. The *E. coli* strains were grown in Luria−Bertani medium, supplemented with 100 μg/mL ampicillin (final concentration). Oligonucleotides were purchased from Operon. DNA dideoxy sequencing confirmed the identity of all plasmids constructed.

Cloning and Expression of Glmt, IlvE, CDAPS3-PCP10, DptI, and LptI. The genes coding for *glmT*, *ilvE*, and the *cdaPS3-PCP10* gene fragment were amplified by PCR from chromosomal DNA of *S. coelicolor* A3(2) (DSM 40783) using the Phusion polymerase (Finnzymes). The genes coding for *dptI* and *lptI* were amplified from *S. roseosporus* (NRRL 11379) and from *S. fradiae* (NRRL 18158) using the same method. According to the manufacturer's protocol for PCR amplification of template DNA with high GC-content (*S. coelicolor* 74%), the dNTP concentrations were increased to 20 mM. Amplification of *glmT* (SCO3215) was carried out using the oligonucleotides 5′-*glmT* (5′-AAA AAA CCA TGG TGA CCG GGG ACG ACG TGC AGG GG) and 3′-*glmT* (5′-AAA AAA AAG CTT TGC CGC CTT CCC GGC GGT GGC CG). The *ilvE* gene (SCO5523) was amplified using the primer combination of 5′-*ilvE* (AAA AAA GGA TCC ATG ACG ACG CCC ACG ATC GAG CTC) and 3′-*ilvE* (AAA AAA AAG CTT TCA GGC CAG CGT GTG CAT CCA CC). The gene fragment *cdaPS3-PCP10* encoding the PCP of module 10 (fragment of SCO3232, *cdaPS3*) was amplified using the oligonucleotides 5′-*cdaPS3-PCP10* (AAA AAA GGA TCC ACC GGC CGG ACC GCG GGC CG) and 3′-*cdaPS3-PCP10* (AAA AAA GCGG CCGC GCC CTT CGC CCC GGC GAG CAC). The gene *dptI* was amplified using the primer combination of 5′-*dptI* (AAA AAA CCA TGG TGA CCG GCG AAA CCC GCA CCA C) and 3′-*dptI* (AAA AAA AAG CTT TGG TTT GCG TCC GTG GGC GAC GA). For amplification of *lptI*, the oligonucleotides 5′-*lptI* (AAA AAA GGA TCC ATG CAG GCG GAT GCA CCG GCG G) and 3′-*lptI* (AAA AAA AAG CTT TCA GGT GGG TGG CTT GTG GGA GAC GG) were used.

After purification and digestion with *Nco*I, *Hind*III (*glmT*, *dptI*), *Bam*HI, *Hind*III (*ilvE*, *lptI*), and *Nco*I, *Not*I (*cdaPS3-PCP10*), respectively, the gene fragments of *glmT*, *ilvE*, *dptI*, and *lptI* were ligated into the corresponding restriction sites of a derivatized pET-28(a+) vector (Novagen), whereas the gene fragment *cdaPS3-PCP10* was similarly ligated into a derivatized pQE30 vector (Qiagen).

Production of Recombinant Enzymes. The pQE30- and pET-28-(a+)-plasmids, containing the gene fragments of interest, were used to transform *E. coli* BL21(DE3) (Novagen). For production of recombinant

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GlmT, DptI, and LptI, the transformed cells were grown at 20 °C to an optical density of 0.5 (600 nm), induced with 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG), and grown for a further 18 h. For production of IlvE and CDAPS3-PCP10, the transformed cells were grown at 30 °C to an optical density of 0.5 (600 nm), induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG), and grown for a further 3 h. The recombinant proteins were purified by Ni-NTA affinity chromatography (Amersham Pharmacia Biotech). Fractions containing the recombinant proteins were identified by SDS-PAGE analysis, pooled, and subjected to buffer exchange into 25 mM HEPES, 50 mM NaCl, pH 7.0 using HiTrap desalting columns (Amersham Pharmacia Biotech). The protein concentration was determined spectrophotometrically using calculated extinction coefficients at 280 nm. After being flash-frozen in liquid nitrogen, the proteins were stored at −80 °C.

General Methylation Assay. The recombinant methyltransferase (GlmT, DptI, or LptI) (5 μM) was incubated for 15 h at 25 °C with different substrates (250 μM) and the methyl group donor SAM (500 μM) in 50 μL of 25 mM HEPES, 50 mM NaCl (pH 7.0). Control reactions were carried out without either methyltransferase or SAM, respectively.

Methylation Assay with Glu-*S*-PCP and Chemoenzymatically Derived CDA. Glu-*S*-PCP and the CDA analogue (nmCDA) were synthesized and incubated with the methyltransferase following the general methylation assay. The Glu-*S*-PCP and CDA analogue synthesis and the analysis of the methylation assays are described in the Supporting Information.

Methylation Assay with Free α-Ketoacids or Amino Acids. Free amino acids or α-ketoacids were incubated with the methyltransferase following the general methylation assay procedure. The reaction was stopped by adding 50 μL of MeOH. The reaction products were analyzed using reversed-phase HPLC/MS on an ESI-Quad 1100(A) Series MSD mass spectrometer (Agilent) using a C18 Nucleodur column (Macherey and Nagel, 250/3, pore diameter of 100 Å, particle size of 3 μm) with the following conditions: water/2 mM triethylamine, 0.3 mL/min, 45 °C. To enhance the sensitivity of the MS detection, the MS spectrometer was used in the single ion mode screening exclusively for the masses of the expected products and substrates. Quantification of substrates and products was carried out by integration of mass extracted signals of the MS spectrometer. Kinetic parameters K_M and k_{cat} were determined by varying the substrate concentration between 10 μM and 2 mM and stopping the reaction by addition of MeOH.

Transamination Assay Using Commercially Available Transaminases. The five following commercially available transaminases were purchased from Sigma to test their ability to transaminate a racemic mixture of synthetic 3-methyl-2-oxoglutarate[19] to the corresponding amino acid: the glutamic-pyruvic transaminase from porcine heart (order number G-8255), the glutamic-oxalacetic transaminase Type I from porcine heart (order number G-2751), the glutamic-oxalacetic-transaminase Type II-A from porcine heart (order number G-7005), the broad-range transaminase from bacterial source (order number T-7684), and the branched-chain amino acid transaminase from bacterial source (order number B-2549). The transaminases (0.005 U/μL) were incubated with the synthetic racemic 3-methyl-2-oxoglutarate (synthesis described elsewere[19]) (1 mM), the donor amino acid corresponding to the particular transaminase (10 mM) (Ala for glutamic-pyruvic transaminase, Asp for glutamic-oxalacetic transaminases and broad-range transaminase, Val for branched-chain transaminase), and pyridoxal-5′-phosphate (PLP) (1.5 mM) in a total volume of 50 μL. To reach a more quantitative conversion, commercially available dehydrogenases (Sigma) (2×10^{-5} U/μL) (L-lactic dehydrogenase for assays with glutamic-pyruvic transaminase, malic dehydrogenase for assays with glutamic-oxalacetic transaminase and broad-range transaminase) and NADH (1.5 mM) were added following the transaminase manufacturer's protocols. These dehydrogenases convert the byproducts L-Ala and L-Asp of the transamination reactions into L-lactate and L-malate, respectively, and therefore switch the equilibrium of the transamination reaction to the product side. Temperatures and buffers were chosen as recommended by the manufacturer. The reaction mixtures were incubated overnight. After reactions were stopped using 50 μL of MeOH, the mixtures were analyzed by reversed-phase HPLC-MS using the single ion mode and the same gradient as described for the methylation assays with free amino acids.

Transamination Assay Using Recombinant IlvE from *Streptomyces coelicolor*. Recombinant IlvE from *Streptomyces coelicolor* (5 μM) was incubated with synthetic racemic 3-methyl-2-oxoglutarate (1 mM) and L-Val (10 mM) in a total volume of 50 μL in 50 mM HEPES (pH 7.5). Reactions were quenched and analyzed in the same way as described for assays with commercially available transaminases. Analysis of assays showed that addition of PLP was not necessary.

Coupled Methylation−Transamination Assay. First, α-ketoglutarate was methylated following the general methylation procedure overnight. In this time, the methylation leading to 3-methyl-2-oxoglutarate occurred quantitatively. The assay was stopped by adding an equal volume of MeOH. The reaction mixtures were stored at −20 °C to precipitate the methyltransferase and subsequently centrifuged at 13 000 rpm and 4 °C. The supernatant was separated from the pellet and lyophilized over night. The resulting solid was redissolved in 50 mM HEPES (pH 7.5) to a theoretical concentration of 3-methyl-2-oxoglutarate of 5 mM, and the absolute concentration was estimated using reversed-phase HPLC-MS. This solution of 3-methyl-2-oxoglutarate was used as a stock for the following transamination assay. In this assay, the enzymatically prepared 3-methyl-2-oxoglutarate (250 μM) was incubated with Val (2 mM) and the recombinant transaminase IlvE from *Streptomyces coelicolor* (8 μM) in 50 mM HEPES (pH 7.5) for 30 min to give a nearly complete conversion to 3-MeGlu. Kinetic characterization of this transamination was done by varying the substrate concentration between 10 μM and 1 mM and by quenching the reaction upon addition of MeOH at different time points. Analysis of reactions was done after dabsyl-derivatization (see below) using reversed-phase HPLC-MS analysis. Dabsyl-amino acids were detected following absorption at 436 nm and by MS spectrometry using the single ion mode. HPLC analysis occurred on a C18 Nucleodur column (Macherey and Nagel, 250/2, pore diameter 100 Å, particle size 3 μm) using the following gradient: 0−30 min, 7−30% acetonitrile/2 mM triethylamine in water/2 mM triethylamine, 0.2 mL/min, 45 °C.

Derivatization and Determination of Configuration of Enzymatically Synthesized 3-MeGlu by HPLC. The dabsyl derivatization of enzymatically prepared and synthetic 3-MeGlu was carried out as described elsewhere.[19,27,28] The lyophilized coupled assay or synthetic 3-MeGlu (concentrations between 10 μM and 1 mM) was dissolved in $NaHCO_3$ (0.15 M, pH 9.0) in a total volume of 400 μL. Dabsyl chloride (15 mM) in acetone (400 μL) was added. The mixture was heated at 70 °C for 20 min and diluted with EtOH (total 2 mL). After centrifugation at 5000*g* for 5 min, the supernatant was analyzed by reversed-phase HPLC-MS 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 μm, 150 × 4.6 mm (Phenomenex), and the following conditions: solvent A was water/1% acetonitrile/0.1% formic acid; solvent B was acetonitrile/1% water/0.1% formic acid. The flow rate was 1 mL/min with a gradient from 20% B to 70% B over 10 min and then increasing to 100% B over the next min. The gradient was then held for a further 4 min.

Results and Discussion

Expression and Purification of GlmT, IlvE, CDAPS3-PCP10, DptI, and LptI. The genes *glmT*, *ilvE* and the gene fragment *cdaPS3-pcp10* from *S. coelicolor*, the gene *dptI* from

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S. roseosporus, and the gene *lptI* from *S. fradiae* were amplified and cloned into expression vectors as described in the Experimental Section. The expression vectors were used to overproduce the recombinant proteins in *E. coli* as His_6- or His_7-tagged fusions (GlmT, 38.5 kDa; DptI, 37.4 kDa; LptI, 39.9 kDa; CDAPS3-PCP10, 12.8 kDa; IlvE, 40.8 kDa), which were purified using Ni-NTA affinity chromatography. SDS-PAGE analysis indicated high purity of the proteins. The protein identity was verified by mass spectrometry (MS) analysis. From 1 L of culture, we obtained the following yields for the single proteins: GlmT, 0.3 mg; DptI, 0.1 mg; LptI, 0.2 mg; CDA3-PCP10, 4 mg; IlvE, 6 mg.

GlmT Does Not Methylate Glu, PCP-Bound Glu, or CDA. Sequence alignments of GlmT and its homologues DptI and LptI reveal a putative *S*-adenosyl methionine (SAM) binding site and show similarity to ubiquinone methyltransferase (UbiE), and other SAM-dependent methyltransferases. Therefore, these proteins were predicted to act as glutamic acid 3-methyltransferases generating 3-MeGlu, which is then activated by the A-domain of the corresponding module.[18] To examine the methylation activity of GlmT in vitro, the recombinant protein was incubated with several possible substrates in presence of the methyl group donor SAM. The tested substrates were free L-Glu and D-Glu, L-Glu, and D-Glu tethered to the recombinant holo-PCP domain (Glu-*S*-PCP) via the natural phosphopantetheine prosthetic group of module 10 from the CDA peptide synthetase 3 (CDAPS3), and a chemoenzymatically synthesized[29] non-methylated CDA analogue (nmCDA, Supporting Information Figure 1). HPLC-MS analysis of enzymatic incubations of all of these substrates detected no formation of methylated free or PCP-tethered Glu stereoisomers or of a methylated CDA analogue, respectively. This suggests that GlmT neither catalyzes methylation of free or PCP-bound Glu nor acts as tailoring enzyme methylating the lipopeptide after release from the synthetase.

Methylation of α-Ketoglutarate. Recent in vivo studies described the re-establishment of 3-MeGlu containing CDA production in a *glmT* deletion mutant of *S. coelicolor* (Δ*glmT*) by feeding with synthetic (2*S*,3*R*)-3-MeGlu,[19] indicating that the A-domain of CDAPS3 module 10 is able to activate and incorporate the (2*S*,3*R*)-3-MeGlu in addition to Glu into the CDA backbone. These findings are consistent with the observation that the amino acid specificity-conferring codes[30] of the corresponding A-domains of CDA-, daptomycin-, and A54145-NRPSs[9] differ from other A-domains that are known to activate Glu, suggesting that they have evolved specificity for 3-MeGlu as well as Glu. In light of this and the fact that GlmT is unable to methylate PCP-tethered Glu, or a Glu-containing cyclic peptide substrate, it can be concluded that methylation is likely to take place prior to the activation by the A-domain. Therefore, we tested further possible methylation scenarios leading to free 3-MeGlu.

In a metabolic flux analysis of CDA biosynthesis, α-ketoglutarate (αKG) was suggested to be methylated by GlmT resulting in 3-methyl-2-oxoglutarate, which is then transaminated to 3-MeGlu.[31] To test this hypothesis, αKG was incubated with GlmT and SAM at 25 °C overnight. In HPLC-MS analyses

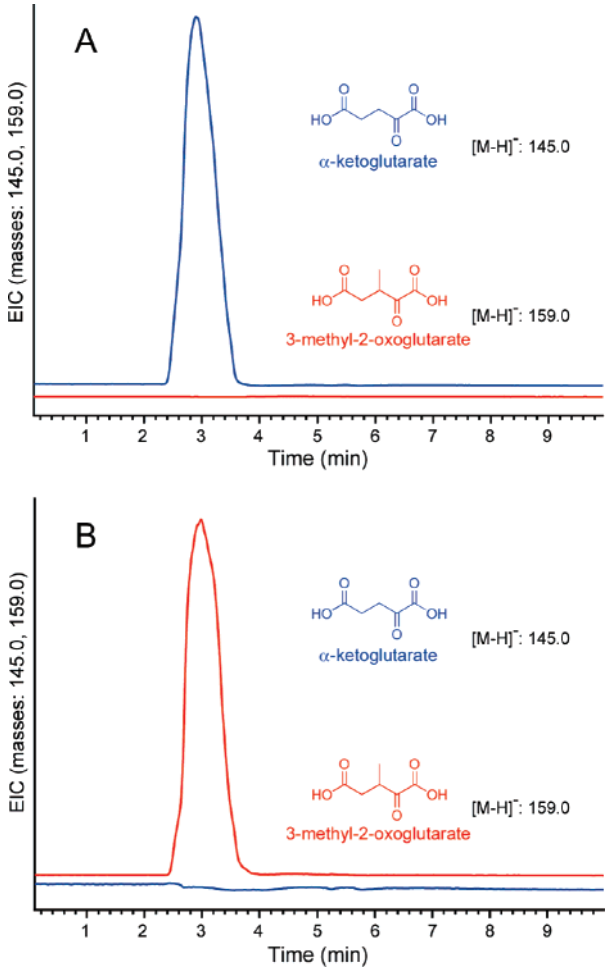

Figure 2. *β*-Methylation of αKG by GlmT. Extracted ion chromatograms of HPLC-MS analyses. The blue traces represent screens for αKG; the red traces represent screens for the methylation product 3-methyl-2-oxoglutarate. (A) Analysis of incubation without GlmT. (B) Analysis of incubation with GlmT.

of this assay, a quantitative conversion of αKG leading to 3-methyl-2-oxoglutarate was observed (Figure 2). Identical results were also obtained using recombinant DptI and LptI from *S. roseosporus* and *S. fradiae*, respectively. Also, no methylation of αKG was observed in control assays in the absence of SAM or the methyltransferases.

These experiments clearly show that GlmT, DptI, and LptI are SAM-dependent α-ketoglutarate methyltransferases catalyzing the transformation of αKG leading to 3-methyl-2-oxoglutarate. From the chemical point of view, the usage of αKG instead of Glu makes sense. Most SAM-dependent methylations occur as an attack of a nucleophilic substrate onto the activated methyl group of SAM following a S_N2 mechanism.[32,33] The direct SAM-dependent *β*-methylation of Glu by this mechanism would require the formation of a highly unstable *β*-carbanion. On the other hand, the formation of resonance-stabilized nucleophilic enolate of αKG in the active site of GlmT is more plausible.

Interestingly, the obtained results verify the proposed function of GlmT based on recent in vivo investigations.[19] These studies

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(33) Mascaro, L., Jr.; Horhammer, R.; Eisenstein, S.; Seller, L. K.; Mascaro, K.; Floss, H. G. *J. Am. Chem. Soc.* **1977**, *99*, 273−274.

showed that 3-MeGlu-containing CDA production in the Δ*glmT* mutant was re-established by feeding with synthetic racemic 3-methyl-2-oxoglutarate.[19] The combined results from the earlier in vivo studies and the in vitro biochemical experiments described here, therefore, definitively confirm that GlmT catalyzes the direct methylation of αKG to give 3-methyl-2-oxoglutarate.

To characterize the methylation reaction of αKG by GlmT more precisely, we determined the kinetic parameters at a SAM concentration of 2 mM by varying the substrate concentration and stopping the enzymatic reactions at appropriate time points. These experiments resulted in a K_M of 62.5 ± 4.8 μM, a k_{cat} of 0.11 ± 0.002 min^{-1}, and a k_{cat}/K_M of 1.78 ± 0.17 min^{-1} mM^{-1}. The observed values are a similar order of magnitude to values reported for NovO- and CouO-catalyzed C-methylations of the aminocoumarin scaffold during the biosynthesis of novobiocin and coumermycin.[34] Furthermore, the observed k_{cat} is within the range reported for *O*-methyltransferases. We also tested other α-keto acids for GlmT-mediated methylation. These α-keto acids were pyruvate, phenylpyruvate, α-ketobutyrate, and oxalacetate. HPLC-MS analyses showed that GlmT is highly specific and exclusively accepts αKG as the cognate substrate.

Transamination of 3-Methyl-2-oxoglutarate. The next goal was to discover how the methylation product 3-methyl-2-oxoglutarate is transaminated to 3-MeGlu in *S. coelicolor*. In the acidic lipopeptide biosynthetic gene clusters, there are no conserved genes present that may encode proteins that are similar to known aminotransferases. Therefore, 3-methyl-2-oxoglutarate transamination is likely to be catalyzed by one or more proteins encoded outside the biosynthetic gene cluster. There are 77 genes encoding known or putative aminotransferases located within the *S. coelicolor* genome [http://streptomyces.org.uk/]. Some of these enzymes could potentially transfer the α-amino group from various amino group donors to 3-methyl-2-oxoglutarate leading to 3-MeGlu. Furthermore, the NADPH-dependent glutamate synthase (SCO1977, SCO2025, SCO2026) and NADP-specific glutamate dehydrogenase (SCO4683),[35] which utilize αKG in the biosynthesis of L-Glu, could also be responsible for the synthesis of 3-MeGlu from 3-methyl-2-oxoglutarate. To identify an aminotransferase, which has the ability to catalyze the transamination of 3-methyl-2-oxoglutarate, we first screened five commercially available transaminases (see Experimental Section) by incubation with synthetic racemic 3-methyl-2-oxoglutarate,[19] pyridoxal-5′-phosphate (PLP), and an appropriate amino acid as amino group donor. HPLC-MS analyses of these reactions revealed that the branched chain amino acid aminotransferase IlvE from bacterial source (Sigma) was able to catalyze the formation of 3-MeGlu in presence of Val (data not shown). Homologues of this transaminase are present in all bacteria. In *S. coelicolor*, the enzyme most similar to IlvE is encoded by the gene SCO5523. We therefore cloned this gene and overproduced the *S. coelicolor* IlvE in *E. coli* as a His_6-tagged fusion and tested this putative transaminase with synthetic racemic 3-methyl-2-oxoglutarate and Val as the amino group donor. HPLC-MS analysis showed the formation of 3-MeGlu (Supporting Information Figure 2). These results clearly point out that IlvE from the primary metabolism of *S. coelicolor* is one candidate that is able to transform 3-methyl-2-oxoglutarate to 3-MeGlu.

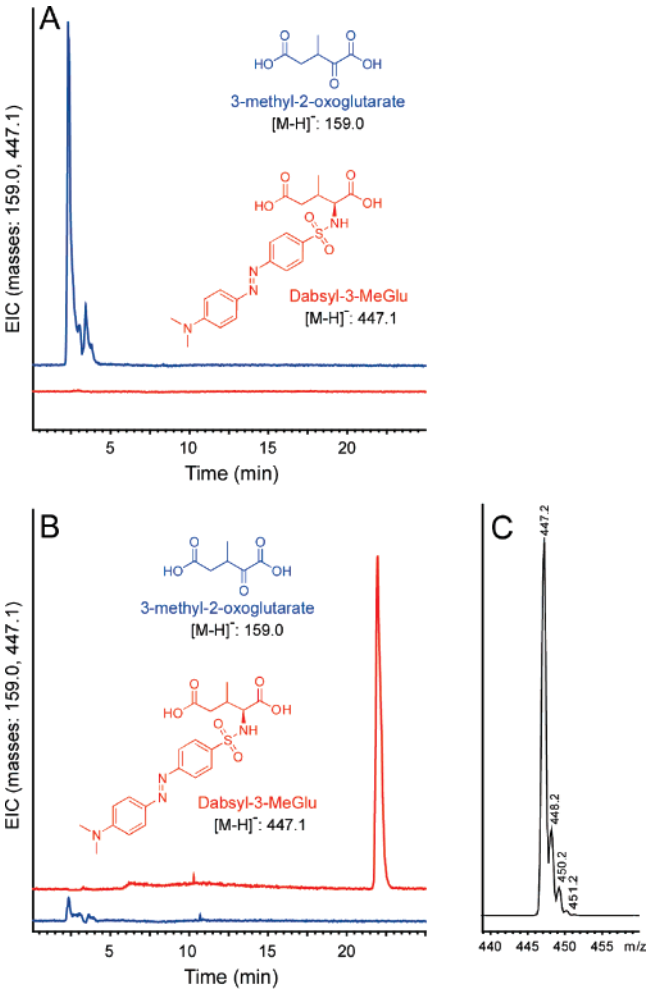

Figure 3. IlvE-catalyzed transamination of enzymatically prepared 3-methyl-2-oxoglutarate. (A,B) Extracted ion chromatograms of HPLC-MS analyses of dabsylated transamination assays measured using the single ion mode. The blue traces represent screens for 3-methyl-2-oxoglutarate; the red traces represent screens for dabsylated 3-MeGlu. (A) Analysis of incubation without IlvE. (B) Analysis of incubation with IlvE. A nearly total conversion of 3-methyl-2-oxoglutarate to 3-MeGlu is observable. (C) MS of enzymatically prepared dabsyl-3-MeGlu measured using the scan mode during MS analysis ($[M - H]^-$ calculated: 447.14).

Encouraged by this observation, we developed a coupled methylation-transamination assay to establish a complete synthetic route to 3-MeGlu in vitro. In the first step, αKG was methylated quantitatively using SAM and GlmT. After separation of the methyltransferase by MeOH-precipitation, the lyophilized product was redissolved and incubated at a concentration of 250 μM with IlvE from *S. coelicolor* (8 μM) and the amino group donor Val (2 mM) for 15 min at 25 °C. After a further lyophilization step, the reaction products were redissolved in $NaHCO_3$ (0.15 M, pH 9.0) and derivatized with dabsyl chloride. This derivatization improves retention and enables separation of the 3-MeGlu diastereomers on reversed-phase HPLC.[19,28] HPLC-MS analysis showed nearly total conversion of 3-methyl-2-oxoglutarate to 3-MeGlu (Figure 3).

In primary metabolism IlvE catalyzes the transfer of the amino group of the hydrophobic amino acids L-Ile, L-Val, and L-Leu onto αKG leading to L-Glu and α-ketoacids. Furthermore, IlvE catalyzes the reverse reaction, in which L-Glu is the amino acid

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substrate. Therefore, IlvE is a transaminase, which shows specificity for hydrophobic amino acids and L-Glu. Because 3-MeGlu and 3-methyl-2-oxoglutarate show structural features from both natural substrates of IlvE, the finding presented here, that IlvE accepts 3-methyl-2-oxoglutarate as a substrate, makes sense. The structural reason of this dual specificity for acidic and hydrophobic amino acids was elucidated by the crystal structure of IlvE from *Escherichia coli*, which was resolved in complex with different substrates.[36,37] This shows that the IlvE binding pocket is able to accommodate both hydrophobic and acidic amino acid side chains.

To further characterize the IlvE-catalyzed transformation of 3-methyl-2-oxoglutarate, the kinetic parameters of this reaction at a fixed concentration of the amino group donor Val (5 mM) were determined, by varying the substrate concentration and stopping the enzymatic reactions at the appropriate time points. The reaction progress was monitored using HPLC-MS after dabsyl derivatization. These investigations resulted in an apparent K_M of 0.196 mM ± 0.403, a k_{cat} of 12.1 s^{-1} ± 0.9, and a k_{cat}/K_M of 3716 ± 557 min^{-1} mM^{-1}. These values are a similar order of magnitude to values determined for IlvE from *E. coli* for αKG in the presence of L-Val (K_M, 1.7 mM; k_{cat}, 19 s^{-1}; k_{cat}/K_M, 660 min^{-1} mM^{-1}).[38]

Stereospecificity of Methylation and Transamination Reaction. To determine the absolute configuration of the 3-MeGlu product of the GlmT/IlvE coupled transformations, the reaction mixture was derivatized with dabsyl chloride. HPLC-MS analysis showed a coelution of the enzymatically prepared dabsylated 3-MeGlu with synthetic dabsylated (2*S*,3*R*)-3-MeGlu (R_t = 9.8 min). On the other hand, at the retention time corresponding to the synthetic dabsylated (2*S*,3*S*)-3-MeGlu (R_t = 10.2 min), there is clearly no product exhibiting the required molecular ions at the detection limits of the instrument (Figure 4).

Given that IlvE from *S. coelicolor* is only able to transaminate the natural (2*S*)-amino acid stereoisomers (Supporting Information Figure 3), these results clearly prove that the enzymatically produced 3-MeGlu exhibits (2*S*,3*R*) absolute configuration. Taking into account that the L-stereospecificity of IlvE is established, these results indicate that GlmT catalyzes the stereopecific transformation of αKG leading exclusively to (3*R*)-3-methyl-2-oxoglutarate, with very high stereospecificity.

The (2*S*,3*R*)-configuration of enzymatically prepared 3-MeGlu is the same as observed for the 3-MeGlu residue in CDA and daptomycin.[19−21] The high degree of protein sequence similarity of the methyltransferases GlmT, DptI, and LptI and of the A-domains for Glu/3Me-Glu activation of CDA, daptomycin, and A54145 NRPSs suggests that A54145 will possess identical absolute configuration. Recently, in vivo results indicated a highly evolved stereospecificity of the module 10 A-domain of CDAPS3. Indeed, production of CDA containing 3-MeGlu in the *S. coelicolor* Δ*glmt* strain could be restored only by feeding the natural (2*S*,3*R*)-3-MeGlu, but not by feeding the (2*S*,3*S*)-diastereomer.[19] The results presented here show that the configuration of the 3-MeGlu residue in acidic lipopeptides is controlled not only by the NRPS selectivity, but also by the stereospecificity of the biosynthetic pathway leading to the 3-MeGlu precursor.

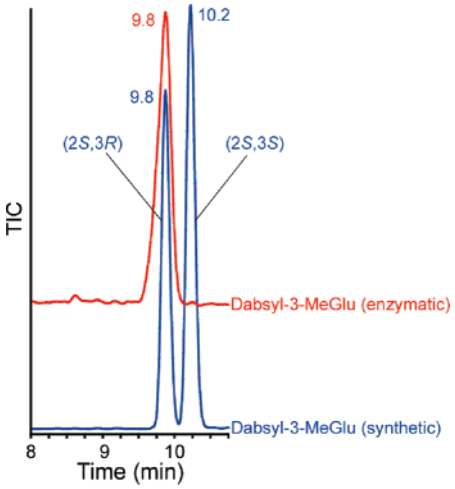

Figure 4. Determination of absolute configuration of enzymatically produced 3-MeGlu. Total ion chromatograms of HPLC-MS analyses. Blue trace: HPLC-MS of a mixture of dabsylated synthetic (2*S*,3*R*)- and (2*S*,3*S*)-3-MeGlu diastereomers. The (2*S*,3*R*)-3-MeGlu ($[M + H]^+$ = 449.2 (449.1 calculated)) elutes at 9.8 min; the (2*S*,3*S*)-3-MeGlu ($[M + H]^+$ = 449.2 (449.1 calculated)) elutes at 10.2 min. Red trace: HPLC-MS of dabsylated enzymatically produced 3-MeGlu. A peak is observed at 9.8 min ($[M + H]^+$ = 449.2 (449.1 calculated)) coeluting with the (2*S*,3*R*)-3-MeGlu diastereomer. In the chromatogram of the enzymatic product, at 10.2 min, there are no products exhibiting the molecular ions corresponding to dabsylated 3-MeGlu at the detection limits of the instrument.

Conclusions

The acidic lipopeptides CDA, daptomycin, and the A54145 lipopeptides all contain a conserved 3-MeGlu residue at equivalent positions within their decapeptide cores. In this study, the stereospecific in vitro biosynthesis of this important 3-MeGlu residue is elucidated, using enzymes from the CDA producer *Streptomyces coelicolor* exclusively. We have recombinantly overexpressed the putative methyltransferase encoding genes *glmT* along with *dptI* and *lptI* from *S. roseosporus* and *S. fradiae*, respectively, and shown that the resulting gene products are all SAM-dependent αKG methyltransferases catalyzing the methylation of αKG leading to 3-methyl-2-oxoglutarate. Furthermore, we screened for transaminases that can catalyze the transformation of 3-methyl-2-oxoglutarate to 3-MeGlu. From this screen, the branched chain aminotransferase IlvE (SCO5523) from *S. coelicolor* was identified as the most efficient catalyst for this reaction, transforming 3-methyl-2-oxoglutarate to 3-MeGlu in the presence of an excess of Val as the amino group donor. The coupling of the methylation and transamination reactions, in vitro, leads to the synthesis of 3-MeGlu starting from αKG. By comparison of retention times of dabsylated 3-MeGlu as a product of the coupled reaction with dabsylated synthetic (2*S*,3*R*)- and (2*S*,3*S*)-3-MeGlu, it was possible to prove the enzymatic product exhibits the (2*S*,3*R*)-configuration, which is the same as that observed for the 3-MeGlu residue in CDA and daptomycin. Taking these results together, we have established a two-step synthesis of an important non-proteinogenic amino acid introducing two new stereocenters with complete stereocontrol, using mild, convenient enzymatic catalysis in aqueous media.

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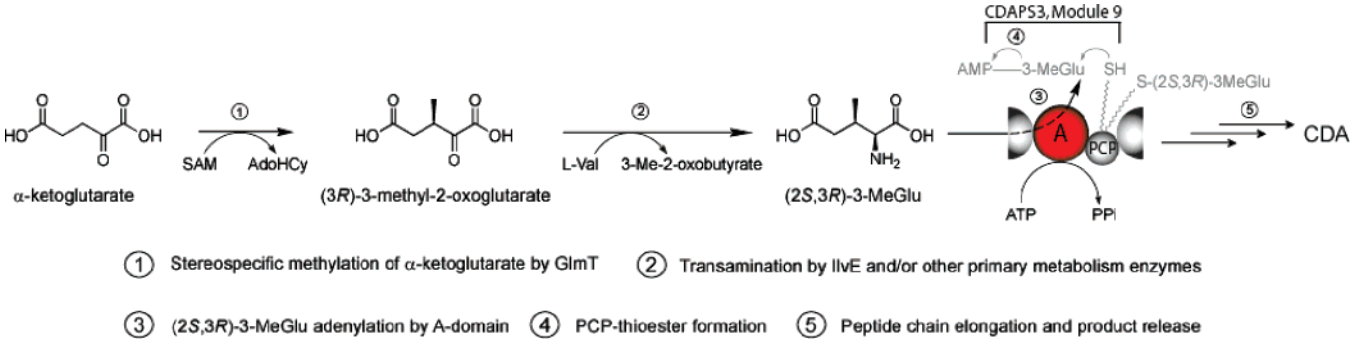

Figure 5. Scheme showing the reaction sequence of (2*S*,3*R*)-3-MeGlu biosynthesis and its incorporation into the CDA lipopeptide.

These biochemical results together with recent in vivo studies[19] on the complementation of the Δ*glmT* mutant of *Streptomyces coelicolor* clarify the mode of biosynthesis and incorporation of (2*S*,3*R*)-3-MeGlu into CDA (Figure 5). Following the biosynthesis of (2*S*,3*R*)-3-MeGlu, this precursor is then recognized by the CDAPS3 module 10 A-domain and incorporated into CDA by the NRPS machinery. Given that DptI and LptI proteins are shown to methylate αKG, in a fashion analogous to that of GlmT, it is likely that the same biosynthetic pathway operates for other 3-MeGlu-containing lipopeptides including daptomycin and A54145.

Furthermore, the results presented here open new ways for the enzymatic synthesis of β-methylated building blocks and for the engineering of acidic lipopeptide biosynthesis. The importance of β-methylation for bioactivity in all acidic lipopeptides points out that this position is an interesting target for variations in the search for new or improved lipopeptides and related nonribosomal peptide natural products.

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Supporting Information Available: LC-MS analysis of IlvE-catalyzed transamination of synthetic racemic 3-methyl-2-oxoglutarate, determination of stereospecificity of IlvE-catalyzed transamination, and further experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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