Precursor-directed biosynthesis of nonribosomal lipopeptides with modified glutamate residues†

Amanda Powell,[a] Majid Al Nakeeb,[a] Barrie Wilkinson[b] and Jason Micklefield*[a]

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Precursor-directed biosynthesis of calcium dependent antibiotics (CDAs) with modified 3-trifluoromethyl and 3-ethyl glutamate residues was achieved by feeding synthetic glutamate analogues to a mutant strain of *Streptomyces coelicolor* impaired in the biosynthesis of the natural precursor (2*S*,3*R*)-3-methyl glutamic acid.

Nonribosomal peptides are structurally diverse secondary metabolites which comprise a wide range of non-proteinogenic amino acids and other components such as carbohydrate, fatty acid and polyketide moieties.[1] The broad spectrum of biological activity exhibited by nonribosomal peptides has resulted in the development of a number of important and widely used therapeutic agents, including daptomycin, a member of the new lipopeptide class of antibiotics.[2] As a consequence, there has been considerable interest in the biosynthetic engineering of new peptide variants with improved properties. Notably, domain and module replacements within the nonribosomal peptide synthetase (NRPS) assembly lines[3] as well as active site modification of adenylation (A)-domains[4] have resulted in new peptide products. In addition, precursor-directed biosynthesis approaches have been developed where deletion of specific genes involved in the biosynthesis of nonproteinogenic amino acids has allowed the incorporation of synthetic precursor analogues into nonribosomal peptides.[5]

Recently, whilst investigating the biosynthesis of the calcium dependent antibiotics (CDAs) from *Streptomyces coelicolor* (Fig. 1),[6] we demonstrated that CDA and related lipopeptide daptomycin possesses (2*S*,3*R*) configured 3-methylglutamate (3-MeGlu) residues.[7] Furthermore deletion of a putative methyltransferase encoding gene *glmT* (SCO3215) from the *cda* biosynthetic gene cluster results in production of Glu rather than 3-MeGlu containing CDAs. By feeding 3-methyl-2-oxoglutarate **1** and (2*S*,3*R*)-3-MeGlu **2** to the mutant Δ*glmT* it was possible to re-establish production of 3-MeGlu containing CDA.[7] This led to the conclusion that GlmT is a methyltransferase that methylates α-ketoglutarate to give (3*R*)-methyl-2-oxoglutarate **1**, which is then transaminated to 3-MeGlu **2** by an aminotransferase (Fig. 1). In this paper we aimed to exploit these findings in order to effect the precursor-directed biosynthesis of CDAs with modified glutamate residues. This is particularly important because the antimicrobial activity and toxicity of CDA, daptomycin and related acidic lipopeptides are known to depend on the nature of the glutamate residue at a conserved position within the peptide core.[7–9]

The *S. coelicolor* MT1110 parental strain produces predominantly 3-MeGlu containing CDAs, with only a small amount of Glu containing CDA variants.[7] It is thus likely that the module 10 A-domain of cdaPS3 can activate (2*S*,3*R*)-3-MeGlu more efficiently than Glu. We thus anticipated that the MT1110-Δ*glmT* mutant strain, which lacks 3-MeGlu production, might selectively incorporate synthetic β-substituted glutamates when supplemented in the growth media, leading to new CDA analogues. To explore this the synthesis of 3-trifluoromethyl glutamic acid, which is sterically similar to the natural precursor, was first undertaken (Scheme 1). Accordingly, trifluoromethyl crotonate **3** was treated with the enolate anion derived from methyl nitroacetate **4**, in a

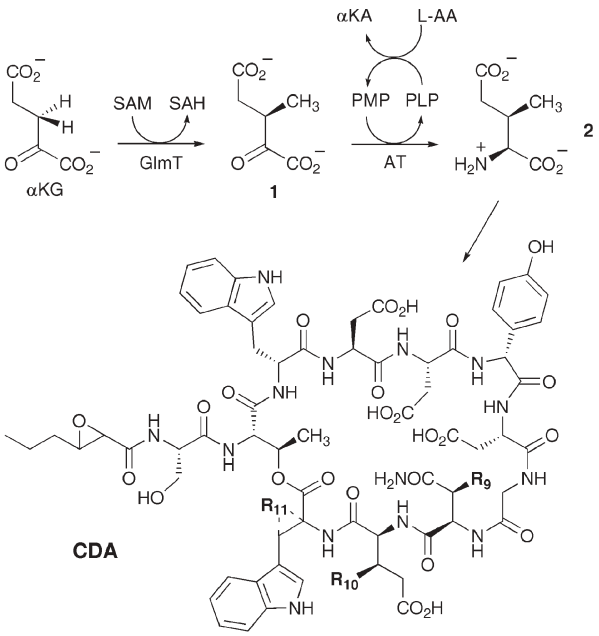

Fig. 1 Biosynthesis of the (2*S*,3*R*)-3-methylglutamic acid precursor[7] of calcium dependent antibiotics (CDAs) produced by the wild type *S. coelicolor*:[5a] CDA1, $R_9 = OPO_3H_2$ and $R_{10} = H$; CDA2, $R_9 = OPO_3H_2$ and $R_{10} = CH_3$; CDA3, $R_9 = OH$, $R_{10} = H$; CDA4, $R_9 = OH$, $R_{10} = CH_3$. In addition the a-series contain *Z*-Δtrp (R_{11} = π-bond) and the b-series contain L-Trp (R_{11} = H,H) at position 11. Proposed structures of new products identified in this work are: CF_3-CDA3b, $R_9 = OH$, $R_{10} = CF_3$ and R_{11} = H,H; CF_3-CDA3a, $R_9 = OH$, $R_{10} = CF_3$ and R_{11} = π-bond; Et-CDA3b, $R_9 = OH$, $R_{10} = CH_2CH_3$ and R_{11} = H,H. GlmT = 2-oxoglutarate-3-methyltransferase; AT = putative amino-transferase enzyme; L-AA = L-amino acid; α-KA = α-ketoacid; SAM = *S*-adenosyl-methionine; SAH = *S*-adenosyl-homocysteine; PLP = pyridoxal-5′-phosphate; PMP = pyridoxamine-5′-phosphate.

[a]*School of Chemistry and Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester, UK M1 7ND. E-mail: Jason.micklefield@manchester.ac.uk; Tel: 306 4509*
[b]*Biotica, Chesterford Research Park, Little Chesterford, Essex, UK CB10 1XL*

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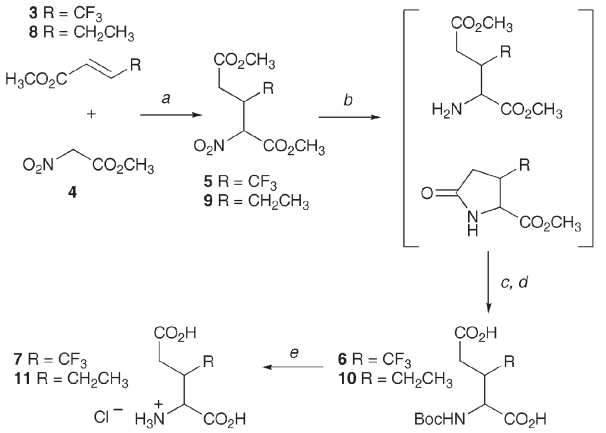

Scheme 1 Synthesis of 3-trifluoromethyl and 3-ethyl glutamic acids. (a) DBU; (b) Raney-Ni, HCO_2H; (c) $(Boc)_2O$, 4-DMAP, Et_3N; (d) LiOH, THF, H_2O; (e) 4 M HCl in 1,4-dioxane.

conjugate addition, to give **5** in 80% yield. Reduction of the nitro group of **5** with Raney nickel in formic acid results in a mixture of 3-trifluoromethylglutamate and the corresponding cyclic 3-trifluoromethylpyroglutamate methyl esters, which were not separated. This mixture was then treated with di-*tert*-butyl dicarbonate which results in *N*-protection and allows the subsequent basic hydrolysis of the intermediate lactam and methyl esters to give the Boc protected 3-trifluoromethyl glutamate **6**. Treatment of **6** with 4 M HCl in dioxane gives the required 3-trifluoromethyl glutamic acid **7** as a racemic mixture of diastereoisomers (89% yield from **5**).

The MT1110-Δ*glmT* mutant strain was then grown in liquid culture, under conditions established previously.[7] After 2 days of growth **7** was added to a final concentration of 750 $\mu g \cdot mL^{-1}$. After a further 4 days' growth, the cultures were then harvested, the supernatant passed over C18 (bond-elute) cartridges and the eluant analysed by electrospray ionisation (ESI) LC-MS. This revealed that the Glu containing CDA3b and CDA3a had been produced as the major products, along with hydrolysis side products that are commonly seen from analysis of the supernatant of this mutant and other strains of *S. coelicolor*. In addition, a minor new product with retention time 7.8 min exhibited protonated, sodiated and potassiated molecular ions in the ESI-MS that correspond to a product with *mw* 1550.3 Da (see ESI). This is consistent with a variant of CDA3b possessing an additional trifluoromethyl group (CF_3-CDA3b, Fig. 1). This new product is clearly not evident, at the threshold of LC-MS detection limits, in the supernatant of MT1110-Δ*glmT* grown under identical conditions in the absence of 3-trifluoromethyl glutamic acid. To further explore the identity of the new product, large-scale fermentation of the MT1110-Δ*glmT*, supplemented with 3-trifluoromethyl glutamic acid **7**, was carried out and the resulting supernatant was purified by RP-HPLC. Due to the low levels of the production, only μg quantities of the new product were isolated. This was not entirely unexpected since we had previously shown that feeding 3-methyl glutamic acid as a racemic mix of diastereoisomers to MT1110-Δ*glmT*, even at high concentrations, results in only low yields of 3-MeGlu containing CDA4a compared with feeding the natural stereoisomer, which restores near normal levels of CDA4a production.[7] Further LC-MS analysis of a fraction containing CF_3-CDA3b (Fig. 2) revealed

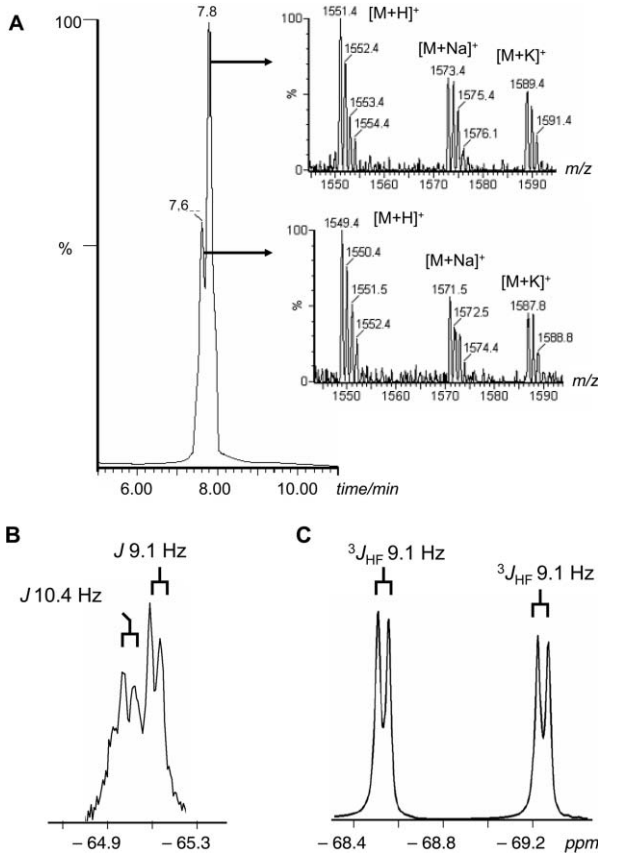

Fig. 2 (**A**) LC-MS analysis of purified extracts from the large scale feeding of 3-trifluoromethyl glutamic acid to MT1110-Δ*glmT*. Shows a major product at 7.8 min which exhibits molecular ions *m/z* 1551.4 ($[M + H]^+$, $C_{67}H_{78}F_3N_{14}O_{26}$ requires 1551.5); 1573.4 ($[M + Na]^+$, $C_{67}H_{77}F_3N_{14}O_{26}Na$ requires 1573.5); 1589.4 ($[M + K]^+$, $C_{67}H_{77}F_3N_{14}O_{26}K$ requires 1589.4) consistent with CF_3-CDA3b. In addition a minor product is evident at 7.6 min which exhibits molecular ions *m/z* 1549.4 ($[M + H]^+$, $C_{67}H_{76}F_3N_{14}O_{26}$ requires 1549.5); 1571.5 ($[M + Na]^+$, $C_{67}H_{75}F_3N_{14}O_{26}Na$ requires 1571.5); 1587.8 ($[M + K]^+$, $C_{67}H_{75}F_3N_{14}O_{26}K$ requires 1587.5) consistent with CF_3-CDA3a. (**B**) ^{19}F NMR signals of CF_3-CDA3b and CF_3-CDA3a products isolated by repeated RP-HPLC. (**C**) ^{19}F NMR signals of a racemic mixture of synthetic 3-trifluoromethyl glutamic acid.

another minor product with a short retention time (7.6 min), which exhibited protonated, sodiated and potassiated molecular ions that correspond to a *mw* 1548.4 Da. This is consistent with a variant of CDA3a possessing an additional trifluoromethyl group (CF_3-CDA3a). In addition, ^{19}F NMR spectroscopy of the HPLC fractions containing the new CDA products shows a major doublet at δ_F −65.2 ppm ($^3J_{HF}$ = 9.1 Hz) along with a second smaller doublet (δ_F −65.0 ppm, $^3J_{HF}$ = 10.4 Hz) which is consistent with a mixture of 3-trifluoromethyl Glu containing CF_3-CDA3b and CF_3-CDA3a (Fig. 2). Indeed the synthetic 3-trifluoromethyl glutamic acid shows a distinct pair of diastereotopic CF_3 signals in the ^{19}F NMR (δ_F −68.5 ppm, $^3J_{HF}$ = 9.1 Hz and δ_F −69.3 ppm, $^3J_{HF}$ = 9.1 Hz).

To further explore the utility of this precursor-directed biosynthesis approach, another precursor analogue, 3-ethylglutamate **11** (3-EtGlu) was synthesised as a mixture of racemic diastereoisomers (**8** → **11**), in a fashion analogous to that described above (Scheme 1). Similarly the synthetic 3-EtGlu product was fed

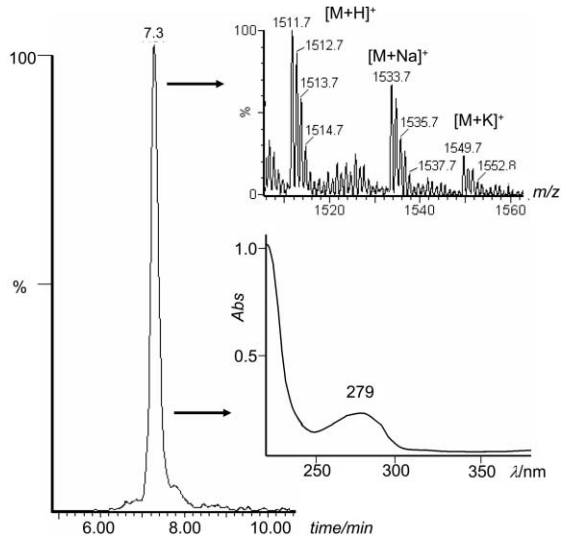

Fig. 3 LC-MS and UV analysis of purified extracts from the large scale feeding of 3-ethyl glutamic acid to MT1110-Δ*glmT*. Shows a product with retention time of 7.3 min which exhibits molecular ions *m/z* 1511.7 ([M + H]$^+$, $C_{68}H_{83}N_{14}O_{26}$ requires 1511.6); 1533.7 ([M + Na]$^+$, $C_{68}H_{82}N_{14}O_{26}Na$ requires 1533.5); 1549.7 ([M + K]$^+$, $C_{68}H_{82}N_{14}O_{26}K$ requires 1549.5) consistent with Et-CDA3b.

to the MT1110-Δ*glmT* and ESI LC-MS again revealed Glu containing CDA3b and CDA3a to be the major products (see ESI). However a small amount of a new product, with retention time 7.3 min, was evident which is not present in the supernatant of MT1110-Δ*glmT* in the absence of 3-EtGlu. This new product exhibited protonated, sodiated and potassiated molecular ions in the ESI-MS corresponding to a *mw* 1510.7 Da, which is consistent with the expected product Et-CDA3b (Fig. 3). HPLC purification of the extracts also enabled the UV spectrum of the new product to be recorded. This shows a typical spectrum for a b-series CDA with λ_{max} 279 nm (in H_2O), with no absorption in the 350 nm region which is evident in the a-series CDA that contain *Z*-dehydrotryptophan residues.[5a] As before yields of the new ethyl-CDA analogue were low and prevented detailed NMR analysis.

It is likely that the low levels of incorporation observed with 3-CF_3Glu and 3-EtGlu are largely due to the specificity of the module 10 A-domain of the CDA NRPS (CdaPS2). To address this we are exploring active site modification[4] of this A-domain in order to broaden substrate specificity and favour activation and incorporation of β-substituted Glu analogues. We anticipate that this may allow sufficient quantities of the product to be isolated for biological testing. In addition, following our earlier identification[5a] and characterisation[7] of the GlmT encoding gene (SCO3215) within the *cda* cluster, an homologue encoded by the gene *dptI* was found within the daptomycin biosynthetic gene cluster.[10] Deletion of *dptI* from the daptomycin producer strain, *Streptomyces roseosporus*, similarly abolishes production of 3-MeGlu containing daptomycin in favour of the Glu variant.[11] Interestingly the parent *S. roseosporus* strain produces daptomycin with a 3-MeGlu residue exclusively, with none of the Glu variant. This suggests that the A-domain responsible for activation of 3-MeGlu in the daptomycin NRPS (DptD) is even more specific for the 3-MeGlu substrate. In addition lipopeptide production in the industrial *S. roseosporus* strain is *ca.* 100 times higher than production of CDAs in *S. coelicolor*. As a result the yields of 3-substituted Glu containing daptomycin analogues produced using this approach with *S. roseosporus* Δ*dptI* should be much higher than the yields observed here with *S. coelicolor* MT1110-Δ*glmT*.

In summary, a precursor-directed biosynthesis approach has been developed which utilises a mutant strain of *S. coelicolor* to incorporate synthetic 3-trifluoromethyl and 3-ethyl glutamate residues into the calcium dependent antibiotics. The similarity between the mode of 3-MeGlu biosynthesis and activation in CDA and daptomycin assembly indicates that this approach can also be applied to the precursor-directed biosynthesis of daptomycin analogues with 3-substituted glutamate residues, which may be more active and less toxic than the parent antibiotic. The emerging threat of clinically relevant pathogens acquiring resistance to daptomycin, from environmental sources,[12] makes the development of new analogues particularly urgent.

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