Electronic supplementary information (ESI)

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

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2-Nitro-3-trifluoromethyl-pentanedioic acid dimethyl ester (5).

1,8-Diazabicyclo[5.4.0]undeca-7-ene (DBU) (6.15 mL, 41.2 mmol) was added to a mixture of methyl 4, 4, 4-trifluoro crotonate **3** (6.35 g, 41.2 mmol) and methyl nitroacetate **4** (3.79 mL, 41.2 mmole) in anhydrous THF (20 mL) under N_2 at room temperature and stirred in the absence of light for 72 h. Aqueous HCl (1.2 M) was then added to adjust the pH to 7.0 and the resultant mixture was extracted with diethyl ether (3 x 150 mL), dried over $MgSO_4$, filtered and evaporated under reduced pressure. Purification by flash chromatography (90:10 toluene/EtOAc) gave **5** (9.0 g, 80 %) as a pale yellow oil. R_f 0.45 (90:10 toluene/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$), δ 2.74-2.93 (2H, m, CH_2), 3.67 & 3.68 (2 x 1.5H, s, OCH_3-diasteroisomers), 3.78 & 3.82 (2 x 1.5H, s, OCH_3-diasteroisomers), 3.94-4.02 (1H, m, C$H$$CF_3$), 5.42 (0.5H, d, J = 6.8 Hz, C$H$$NO_2$-diasteroisomers), 5.53(0.5H, d, J = 3.8 Hz, C$H$$NO_2$-diasteroisomers); ^{13}C NMR (100.6 MHz, $CDCl_3$), δ 29.7 & 29.8 (CH_2-diastereoisomers), 41.3 & 41.7 (2 x q, J = 28.5 Hz , $C$$HCF_3$-diastereoisomers), 53.0, 53.1, 54.6 & 54.8 (4 x O$C$$H_3$-diastereoisomers), 84.4 & 84.5 (2 x $C$$HNO_2$-diastereoisomers), 121.3 & 129.7 (2 x q, J = 280.5 Hz $C$$F_3$-diastereoisomers), 162.9, 170.3 & 170.4 (3 x $C$$O_2CH_3$-diastereoisomers); ^{19}F NMR (188.31 MHz, $CDCl_3$) –70.2 (2 x 1.5F, d, J = 9.0 Hz, $CHCF_3$-diastereoisomers), –70.3 (2 x 1.5F, d, J = 9.0 Hz, $CHCF_3$-diastereoisomers); LRMS-EI/CI (*m/z*): 291 ([M+NH_4]$^+$, 100 %); HRMS-EI/CI (*m/z*): [M+NH_4]$^+$ calc'd for $C_8H_{14}F_3N_2O_6$, 291.0798. Found 291.0802; IR (neat): ν 1745 (C=O), 1364 & 1574 (C-NO_2).

3-Trifluoromethyl glutamic acid hydrochloride salt (7). Raney-Ni (2.4 g) was added to the dimethyl ester **5** (9.0 g, 33.0 mmol) in anhydrous CH_3OH (60 mL) and HCO_2H (50 mL) and the mixture stirred for 48 h at room temperature under N_2. The reaction mixture was then filtered through celite and the precipitate was washed with CH_3OH and EtOAc. The solvent was evaporated under reduced pressure. H_2O (30 mL) was added to the residue followed by saturated aqueous $NaHCO_3$ until the resulting solution was pH 8.0. The mixture was then extracted with diethyl ether (3 x 150 mL) and EtOAc (3 x 150 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The resulting residue was dissolved in anhydrous CH_2Cl_2 (40 mL) under N_2 at 0 ˚C and di-*tert*-butyl dicarbonate (7.54 g, 34.6 mmol), triethylamine (4 mL, 28.8 mmol) and 4-DMAP (3.52 g, 28.8 mmol) were then added. The mixture was allowed to warm to room temperature and stirred for 18h. H_2O (150 mL) was then added and the mixture extracted with CH_2Cl_2 (3 x 250 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was then separated by flash chromatography and the fractions eluting with 70:30 Hexane/EtOAc, where collected and evaporated under reduced pressure. LiOH (1.75 g, 73.25 mmol) in H_2O (10 mL) was then added to the residue in THF (25 mL) at 0 ˚C and the mixture was stirred for 18 h. H_2O (100 mL) was then added and the solution was adjusted to pH 4.0 with CH_3CO_2H and extracted with EtOAc (3 x 150 mL), dried over $MgSO_4$, filtered, and evaporated under reduced pressure to provide **6** (5 g, crude yield) as a pale yellow solid. The solid **6** (5g, 15.9 mmol) was then treated with a solution of 4M HCl in 1,4-dioxane (50 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 (20 mL) was added, and the mixture was washed with CH_2Cl_2 (3 x 25 mL). The aqueous extract were evaporated under reduced pressure and the residue was triturated with toluene to give to give 3-trifluoromethyl glutamic acid HCl salt **7** (3.03 g, 89 % from **5**) as a pale yellow hygroscopic solid. ^{1}H NMR (400 MHz, D_2O) δ 2.85-2.93 (2H, m, CH_2), 3.62-3.65 (1H, m, CHCF$_3$), 4.39 (0.5H, d, J = 3.4 Hz, CHCO$_2$H-diastereoisomers), 4.43 (0.5H, d, J = 2.4 Hz, CHCO$_2$H-diastereoisomers), ^{13}C NMR (100.6 MHz, D_2O) δ 29.7 & 30.2 (CH_2-diastereoisomers), 40.0 & 41.0 (2 x q, J = 27 Hz, CHCF$_3$-diastereoisomers), 51.4 & 51.7 (CHCO$_2$H-diastereoisomers), 124.5 &127.5 (2 x q, J = 280 Hz, CF$_3$- diastereoisomers), 169.4, 169.6, 173.6 & 173.8 (CH_2CO$_2$H-diastereoisomers); ^{19}F NMR (188.31 MHz, D_2O) δ –68.5 (1.5F, d, $^3J_{HF}$ = 9.1 Hz, CHCF_3-diastereoisomers), - 69.3 (1.5F, d, $^3J_{HF}$ = 9.1 Hz, CHCF_3-diastereoisomers). LRMS-ESI (-) (*m*/*z*): 214 ([M-H$^+$]$^-$, 38 %); HRMS-ESI (-) (*m*/*z*): [M-H$^+$]$^-$ calc'd for $C_6H_7F_3NO_4$ 214.0333. Found 214.0325.

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3-Ethyl-2-nitro-pentanedioic acid dimethyl ester (9) 1,8-Diazabicyclo[5.4.0]undeca-7-ene (DBU) (5 mL, 33.6 mmol) was added to a mixture of methyl trans-2-pentenoate **8** (8 mL) and methyl nitroacetate **4** (4 g, 33.6 mmol) in anhydrous CH_3CN (10 mL) under N_2 at room temperature and stirred in the absence of light for 72 h. Aqueous HCl (1.2 M) was then added to adjust the pH to 7.0 and the resultant mixture was extracted with diethyl ether (3 x 250 mL), dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (90:10 toluene/EtOAc) gave **9** (6.45 g, 82 %) as a pale yellow oil. R_f 0.59 (90:10 toluene/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 & 1.04 (2 x 1.5H, dt, J = 7.5, 15.5 Hz, CH_3-diastereoisomers), 1.45-1.64 (2H, m, CH_2CH$_3$), 2.49-2.69 (2H, m, CH_2CO$_2$CH$_3$), 2.78-2.85 (1H, m, CHCH$_2$CH$_3$), 3.72 & 3.73 (2 x 1.5H, s, OCH$_3$-diastereoisomers), 3.86 & 3.87 (2 x 1.5H, s, OCH$_3$-diastereoisomers), 5.43 (0.5H, d, J = 6.5 Hz, CHNO$_2$-diastereoisomers), 5.44 (0.5H, d, J = 5.5 Hz, CHNO$_2$ -diastereoisomers); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 11.6 & 11.7 (CH$_2$*C*H$_3$-diastereoisomers), 23.7 & 23.8 (*C*H$_2$CH$_3$-diastereoisomers), 33.9 & 34.4 (*C*H$_2$CO$_2$CH$_3$-diastereoisomers), 38.1 & 38.3 (*C*HCH$_2$CH$_3$-diastereoisomers), 52.4, 53.9 & 53.9 (3 x O*C*H$_3$-diastereoisomers), 89.5 & 89.9 (*C*HNO$_2$-diastereoisomers), 164.6, 164.9, 172.4 & 172.5 (4 x *C*O$_2$CH$_3$); LRMS-ESI (*m/z*): 256([M+Na]$^+$, 100 %), HRMS-ESI (*m/z*): [M+Na]$^+$ calc'd for $C_9H_{15}NO_6$ Na 256.0792 Found 256.0786. IR (neat): ν 1732 (C=O), 1368 & 1563 (C-NO$_2$).

3-Ethyl glutamic acid hydrochloride salt (11). Raney-Ni (2.0 g) was added to the dimethyl ester **9** (6.7 g, 28.7 mmol) in anhydrous CH_3OH (50 mL) and HCO_2H (50 mL) and the mixture stirred for 48 h at room temperature under N_2. The reaction mixture was then filtered through celite and the precipitate was washed with CH_3OH and EtOAc. The solvent was evaporated under reduced pressure. H_2O (25 mL) was added to the residue followed by saturated aqueous $NaHCO_3$ until the resulting solution was pH 8.0. The mixture was then extracted with diethyl ether (3 x 250 mL) and EtOAc (3 x 250 mL). The combined organic extracts dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The resulting crude amine was dissolved in anhydrous CH_2Cl_2 (40 mL) under N_2 at 0 ˚C and di-*tert*-butyl dicarbonate (9.67 g, 44.3 mmol), triethylamine (4.1 mL, 29.5 mmol) and 4-DMAP (3.6 g, 29.5 mmol) were then added. The reaction mixture was allowed to warm to room temperature and stirred for 18h. H_2O (150 mL) was then added and the mixture was extracted with CH_2Cl_2 (3 x 250 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was separated by flash chromatography and the fractions eluting in 70:30 Hexane/EtOAc, were evaporated under reduced pressure. The residue was then dissolved in THF (25 mL) at 0 ˚C and treated with LiOH (1.75 g, 73.0 mmol) in H_2O (11.0 mL) and stirred at room temperature for 18 h. H_2O (150 mL) was then added and the solution was adjusted to pH 4.0 with CH_3CO_2H and extracted with EtOAc (3 x 250 mL), dried over $MgSO_4$, filtered, and evaporated under reduced pressure to

provide **10** (4.3 g, crude yield) as a white solid. **10** (4.3 g, 15.6 mmol) was then treated with a solution of 4M HCl in 1, 4-dioxane (40 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 (25 mL) was added, and the mixture was washed with CH_2Cl_2 (3 x 25 mL). The aqueous extract was evaporated under reduced pressure and the resulting solid was triturated with toluene to give 3-ethyl glutamic acid HCl salt **11** (2.33 g, 85%) as a white hygroscopic solid. ^{1}H NMR (400 MHz, D_2O) δ 0.82 (3H, t, J = 7.4 Hz, CH_3), 1.20-1.44 (2H, m, CH_2CH$_3$), 2.32 (1H, m, CHCH$_2$CH$_3$), 2.46 (2H, d, J = 6.5 Hz, CH_2CO$_2$H), 4.04 (0.5H, d, J = 3.5 Hz, CHCO$_2$H-diastereoisomers), 4.09 (0.5H, d, J = 3.2 Hz, CHCO$_2$H-diastereoisomers); ^{13}C NMR (100.6 MHz, D_2O) δ 11.0 (*C*H$_3$), 22.5 & 23.2 (*C*H$_2$CH$_3$-diastereoisomers), 34.5 & 34.9 (*C*H$_2$CO$_2$H-diastereoisomers), 37.4 & 37.5 (*C*HCH$_2$-diastereoisomers), 55.3 & 55.7 (*C*HCO$_2$H-diastereoisomers), 171.5, 171.6, 176.5 & 176.6 (CH*C*O$_2$H-diastereoisomers); LRMS-ESI (*m/z*): 176([M+H]$^+$, 100 %); HRMS-ESI (*m/z*): [M+H]$^+$ calc'd for $C_7H_{14}NO_4$ 176.0917. Found, 176.0920.

***S. coelicolor* MT1110-Δ*glmT* small-scale liquid cultures and substrate feeding experiments.**

Small-scale liquid feeding experiments were carried out in SV2 media (total volume 7 mL)[5a] inoculated with one 5 mm plug from a 3-day R5$^+$ [Reference S1] agar plate of *S. coelicolor* MT1110-Δ*glmT*. The cultures were then incubated at 28 ˚C, 180 rpm, for 2 days. Filter-sterilized aqueous solutions of substrates (50 μg.mL^{-1} to 1.0 mg.mL^{-1} range) were then added to the cultures. After a total of 6 days fermentation, the cultures were harvested as described previously[7] and extracts were then analyzed by LC-MS.

***S. coelicolor* MT1110-Δ*glmT* large-scale feeding experiments**

Large-scale liquid feeding experiments were carried out in SV2 media (300 mL) inoculated with 4-5 10 mm plug from a 3-day R5$^+$ agar plate of *S. coelicolor* MT1110-Δ*glmT*. The cultures were then incubated at 28 ˚C, 180 rpm, for 2 days. Filter-sterilized aqueous solutions of the substrate trifluoromethyl glutamic acid or ethyl glutamic acid (100 mg.mL^{-1}) were then added to give a final concentration of 750 μg.mL^{-1}. After a total of 6 days fermentation, the cultures were harvested as described previously.[7] In total 2.4 g of ethyl glutamic acid was supplied to a 2.7 L culture of MT1110-Δ*glmT* (in 300 mL batches). Similarly a total of 2.9 g trifluoromethyl glutamic acid was supplied to a 3 L MT1110-Δ*glmT* (in 300 mL batches). Extracts were then analyzed by LC-MS

Analysis of CDA Extracts Using LC-MS

LC-MS analysis was carried out on a Micromass LCT orthogonal acceleration time of flight mass spectrometer, equipped with an electrospray ionisation source run in positive mode (scanning from 700 to 1700 *m*/*z*) combined with a Waters 2790 Separation module. Gradient elution was carried out using a reversed phase C-18 150 x 4.6 mm 3 μm Phenomenex column. 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 80 % A and 20 % B, increasing to 70 % B over 10 min and increased to 100 % B over the next min and held for a further 4 min.

HPLC purification of CDA Extracts.

HPLC of CDA extracts were carried out on a Varian Prostar instrument, equipped with Prostar 335 photodiode array detector. Detection of CDA peptides was by UV at $\lambda = 280$ nm and $\lambda = 350$ nm. Purification of the crude extracts was achieved by semi-preparative reversed phase HPLC: Phenomenex C-18 5 μm, 250 x 10 mm column. Solvent A was H_2O with 0.1 % HCO_2H and solvent B was acetonitrile with 0.1 % HCO_2H. The flow rate was 5 $mL.min^{-1}$; starting with 0 % B and 100 % A, increasing to 100% B over 30 min and then held for a further 5 min. Separate 1 min fractions were collected over the program. Fractions with peaks of interest were pooled and evaporated under reduced pressure. Fractions were analysed by LC-MS to determine the presence of CDA. Fractions contain novel CDAs were subjected to further HPLC purification using the same column, solvents, instrument and U.V detection as stated above with the following gradient: 70 % A and 30 % B, which increased to 40% B over 35 min with a further increase to 60 % B in the following 5 min.

Supplementary References

[S1] T. Kieser, M. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. **2000**. Practical Streptomyces genetics. The John Innes Foundation, Norwich, U.K.

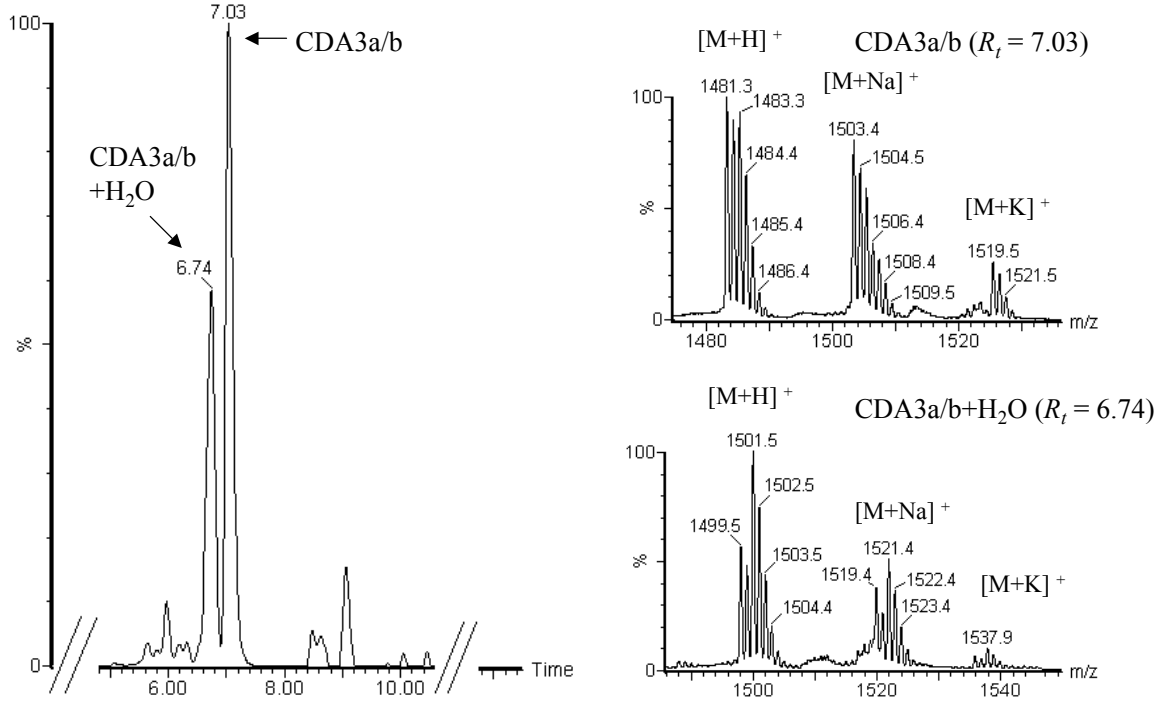

Figure S1. LC-MS chromatograph of crude extracts from the control Δ*glmT*, shows production of CDA3a and CDA3b as the major product co-eluting (R_t = 7.03 min) along with a minor amount of the hydrolysed (linear) forms of CDA3a and CDA3b co-eluting (R_t = 6.74 min). There are no products with masses relating to a possible CF_3-CDA3b, CF_3-CDA3a or Et-CDA3b present in this and extracted mass chromatograms at the detection limits of the instrument. Note the ratio of CDA3a/3b is higly variable, depending on the volume of fermentation, which may be due to different levels of oxygen.

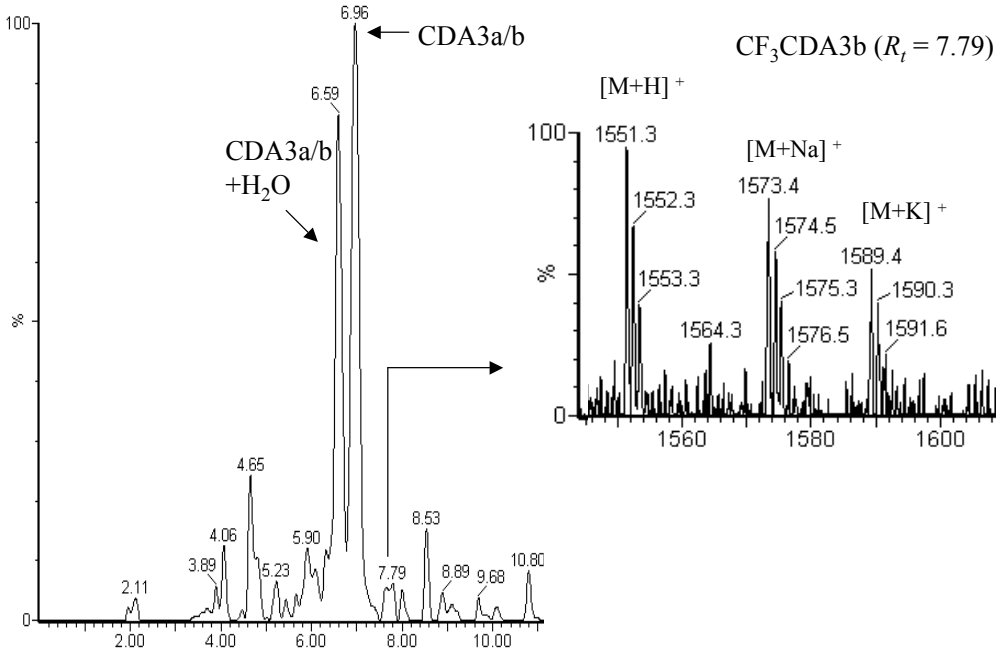

Figure S2. LC-MS chromatograph of crude extracts from the Δ*glmT* supplemented with 3-trifluoromethyl glutamic acid (750 μg.mL^{-1}) shows production of CF_3-CDA3b (R_t = 7.79 min) with the corresponding protonated, sodiated and potassiated molecular ions along with CDA3a/b (R_t = 6.96 min) and the hydrolysed (linear) forms of CDA3a/b at R_t = 6.59 min.

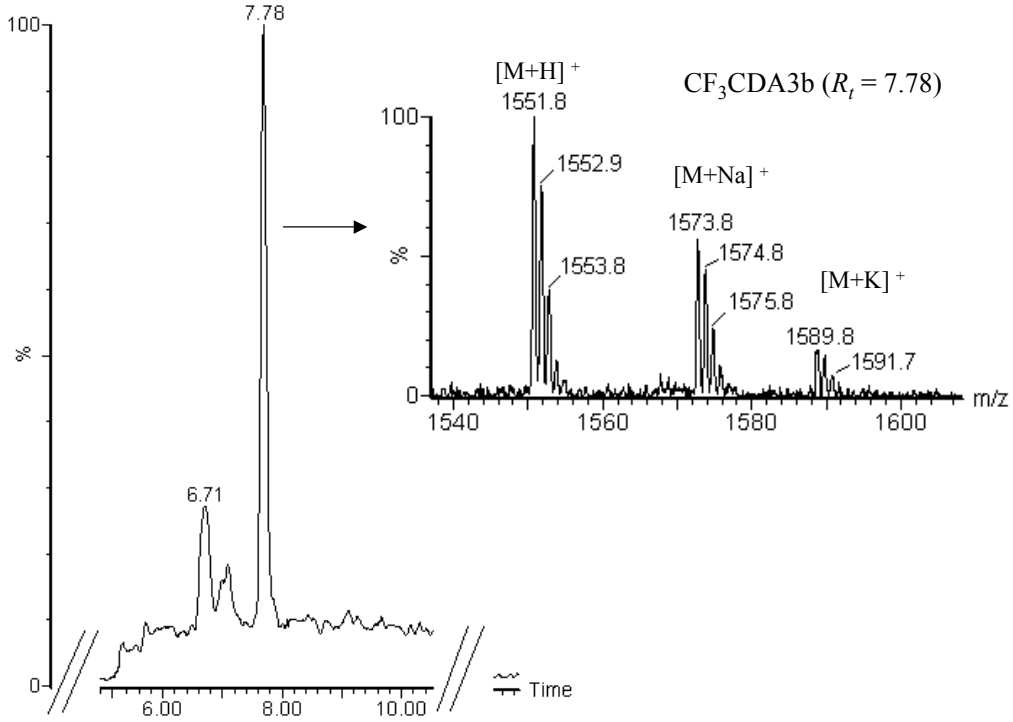

Figure S3. Mass Extraction (*m/z* = 1551.5) LC-MS chromatograph of crude extracts from another feeding experiment where Δ*glmT* which was supplemented with 3-trifluoromethyl glutamic acid (750 μg.ml^{-1}). Again this shows production of CF_3-CDA3b (R_t = 7.78 min).

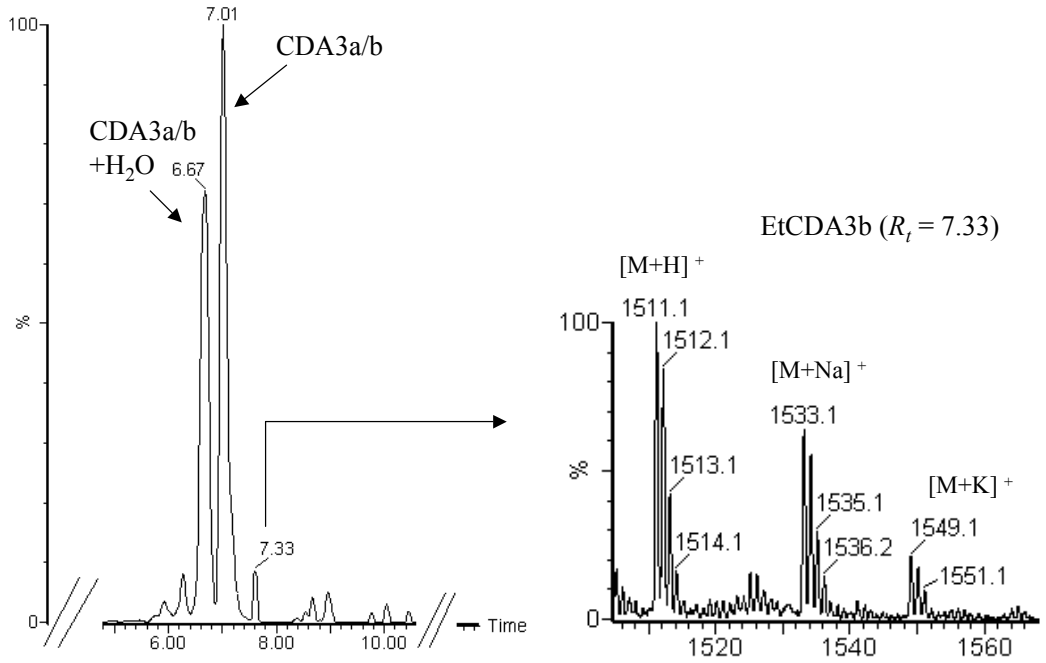

Figure S4. LC-MS chromatograph of crude extracts from the Δ*glmT* supplemented with 3-ethyl glutamic acid (900 μg.mL^{-1}) shows production of Et-CDA3b (R_t = 7.33 min) with the corresponding protonated, sodiated and potassiated molecular ions along with CDA3a/b (R_t = 7.01 min) and the hydrolysed (linear) forms of CDA3a/b at R_t = 6.67 min.

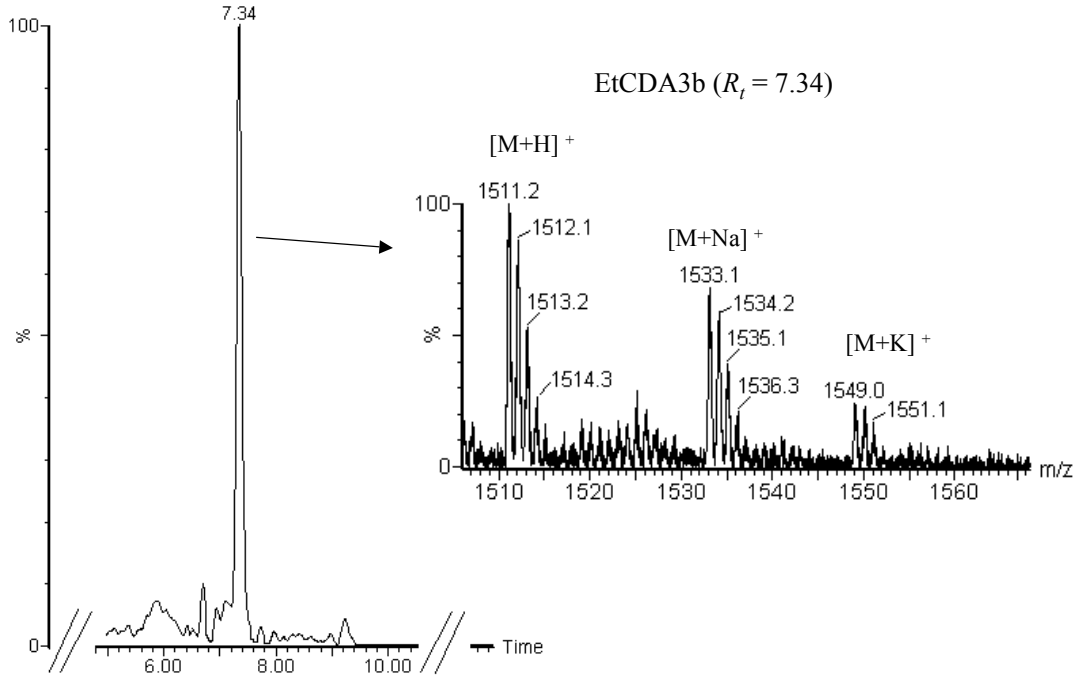

Figure S5. Mass Extraction (*m/z* = 1511.5) LC-MS chromatograph of the same extracts from the Δ *glmT* supplemented with 3-ethyl glutamic acid (900 μg.ml^{-1}) shows production of EtCDA3b (R_t = 7.34 min).